Adenocarcinoma of the Lung:  
An Exploration of the Relationships Between Histopathology, Molecular Pathology and Inflammatory Markers and Their Relationship to Patient Outcomes

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This thesis is dedicated to

my wife Rachel

and our children

Patrick and Alexis
Abstract

Lung cancer remains the most common cause of cancer related death worldwide, with nearly 1.4 million deaths in 2008 globally. Adenocarcinoma is the most common type of lung cancer, and its frequency compared to other histologic subtypes is increasing. The simplicity of the label “adenocarcinoma” hides its significant pathologic and clinical heterogeneity. This thesis explores a number of clinicopathologic correlates in lung adenocarcinoma specimens obtained from patients treated at St Vincent’s Hospital in Melbourne, Australia.

In 2011 the International Association for the Study of Lung Cancer (IASLC), the American Thoracic Society (ATS) and the European Respiratory Society (ERS) proposed a new classification system for pulmonary adenocarcinoma. This was subsequently adopted in the 2015 edition of the World Health Organisation Classification of Tumours of the Lung, Pleura, Thymus and Heart. Multiple groups demonstrated that the new classification had prognostic significance following resection of pulmonary adenocarcinoma independent of stage. The impact of the classification in metastatic disease was not known.

This thesis found that it was possible to identify the adenocarcinoma patterns of solid with mucin, papillary, micropapillary and acinar in each specimen taken from a metastatic site and semi-quantitatively assess each component. Further, the identification of a major pattern was not prognostic, but did predict for differences in survival time for patients treated with systemic therapy. The worst outcomes were observed for patients with tumours with a major solid pattern. The major solid pattern was also found to have infrequent occurrence of activating epidermal growth factor receptor (EGFR) mutations. As this is the first time that this novel finding has been reported. Validation from other groups is required.
The presence of the IASLC/ATS/ERS classification as a robust new tool with clinical relevance has led to further research to define other clinicopathologic correlates. Oncogene driver mutations in genes such as *EGFR* and Kirsten RAS (*KRAS*) are critical in selection of therapy in advanced disease. This thesis examined relationships between adenocarcinoma subtype and mutation status for patients who had resected lung adenocarcinoma. Patients with solid predominant adenocarcinoma were significantly less likely to have *EGFR* mutations, while *KRAS* mutation was a frequent event in invasive mucinous adenocarcinoma. No other significant associations were found. The findings were consistent with those recently reported by other groups from centres located in predominantly Caucasian countries.

*EGFR* inhibition and the discovery of *EGFR* mutations was the starting point for a major change in the approach to treatment of advanced lung adenocarcinoma, however resistant to treatment occurs. It had been suggested that upregulation of phosphorylated STAT3 (pSTAT3) via interleukin 6 (IL6) and Janus Kinase (JAK) may be linked to *EGFR* mutation status in the absence of treatment with *EGFR* tyrosine kinase inhibitors and therefore may offer a rational target to delay resistance to such therapies. In the patient cohort studied the presence of *EGFR* or *KRAS* mutation status did not enrich for activation of IL6, JAK1 or pSTAT3 as determined by immunohistochemistry. Further, there was no clinicopathologic or prognostic correlates of note found by the IL6, JAK1 or pSTAT3 activation state. The assessment of IL6, JAK1 and pSTAT3 in the same samples and by two methods to assess positivity was a unique feature of this study.

In conclusion this contributes new knowledge on the relevance of pathologic subtyping in advanced lung adenocarcinoma. It confirms and consolidates recent reports oncogene mutation status and adenocarcinoma subtype following surgical resection. It examines the IL6 / JAK1 / pSTAT3 pathway in detail in resected pulmonary adenocarcinoma. Translational research that explores why adenocarcinoma subtypes have different outcomes by treatment may allow clinicians to direct therapies differently or unlock new pathways for targeting lung adenocarcinoma with therapeutic effect.
Declaration

This is to certify that

(i) this thesis comprises only my original work towards the Doctor of Medical Science except where indicated in the preface

(ii) due acknowledgement has been made in the text to all other material used

(iii) the thesis is less than 100 000 words in length, exclusive of tables, maps, bibliographies and appendices

Signature: .................................................................
Preface

All of the work in this thesis is my own, with the following exceptions whom I gratefully acknowledge:

All anatomical pathology assessments were performed by A/Prof Prudence Russell, Anatomical Pathologist, at the Department of Pathology, St Vincent’s Hospital, Melbourne (Chapters 2, 3 and 4)

The \textit{EGFR} and \textit{KRAS} mutation analysis was performed by Dr Hongdo Do, postdoctoral researcher, formerly of Peter MacCallum Cancer Centre and now of the Olivia Newton-John Cancer Research Institute. The methods relating to this process were written by him and used in this thesis with permission (Chapters 2, 3 and 4).

Tissue Microarrays were constructed by David Byrne, Research Assistant and Richard Young, Research Officer, both of Peter MacCallum Cancer Institute. (Chapter 4)

Preparation of Slides for IL6, gp130, JAK1 and pSTAT3 assessment was performed by Shou Chen, Grade 1 Scientist, Department of Pathology, St Vincent’s Hospital Melbourne (Chapter 4)

Statistical Analysis was performed in collaboration with A/Prof Vijaya Sundarararjan, Physician and Biostatistician, Department of Medicine, University of Melbourne
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Successful completion of this thesis would not have been possible without the contributions of Dr Hongdo Do (in the lab of A/Prof Alex Dobrovic) and A/Prof Vijaya Sundararajan.

Finally and most importantly I would like to acknowledge and thank my family. To my children, Patrick and Alexis, for being blissfully unaware of the need to complete my thesis and dragging me away into your adventures.

And to my wife Rachel. She has stood beside me and contributed all the way through basic training, the move to Melbourne for advanced training, my higher degree and the final years of completing this thesis at home in Perth. My full and successful life would not be possible without your love and support. Thank you.
Publications and Presentations

Publications


Presentations


Presentation, 4th Australian Lung Cancer Conference, October 2014, Brisbane, Australia.

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19. Overall Survival by low or high staining for pSTAT3 as defined by the H Score
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<td>95% CI</td>
<td>95% Confidence Interval</td>
</tr>
<tr>
<td>AC</td>
<td>Adenocarcinoma</td>
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<tr>
<td>AIS</td>
<td>Adenocarcinoma in situ</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
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<tr>
<td>ALK</td>
<td>Anaplastic Lymphoma Kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>ATS</td>
<td>American Thoracic Society</td>
</tr>
<tr>
<td>BAC</td>
<td>Bronchioalveolar carcinoma</td>
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<tr>
<td>Bcl2</td>
<td>B-cell chronic lymphocytic leukaemia/lymphoma 2</td>
</tr>
<tr>
<td>BIM</td>
<td>Bcl-2 like 11</td>
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<tr>
<td>bp</td>
<td>Base pairs</td>
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<tr>
<td>CH</td>
<td>Colony hybridisation</td>
</tr>
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<td>CHEK2</td>
<td>Checkpoint Kinase 2</td>
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<tr>
<td>CIR</td>
<td>Cumulative Index of Recurrence</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CRT</td>
<td>Chemoradiotherapy</td>
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<tr>
<td>CT</td>
<td>Computer tomography</td>
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<tr>
<td>DFS</td>
<td>Disease free survival</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DSS</td>
<td>Disease specific survival</td>
</tr>
<tr>
<td>ECOG</td>
<td>Eastern Co-operative Oncology Group</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EML-4</td>
<td>Ectoderm Microtubule associated like protein</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>EORTC</td>
<td>European Organisation for Research and Treatment of Cancer</td>
</tr>
<tr>
<td>ERS</td>
<td>European Respiratory Society</td>
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<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin embedded</td>
</tr>
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<td>gp130</td>
<td>Glycoprotein 130</td>
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<tr>
<td>GSTM1</td>
<td>Glutathione S-transferase M1</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>GTP</td>
<td>Guanine triphosphate</td>
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<tr>
<td>GWA</td>
<td>Genome Wide Association</td>
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<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<tr>
<td>HHT</td>
<td>Homoharringtonine</td>
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<td>HR</td>
<td>Hazard Ratio</td>
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<td>HREC</td>
<td>Human Research Ethics Committee</td>
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<tr>
<td>HRM</td>
<td>High resolution melt</td>
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<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
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<tr>
<td>IASLC</td>
<td>International Association for the Study of Lung Cancer</td>
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<tr>
<td>ICD</td>
<td>International Classification of Diseases</td>
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<td>IDEAL</td>
<td>Iressa Dose Evaluation in Advanced Lung Cancer</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>IL6</td>
<td>Interleukin 6</td>
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<td>IMA</td>
<td>Invasive mucinous adenocarcinoma</td>
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<tr>
<td>iMDT</td>
<td>Multidisciplinary Tumour Database</td>
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<td>IPASS</td>
<td>Iressa Pan Asia Study</td>
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<tr>
<td>JAK</td>
<td>Janus Kinase</td>
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<tr>
<td>K</td>
<td>Kappa</td>
</tr>
<tr>
<td>Kd</td>
<td>Dissociation constant</td>
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<tr>
<td>KRAS</td>
<td>Kirsten Rat Sarcoma</td>
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<tr>
<td>LACE</td>
<td>Lung Adjuvant Cisplatin Evaluation</td>
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<td>LNA</td>
<td>Locked nuclei acid</td>
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<tr>
<td>LPA</td>
<td>Lepidic predominant adenocarcinoma</td>
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<td>M</td>
<td>Metastasis</td>
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<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>mBAC</td>
<td>Mucinous bronchioalveolar carcinoma</td>
</tr>
<tr>
<td>MDT</td>
<td>Multidisciplinary Team</td>
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<tr>
<td>MEK</td>
<td>Mitogen protein activated kinase kinase</td>
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<td>MIA</td>
<td>Minimally Invasive Adenocarcinoma</td>
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<td>MP(A)</td>
<td>Micropapillary (adenocarcinoma)</td>
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<td>MPE</td>
<td>Malignant pleural effusion</td>
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<tr>
<td>MRI</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>MSKCC</td>
<td>Memorial Sloan Kettering Cancer Centre</td>
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<td>MST</td>
<td>Median survival time</td>
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<tr>
<td>N</td>
<td>Node</td>
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<tr>
<td>nM</td>
<td>Nanomoles</td>
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<tr>
<td>nmBAC</td>
<td>Non-mucinous bronchioalveolar carcinoma</td>
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<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
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<td>OR</td>
<td>Odds Ratio</td>
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<td>P6</td>
<td>Pyridone 6</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PD-1</td>
<td>Programmed cell death protein 1</td>
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<tr>
<td>PD-L1</td>
<td>Programmed cell death ligand 1</td>
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<td>PET</td>
<td>Positron emission tomography</td>
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<td>PIAS</td>
<td>Protein Inhibitor of Activated STAT3</td>
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<td>PMCC</td>
<td>Peter MacCallum Cancer Centre</td>
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<td>PORT</td>
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<td>PFS</td>
<td>Progression Free Survival</td>
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<td>PS</td>
<td>Performance Status</td>
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<td>pT</td>
<td>Pathologic tumour stage</td>
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<td>QLQ</td>
<td>Quality of Life Questionnaire</td>
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<td>QoL</td>
<td>Quality of Life</td>
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<td>RECIST</td>
<td>Response Evaluation Criteria in Solid Tumours</td>
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<td>RR</td>
<td>Relative Risk</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
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<td>SARMS</td>
<td>Scorpion Amplification Refractors Mutation System</td>
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<td>SCLC</td>
<td>Small cell lung cancer</td>
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<td>SEER</td>
<td>Surveillance, Epidemiology and End Results</td>
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<td>SqCC</td>
<td>Squamous Cell Carcinoma</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>SOCS</td>
<td>Suppressors of Cytokine Signalling</td>
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<td>STAT</td>
<td>Signal Transducers and Activators of Transcription</td>
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<tr>
<td>T</td>
<td>Tumour</td>
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<tr>
<td>TERT</td>
<td>Human Telomerase Reserve Transcriptase</td>
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<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour, Nodes, Metastasis</td>
</tr>
<tr>
<td>TTF-1</td>
<td>Thyroid Transcription Factor 1</td>
</tr>
<tr>
<td>UICC</td>
<td>International Union for Cancer Control</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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CHAPTER 1

INTRODUCTORY COMMENTS ON LUNG CANCER
1.1 Epidemiology

1.1.1 Incidence and Mortality

Lung cancer is a major cause of morbidity and mortality. It is estimated that in 2008, cancers of the lung and bronchus accounted for 1 095 200 new cases (1st) and 951 000 cancer deaths (1st) in men, and 513 600 new cases (4th) and 427 400 deaths (2nd) in women [1]. The Australian data for 2007 shows that while lung cancer was the 4th most common new cancer diagnosis, it was the most common cause of cancer mortality [2]. The 5-year overall survival for lung cancer is 12% for men and 16% for women [3].

In Australia the mean age for diagnosis of lung cancer is 71 in males and 70 in females [2]. Less than 5% of lung cancers are diagnosed in patients less than 50 years of age, while more than 80% are diagnosed in those aged 60 or older. Increasing age is associated with worse lung cancer survival. Victorian data shows that the 5-year survival for patients falls from 37% for patients aged less than 44, to 20% between the ages of 45 – 64, to 15% between the ages of 65 – 74, and to 8% for patients aged older than 75 (p<0.01) [3].

In Australia lung cancer is more common in male patients who live in remote areas compared to cities and regional areas, while no difference is seen for female patients. Mortality rates are higher in remote areas, but this may partly be explained (although not excused) by the higher proportion of indigenous people living in these areas [2]. Remoteness is also associated with higher rates of smoking in Australia, and may also have impacts on timely access to treatment. Lower socioeconomic status is associated with increased risk for lung cancer, with a 1.5 times increased risk between the lowest and highest quintiles in males, and a 1.2 times increased risk for females. As a result,
the mortality rates are 1.5 times higher in males, and 1.3 times higher in females comparing the lowest to the highest socioeconomic groups.

Rates of lung cancer diagnosis are 1.6-1.7 times higher in Australian indigenous patients when compared to non-indigenous patients. The rates of mortality are also much higher, with indigenous males being 1.6 times more likely to die and indigenous females being 1.9 times more likely to die from their lung cancer diagnosis compared to other patients.

### 1.1.2 Aetiology

#### 1.1.2.1 Tobacco

Tobacco had been postulated as a causative factor in the development of lung cancer as early as 1912 (cited in [4]). A rise in the rate of lung cancer had been observed from the 1920s to the 1940s, with various theories promoted to explain the cause. Two groups published elegant papers in 1950 that more clearly demonstrated the link. Wynder and Graham reported on patients from the USA with bronchogenic carcinoma in comparison to patients from the general hospital population [4]. They found rates of “heavy smoking or greater” at 96% amongst men with lung cancer as compared to 74% amongst those without lung cancer. Further, lung cancer in never or minimal smokers was rare. Doll and Hill published a similar case-control study on patients from the UK [5]. In their case-control cohort the rates of non-smoking amongst men with lung cancer was only 0.3% as compared with the controls of the general hospital population at 4.2%. In women the figures were 32% and 53% respectively. Doll and Peto followed a cohort of male doctors in the UK and showed a clear correlation between lung cancer (as well as a number of other conditions) and smoking history [6]. Further, they were able to show that duration of smoking cessation reduced the risk of lung cancer over time. Tobacco consumption is a risk not only for the individual consuming
it but also to those with passive or involuntary exposure with increased rates of a number of malignancies seen [7].

Smoking cessation remains an important public health measure. In Australia strategies to reduce rates of tobacco smoking include media campaigns and public education, price signals through increasing taxation, targeted actions in groups with a high prevalence of smoking, reduction in advertising of tobacco products, and reduction in exemptions to smoke-free settings [8]. In December 2012 Australia became the first country to introduce plain packaging for tobacco packs, with multiple studies showing beneficial effects on the behaviour of smokers and government data showing a reduction in the total number of smokers (reviewed in [9]). Lung cancer mortality has been found to rise with increasing tobacco exposure within a country and fall after a period of decreasing consumption [1, 10].

1.1.2.2 Asbestos

A link between asbestos exposure and the subsequent development of lung cancer was first suggested by Lynch and Smith in 1935 [11]. Doll subsequently examined outcomes for 113 men who had had greater than 20 years exposure to asbestos in their working life and compared their rates of death by cause to that reported for all men in England and Wales [12]. He reported that the rate of death from lung cancer was 10-fold higher than that expected for men with such exposure. On the weight of mounting evidence asbestos was recognised as a human carcinogen by the International Agency for Research on Cancer (IARC) in 1987 [13]. The largest increases were seen for mesothelioma (which is almost exclusively related to asbestos exposure), lung cancer and laryngeal cancer.

The first effort to investigate the interplay between asbestos exposure and tobacco consumption was made by Hammond et al who reported their results in 1979 [14].
They compared a cohort of insulation workers (who almost all had significant asbestos exposure) to a group of volunteers in a prospective epidemiological study of the American Cancer Society (with limitations on this inclusion in this group to attempt to control for potential confounders). The authors were careful to acknowledge the inherent limitations of their study in presenting their findings. Despite this they demonstrated that increasing exposure to cigarette smoke amongst workers exposed to asbestos was associated with multiplicative effects on the risk of lung cancer mortality. Subsequent reviews and meta-analysis have confirmed that the increased risk holds true over multiple studies, although there is disagreement as to whether the interactions is multiplicative (as found by Lee [15]) or additive (as reported by Ngamwong et al [16]).

1.1.2.3 Other Carcinogens

Multiple agents are associated with increased risk of development of pulmonary malignancies. The potential for such exposures to lead to lung cancer were identified from as early as the 1950s in a report from Wynder and Graham [17]. At present the IARC lists twenty seven further substances which are known to contribute to the development of pulmonary malignancy, including exposure to some metals (such as aluminium production, arsenic and inorganic arsenic compounds, beryllium and cadmium), different types of coal exposure, several forms of radiation, several industries and outdoor air pollution [18].

1.1.2.4 Genetic Susceptibility – Family History and Known Mutations

While tobacco smoking is clearly implicated as the major causative factor in development of lung cancer, it is well noted that some smokers develop cancer while others do not, despite similar levels of exposure. A number of genetic factors have been found that influence the likelihood of developing lung cancer.
The role of a positive family history is noted in a number of studies. Matakidou et al carried out a systemic review of papers of the relationship of family history to lung cancer development in 2005, including 28 eligible papers [19]. Twenty-seven of the papers found an association, of which statistical significance was reached in twenty-one. The pooled relative risk (RR) for a person with a positive family history was 1.82 (95% confidence interval [CI] 1.58 – 2.10) although significant heterogeneity was found between studies. Lissowska et al conducted a case-control study and found that risk of lung cancer increased by both the presence of and increasing number of first degree relatives with a lung cancer diagnosis [20].

Population based studies have also demonstrated increased familial risk. A study of the population of Iceland showed significantly increased relative risk for first degree relatives of patients with cancer, with a statistical increase in risk seen as far out as third degree relatives [21]. The study also showed that the risk was further increased if a family member was diagnosed below the age of sixty. The increase in risk was not attributable to tobacco consumption. Lindelof and Eklund examined rates of familial risk in the larger National Cancer Registry of Sweden in a case-control fashion [22]. The strongest familial associations were seen for cancers of the eye, thyroid and testis and Hodgkin’s lymphoma. The familial risk for lung cancer was increased by an odds ratio of 1.9 (95% CI 1.6 – 2.4) for cases compared with controls – a rate similar to that seen in the Icelandic population.

A number of groups have investigated potential changes in the genome that may account for changes in risk. To date only one protective change has been found in the genome that reduces the likelihood of developing of lung cancer – missense mutation in Checkpoint Kinase 2 (CHEK2). CHEK2 is an enzyme that is activated as a result of DNA damage (as reviewed in [23]). Mutations in this gene have been associated with an increased risk of a number of cancers (as reviewed in [24]). In a case-control study Brennan et al found that missense mutation in the CHEK2 enzyme led to a protective effect against the lung carcinogenicity promoted by tobacco smoking (Relative Risk
In light of these findings Cybulski et al extended a prior analysis to include a new total of 895 patients with lung cancer and 6391 controls (an increase on a previous total of 272 cases and 4000 controls) [24]. They showed that CHEK2 mutations were associated with a decreased likelihood of lung cancer at an odds ratio (OR) of 0.3 (95% CI 0.2 – 0.5, p<0.00001).

Loss of function mutation in glutathione S-transferase M1 (GSTM1) has also been implicated in lung cancer development. Loss of function leads to a decreased ability to clear carcinogens, theoretically explaining the increased risk of lung cancer. McWilliams et al conducted a meta-analysis of case-control studies published from 1984 – 1995 [25]. They found that GSTM1 deficiency led to an increased risk of lung cancer (OR 1.41, 95% CI 1.23 – 1.61, p<0.0001). Similar results were found by Shi et al in an analysis limited to Chinese patients (OR 1.54, 95% CI 1.31 – 1.80, p<0.001) [26]. Bennett et al examined GSTM1 activity in a group of 106 never smoking white women who had developed lung cancer [27]. They looked at environmental tobacco smoke exposure, and found that those who lacked GSTM1 were at much higher risk of developing lung cancer from such exposure.

Lung cancer due to germline mutations following Mendelian inheritance patterns is uncommon. Familial syndromes in which lung cancer is observed include p53 mutation (Li-Fraumeni syndrome) [28-30], carriers of a mutation in the retinoblastoma gene [31], xeroderma pigmentosum [32], Bloom’s syndrome [33] and Werner’s syndrome [34].

1.1.2.5 Genetic Susceptibility – Genome Wide Associations

Genome wide association (GWA) studies have been conducted by several groups to identify areas of genomic variation associated with higher rates of lung cancer development. An area on 15q25 has been identified in four GWA studies as being associated with an increased susceptibility to lung cancer [35-38]. Two genes for
nicotinic acid receptor subunits are found in this location, and have been previously linked to nicotine dependence. Two studies found that differences at 15q25 were associated with tobacco consumption [36, 37]. Hung et al found that polymorphisms at 15q25 also increased the risk of lung cancer in never smokers [35]. A separate study from Wang et al examining never smokers only did not find an association with 15q25 polymorphism and lung cancer risk [39].

Chromosome 6q has been implicated with an increased risk for lung cancer in a study from Amos et al [40]. Non-carriers of changes at this allele demonstrated a clear dose-response relationship between tobacco exposure and increasing risk of lung cancer. For carriers of the affect allele tobacco exposure led to an increased risk of lung cancer, but this was stable despite increasing levels of exposure (that is any tobacco consumption significantly increased risk, and to a level higher than that of non-carriers). You et al identified the gene RGS17, a gene involved in G-protein signalling, as the likely gene at this site, as well as demonstrating its over expression in lung cancer samples and cell lines [41].

A locus at chromosome 6p has been described by two groups. Hung et al demonstrated this area as a region of interest in a GWA study in 2008 [35]. However it was not possible to identify the likely causative gene(s). Wang et al further identified 6p21 as a region associated with lung cancer risk [38]. They noted two potential genes that may be involved at this region – BAT3, a gene associated with apoptosis; and MSH5, a gene associated with mismatch repair. In a further study Wang et al reported that polymorphisms in 6p21 were not associated with increased lung cancer risk in never smokers [39].

McKay et al found a region at 5p15 implicated in lung cancer risk [42]. Two genes are located in this region, and it is thought that the TERT (human telomerase reverse transcriptase) gene is most likely to be responsible given its role in maintenance of
telomere length. Wang et al also found that polymorphism at this allele was associated with increased risk of lung cancer in never smokers [38].

The International Lung Cancer Consortium performed a large case-control study in an attempt to validate the regions previously identified in GWA studies of lung cancer risk [43]. The study included nine cohorts from North America, eight from Europe and four from Asia. The study was able to confirm the association of polymorphisms at 15q25 and lung cancer risk for white patients, while no such association was found in patients from Asian cohorts. Further, for white patients the association between polymorphisms and cancer risk was found for smokers only. Polymorphisms at 5p15 were significant across all studies with no significant heterogeneity found. The study was unable to replicate the previous findings with regards to 6p21, with no significant difference found between polymorphisms and the rate of lung cancer. Finally, the study examined the effect of an increasing number of “risk alleles” with regards to lung cancer risk. As the number of risk alleles increased (from zero to six) the odds ratio for lung cancer also increased.

GWA studies have found a number of putative sites of genetic polymorphisms that may account for increased risk of lung cancer in the general population. These differences are important to recognise as they may unlock strategies for prevention or treatment of lung cancer. While the data goes some way to explaining why “some people get cancer” while others don’t it is not a practical test to apply in routine practice at present – prevention of tobacco consumption remains the most effective protection against lung cancer.

1.2 Histopathologic Classification and Staging

For a patient with a suspected or proven malignancy a number of factors influence the planned management. Two key pieces of information are required in order to determine what treatment course(s) are available for a patient having due regard to their personal
circumstances, comorbidities and fitness. Firstly, a tissue diagnosis is required to
determine whether or not a process is malignant, and if so what type of malignancy it is.
Secondly, determining the distance of spread of the tumour ("staging") is required for
information on prognosis and decision on the most appropriate treatment modalities.

1.2.1 Histopathology

The appropriate diagnosis of almost all malignant processes requires the involvement of
a trained pathologist to assess tissue specimens via combinations of cytologic,
histopathologic and molecular tests. The spectrum of tumours of the lung is wide and
includes epithelial tumours, including adenocarcinoma, squamous cell carcinoma and
neuroendocrine tumours, mesenchymal tumours, lymphohistiocytic tumours and
tumours of ectopic origin. In addition to this is the recognition of non-malignant
conditions that may mimic invasive malignancy, and malignant tumours that have
metastasised to the lung. The criteria for diagnosing malignancy in the lung have
recently been updated with the release of the fourth edition of the World Health
Organisation (WHO) Classification of Tumours of the Lung, Pleura, Thymus and Heart
[44]. The importance of histology in treatment decisions is discussed in detail in
Chapter Two (page 64).

1.2.2 Staging and Prognosis

The most influential predictor of prognosis in non-small cell lung cancer (NSCLC) is
the stage of the tumour. Accurate staging is required to guide patient treatment.
Clinical stage is determined on the basis of diagnostic investigations including physical
examination and imaging studies (including plain x-ray, ultrasound, computed
tomography [CT] scan, magnetic resonance imaging [MRI], bone scintigraphy, and
positron emission tomography [PET] scan). Pathologic staging is assigned on the basis
of resection specimens, or invasive procedures such as biopsy or surgery where the specimen confirms the highest stage suspected on the basis of previous investigation.

The AJCC Cancer Staging Manual prepared by the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC) provides direction for lung cancer staging. The seventh Edition of the Cancer Staging Manual, published in 2010, is the most recent iteration [45]. The tumour stage is assigned on the basis of the features of the primary tumour (T), presence regional lymph node metastases (N) and presence of distant metastases (M). The research to support the eighth edition of the TNM classification for lung cancer has been published and will supersede the previous seventh edition [46]. The database for the newest edition incorporates data from 94708 patients treated across 46 institutions in 20 countries.

The proposed T descriptors for the eighth edition of the TNM are listed in table 1. There were 13012 patients assigned on clinical grounds, and 30018 patients assigned on pathologic grounds [47]. The majority of patients had node negative tumours (74%) and most patients included came from Asian institutions (79%). Major changes from the seventh edition include the following:

- Recognition of the importance of increasing tumour size such that T descriptors are now measured in 1cm increments up to 5cm
- Reclassifying tumours greater than 5cm in size from T2b to T3
- Reclassifying tumours greater than 7cm in size from T3 to T4
- Reclassifying tumours previously described purely on the basis of proximity to the carina being less than 2cm from T3 to T2 (as long as the carina is not involved)
- Post obstructive atelectasis or pneumonitis is now a T2 tumour
- Reclassifying diaphragmatic invasion to T4 (from T3)
- Deleting mediastinal pleural invasion as a T3 descriptor given its rarity
### Table 1: Proposed T Descriptors for the 8th Edition of the TNM staging system [47]

<table>
<thead>
<tr>
<th>TX</th>
<th>Primary tumour unable to be assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>No evidence of primary tumour</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>T1</td>
<td>Tumour ( \leq 3 \text{ cm} ) in greatest dimension surrounded by lung or visceral pleural without bronchoscopic evidence of invasion more proximal than the lobar bronchus</td>
</tr>
<tr>
<td>T1a</td>
<td>Tumour ( \leq 1 \text{ cm} ) in greatest dimension</td>
</tr>
<tr>
<td>T1b</td>
<td>Tumour ( &gt;1 \text{ cm but} \leq 2 \text{ cm} ) in greatest dimension</td>
</tr>
<tr>
<td>T1c</td>
<td>Tumour ( &gt;2 \text{ cm but} \leq 3 \text{ cm} ) in greatest dimension</td>
</tr>
<tr>
<td>T2</td>
<td>Tumour ( &gt;3 \text{ cm but} \leq 5 \text{ cm} ) or with any of the following features</td>
</tr>
<tr>
<td></td>
<td>Invades visceral pleura</td>
</tr>
<tr>
<td></td>
<td>Associated with atelectasis or obstructive pneumonitis</td>
</tr>
<tr>
<td>T2a</td>
<td>Tumour ( &gt;3 \text{ cm but} \leq 4 \text{ cm} ) in greatest dimension</td>
</tr>
<tr>
<td>T2b</td>
<td>Tumour ( &gt;4 \text{ cm but} \leq 5 \text{ cm} ) in greatest dimension</td>
</tr>
<tr>
<td>T3</td>
<td>Tumour ( &gt;5 \text{ cm but} \leq 7 \text{ cm} ) or invasion into any of the following</td>
</tr>
<tr>
<td></td>
<td>Parietal Pleura</td>
</tr>
<tr>
<td></td>
<td>Chest wall (including superior sulcus tumours)</td>
</tr>
<tr>
<td></td>
<td>Phrenic nerve, parietal pericardium</td>
</tr>
<tr>
<td></td>
<td>Separate tumour nodules in the same lobe</td>
</tr>
<tr>
<td>T4</td>
<td>Tumour ( &gt;7 \text{ cm in size} ) or with invasion of any the following</td>
</tr>
<tr>
<td></td>
<td>Diaphragm</td>
</tr>
<tr>
<td></td>
<td>Heart</td>
</tr>
<tr>
<td></td>
<td>Great vessels</td>
</tr>
<tr>
<td></td>
<td>Trachea</td>
</tr>
<tr>
<td></td>
<td>Recurrent Laryngeal Nerve</td>
</tr>
<tr>
<td></td>
<td>Esophagus</td>
</tr>
<tr>
<td></td>
<td>Vertebral body</td>
</tr>
<tr>
<td></td>
<td>Carina</td>
</tr>
<tr>
<td></td>
<td>Separate tumour nodule(s) in a different ipsilateral lobe</td>
</tr>
</tbody>
</table>
Data for the N descriptors is based on information from 38910 patients for the cN status and 31426 patients for the pN status [48]. The majority of patients were submitted from Japanese centres (59% and 75% respectively). The importance of accurate identification of the location of involved lymph nodes is assisted by standardised terminology as provided by the International Association for the Study of Lung Cancer (IASLC) lymph node map [49]. The location of the involved lymph nodes remains unchanged from that described in the seventh edition [45]. However within the descriptors for N1 and N2 the number of stations involved has been found to be important, such that the new descriptors are as listed in table 2.

Table 2: Proposed N Descriptors for the 8th Edition of the TNM staging system [48]

<table>
<thead>
<tr>
<th>NX</th>
<th>Regional lymph nodes cannot be assessed</th>
</tr>
</thead>
<tbody>
<tr>
<td>N0</td>
<td>No regional lymph node metastases</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>N1a</td>
<td>Involvement of a single pN1 nodal station</td>
</tr>
<tr>
<td>N1b</td>
<td>Involvement of multiple pN1 nodal stations</td>
</tr>
<tr>
<td>N2</td>
<td>Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)</td>
</tr>
<tr>
<td>N2a</td>
<td>Involvement of a single pN2 nodal station without pN1 (skip pN2)</td>
</tr>
<tr>
<td>N2b</td>
<td>Involvement of a single pN2 nodal station with pN1</td>
</tr>
<tr>
<td>N2c</td>
<td>Involvement of multiple pN2 nodal stations</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>

The changes to the M descriptors proposed for the eighth edition of the TNM staging system have been minimal (table 3) [50]. The new descriptors recognise the importance of the burden of extrathoracic metastatic disease and the concept of “oligometastatic” disease where a metastasis is confined to a single distant site. Recommendations for further study are made to investigate:
- For patients with M1b disease whether the site of metastasis affects prognosis
- For patients with M1c disease whether the number of metastases and the number of involved organs affects prognosis

**Table 3: Proposed M Descriptors for the 8th Edition of the TNM staging system [50]**

<table>
<thead>
<tr>
<th>M0</th>
<th>No distant metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
<tr>
<td>M1a</td>
<td>Separate tumour nodule(s) in a contralateral lobe with pleural nodules or malignant pleural (or pericardial) effusion</td>
</tr>
<tr>
<td>M1b</td>
<td>A single distant metastasis in a single organ</td>
</tr>
<tr>
<td>M1c</td>
<td>Multiple distant metastases either in a single organ or in multiple organs</td>
</tr>
</tbody>
</table>

As a result of the data analysed for the new T, N and M descriptors the IASLC Lung Cancer Staging Project has developed a revision of the stage groupings [51]. Data was obtained for both clinically and pathologically staged cases. The proposed stage groupings are listed below, with increasing stage indicating poorer survival outcomes (table 4).
Table 4: Proposed Stage Groupings for the 8th edition of the TNM Classification for Lung Cancer [51]

<table>
<thead>
<tr>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occult Carcinoma</td>
<td>X</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>is</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IA1</td>
<td>1a(mi)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IA2</td>
<td>1b</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IA3</td>
<td>1c</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IB</td>
<td>2a</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IIA</td>
<td>2b</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IIIB</td>
<td>1a-c</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2a-b</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IIIA</td>
<td>1a-c</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2a-b</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0-1</td>
<td>0</td>
</tr>
<tr>
<td>IIIB</td>
<td>1a-c</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2a-b</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>IIIC</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>IVA</td>
<td>Any</td>
<td>Any</td>
<td>1a</td>
</tr>
<tr>
<td></td>
<td>Any</td>
<td>Any</td>
<td>1b</td>
</tr>
<tr>
<td>IVB</td>
<td>Any</td>
<td>Any</td>
<td>1c</td>
</tr>
</tbody>
</table>
1.3 Treatment of Non-Small Cell Lung Carcinoma

1.3.1 Early stage NSCLC

The treatment of early stage NSCLC is aimed at removal or eradication of the primary tumour (local treatment). In the setting of patients treated surgically this may be followed by adjuvant chemotherapy and on occasion adjuvant radiotherapy which is aimed at reducing the likelihood of subsequent recurrence either locally or distantly. Treatment is delivered with curative intent – that is to render the patient permanently free of their lung cancer.

1.3.1.1 Local Treatment

Surgery remains the gold standard in curative strategies for early stage NSCLC for appropriately selected patients [52]. As noted in the guidelines of the American College of Chest Physicians this is best carried out in the setting of surgeons with specialist expertise in thoracic surgery in a high volume centre [52]. At present the use of sublobar resection is limited to patients who would not tolerate an anatomical lobectomy. There is significant interest in identifying a cohort of patients in whom sub-lobar resection could be safely used as standard of care, however high level evidence to identify such a group has not yet been achieved [53].

An increasing number of options are available for patients with early stage disease who are not fit for surgery or choose not to have surgery. These include stereotactic body radiotherapy (also known as stereotactic radiosurgery) [54], locally ablative therapies such as radiofrequency ablation [55] or long course radiotherapy at standard fractions [56]. The choice of treatment for those not fit for anatomical resection depends on clinician assessment, patient preferences and the local experience available.
The first major meta-analysis of chemotherapy in NSCLC was completed in 1995 by the NSCLC Collaborative Group [57]. Prior to this, the authors noted that the size of trials being conducted in NSCLC were too small to detect moderate differences, with approximately half of the conducted trials recruiting fewer than 100 patients. The meta-analysis included individual data on patients who had undergone curative resection and who were enrolled in randomised trials involving adjuvant cytotoxic therapy. Trials were divided into 4 groups based on the therapy administered (cisplatin based; prolonged alkylating agent; trials with vinka alkaloid or etoposide but not cisplatin; other regimens). Trials of older regimens demonstrated an increased risk for the addition of chemotherapy compared to surgery alone (hazard ratio [HR] 1.15, p=0.005). Trials using cisplatin-based therapy showed no significant difference comparing chemotherapy plus surgery to surgery alone, but suggested a beneficial effect (HR 0.87, p=0.08).

The landmark Lung Adjuvant Cisplatin Evaluation (LACE) meta-analysis cemented the role of adjuvant cisplatin-based chemotherapy [58]. This meta-analysis included randomised studies of cisplatin-based adjuvant chemotherapy compared with no adjuvant systemic therapy (the use of adjuvant radiotherapy was optional). Five studies were included [59-63]. The LACE meta-analysis included 4584 patients. Significantly overall survival was improved with an 11% reduction in risk of death (HR 0.89; 95% CI 0.82 – 0.96; p=0.005), translating to an absolute benefit of 5.4% at 5 years. A similar effect on disease free survival (DFS) was also seen (HR 0.84, 95% CI 0.78 – 0.91, p<0.001). No significant heterogeneity was seen between trials. Of note, while the rate of lung cancer deaths was lower in the chemotherapy arms (HR 0.83, 95% CI 0.76 – 0.90, p<0.001), non-lung cancer deaths from cardiac and respiratory causes as well as chemotherapy toxicity was higher (HR 1.36, 95% CI 1.10 – 1.69, p=0.004). Furthermore, chemotherapy was detrimental to patients with an Eastern Co-operative Oncology Group (ECOG) performance status (PS) of 2, and in patients with stage IA disease. The strongest evidence was seen for the combination of cisplatin with
vinorelbine, as further demonstrated in a pre-planned sub-study of the data from the LACE meta-analysis [64]. Adjuvant chemotherapy with cisplatin and vinorelbine is the standard of care for patients of ECOG PS 0 or 1 following resection of stages II or III NSCLC.

Some differences exist in inclusion criteria for the studies used in the LACE meta-analysis. Of the five studies, only JBR-10 required a minimum surgical resection of a lobectomy, and lymph node dissection was mandated [62]. JBR-10 also required CT staging, with other studies noting only “pathologic staging” in the text of their manuscripts. Three studies (IALT, ANITA and ALPI) allowed less than pneumonectomy and did not comment on nodal sampling requirements or allowed patients who had not had nodal sampling [59, 60, 63]. The Big Lung Trial makes no mention of the type of surgery received, other than that it was performed “with curative intent” [61]. Patients on the Big Lung Trial who received their systemic therapy in a neoadjuvant fashion were excluded from the LACE meta-analysis.

Some studies into the adjuvant use of Epidermal Growth Factor Receptor (EGFR) tyrosine kinase inhibitors (TKIs) following resection of NSCLC have been reported, with several more ongoing. The BR19 (gefitinib) and RADIANT (erlotinib) trials tested EGFR TKIs in an unselected NSCLC population post-surgical resection, with adjuvant chemotherapy not being mandated [65, 66]. Neither trial showed a disease free survival benefit in the overall population. In BR19, the number of patients with EGFR mutant disease was too small to make any comment on gefitinib as an adjuvant strategy in this group [65]. As the RADIANT study failed to meet its’ primary efficacy endpoint, analysis of the EGFR mutant subgroup was descriptive only [66]. There was a trend to improved DFS in the erlotinib group (median 46.4 months vs placebo 28.5 months) for patients with EGFR mutant disease, with statistical significance unable to be claimed due to hierarchical statistical testing and a non-significant primary endpoint.
The first planned trial of \textit{EGFR} TKIs in a surgically resected population with \textit{EGFR} mutant lung cancer was recently reported [67]. In the ADJUVANT study four cycles of cisplatin and vinorelbine were compared with up to 24 months of gefitinib in a Chinese cohort. The authors showed a significant improvement in DFS with a median of 28.7 months for patients receiving gefitinib compared to 18.0 months with those receiving chemotherapy (HR 0.60, 95% CI 0.42 – 0.87, p=0.005). However, in this study the three year DFS was poor in both arms (gefitinib 34% vs chemotherapy 27%) and almost all patients had relapsed at 48 months. Hopefully the manuscript of the study may shed some light on why these poor outcomes were seen. Overall survival data remain immature.

A number of studies of adjuvant targeted therapies in a biomarker selected populations are ongoing, including use of gefitinib [68], erlotinib [69], icotinib [70], osimertinib [71] and crizotinib [69].

1.3.1.3 Adjuvant Treatment - Radiotherapy

The role of adjuvant radiotherapy in resected NSCLC is not well defined. Frequently it is used in situations where incomplete resection of the primary tumour has occurred, and microscopic or macroscopic disease remains [72]. More controversial is its role in resected stage III NSCLC. The Post-Operative RadioTherapy (PORT) trialists’ group addressed the role of radiotherapy in the first meta-analysis on the subject in 1998 [73]. Nine randomised trials including 2128 patients were included. The addition of PORT to surgery resulted in an increase in the risk of death (HR 1.21, 95% CI 1.08 – 1.34, p=0.001). On analysis by stage, the greatest detriment was to patients with stage I disease, with no clear evidence of harm or benefit for patients with stage III disease.

In 2006 an analysis of the use of PORT and disease outcomes was performed using data collected in the Surveillance, Epidemiology and End Results (SEER) program database
This analysis included patients over the age of 21 with stage II or III disease who had undergone lobectomy or pneumonectomy, had N0 – N2 disease, and who had survived four or more months after surgery. A total of 7465 patients were analysed. The major finding of note from this paper was the significant harm in terms of overall survival caused to patients receiving PORT for N0 (HR 1.176, 95% CI 1.005 – 1.376, p=0.0435) or N1 disease (HR 1.097, 95% CI 1.015 – 1.186) compared to the survival benefit achieved for patients with N2 disease undergoing PORT (HR 0.855, 95% CI 0.762 – 0.959, p=0.0077). On the basis of this data, patients with higher risk N2 resected disease (for example – multiple nodal stations involved) are considered for PORT on a case-by-case basis. A prospective randomised study being conducted in France (Lung ART) is attempting to provide level 1 evidence on the role of PORT for resected N2 disease, however it is many years before the expected completion of data collection [75, 76].

1.3.2 Locally Advanced NSCLC

A proportion of patients present with disease that is not amenable to surgery at presentation, but in whom there is no evidence of distant metastatic disease. After appropriate consideration in multidisciplinary setting such patients may be offered definitive chemoradiotherapy, or less commonly and in a highly selected manner neoadjuvant treatment may be offered prior to surgery. In such patients the aim is still to treat with curative intent, however the likelihood of local or distant failure is significantly higher.

1.3.2.1 Definitive ChemoRadiotherapy (CRT)

Definitive radiotherapy has been offered for patients with unresectable locally advanced disease and the absence of distant metastatic spread. Historically survival outcomes for these patients have been poor despite treatment being delivered with curative intent.
As a result multiple clinical trials have been undertaken looking at the role and timing of systemic therapy in addition to radiotherapy for such patients. A Cochrane Systematic Review published in 2010 examined the evidence available for the addition of systemic therapy [78]. The review demonstrated that the addition of systemic therapy to radiotherapy reduced the risk of death (HR 0.71, 95% CI 0.64 – 0.80, I² 0%) and improved progression free survival (PFS) (HR 0.69, 95% CI 0.58 – 0.81, I² 45%). A meta-analysis from Auperin et al examined studies of concurrent as compared with sequential chemoradiotherapy [79]. Six trials met eligibility criteria including 1205 patients. Three of these trials were small in size contributing 279 patients in total, and further these trials only used single agent platinum as the systemic therapy. Despite this, concurrent therapy was associated with better overall survival (HR 0.84, 95% CI 0.74 – 0.95).

The addition of further systemic therapy has been trialled in order to improve treatment outcomes. Vokes et al reported that the use of induction chemotherapy prior to CRT added toxicity with no survival benefit found [80]. Similarly, two phase III trials [81, 82] and a pooled analysis [83] failed to demonstrate any benefit for the addition of consolidation chemotherapy following CRT. A further meta-analysis of individual patient data from five phase II trials was undertaken by van Houtte et al [84]. These trials included either induction or consolidation chemotherapy with CRT as compared to CRT alone, and again no survival advantage was seen with additional systemic therapy.

Where patients are not fit for concurrent CRT then radical radiotherapy alone, sequential CRT, palliative intent XRT or systemic therapy and symptom management alone are all potential treatment options.
1.3.2.2 Neoadjuvant Treatment Prior to Surgery

For highly selected patients neoadjuvant therapy prior to surgery may be considered and is best undertaken in the setting of a multidisciplinary team. At present this approach is limited to patients where there is known but potentially resectable stage III disease. This treatment approach is designed to decrease the loco-regional failure rates following chemoradiotherapy with the subsequent aim of improving survival [85]. Alternatively neoadjuvant chemotherapy followed by surgery may be used, with omission of radiotherapy. There are limited studies and analyses examining such an approach. As noted in the European Society of Medical Oncology consensus guidelines trials for patients with this condition are highly selective and contain small patient numbers. Application of results from such trials require careful patient selection [86].

Song et al performed a meta-analysis of trials examining neoadjuvant chemotherapy followed by surgery compared to surgery alone in NSCLC [87]. For trials including patients with all stages of disease there was significant heterogeneity of outcomes. A further analysis was performed and limited to the seven trials enrolling patients with stage III disease only. In this setting there was a significant improvement in overall survival outcomes for the use of chemotherapy with a pooled HR of 0.77 (95% CI 0.68 – 0.87, p<0.01). The NSCLC Meta-Analysis Collaborative Group subsequently published a meta-analysis of 15 trials based on individual patient data [88]. Of these trials ten gave preoperative chemotherapy only while in five trials postoperative chemotherapy could also be given. Based on outcomes for 2385 patients the use of preoperative chemotherapy resulted in a 13% reduction in the risk of death (HR 0.87, 95% CI 0.78 – 0.96, p=0.007) translating to a 5% absolute benefit. There was no statistically significant heterogeneity across trials despite wide differences in methods. Interestingly the improvement in survival outcomes were similar to that reported in the LACE meta-analysis [58].
Multiple groups have examined the role of various approaches of chemotherapy, radiotherapy and surgery in the setting of (potentially) resectable stage III disease. Unfortunately many of these trials have struggled either due to lack of funding or slow accrual leading to early termination of the study. The studies discussed here had the largest number of patients randomised. Van Meerbeeck et al published their results in 2007 [89]. In this study patients were given three cycles of induction platinum and if response was observed they were then randomised to surgery or definitive radiotherapy. No difference was seen in progression free or overall survival outcomes by treatment arm.

Albain et al treated all patients in their study with radiotherapy and concurrent cisplatin and etoposide [90]. Patients were randomised to complete radiotherapy or proceed to surgery. All patients received two cycles of consolidation chemotherapy. Patients in the surgical arm had a longer PFS (12.8 vs 10.5 months, HR 0.77, 95% CI 0.62 – 0.96, p=0.017) but no difference in overall survival. More treatment related deaths were observed in the surgical arm, predominantly occurring in patients who required a pneumonectomy. The ESPATUE trial was similar in design [91]. Patients received induction chemotherapy then chemoradiotherapy. Patients were randomised to completing full course of chemoradiotherapy or a truncated course followed by surgical resection. No difference was seen between treatment arms although the authors noted that overall the outcomes were “excellent”. This again suggests that a highly selected population were enrolled.

Two studies have examined whether radiotherapy is necessary in addition to chemotherapy prior to surgery. Pless et al gave patients induction chemotherapy with cisplatin and docetaxel [92]. Following this patients either had radiotherapy and then surgery or proceeded straight to surgery. Again, no differences were observed in event free or overall survival. Thomas et al followed a similar study design [93]. In their study patients who received radiotherapy preoperatively (in addition to their chemotherapy) had a small increase in the rate of complete resection and downstaging.
of involved mediastinal lymph nodes. Further, those who were downstaged had better survival outcomes. Despite this in the overall analysis no survival benefits were seen for trimodality therapy over chemotherapy and surgery alone.

These studies show that multi-modality therapy in stage III disease is appropriate with a view to improving survival outcomes. However aggressive trimodality approaches are only appropriate for highly selected patients treated in experienced centres [86].

1.3.3 Systemic Therapy in Advanced Disease

1.3.3.1 Chemotherapy

Cytotoxic chemotherapy has remained the mainstay of treatment for advanced NSCLC for many years. Older trials of chemotherapy used agents that were either poorly tolerated or are now known to lack significant efficacy in this condition. A meta-analysis published in 1995 noted that “there was considerable pessimism about the role of chemotherapy in NSCLC” at the start of their analysis [57]. The meta-analysis suggested that long term alkylating agents were detrimental. Conversely, cisplatin based chemotherapy resulted in a significant improvement in overall survival, increasing the number of patients alive at one year from 5% to 15%, and increasing the median survival by approximately six weeks (HR 0.73, p<0.0001).

The second generation regimen cisplatin and etoposide became one of several regimens used on the basis of available trial data [94-96]. It was subsequently chosen as the control arm in a trial assessing whether better outcomes could be achieved with the combination of cisplatin and paclitaxel (with paclitaxel being administered at two dose levels) [97]. Bonomi et al showed that the pooled data for both dose levels of cisplatin/paclitaxel as compared to cisplatin/etoposide produced a small but statistically significant survival advantage (p=0.048). The trial struggled to reach significance in
part due to patients on the control arm surviving longer than was anticipated during the planning of the trial.

Schiller et al conducted a well-known phase III trial through ECOG to examine a number of new platinum doublet combinations [98]. Cisplatin/paclitaxel was used as the control arm, with three experimental arms – cisplatin/docetaxel; cisplatin/gemcitabine; and carboplatin/paclitaxel. All arms had very similar response rates and survival times. There are a large number of third generation regimens available that combine a platinum agent (cisplatin or carboplatin) with a second drug including albumin-bound paclitaxel, paclitaxel, docetaxel, gemcitabine or vinorelbine [99]. As such selection of treatment is based on patient preferences and side effect profile. Further, many of these drugs can used as single agent therapies in the second line and beyond.

Subsequent gains with cytotoxic therapies alone or in combination have been modest. Scagliotti et al demonstrated a modest improvement for overall survival for patients with non-squamous NSCLC treated with cisplatin/pemetrexed compared to the control arm of cisplatin/gemcitabine (HR 0.81, 95% CI 0.70 – 0.94, p=0.005) [100]. Conversely, patients with SqCC had inferior survival outcomes with cisplatin/pemetrexed (HR 1.23, 95% CI 1.00 – 1.51, p=0.05). This was the first study to show differences in overall survival outcomes when the chemotherapy choice was based on the histologic type of NSCLC.

Two studies have also shown that a maintenance strategy using pemetrexed immediately following first line platinum doublet chemotherapy (a non-pemetrexed based regimen in the JMEN trial; cisplatin/pemetrexed in the PARAMOUNT trial) led to improved survival rather than observation and commencement of treatment at progression [101, 102].
Evidence exists for two monoclonal antibodies in combination with chemotherapy however uptake of these treatments have been limited outside of North America. Low uptake in Australia has been driven by the inability of the sponsoring pharmaceutical companies to gain approval for reimbursement for their medications through the Pharmaceutical Benefits Scheme, rendering such medication unaffordable for the vast majority of patients. Sandler et al examined the addition of the vascular endothelial growth factor (VEGF) inhibitor bevacizumab to the combination of carboplatin and paclitaxel in comparison to carboplatin and paclitaxel for patients with non-squamous NSCLC [103]. This randomised trial demonstrated a two month improvement in median overall survival with the addition of bevacizumab (12.3 months vs 10.3 months, HR 0.79, 95% CI 0.67 – 0.92, p=0.003). The subsequent POINTBREAK trial compared the experimental arm of carboplatin, pemetrexed and bevacizumab followed by pemetrexed and bevacizumab maintenance in comparison to the reference arm carboplatin, paclitaxel and bevacizumab followed by maintenance bevacizumab [104]. No difference in overall survival, the primary endpoint, was observed between the two study arms (experimental 12.6 months vs reference 13.4 months; HR 1.00 (95% CI 0.86 – 1.16, p=0.949).

For patients with squamous cell carcinoma the epidermal growth factor receptor (EGFR) monoclonal antibody necitumumab has been found to improve survival outcomes in combination with cisplatin and gemcitabine. In the phase III SQUIRE trial addition of necitumumab to the chemotherapy backbone resulted in a modest improvement in median overall survival (11.5 months vs 9.9 months, HR 0.84, 95% CI 0.74 – 0.96, p=0.01) [105]. This was similar to the survival improvement seen with another EGFR monoclonal antibody, cetuximab, in addition to chemotherapy for patients with NSCLC in the FLEX trial [106].

It is important to note that a number of patients diagnosed with advanced NSCLC are either of advanced age or poor performance status. Despite this appropriately selected patients still benefit from third generation platinum doublet chemotherapy as compared
to single agent chemotherapy. A trial from the French Thoracic Oncology Group (Intergroupe Francophone de Cancérologie Thoracique) showed a significant benefit for the combination of carboplatin and weekly paclitaxel over the control arm of single agent gemcitabine or vinorelbine (median OS 10.3 months vs 6.2 months, HR 0.64, 95% CI 0.52 – 0.78, p<0.001) [107]. Single agent gemcitabine or vinorelbine offer good choices for active therapy where patients are not fit for platinum doublet chemotherapy regimens [108, 109].

1.3.3.2 Oncogene Targeted Therapy

The discovery of activating mutations in EGFR heralded a major shift in the diagnostic and therapeutic approach to NSCLC [110, 111]. This is discussed in detail in chapter three (page 141). Exploration of the lung cancer genome has revealed an increasing number of targetable oncogene mutations in pulmonary adenocarcinoma [112]. Randomised clinical trials have been conducted and completed for patients with EGFR mutations and ALK translocations. The EGFR TKIs gefitinib, erlotinib and afatinib have all been compared to first line platinum doublet chemotherapy in a number of clinical trials [113-119]. Across these studies the median PFS for EGFR TKI treatment ranged from 9.2 to 13.1 months, whereas that for standard chemotherapy ranged from 4.6 to 6.9 months. Outcomes for all studies were highly statistically significant and clinically meaningful (detailed further in Table 15, page 147). Recently the results from the AURA3 trial have been released [120]. This study used the third generation EGFR TKI osimertinib in patients who developed resistance to their first line TKI through the secondary EGFR point mutation T790M. The study showed that osimertinib increased median PFS from 4.4 months for platinum (cisplatin or carboplatin) with pemetrexed chemotherapy to 10.1 months for osimertinib (HR 0.30 [95% CI 0.23 – 0.41], p<0.001), marking a new standard of care for this patient population. The response rate improved from 31% for chemotherapy to 71% with osimertinib.
Therapy for patients with ALK translocations have also vastly improved through the advent of TKIs. Building on the activity seen in phase I and II trials, crizotinib was compared to standard second line chemotherapy (pemetrexed or docetaxel) for patients whose tumours had ALK translocations, and had previously had platinum doublet chemotherapy [121]. When compared to chemotherapy in the Profile 1007 study, crizotinib showed improved PFS (7.7 months vs 3.0 months; HR 0.49 [95% CI 0.37 – 0.64]; p<0.001), response rate (65% vs 20%, p<0.001), and improved patient reported outcomes. The Profile 1014 study compared crizotinib to cisplatin and pemetrexed for first line treatment of patients with ALK translocated tumours. A limitation of this study was the decision to not provide maintenance pemetrexed chemotherapy beyond six cycles of first line chemotherapy in the control arm [122]. Crizotinib was associated with improved PFS (median 10.9 months vs 7.0 months; HR 0.45 [95% CI 0.35 – 0.60], p<0.001), response rate (74% vs 45%, p<0.001) and patient reported outcomes compared to chemotherapy.

The number of TKIs in testing for ALK translocated lung cancer is impressive. Recently, ceritinib demonstrated increased efficacy over chemotherapy for patients with prior treatment with crizotinib and platinum-doublet chemotherapy (ASCEND-5 trial [123]). The median PFS was improved (5.4 vs 1.6 months; HR 0.49, p<0.001). Quality of life was also improved as compared to chemotherapy, with the exception of measures relating to gastrointestinal symptoms, reflecting increased gastrointestinal toxicities of ceritinib. Ceritinib was also compared to platinum and pemetrexed chemotherapy as first line therapy for treatment naive ALK translocated lung cancer (ASCEND-4 trial [124]). Ceritinib improved the median PFS to 16.6 months as compared with chemotherapy at 8.1 months, HR 0.55 (95% CI 0.42 – 0.73, p<0.00001). Importantly, ceritinib also protected against progression in the CNS.

The first trial of a new generation ALK TKI, alectinib, as compared to crizotinib has also been published in 2016. The J-ALEX trial enrolled Japanese ALK inhibitor treatment naïve-patients with ALK translocations in their tumours [125]. The median
PFS for alectinib was not reached (lower limit of 95% CI 20.3 months) as compared to crizotinib at 10.2 months (95% CI 8.2 – 12.0) with a hazard ratio of 0.34 (95% CI 0.17 – 0.71, p<0.0001). Outcomes from the global ALEX study have also recently been reported [126]. The study design was the same as J-ALEX, and patients were enrolled globally. Similar results were reported, with the median PFS for alectinib not being reached at the time of data analysis, compared with crizotinib at 11.1 months (HR 0.47, 95% CI 0.34 – 0.65, p<0.001). In both J-ALEX and ALEX, alectinib provided superior CNS disease control compared with crizotinib. Further promising agents for ALK translocated lung cancer are in development and early phase trials, including brigatinib [127] and lorlatinib [128].

As further investigation of the lung cancer genome is performed the number of actionable or potentially actionable somatic mutations and alterations found is increasing [112]. Table 5 outlines some the changes described as well as active agents against these changes.
### Table 5: Actionable or potentially actionable mutations found in the lung adenocarcinoma genome. MDR – median duration of response

<table>
<thead>
<tr>
<th>Gene</th>
<th>Frequency</th>
<th>(Potential) Targeted Therapy</th>
<th>Sample Size</th>
<th>Response Rate</th>
<th>Median PFS / Note</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ROS1 proto-oncogene receptor tyrosine kinase fusions</strong></td>
<td>1-2% [129]</td>
<td>Crizotinib [130]</td>
<td>50</td>
<td>72%</td>
<td>19.2 months</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Crizotinib [131]</td>
<td>30</td>
<td>80%</td>
<td>9.1 months</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lorlatinib [132]</td>
<td>11</td>
<td>54%</td>
<td>Phase 1 study</td>
</tr>
<tr>
<td><strong>Rearranged during transfection (RET) rearrangement</strong></td>
<td>1-2% [129]</td>
<td>Alectinib [133]</td>
<td>20</td>
<td>28%</td>
<td>Case Reports only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cabozantinib [134]</td>
<td>20</td>
<td>28%</td>
<td>7 months</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vandetanib [135]</td>
<td>17</td>
<td>53%</td>
<td>4.7 months</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lorlatinib [136]</td>
<td>25</td>
<td>54%</td>
<td>7.3 months</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sunitinib [137]</td>
<td></td>
<td></td>
<td>Case Report</td>
</tr>
<tr>
<td><strong>HER2 mutant lung cancer</strong></td>
<td>2% [138]</td>
<td>Dacomitinib [139]</td>
<td>30</td>
<td>10%</td>
<td>3 months</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Afatinib [140, 141]</td>
<td></td>
<td></td>
<td>Case series</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lapatinib [141]</td>
<td></td>
<td></td>
<td>Case series</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Neratinib [141]</td>
<td></td>
<td></td>
<td>Case series</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trastuzumab + chemotherapy [141]</td>
<td></td>
<td></td>
<td>Case series</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Trastuzumab emtansine [142]</td>
<td></td>
<td></td>
<td>Case Report</td>
</tr>
<tr>
<td><strong>BRAF Mutation</strong></td>
<td>2-3% [143, 144]</td>
<td>Dabrafenib [145]</td>
<td>78</td>
<td>33%</td>
<td>5.5 months</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dabrafenib / Trametinib [146]</td>
<td>57</td>
<td>63%</td>
<td>9.7 months</td>
</tr>
<tr>
<td><strong>MET Exon 14 Skipping Mutation</strong></td>
<td>3-4% [147, 148]</td>
<td>Crizotinib [149-151]</td>
<td></td>
<td></td>
<td>Case Reports</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cabozantinib [150]</td>
<td></td>
<td></td>
<td>Case Reports</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MGCD265 [152]</td>
<td></td>
<td></td>
<td>Case Reports</td>
</tr>
<tr>
<td><strong>MET Amplification</strong></td>
<td>2-4% [153]</td>
<td>Crizotinib [154]</td>
<td>12</td>
<td>33%</td>
<td>35 weeks (MDR)</td>
</tr>
</tbody>
</table>
The knowledge of whether a patient has a targetable oncogene mutation allows for the selection of appropriate therapy. In a cohort of 1007 patients Kris et al demonstrated that patients with lung adenocarcinoma and an oncogenic driver mutations have no survival difference compared with those lacking such mutations if non-targeted therapy is used [155]. However, when patients with a targetable oncogenic mutation received appropriately targeted therapy their survival outcomes are improved.

A number of oncogenes have been described in SqCC. Despite significant efforts no oncogene targeted therapies have been successfully developed for this patient cohort yet [156, 157].

1.3.3.3 Immunotherapy for NSCLC

Immune check point inhibitors represent the next major advance in the treatment of NSCLC. To date the greatest success in improving patient survival has been seen in agents targeting the interaction between programmed cell death protein 1 (PD-1) and its ligand programmed cell death ligand 1 (PD-L1). Okazaki and Honjo provided a detail review of the discovery of PD-1 and PD-L1 in translational research and the potential clinical relevance [158]. PD-1 was first discovered in 1992 in the search for molecules that were active in the programmed cell death pathway. However, the function of PD-1 was not able to be determined until PD-1 deficient mice could be engineered. These mice developed autoimmune conditions of a similar nature to lupus, thus demonstrating the presence of PD-1 was essential for normal immune regulation. The identification of PD-1 allowed further pre-clinical work that lead to the discovered of its ligands, subsequently named PD-L1 and PD-L2. Genetic variations in nuclei acid sequences (single nucleotide polymorphisms [SNPs]) were found to be more frequent in patients with autoimmune conditions than those without. Manipulation of the immune system to upregulate the PD-1 / PD-L1 or PD-L2 interaction was proposed a potential way to treat autoimmune conditions. Immunohistochemical studies also showed the presence of staining for PD-1, PD-L1 and PD-L2 in a broad range of solid tumours, and thus
blockade of the pathway to stop immune downregulation was proposed as a potential therapeutic strategy for solid tumours.

The first phase III studies to be published compared the PD-1 inhibitor nivolumab with docetaxel in the 2nd line treatment of platinum refractory squamous NSCLC [159] and non-squamous NSCLC [160]. Both studies showed clinically and statistically significant improvements in overall survival (squamous: HR 0.59, 95% CI 0.44 – 0.79, p<0.001; non-squamous: HR 0.73, 95% CI 0.59 – 0.89, p=0.002). Both of these studies accepted patients regardless of the level of staining for programmed cell death ligand 1 (PD-L1) in tumour by immunohistochemistry (IHC). In these studies PD-L1 expression was assessed by the 28-8 clone on the Dako automated platform.

The KEYNOTE-010 study compared another PD-1 inhibitor, pembrolizumab, to docetaxel chemotherapy in the second line setting [161]. In this trial two dose levels of pembrolizumab were investigated, but the trial limited enrolment to patients with PD-L1 expression via IHC in their tumours. Overall survival was significantly longer for pembrolizumab than docetaxel at the selected dose of 2mg/kg (HR 0.71, 95% CI 0.58 – 0.88, p=0.0008). For those patients with PD-L1 IHC staining of at least 50%, as measured using the 22C3 antibody on the Dako system, the survival advantage was noticeably higher (HR 0.54, 95% CI 0.38 – 0.77, p=0.0001).

As immunotherapy has been successful in the second line setting new trials have been developed of these agents in the first line setting. Two phase III trials have subsequently been reported (both excluding patients whose tumours had EGFR mutations or ALK translocations). In the Keynote-024 trial single agent pembrolizumab was compared to platinum doublet chemotherapy for patients whose tumours had PD-L1 staining of ≥50% by immunohistochemistry with the 22C3 assay on the Dako platform [162]. This trial showed that patients treated with pembrolizumab had improved PFS (median PFS 10.3 vs 6.0 months; HR 0.50 [95% CI 0.37 – 0.68].
p<0.001) and improved OS (HR 0.60 [95% CI 0.41 – 0.89], p=0.005). A similar trial, Checkmate-026, compared the efficacy of nivolumab with standard platinum doublet chemotherapy [163]. This trial included patients with PD-L1 expression ≥1% as measured by the Dako 28-8 IHC assay. The trial show no difference in either PFS or OS between arms, in contrast to the Keynote-024 study. Further, no difference was seen between arms for patients with the highest levels of PD-L1 expression by IHC (≥50% or ≥75%). The explanation for differences between the trials is not clear, and the outcomes of further studies are awaited.

The PD-L1 inhibitor atezolizumab has shown promising results in phase II trials in both the first line and in the second line compared to docetaxel [164, 165]. Evidence of improved efficacy of atezolizumab over chemotherapy was recently demonstrated in the phase III OAK trial. Patients were treated in the second or third line with standard chemotherapy with docetaxel or the experimental arm of atezolizumab [166]. Atezolizumab improved overall survival for patients with any expression of PD-L1 on either tumour cells or immune cells as measured by the SP142 immunohistochemical assay. The PD-L1 inhibitors darvalumab and avelumab are also being tested in this space with promising results in early phase trials [167, 168].

The immunohistochemical expression of PD-L1 has been suggested as a logical biomarker to guide patient selection. Unfortunately its presence or absence has not proven to be a completely reliable predictor of responsiveness to therapy [169, 170]. In comparison to oncogene mutations which are present in the DNA of the cancer cell and are usually stably expressed in the absence of selective pressure, PD-L1 expression is dynamic. PD-L1 expression is also heterogeneous which may lead to false-positive or false-negative results. An example of this issue is the difference in exploratory outcomes by PD-L1 status for patients treated with nivolumab on the Checkmate 017 (Squamous Cell NSCLC, [159]) and Checkmate 057 (Non-Squamous NSCLC, [160]) studies. In the 017 study there was no clear difference in OS or PFS outcomes for the presence of absence of PD-L1 staining at various levels examined (1%, 5% and 10%).
In the 057 study there were greater response rates and a trend to improved OS and PFS for patients whose tumours had PD-L1 expression when treated with nivolumab. Conversely, outcomes were similar for patients treated with nivolumab as compared with docetaxel when PD-L1 staining was classified as negative.

Analytic issues may also effect PD-L1 IHC expression. These may include the time from acquisition of the tissue and the transport medium used prior to arrival the laboratory, handling of the specimen in the lab, and the age of the tissue being assessed (fresh vs archival specimens). Treatment of a tumour (whether by systemic therapy or locally ablative techniques such as radiotherapy) may also affect the result. To date five separate antibodies to PD-L1 have been developed for IHC assessment, with each binding to different domains on the PD-L1 protein. PD-L1 assessment in each of the clinical trials has varied. The antibody used, the level of staining considered positive and the cells assessed (tumour cells and/or infiltrating immune cells) has made cross trial comparison of efficacy difficult.

The Blueprint Project marks a combined effort between two scientific bodies, four pharmaceutical companies and two diagnostics companies. This project looks to examine the similarities and differences between four of the available diagnostic antibodies on lung cancer specimens. Preliminary results from a phase I study have been released [171]. When the four antibodies were tested on the same tumour samples, concordance of positivity or negativity was achieved in only 19 of the 39 samples tested. Identification of a predictive biomarker remains important. Identifying those patients who will respond to single agent therapy allows them effective treatment with a modest side effect profile. For those patients who are unlikely to benefit from single agent therapy it may either spare the expense associated with PD-1 and PD-L1 inhibitors, or it may direct them towards clinical trials of combination therapies (e.g. chemotherapy and PD-1/PD-L1 inhibitor combined / combination immunotherapy / combination with small molecule TKIs) or other novel treatment approaches. Immunotherapeutic approaches are also being trialled as a neoadjuvant treatment prior
to surgery, as an adjuvant therapy following surgery, following chemoradiotherapy, and in the first line setting either as a single agent, in combination with chemotherapy or in combination with other immunotherapeutic agents.

1.3.3.4 Differences in options for systemic treatment – Chemotherapy, Oncogene Targeted Therapy and Immunotherapy

As noted in section 1.3.3.1 chemotherapy has remained the backbone of systemic treatment options in advanced NSCLC. In general likelihood of obtaining a radiographic response and the duration of such response have been modest [98]. Prolonged responses to treatment have been rare. Further, there was little to guide the selection of which systemic therapy regimen was best, with the noted exception of the differentiation in use of pemetrexed by squamous vs non-squamous histology [100]. Decisions were limited to patient and clinician preference, the relevant side effect profile of the agent(s) selected and the administration schedule and number of visits. Other than these factors there was little to differentiate between choices.

As a general rule clinicians have focused on the headline results from clinical trials in NSCLC – primarily looking at gains in survival outcomes (be that OS or DFS) and the associated toxicities that come with treatment. In a patient population who are frequently frail or have comorbidities the effects of a treatment on quality of life are especially important. Fernandez-Lopez et al performed a review of phase III randomised controlled trials in advanced NSCLC comparing two or more systemic therapies [172]. Seventy-six studies from the years 2002 to 2012 were included in the final analysis. Interestingly 46 trials (60.5%) included assessments of quality of life (QoL). Of these ten studies (21.7%) reported a statistically significant improvement in QoL between the control arm and the experimental arm.

Quality of life outcomes are of particular importance in advanced disease where the goal is improve survival while stabilising or reducing the symptom burden. In elderly
patients (≥70 years of age and of ECOG PS ≤2) the ELVIS trial and the MILES trial both showed that single agent vinorelbine or single agent gemcitabine were both well tolerated and improved quality of life as well as survival [108, 109]. The IFCT-0501 trial (discussed on page 47) showed similar quality of life outcomes for elderly patients with an ECOG PS of 0 to 2 treated with either single agent chemotherapy or with more intense chemotherapy with a platinum doublet [107].

The importance of two recent advances in the systemic treatment of advanced NSCLC cannot be understated. The first major advance is the testing of tumour samples for oncogene driver mutations. Many oncogenic mutations allow selection of oncogene targeted therapy that is generally administered orally with modest or minimal toxicity and with longer PFS times than are seen with chemotherapy. Further, patients treated with these agents have better QoL than those treated with chemotherapy in the randomised trials. To date the best examples are treatment for tumours with EGFR mutations and ALK translocations or fusions. As noted in chapter 3.1.3 (page 145) the PFS for patients treated with EGFR inhibitors is significantly longer for patients treated with EGFR TKIs as compared to standard platinum doublet chemotherapy. Health related QoL is also significantly improved for patients receiving EGFR TKIs as compared with chemotherapy in the five studies where robust QoL data was collected (NEJ002, IPASS, OPTIMAL, LUX-Lung 3 and LUX-Lung 6 – as reviewed by Sebastian et al [173]). For patients with ALK translocations PFS is improved with the first generation ALK TKI crizotinib as compared with chemotherapy with cisplatin and pemetrexed (10.9 months vs 7.0 months; HR 0.45 [95% CI 0.35 – 0.60], p<0.001). Health related QoL was better for patients treated with crizotinib. When examining patients treated with TKIs for these two mutations there are a subset who are able to remain on therapy for a prolonged period (in some cases years) and this is rarely observed with cytotoxic chemotherapy. In the case of both EGFR and ALK TKIs new agents that target known resistance mechanisms are also now available, and allowing patients to sequence from one inhibitor to another.
The introduction of immunotherapy marks the second major paradigm shift. As previously discussed there is a lack of a reliable biomarker to judge who will and won’t respond well to single agent immunotherapy. The area of caution to date has been in the treatment of patients with significant underlying autoimmune conditions, where the use of an immune stimulating agent such as a PD-1 or PD-L1 inhibitor may result in a flare of their underlying condition. Patients with EGFR mutations also represent a group where the optimal place of immunotherapy has yet to be found. A meta-analysis by Lee et al of the Checkmate 057, Keynote 010 and POPLAR studies found that there was significant OS benefit for patients without an EGFR mutation when comparing PD-1 or PD-L1 blockade with chemotherapy (HR 0.66 [95% CI 0.58 – 0.76, p<0.001]) [174]. Patients with EGFR mutations had no difference in OS outcomes when treated with immune checkpoint therapy as compared with docetaxel (HR 1.05 [95% CI 0.70 – 1.55, p=0.80]). The test for interaction between treatment arm and mutation status was statistically significant (p=0.03).

In addition to the survival advantage obtained in most studies by single agent PD-1 or PD-L1 inhibition, the treatment has a better toxicity profile when compared to single agent 2nd line chemotherapy such as docetaxel, or in comparison to first line platinum doublet chemotherapy. This finding has been repeated across multiple studies [159-163, 166]. Despite the accumulating scientific evidence of efficacy there has been a paucity of quality of life data presented. Health related quality of life outcomes from the Keynote-024 study (pembrolizumab vs first line platinum doublet chemotherapy in patients with a tumour PD-L1 level of ≥50%) were recently presented at the 2016 World Conference on Lung Cancer [175]. In this study quality of life (QoL) was measured using the European Organisation for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire (QLQ) C30 (general module) and QLQ-LC13 (lung cancer specific modules). General QoL improved from baseline to the week 15 assessment for patients on pembrolizumab, but remained stable for patients on chemotherapy. The difference was statistically significant (p=0.002) and clinically meaningful. The proportion of patients with improved QoL was higher for pembrolizumab treated patients (40% vs 26.5%). Fewer patients has deterioration in
lung cancer symptoms as measured by the QLQ-LC13 on pembrolizumab (30% vs 39%) and the time to deterioration was also significantly longer (HR 0.66, 95% CI 0.44 – 0.97, p=0.029). Results from other studies are yet to be presented in detail, but anecdotally the clinical experience with these agents matches the trial data.

1.3.4 Palliative Care

Symptom control is particularly important in the care of patients with advanced lung cancer. Despite advances in systemic therapy many patients become or remain unwell and succumb to their illness and its treatment. Work conducted in the early 1990s by the Medical Research Council in the UK examined the prevalence of symptoms for patients with small cell lung cancer (SCLC) and NSCLC enrolled in two clinical trials [176]. The most common symptoms at presentation included worrying, anxiety, tiredness, lack of energy, loss of appetite, sleep disturbance, shortness of breath and cough. Lutz et al reported on a cohort of 69 consecutive patients with advanced lung cancer being referred to radiation oncology [177]. As these patients were being referred for palliative radiotherapy all had significant symptoms. Symptom levels were significantly higher for patients in the last three months of their life as compared to those who lived for four to six months.

Temel et al conducted an important and now frequently cited study examining early referral to palliative care (within eight weeks of diagnosis) as compared with standard of care in patients with new diagnosed NSCLC [178]. Importantly enrolment was offered to reasonably well patients of ECOG performance status 0-2. The study found that patients in the experimental arm reported significantly better quality of life, lower rates of depression at 12 week assessment, less aggressive care at the end of life and were more likely to have their preferences for care documented. Conversely, rates of service use (such as hospice care) were higher. The study generated an interesting finding of improvement in survival outcomes for those patients receiving early
palliative care. In view of the important finding similar trials are now being established to confirm the effect.

1.4 Conclusion

Lung cancer remains the most common cause of cancer related death worldwide, with poor long term survival. Tobacco consumption is the most common reversible risk factor, and there is a strong association between its use and the development of lung cancer. Asbestos and other carcinogens also contribute to this risk. Over and under expression of several genes has been demonstrated to modify the risk of development of lung cancer in GWA studies. There is an increasing number of patients developing lung cancer in the absence of a history of tobacco consumption.

The major aim of diagnosis in lung cancer is to attempt to detect the condition at its earliest stages where treatment may be delivered with curative intent. This may mean surgery (with the consideration of adjuvant systemic therapy), local therapy such as radiofrequency ablation or stereotactic radiotherapy, or radical radiotherapy delivered concurrently with chemotherapy. Where curative intent therapy is not possible the goals of treatment are to palliate symptoms, control the malignant process, and improve longevity while protecting or improving quality of life. The role and importance of anatomical and molecular pathology in confirming the diagnosis of a lung cancer type to allow use of the most appropriate therapies cannot be understated.
Incremental gains in patient outcomes have been possible through:

- The development of screening programs for people at the highest risk of developing lung cancer, with the aim of early intervention
- Improvements in techniques for local and locally advanced disease including surgery, radiotherapy and radiofrequency ablation
- Improvements in systemic therapy, which have been modest at best during the early of cytotoxic chemotherapy, as well as improving understanding of the tolerability of various regimens
- Increasing intervention from specialist palliative care teams to provide symptom control in a high symptomatic population who often have comorbidities.

The discovery of “druggable” oncogene driver alterations, such as \( \text{EGFR} \) mutations, \( \text{ALK} \) fusions and \( \text{ROS1} \) fusions, marked the first major advance in systemic treatment in decades. More recently these have been followed by the introduction of effective immunotherapies to clinical practice. Both these therapeutic modalities offer the potential of prolonged response with minimal or modest toxicity for appropriately selected patients.

This thesis examines clinicopathologic outcomes for patients treated for lung adenocarcinoma at St Vincent’s Hospital, Melbourne. Chapters two and three concentrate on the application of a pathologic classification of histologic subtypes of lung adenocarcinoma. This classification was proposed by the IASLC, ATS and ERS, and it was subsequently adopted into the fourth WHO classification of Tumours of the Lung, Thymus, Pleura and Heart [44, 179].

Chapter two examines pulmonary adenocarcinoma pathologic subtypes at metastatic sites obtained from a surgical procedure. Substantial work and the publication of a number of studies was required to allow development of the new classification of pulmonary adenocarcinoma subtypes following resection of the primary tumour [44,
Historically, there has only been one other paper on the importance of pulmonary adenocarcinoma patterns at metastatic sites, however that worked dated from 1988 [180]. This work (and its associated publication) is unique as it is the first time that such patterns have been studied at purely metastatic sites [181]. The data is examined to see whether pathologic subtyping at metastatic sites may be of prognostic importance, and whether there are any associations with oncogenic mutations in \textit{EGFR} and \textit{KRAS}.

In Chapter Three the thesis examines outcomes for a cohort of patients who had undergone surgical resection with curative intent for early or locally advanced lung adenocarcinoma. The main aim is to examine the rates of \textit{EGFR} and \textit{KRAS} mutations by pathologic subtype and discover if there are any correlations that point to a subgroup being enriched for or unlikely to have a particular mutation. Further, the potential interactions between tumour subtype, oncogene mutation and overall survival are explored. At the time of conception of this study, the interplay between pathologic subtype and oncogenic mutation status was not known. To date the majority of studies published on this subject have originated from Asian cohorts (n=12), where the rate of \textit{EGFR} mutations is significantly higher than in a Caucasian cohort. This study adds to the four other studies in Caucasian cohorts as discussed in Chapter Three.

Inhibition of \textit{EGFR} mutant lung cancer with \textit{EGFR} TKIs has proved a successful strategy for appropriated selected patients with advanced disease. These therapies are well tolerated and often have prolonged responses. An important goal of translational research is to understand how resistance to a therapy may develop in order to find new ways to prolong the time a patient is on a given line of treatment. The pathway involving interleukin-6 (IL-6), Janus Kinase (JAK) and Signal Transducers and Activators of Transcription 3 (STAT3) is a potential pathway through which resistance to \textit{EGFR} inhibition may occur in advanced disease.
In Chapter Four, a detailed assessment of the activation of IL6, JAK1 and phosphorylated STAT3 (pSTAT3) is made by immunohistochemistry in lung adenocarcinoma obtained at curative intent surgical resection. Much of the previous work has focused on cell line and xenograft studies. There are significant gaps in the available studies based on clinical specimens. Many of the studies examining pSTAT3 expression have heterogeneity of the pathologic subtypes assessed, with mixtures of adenocarcinoma, squamous cell carcinoma and other histologies. Amongst those studies limiting their assessment to pulmonary adenocarcinoma, there has been no consistency of methods used to assess for positivity of pSTAT3 by IHC. Prior studies have also not assessed all three components of the IL6, JAK and STAT3 pathway together.

The study looks at clinical and pathologic correlations of IL6, JAK1 and pSTAT3 activation as measured by IHC in a clinical cohort. This study assesses the largest number of cases of cases stained for IL6, gp130 and JAK1 reported in the literature. It also attempts to account for inconsistency of methods of assessment of pSTAT3, by adopting two methods used in comparable studies. The study particularly seeks to find whether the presence of an EGFR mutation enriches for activity of the IL6 / JAK1 / pSTAT3 pathway in the absence of systemic therapy when compared to samples with KRAS mutations and samples that are wild type for both of these oncogenes. It is also able to examine clinicopathologic correlations of IL6, JAK1 and pSTAT3 expression, together with survival outcomes.

This thesis makes a contribution to the understanding of the pathology of lung adenocarcinoma and clinical associations.
CHAPTER 2

ADENOCARCINOMA SUBTYPING IN ADVANCED DISEASE
2.1 Literature Review

The need for sophistication and precision in lung cancer diagnosis has increased over the last decade. Improvements in classification schemes may be driven by a number of factors such as appreciation of differences in the pathological appearance, differences in biological behaviour and natural history of a tumour type, or the recognition of tumour features that predictive efficacy or toxicity from the various available treatment modalities. Ultimately a new classification scheme should lead to improved treatment outcomes. This chapter focuses on application of recommendations in the 2011 classification of the IASLC, American Thoracic Society (ATS) and European Respiratory Society (ERS) for pulmonary adenocarcinoma, and whether these classifications provide additional prognostic information in patients diagnosed with advanced disease [179].

2.1.1 Selection of Cytotoxic Therapy in Advanced Lung Cancer – Role of Pathology

Historically, lung cancer was treated as a single entity. When recognised at an “early stage” radical surgery was employed because the disease was thought to be surgically resectable at this point. The most frequent operation was pneumonectomy. Many patients undergoing thoracotomy were found to have unresectable disease, due mostly to limitations in the available preoperative imaging modalities that time. The underlying pathologic subtype of lung cancer did not alter this approach [182, 183]. In 1965 Borrie published a series that demonstrated that deaths occurred early from distant metastatic disease despite what was considered to be “successful surgery”. Borrie’s series also showed different biological behaviour between tumour subtypes [183]. The subsequent recognition of small cell lung carcinoma (SCLC) as a biologically distinct entity led to clinical trials showing the apparent superiority of radical radiotherapy over surgery in early stage SCLC in the 1960s [184, 185]. The distinction of SCLC from NSCLC remains critical today given the difference in prognosis and treatment modalities. In 1976 the demonstration of different responses to systemic chemotherapy
on the basis of histologic subtype in advanced NSCLC was first reported. Patients with adenocarcinoma had a statistically significant improvement in median survival when treated with three drug regimens as compared to two drug regimens (201 vs 118 days, p=0.007). No improvement was seen for patients with other histologic types including SCLC, epidermoid carcinoma (squamous cell carcinoma [SqCC]) and large cell carcinoma [186].

Analysis of the SEER database from 1973 to 1987 identified improved 5-year survival for patients with localised adenocarcinoma of the lung compared to SqCC of the lung. In this same analysis there were minimal differences in survival outcomes for patients with regional disease, and no difference for patients with metastatic disease [187]. As recently as the early 2000s, clinical trials of systemic therapy for advanced NSCLC did not differentiate on the basis of histology. The mainstay of therapy was a third generation platinum based doublet (the combination of cisplatin or carboplatin with either gemcitabine, docetaxel, paclitaxel or a vinka alkaloid) [98, 188, 189].

Differentiation between pulmonary adenocarcinoma and squamous cell carcinoma in advanced disease has had increasing relevance in recent years with newer cytotoxic and targeted therapies. In a phase III trial comparing the combination of cisplatin/gemcitabine with cisplatin/pemetrexed an interaction was noted between survival outcomes and the histologic subtype (as discussed in Chapter 1, page 45) [100]. A detailed understanding of the mechanism of the drug pemetrexed, together with the increased expression in one of its target enzymes, thymidylate synthase, in SqCC allowed explanation of the observed difference [100, 190].

The differentiation between adenocarcinoma (AC) and SqCC is also important with regards to the potential to cause harm. A standard of care for patients with advanced NSCLC is the cytotoxic combination carboplatin and paclitaxel. Clinical trials progressed with the addition of bevacizumab to this combination. Bevacizumab acts as
a vascular endothelial growth factor (VEGF) trap, binding to VEGF and preventing it from interacting with its receptor. During a phase II trial it was found that for patients with squamous histology there was a marked increase in the risk of major haemorrhage, and for some of the trial participants the outcome was fatal. Therefore patients with squamous histology were excluded from the subsequent phase III trial [191, 192] and the addition of bevacizumab to carboplatin/paclitaxel is a standard of care for patients with non-squamous histology only in some countries. In light of safety and efficacy concerns, many contemporary clinic trials require the histologic subtype of NSCLC either for stratification prior to randomisation, or as part of the inclusion/exclusion criteria.

In addition the separation of histologic subtypes of NSCLC in patients with advanced disease is necessary due to the identification of oncogenic drivers of lung cancer, including EGFR mutations, Kirsten rat sarcoma virus (KRAS) mutations and echinoderm microtubule associated like protein (EML4) - anaplastic lymphoma kinase (ALK) translocations and their associations with adenocarcinoma histology. Furthermore targeted agents are available for tumours with EGFR mutations and EML4-ALK translocations on the basis of phase III trials, and many other mutated oncogenes can also be targeted with small molecule inhibitors (see section 1.3.3.2 – page 47).

2.1.2 Adenocarcinoma – Improving Classification in Resection Specimens

The classification of pulmonary adenocarcinoma has been sequentially improved by observations from pathologists and clinicians, matching histologic features with either good or poor long-term prognosis. The anatomical pathology guidelines for lung cancer have been prepared by consensus by expert pulmonary pathologists, with the first edition published by the WHO in 1967, with updates in 1981, 1999 and a revision in 2004[193-196]. Throughout all iterations, adenocarcinoma has been described as a malignant tumour with formation of gland-like structures. An international
multidisciplinary collaboration between the IASLC, the ATS and the ERS led to a new classification scheme for pulmonary adenocarcinoma in 2011 [179]. The authors note that a detailed classification is required as adenocarcinoma is the most common type of lung cancer, there is “widely divergent clinical, radiologic, molecular and pathologic” behaviour of lung adenocarcinoma, and significant resources are being devoted to research in the field. Therefore, the classification is required to “assist in determining patient therapy and predicting outcome”. The recommendations of the IASLC/ATS/ERS classification were subsequently adopted in the new 4th Edition of the WHO Classification of Tumours of the Lung, Pleura, Thymus and Heart in 2015 [44].

2.1.2.1 World Health Organisation Classifications of Lung Tumours

The historical background for the first WHO classification of lung tumours, published in 1967, is discussed in detail in the preface to the classification [193]. In summary, the first congress for disease classification in general took place in 1853 in Brussels. Responsibility for disease classification passed to the Health Committee of the League of Nations in 1927, and subsequently to the WHO in 1947. The WHO organised for a large-scale effort of classification of human tumours in 1956. The aim of these efforts was to “develop histological definitions of cancer types and to facilitate the wide adoption of a uniform nomenclature”. Meetings for the first edition for lung cancers (1967) commenced in 1958, with the majority of the classification based on the efforts of Professor Levi Kreyberg from Oslo, and Professors AA Liebow and EA Uehlinger, from the USA. Prior to the recent 2015 edition classifications were developed by pathologists, to be used by pathologists and with limited consideration of the needs of practicing clinicians.

The 1967 WHO classification [193] described AC as a malignancy with the formation of tubules and gland-like structures, with or without the presence of papillary growth and mucin production. The diagnosis of AC could be made even if only a small part of the tumour demonstrated these morphologic features, on the basis that a focus of AC
maybe a remnant of the tumour’s origin in an otherwise poorly differentiated lesion. In other words, the diagnosis of adenocarcinoma was based upon the most “highly differentiated cell type” present, regardless of poorly differentiated elements within the same tumour. The classification described adenocarcinoma as a bronchogenic tumour, and bronchioalveolar carcinoma (BAC) as being highly differentiated, often papillary and with spread through the airspaces. In this classification BAC was defined as an invasive lesion, though subsequently this definition was changed in the 1999/2004 WHO classifications. Solid tumours with mucin production were included with large cell carcinomas, and not identified as adenocarcinoma. A combined epidermoid carcinoma and adenocarcinoma category was also recognised, that is now referred to as “adenosquamous” carcinoma.

The second WHO classification of lung tumours was published in 1981 [194]. The 1981 WHO classification included benign tumours, termed adenomas, and carcinoma in situ for the first time, albeit with only brief discussion. The 1981 WHO classification opined that most tumours should be able to be diagnosed on the basis of haematoxylin and eosin stains only. The need to differentiate primary lung tumours from tumours metastatic to the lung was noted. Tumours with a solid pattern which showed mucin production were moved out of the large cell carcinoma category and became the fourth adenocarcinoma subtype, with the recommendation that a mucin stain should be used to differentiate these tumours from “true” large cell carcinomas. The other adenocarcinoma subtypes were those included in the 1967 WHO classification and consisted of acinar, papillary, and BAC. It was noted that BAC had different biologic behaviour based on the presence or absence of mucus secretion. Classification of these adenocarcinoma subtypes was recommended to be made by a pathologist on the basis of “the predominant cell type” present.

The 3rd edition in 1999 saw a great expansion in the amount of detail provided to the reader [195]. The definition of BAC was changed from a tumour with lepidic growth and invasive patterns to a tumour with no evidence of stromal, vascular or pleural
invasion; that is a tumour with pure lepidic growth and no invasion. This marked a departure from the original definitions in both the 1967 and the 1981 WHO classifications. If evidence of invasion was seen the tumour was to be described as AC of mixed subtypes. This latter term, adenocarcinoma of mixed subtypes, was introduced into the 1999 WHO classification to denote an AC with two or more histologic patterns, and so essentially described tumours which would have been classified as BAC prior to 1999. Over the ensuing years as a result of the change in definition of BAC from a tumour subtype with invasion to one without, the use of the term “BAC” lead to considerable confusion amongst clinicians, researchers and even pathologists.

In the 1999 WHO classification the use of IHC markers to distinguish primary lung AC from metastatic spread to the lung was advocated. The first discussion of the molecular basis of AC was made, although it was limited to \textit{KRAS} mutations only.

The 3\textsuperscript{rd} edition maintained the use of the various histologic subtypes that included acinar, papillary, solid with mucin production, and BAC (which could be mucinous or non-mucinous). Rare AC variants were introduced, including fetal AC, colloid AC, mucinous cystadenocarcinoma, signet-ring AC and clear cell AC. It was noted that subclassification was fraught with difficulty due to tumour heterogeneity, and tumours composed of a single subtype were rare. Hence the newly introduced term, AC of mixed subtypes became the most frequently diagnosed AC subtype, encompassing 80-90\% of all tumours diagnosed.

An update to the third edition was published in 2004 [196]. There was little change in the subclassification of AC compared to the 1999 WHO classification, apart from moving AC of mixed subtypes to the top of the list of subtypes and variants as a reflection of its frequency. For the first time there was detailed discussion of the epidemiology and aetiology of lung cancer as a whole, and description of the genetics and molecular pathogenesis of lung cancer. Additionally macroscopic and cytologic
descriptions of AC were provided. The importance of histopathologic assessment of the entire tumour in cases of small peripheral AC was emphasised. Finally, it was recommended that if a tumour was diagnosed as AC of mixed subtypes, the different subtypes present should be included in the final histology report.

The most recent 4th edition was released in 2015 and is discussed further below [44].

2.1.2.2 Short comings of the WHO classification up to 2004 – early stage disease

Two major deficiencies existed in the WHO classifications of lung tumours prior to the release of the 2015 iteration. The first of these is the use of the term “mixed subtype(s)”. As noted above, a number of histologic subtypes were described in the 1999 and 2004 WHO classifications [195, 196]. The mixed subtype was added in 1999 and retained in 2004. This subtype was added in an attempt to demonstrate the marked heterogeneity of lung adenocarcinoma. However, when examining cohorts of patients the mixed subtype accounted for between 80 to 95% of all specimens [197-201]. Given that this encompasses most patients who have undergone resection of early stage AC, no further useful clinical information is gained from the pathologic label “AC of mixed subtypes”. As will be discussed in subsequent sections, widely varied clinical behaviour and survival patterns were noted within patients meeting the description of pulmonary AC of mixed subtypes, from rapid relapse and death due to metastatic disease through to prolonged survival and cure from surgical intervention.

Secondly, in addition to the high prevalence of the “mixed subtypes”, significant confusion developed in relation to the meaning of the diagnostic term BAC by clinicians and pathologists alike. In the pathologic meaning BAC was strictly redefined in the 1999/2004 WHO classification as being a tumour with pure lepidic growth without evidence of invasion of stroma, blood vessels or pleura [195]. Despite this the term was used quite loosely by some pathologists and many clinicians – it could
encompass lesions that met the strict definition of BAC with pure lepidic growth; it could be used for lesions that were predominantly lepidic with a focus of invasive AC; or it could be used to describe a small lepidic component on the edge of a predominantly invasive AC [202]. A variety of radiologic patterns that occurred on CT scans were labelled “BAC” despite significant variations in appearance. Small peripheral lesions with ground glass opacification were seen with or without a solid component in early stage resectable disease. A multifocal presentation with widespread pulmonary metastases and a lack of distant metastases also existed and attracted the description of “advanced BAC” [203, 204]. Difficulties with understanding and applying the new strict pathologic definition of “BAC” also lead to problems with clinical trial design and inclusion criteria as evidenced by reviews of surgical and systemic therapy efforts for this condition [205, 206]. A measure of the spectrum of pathologies encountered under the term “BAC” is the separation of cases that previously received this descriptor into one of five new descriptive categories in the IASLC/ATS/ERS classification [179].

2.1.2.3 Impetus for the Development of the IASLC/ATS/ERS classification

The introduction to the IASLC/ATS/ERS classification outlines a number of reasons for its development which are aimed to address problems and short comings with the prior WHO classifications. The driving reasons cited in the document include –

- The development of a classification that moves from being purely pathological to being useful for pathologists and clinicians alike
- The need for a strategy to manage small biopsy and cytology specimens in advanced disease such that accurate pathologic assessment can occur for the selection of therapy(ies) and appropriate molecular tests are performed
- The recognition of a correlation between imaging features and pathologic findings on chest CT, which “allows new opportunities for imaging studies to be used by radiologists, pulmonologists, and surgeons for predicting the
histologic subtype of adenocarcinomas, patient prognosis, and improve preoperative assessment for choice of timing and type of surgical intervention”.

2.1.3 The New IASLC/ATS/ERS Classification for Adenocarcinoma – Resection Specimens

As previously noted, adenocarcinoma covers a spectrum of lung cancers with pathologic and clinically heterogeneity. The 2004 WHO classification failed to capture this in a way that was meaningful for pathologists and clinicians [196, 207]. The new classification was developed with input from pathologists and clinicians representing the International Association for the Study of Lung Cancer, the American Thoracic Society and the European Respiratory Society. The rationale for a new classification, methods for reaching consensus and definitions of each subtype are described in detail in the landmark paper published in the Journal of Thoracic Oncology, and summarised here [179].

The authors comment that “a widely divergent clinical, radiologic, molecular and pathologic spectrum exists within lung adenocarcinoma”. A lack of consistency in the use of definitions led to difficulty in comparing clinical studies. To overcome some of these issues a multidisciplinary approach was taken to development of the new classification. A literature review identified 312 articles on the topic of lung adenocarcinoma which formed the evidence base for the new guidelines. These papers were assessed by the GRADE (Grades of Recommendation, Assessment, Development, and Evaluation) system [208, 209]. Meetings were held between March 2008 and December 2009 to “discuss issues related to lung adenocarcinoma classification” and to develop the new IASLC/ATS/ERS classification of lung adenocarcinoma [179].

The following definitions were produced for resected specimens in solitary adenocarcinomas measuring ≤ 3cm and without lymph node metastasis:
- Adenocarcinoma in situ (AIS) – a small (≤ 3cm) solitary AC with pure lepidic growth

- Minimally invasive adenocarcinoma (MIA) - a small (≤ 3cm) solitary AC predominantly consisting of lepidic growth, with small foci of invasive AC measuring ≤ 5mm
  - Complete histologic assessment of the tumour is required for the diagnosis of AIS or MIA to be made

- For tumours ≤3cm in size but with an invasive component of > 5mm the tumour is classified as an invasive adenocarcinoma, with the predominant subtype described as discussed in the next paragraph.

For invasive adenocarcinoma guidelines recommend comprehensive histologic subtyping, identifying each component present in 5% increments, with diagnosis of the tumour type according to the predominant subtype seen. The major diagnostic classifications for invasive adenocarcinoma are:

- Lepidic predominant AC (LPA) – a tumour in which there is predominant lepidic growth with the tumour measuring greater than 3 cm in size or with an invasive focus of greater than 0.5mm

- Acinar predominant

- Papillary predominant

- Micropapillary predominant – newly added to the range of subtypes described in the 1999/2004 WHO classification [195] given the association of this histologic pattern with a poor prognosis

- Solid predominant
The following rare variants of invasive lung AC were also defined:

- Invasive mucinous AC
- Fetal AC
- Colloid AC
- Enteric AC

The new IASLC/ATS/ERS classification system makes two major recommendations that mark a departure from former practice for the reasons discussed in Section 2.1.2.2 (Shorting comings in the WHO classification – early stage disease). Firstly, a recommendation is made for ‘discontinuing the use of the term “BAC”’. Secondly, the reporting of invasive adenocarcinoma by the predominant histologic subtype results in the abandonment of the term “adenocarcinoma with mixed subtypes”.

2.1.3.1 Studies on Pathologic Subtypes Predating the IASLC/ATS/ERS Classification

The following paragraphs describe the evidence that lead to the description of pathologic subtypes in the IASLC/ATS/ERS classification.

2.1.3.1.1 Adenocarcinoma in situ / Minimally Invasive Adenocarcinoma

It was first recognised in 1980 by Shimosato et al [210] that a proportion of patients with small peripheral lung cancers were cured by surgery alone. Their study included 48 patients with small peripheral AC and focused on the size of central scar in the resected tumour. They graded central fibrosis in each tumour from 1-4 grades (1 – no/minimal desmoplasia; 2 – fibroblastic tissue with a small amount of collagen; 3 – fibroblastic tissue with moderate/abundant collagen; 4 – hyalinised tissue). The 5-year overall survival for patients with a tumour with grade 1 fibrosis was 100%; grade 2 fibrosis was
87.5%; grade 3 fibrosis 13.3%; and grade 4 fibrosis 18.2%. Shimosato’s work demonstrated that increasing amounts of fibrosis in adenocarcinoma were associated with worse survival, most likely due to the increasing proportion of invasive tumour.

The seminal study on small peripheral AC was published by Noguchi et al in 1995 [211]. The authors examined 236 small peripheral ACs (pT ≤20mm) and divided the tumours into two groups based on the degree of “replacement growth” within each tumour. Replacement growth referred to lepidic growth or BAC. Tumours with replacement growth were further divided into 3 groups based on the amount of central fibrosis. Tumours without replacement growth were classified according to the main histologic pattern seen. A classification was developed, with tumours designated alphabetically from A to F. Noguchi A tumours were localised BAC, tumours with 100% lepidic growth, whilst Noguchi B tumours were localised BAC with a central area of fibrotic collapse. Noguchi C tumours were localised BAC with foci of “active fibroblastic proliferation” or in other words invasive tumour patterns with destructive desmoplastic stroma. Noguchi D to F represented invasive ACs with Noguchi D being poorly differentiated, Noguchi E being tubular/acinar and Noguchi F being papillary. When the classification was used to assess 5-year overall survival, the authors found that Noguchi types A and B tumours had 100% 5-year survival and no lymph node metastases. In comparison patients with Noguchi type C tumours had 74.8% 5-year overall survival, and patients with Noguchi types D-F combined had 52.4% 5-year overall survival.

Several other groups have replicated and built on the landmark work of Noguchi et al. In 2000, Yokose et al examined 200 small peripheral ACs (pT ≤20mm) and reported that in tumours with greater than 75% lepidic component and an invasive focus ≤5mm, 5-year overall survival was 100% (n=66) [212]. Maeshima et al found that if the invasive component was ≤10mm in tumours with pT≤20mm overall survival was 100% at 5 years [213]. Suzuki et al reported that when tumour scar was ≤5mm, 5-year overall survival was 100% [214]. More recently, Vazquez et al [215] showed that for lesions
less than 3cm in size with pure lepidic growth, or where the tumour consisted of 90-99% lepidic growth (that is ≤3mm of invasive focus) overall survival was 100% at 5 years.

Some other attempts to emulate the findings of Japanese groups were made in Western centres. Breathnach et al reviewed 138 patients with stage I lung adenocarcinoma – 33 patients with BAC as per the WHO 1999 definition, and 105 with AC other than BAC [216]. No difference was found in 5-year disease free survival between BAC and AC in this study. These findings could potentially be explained by the presence of patients with the former diagnosis of “mucinous BAC” amongst the BAC cohort, however this information is not provided by the authors. Furak et al also attempted to examine the outcomes of patients with a diagnosis of BAC [217]. They accepted a diagnosis of BAC as having a tumour comprising of at least 50% lepidic growth. It is unclear why this definition was adopted given the redefinition in the 1999 WHO classification. As a result, their 5-year overall survival rate was poor at 61.9%. Rena et al examined 28 patients with stage I BAC as per the 1999 WHO classification in comparison to 80 patients with stage I adenocarcinoma [218]. Comparing patients with stage IA BAC (now AIS/MIA) with those with stage IA invasive AC, there was a significant difference in 5-year disease free survival (93 vs 58%, p=0.043). A numerical but not statistically significant difference was seen between patients with stage IB BAC (now LPA) compared with stage IB invasive AC (61 vs 32.5%, p=0.064). The findings of Rena et al are consistent with Noguchi’s prior work.

Two papers published in 2007 and 2009 provide the most recent information with definitions that are closest to those eventually adopted in the IASLC/ATS/ERS classification [179, 219, 220]. In both papers, patients included had small tumours with either pure lepidic growth, or lepidic growth with invasion ≤5mm. Five-year survival for patients with pure lepidic growth was 100% in both papers. In the paper by Yim et al [219] 5-year overall survival for patients with ≤5mm of invasion was 100%. Borczuk
et al found excellent outcomes with MIA (median survival not reached), however 5-year overall survival rate was less than 100% [220].

In summary the work of many groups has provided evidence for resected tumours now defined as AIS and MIA. Shimosato et al first showed that prognosis was related to the degree of central fibrosis, likely reflecting the component of invasive adenocarcinoma [210]. Subsequently Noguchi et al defined AIS after showing that tumours less than 20mm in size comprising 100% replacement growth were in situ carcinoma [211]. This work also supported that of Shimosato et al in associating increasing invasive carcinoma with worse survival outcomes. The three studies from Yokose et al [212], Maeshima et al [213], and Suzuki et al [214] showed that a small invasive focus (≤5mm) in an otherwise lepidic tumour did not affect that 100% survival rate, thus defining tumours now labelled MIA. Two of the initial studies from Western groups were unable to replicate the outcomes seen in Japanese studies [216-218], while the third had similar results, although with less than 100% survival for what would now be called AIS / MIA [218]. These results were subsequently refuted by Yim et al [219] and Borczuk et al [220] who showed that Western patients with AIS or MIA had the same excellent survival outcomes as patients from Asian cohorts.

2.1.3.1.2 Lepidic Predominant Adenocarcinoma

This category is formally defined and included in the IASLC/ATS/ERS guidelines for the first time [179]. It includes tumours that have predominant lepidic growth, but do not qualify as MIA either due to the total size of the lesion being >30mm, the invasive component being >5mm, the presence of visceral pleural or lymphovascular space invasion or the presence of tumour necrosis. In the 2015 WHO classification the morphologic invasion pattern of “spread through air spaces” also elevates tumours that would have been otherwise classified as AIS or MIA into the category of LPA [44]. As this category is new there are no papers that predate the publication of the new classification (recent publications are reviewed below). As noted in the discussion on
the WHO classifications, these tumours were referred to as mixed adenocarcinoma with bronchioalveolar features.

Ohtaki et al reviewed 504 consecutive patients with resected AC. They classified patients according to the 2004 WHO classification, and also noted the predominant subtype. Those patients with tumours with predominant “BAC” growth had better survival compared to those patients whose predominant subtype was papillary, acinar or solid [221]. Anami et al studied 139 patients with small AC (<2cm). They demonstrated that patients whose tumours consisted of greater than 50% lepidic growth had improved overall survival compared to those whose tumours consisted of less than 50% lepidic growth [222].

Terasaki et al examined a cohort of 441 patients with resected pulmonary AC and divided tumours into groups based on the amount of BAC component present [198]. The tumours were grouped into those with “pure BAC”, those with “mixed BAC” and those that were purely invasive without any BAC component present. They further divided tumours with “mixed BAC” (that is, a mixture of lepidic growth and invasive AC) into two groups according to the size of invasion present in the tumour, including those with invasion ≤ 5mm and those with invasion > 5mm. Of those with a focus of invasion > 5mm there were more lymph node metastases found but unfortunately data to enable survival analysis were not presented in the final manuscript. Sakao et al examined 82 tumours of ≤ 2cm in size with variable invasive components and found higher rates of lymph node metastases and pleural invasion if there was minimal or no BAC component in the tumour [223]. In tumours with a significant lepidic component, the rates of lymph node metastases and pleural invasion were lower. Borczuk et al demonstrated a difference in the rates of lymph node metastases between tumours with no/minimal invasion, those with invasion of ≥6mm and those with purely invasive tumours [220]. A numerical difference in survival was seen between mixed and purely invasive tumours which failed to reach statistical significance. Yim et al [219] found
similar results to Borczuk et al [220] with regards to the frequency of lymph node metastases according to invasive size and amount of lepidic component present.

### 2.1.3.1.3 Acinar Adenocarcinoma

There is little literature focusing directly on acinar subtype AC. There are two possible explanations for this. First, the definition of AC according to 2004 WHO classification is a “malignant epithelial tumour with glandular differentiation”[196]. Of all AC subtypes, the morphologic appearance of acinar pattern AC most closely resembles this definition of AC. Secondly, researchers and clinicians aim to identify groups of patients with better or worse prognoses from the “average” patient with a given condition.

Only two groups have directly mentioned the pathologic features of acinar tumours in comparison to others. Ohtaki demonstrated that survival for acinar predominant tumours was similar to that of papillary predominant tumours, superior to that of solid predominant tumours, and inferior to “BAC” predominant tumours [221]. Motoi et al demonstrated that acinar predominant tumours tended to be a smaller size and patients were of a younger age when compared to patients’ whose tumours did not have the predominant acinar subtype [197]. Despite the acinar patterns being prominent in pulmonary adenocarcinoma the body of literature is small.

### 2.1.3.1.4 Cribriform Adenocarcinoma

Cribriform predominant tumours represent a recently described adenocarcinoma subtype. They were not included in the IASLC/ATS/ERS classification or the 2015 4th Edition of the WHO classification of Lung Tumours, but as further studies emerge this may change [44, 179]. The cribriform pattern is found in tumours from other solid organs, and is first mentioned in relation to lung adenocarcinoma by Noguchi et in 1995 [211]. Okudela et al again raised the cribriform pattern as an entity in 2010. In their
paper they include the cribriform pattern as part of the same family as the acinar pattern, and this recommendation held in the IASLC/ATS/ERS classification [179, 224]. Kadota et al provided a succinct definition explaining the differences between the cribriform pattern, acinar pattern and solid pattern as follows [225]:

Cribriform pattern tumours had invasive fused tumour glands with back-to-back, poorly formed glandular spaces lacking intervening stroma or invasive tumour nests that produce small glandular lumina without solid components. These tumours sometimes have a ‘cookie-cutter’ pattern of glandlike spaces. In contrast, the usual acinar pattern had well-defined individual tumour glands with well-formed glandular lumina. Solid pattern tumours had invasive solid tumour nests without glandular space.

Kadota et al reviewed the slides from 1038 patients with resected stage I adenocarcinoma from Memorial Sloan Kettering Cancer Centre (Moreira et al reported on a subset of this group [226]). The cribriform pattern was seen in 25% of cases and was the predominant subtype in 4% of cases. The addition of the cribriform pattern changed 46 patients from acinar predominant to cribriform predominant, three to lepidic predominant, three to papillary predominant and three to solid predominant. Patients with cribriform predominant tumours had a five year freedom from recurrence of 70%, as compared to acinar predominant 87%, papillary predominant 83%, micropapillary predominant 62%, solid predominant 70%, invasive mucinous adenocarcinoma (IMA) 77% and colloid predominant 71%. Furthermore, patients with acinar predominant tumours with a greater than 10% cribriform component had five year freedom from recurrence at 74% as compared to those patients with acinar predominance with less than 10% cribriform pattern at 90%.

Xu et al had earlier published on the clinicopathologic associations of cribriform predominant tumours [227]. They found that in comparison to acinar predominant tumours, cribriform predominant tumours were more likely to have aggressive features
including higher mitotic rate, vascular invasion, higher nucleolar grade and more necrosis. Warth et al independently confirmed the findings with regards to survival outcomes [228]. In their paper the rate of the cribriform predominant pattern was also 4%. No clinicopathologic correlations were found. Cribriform predominant tumours had better median survival outcomes than solid and papillary predominant tumours while being worse than acinar and micropapillary predominant tumours. The median relapse free survival for the cribriform pattern was the worst of all predominant subtypes.

The work of both groups reporting survival outcomes would support the separate identification of cribriform predominant tumours in future classifications, however further confirmation of these findings may be required.

2.1.3.1.5 Solid Adenocarcinoma

Several studies identify solid subtype AC as a poor prognostic pattern. In 1982 Chung et al examined a series of 96 patients treated with surgical resection only for NSCLC, including 46 patients with AC. [229]. The tumours were divided into different grades based on the amount of solid pattern present as follows: grade 1, no solid component; grade 2, solid component mixed with other AC patterns; and, grade 3, almost all solid tumour with mucin production. The authors examined 2 year overall survival by grade and by lymph node involvement. The pathologic stage of lymph node involvement was not specified. Patients with grade 3 tumours had a 2-year survival rate of 44% if node negative and 0% if node positive, whilst patients with grade 1 tumours were all node negative and had 94% 2-year survival. Patients with grade 2 tumours had 63% 2-year survival if node negative and 33% 2-year survival if node positive.

Riquet et al reviewed 1139 patients with resected NSCLC, of whom 565 had AC and 574 had SqCC [230]. They separated patients with AC on the basis of the presence or
absence of a solid with mucin component. The presence of solid with mucin AC led to the worst 5-year OS of 36.8%, compared to 50.2% 5-year OS for SqCC and 58.1% 5-year OS for AC lacking a solid component. The tumour type was a significant factor for overall survival on multivariate analysis. Other significant factors on multivariate analysis included higher T stage, higher N stage and older age. Motoi et al also found a number of pathologic and clinical features that were associated with a major solid component in AC including larger tumour size, smoking history, worse tumour grade and presentation at more advanced stage [197]. Most notably, the 5-year overall survival was much poorer for patients with solid predominant AC in comparison to non-solid predominant tumours (31.8 vs 65.5%, p=0.001).

In the study from Yokose et al examining 200 of small lung ACs less than ≤3cm, a significant solid component (>25%) was associated with a worse outcome (p<0.001) [212]. Ohtaki et al studied 504 patients with resected AC and found that with those with solid predominant histology, 5-year OS was worse than that seen with papillary, acinar and BAC predominant histologies [221]. Furthermore, they found that the presence of any solid component in an AC resulted in worse 5-year overall survival than if there was no solid component present (p<0.001). Five-year overall survival was 60.3% if a solid component was present versus 87.5% if a solid component was absent (p=0.024 on multivariate analysis). In a smaller series investigating 85 patients with AC those with ≥90% solid component had a 21.6 months median overall survival in comparison to 69.4 month overall survival for tumours with <90% solid component (p=0.049 on multivariate analysis) [231].

The work of all groups reported here demonstrate that the solid pattern is aggressive and associated with poor survival outcomes. The appreciation of poorer outcomes for patients with resected solid predominant adenocarcinoma may allow for the research and development of new postoperative adjuvant therapies. Such patients would have the biggest potential gains from a reduction in recurrence rates and improved survival outcomes.
Papillary Adenocarcinoma

The 1997 study from Silver and Askin contributed greatly to the definition of papillary AC [232]. They noted that many prior studies describing a papillary component in lung AC had lacked a definition. The aim of their study was to demonstrate that papillary AC was a separate entity in its own right, and not a variant of “BAC”. They defined papillary AC as “being characterized by the formation of papillary structures supported by central fibrovascular cores with complicated secondary and tertiary branches and tufts” that had to comprise at least 75% of the total tumour. They identified 31 cases in which follow up was available in 28 patients. In their series a history of smoking was common, seen in 86% of patients and the behaviour of disease was aggressive, with recurrence or metastases in 20 patients (71%) during follow up, with median time to recurrence of 14 months (range 1-100 months).

In the series from Yokose et al examining 200 small lung ACs of ≤30mm, tumours with a papillary component of >25% were associated with an unfavourable overall survival outcome on multivariate analysis (p=0.043), with vascular invasion being the only other significant factor [212]. As mentioned previously, Ohtaki et al found papillary ACs to have an intermediate prognosis, with similar outcome to acinar histology, better outcome than solid histology, and worse outcome than “pure BAC”. Motoi et al reported that papillary predominant ACs had better 5-year overall survival in stage I tumours compared to non-papillary predominant subtypes (95.8% vs 71.6%, p=0.047), a finding which is in contrast to that found in the study from Yokose et al. Finally Aida et al examined 147 AC specimens. Using the definition proposed by Silver and Askin, they did not demonstrate any positive or negative prognostic value of papillary histology in comparison with other AC types [232, 233].

These studies do not give a clear indication of the likelihood of relapse of tumours with a papillary pattern given their conflicting results.
2.1.3.1.7 Micropapillary Adenocarcinoma

Building on the description of papillary adenocarcinoma by Silver and Askin [232], Amin et al were the first to investigate the significance of the micropapillary pattern in pulmonary adenocarcinoma [234]. The micropapillary (MP) subtype had previously been associated with aggressive clinical behaviour and poor prognosis in primary tumours of other organs including the breast [235, 236], and urinary system [237, 238]. Amin et al noted that the proportion of MP in the overall tumour often seemed small, but that metastatic deposits frequently displayed this type of AC pattern [234]. MP AC differs from papillary AC in that the tiny tufts of MP pattern lack a fibrovascular core, consisting of clusters of malignant cells only. The micropapillary tufts can be seen apparently floating free in alveolar spaces or within variably thick walls of connective tissue [234, 239-248]. Since the recognition of this subtype, a large number of groups have looked retrospectively at pathological features and outcomes of patients with a MP component on pathologic examination of their surgical specimens.

The micropapillary pattern may only be present in a small proportion of tumour tissue. In a small series by Maeda et al no MP component was detected on preoperative biopsies, despite the pattern being found in the subsequent sample from surgical resection [246]. Exactly what constitutes micropapillary pattern adenocarcinoma has varied between studies, with some authors diagnosing MP pattern adenocarcinoma if there is greater than 1% of the primary tumour comprises an MP pattern [241, 245, 246], whilst some have used greater than 5% [234, 239, 242, 247] and still others greater than 10% [240, 248]. This has led to confusion in the literature and makes cross study comparisons difficult.

Important findings of various studies investigating MP AC are listed in Table 6. Three studies are case series, and as such do not include survival analysis other than to pass comment that the MP pattern appeared to have poorer outcomes compared to historical survival rates [234, 241, 242]. In most of the other studies overall survival for patients
with the MP subtype was worse when compared to an MP negative group. In addition, Zhang et al reported that as the amount of MP subtype increased in the tumour, the survival outcome was worse [245]. Patients with MP pattern AC were more likely to have lymph node metastases, pleural invasion, lymphatic invasion and vascular invasion. Three studies also found that recognition of MP pattern in surgically resected tumours frequently led to upstaging in comparison to tumours without an MP pattern – that is that the pathological stage on assessment of the operative specimen was greater than that expected on the basis of the preoperative clinical stage. It follows that the prognosis and use of adjuvant treatments may will also change [239-241].

One question that arises from these studies is the weight to be assigned to the pathologic stage by TNM versus the importance of the presence of the MP subtype. Four groups note that the survival from pathologic Stage I tumours is worse if the MP subtype is present than when it is absent [239, 240, 243, 248]. Of these, Miyoshi et al [239] and Makimoto et al [240] found that when lymph node spread had occurred (patients with pathologic stages II and III), there was no longer a survival difference based on the presence or absence of an MP component. Likewise, in their study of micrometastatic lymph node spread, Roh et al [247] found that the presence of micrometastatic spread predicted a worse outcome, while the MP subtype did not – MP tumours are more likely to spread, but their natural history once metastatic disease is established is not known from these studies. It may be that they metastasize early but are not necessarily more aggressive once stage IV disease is established. As such, it is not known whether recurrence after early stage tumour as compared to de novo stage IV disease with MP pattern has a poorer natural history. Furthermore, these papers did not set out to consider the influence of other poor prognostic subtypes such as solid AC with mucin production [200, 249]. It may be that as with early disease, in stage IV disease there is a cluster of “bad actors” with an aggressive phenotype (possibly MP and solid with mucin) as compared to those with a less aggressive phenotype (possibly acinar and papillary).
<table>
<thead>
<tr>
<th>First Author</th>
<th>Portion Positive*</th>
<th>Stages Included</th>
<th>Survival Outcome</th>
<th>Diff**</th>
<th>Lymph Node Metastases</th>
<th>Perineural Invasion</th>
<th>Lymphatic Invasion</th>
<th>Vascular Invasion</th>
<th>History of Smoking</th>
<th>Upstaging ***</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amin [234]</td>
<td>5%</td>
<td>Any MP - all stages</td>
<td>Poor - no comparator</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Miyoshi [239]</td>
<td>5%</td>
<td>All</td>
<td>Stage 1 - worse survival; Stage 2/3 - no difference</td>
<td>More common</td>
<td>More common</td>
<td></td>
<td>More common</td>
<td></td>
<td>More common in smokers</td>
<td>Yes</td>
</tr>
<tr>
<td>Makimoto [240]</td>
<td>10%</td>
<td>T&lt;20mm</td>
<td>Stage 1 - worse survival; Stage 2/3 - no difference</td>
<td>More common</td>
<td>More common</td>
<td></td>
<td>More common</td>
<td></td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Tsutsumida [241]</td>
<td>1%</td>
<td>&quot;Non BAC&quot;, T &lt;30mm</td>
<td>MP worse survival</td>
<td>Better‡</td>
<td>More common</td>
<td>No difference</td>
<td>More common</td>
<td>No difference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sanchez Mora [242]</td>
<td>5%</td>
<td>Path Stage 1</td>
<td>MP worse survival</td>
<td>Worse</td>
<td></td>
<td></td>
<td>More common</td>
<td></td>
<td></td>
<td>No difference</td>
</tr>
<tr>
<td>Kamiya [243]</td>
<td>Any</td>
<td>All stages</td>
<td>MP worse overall MP worse in Stage 1</td>
<td>More common</td>
<td>More common</td>
<td>More common</td>
<td>More common</td>
<td>More common</td>
<td>More common in smokers</td>
<td></td>
</tr>
<tr>
<td>Kuroda [244]</td>
<td>Any</td>
<td>All stages</td>
<td>Poor - no comparator</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Zhang [245]</td>
<td>1%</td>
<td>All stages</td>
<td>MP worse survival†</td>
<td>Worse</td>
<td>More common</td>
<td>More common</td>
<td>More common</td>
<td>More common</td>
<td>More common in smokers</td>
<td></td>
</tr>
<tr>
<td>Maeda [246]</td>
<td>1%</td>
<td>Clinical Stage 1</td>
<td>Poor - no comparator</td>
<td></td>
<td></td>
<td>More common</td>
<td>More common</td>
<td>More common</td>
<td></td>
<td>No difference (small sample)</td>
</tr>
<tr>
<td>Roh [247]</td>
<td>5%</td>
<td>Path Stage 1</td>
<td>Worse if micrometastases found No difference if MP</td>
<td></td>
<td>Micromets more common</td>
<td></td>
<td>More common</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Kawakami [248]</td>
<td>10%</td>
<td>pT1</td>
<td>Path Stage 1 - MP worse survival</td>
<td></td>
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</table>

Table 6: Papers describing the pathological findings and behaviour of the micropapillary subtype of adenocarcinoma of the lung

* Smallest portion of tumour displaying MP subtype to be considered significant  ** Tumour differentiation† Survival worse with increasing proportion of MP subtype in the tumour  *** Presence of a micropapillary component associated with a higher pathologic stage than anticipated on the basis of preoperative clinical staging  ‡ Better differentiation a function of author’s definition
2.1.3.1.8 Invasive Mucinous Adenocarcinoma

The addition of the category IMA to the IASLC/ATS/ERS guidelines provides for a more appropriate classification of tumours [179]. Previously this type of pulmonary adenocarcinoma was described as mucinous BAC. The WHO classifications of Lung Tumours in 1999 and 2004 recognised three groups of BAC – non mucinous, mucinous and mixed (combining portions of the first two types) [195, 196].

The medical literature from the 1940s onwards includes many articles on BAC. In 1984 Manning et al first discussed BAC as being of two major subtypes [250]. “Type 1” cells consisted of “tall columnar cells with basally located bland nuclei; intracellular and extracellular mucin are abundant”. “Type 2” cells were noted to “vary from columnar to cuboidal with varied sized dark-staining nuclei with enlarged nucleoli” and minimal mucin production. Of the 42 cases they reviewed, nine were of “type 1”, 25 were of “type 2” and eight were a mixture of both. Patients with “type 1” cells were more likely to present with non-localised disease both clinically and on pathologic grounds. Overall survival for patients with “type 1” disease was significantly worse than those with “type 2”, with the caveat that the study was only of small numbers, and there was no control for other factors influencing survival. “Type 1” cells represent the old definition of mucinous BAC, now known as IMA and “Type 2” cells represent either Clara cells or pneumocytes which are the putative cells of origin of non-mucinous lepidic growth. For reasons that aren’t clear this work wasn’t further acknowledged in the literature of the day resulting in a delay in the widespread recognition of the clinical and pathologic difference between IMA and non-mucinous lepidic patterns.

Two subsequent papers examined the survival of patients with historically defined mucinous BAC in comparison to non-mucinous BAC [217, 251]. Furak et al failed to find a difference in 5-year overall survival between mucinous and non-mucinous BAC, while finding the outcome of patients with mixed BAC was significantly worse [217]. However, this study did not adopt a strict definition of BAC, and allowed patients with up to 50% of the surgical specimen containing a recognised invasive non-BAC pattern. Casali et al studied 40 patients with surgically resected BAC, representing 3% of all surgical resections for NSCLC in their
centre over the study period [251]. Five-year overall survival was significantly worse for patients with mucinous or mixed BAC compared to non-mucinous BAC (p=0.021). In multivariate analysis, the histologic type maintained significance, along with the clinical stage (stage IA/IB vs higher stages).

In 2008 Garfield et al published a paper arguing that there were two types of “BAC” that should be recognised [252]. They made this assertion on the basis that there were differences between “mucinous” and “non-mucinous” BAC beyond the differences in morphological appearance. They summarised the immunohistochemical findings from 10 papers. Tumours from both mucinous and non-mucinous BAC stained positively for cytokeratin-7. Thyroid transcription factor-1 staining was seen in only 24% of tumours of mucinous BAC subtype in comparison to 88% of tumours with non-mucinous BAC. Conversely, cytokeratin-20 staining was seen in 53% of tumours with mucinous BAC, but only 3% of patients with non-mucinous BAC.

The evidence provided by these papers demonstrated that IMA was a distinct entity by morphology, ancillary tests and clinical behaviour, thus justifying its’ separate recognition in the IASLC/ATS/ERS classification scheme.

2.1.3.1.9 Variant Subtypes

Along with IMA, three other uncommon forms of invasive AC are included in the IASLC/ATS/ERS classification [179]. These are the colloid, enteric and fetal adenocarcinoma variants.

Colloid ACs demonstrate “extracellular mucin in abundant pools” with destruction of the alveolar architecture. Mucin-producing tumour clusters are seen within the pools of mucin and also lining the walls of some cystic spaces [253, 254]. Rossi et al report a series of 13 patients from their centre with colloid AC. In their series colloid AC accounted for 0.24% of resected lung cancers. Eleven of these patients (84.6%) were alive and disease free at a
median follow up of 26 months (range 9 – 95 months) [253]. They suggest that there are two
types of colloid adenocarcinoma on the basis of cells types with either signet ring or goblet
cell morphology and differential staining for the IHC stains CDX2 and MUC2. All 11
patients with goblet cell type tumours which were CDX2 and MUC2 positive were alive and
well. However, the two patients with signet ring type tumours which were CDX2 and MUC2
negative suffered early disease recurrence and died despite surgery and adjuvant
chemotherapy. Rossi et al refer to papers published by 12 other groups reporting minimal to
no mortality from this condition. The notable exception is from an early series of 19 patients
by Moran et al. They presented 24 cases of colloid AC, and of the 19 patients with clinical
follow up available eight had died of disease [254].

Enteric ACs generally present as solitary lung lesions with or without spread to regional
lymph nodes. Pathologically they may be indistinguishable from a colorectal metastasis on
the grounds of light microscopy alone. The heterogeneity that is seen in pulmonary AC is
frequently present in pulmonary enteric ACs and may act as a clue to the correct diagnosis.
Clinical investigations must fail to find a primary tumour in the large bowel. Reports and
series of enteric ACs are uncommon, and as such it is not possible to provide a reliable
estimate of prognosis [255-257]. Variable patterns of immunostaining can be seen for
thyroid transcription factor-1 (TTF-1), cytokeratin-7, cytokeratin-20 and CDX2 (a marker
which is frequently expressed in colorectal carcinomas) [255, 256]. Generally metastatic
colorectal tumours positively stain for cytokeratin-20 and CDX2, and negatively for TTF-1
and cytokeratin-7. Li et al do report one case where the features on light microscopy staining
pattern for the pulmonary enteric AC was identical to that typically seen in metastatic
colorectal carcinoma, with the diagnosis only being made on clinical grounds [257].

Fetal ACs are well differentiated tumours with tubular growth that resemble fetal lung.
Patients with fetal ACs tend to be of younger age, present with a smaller primary tumour, are
unlikely to have lymph node metastases, and generally have good survival [258-260].
2.1.3.2 Evidence supporting the new IASLC/ATS/ERS classification for lung adenocarcinoma in resected disease

The IASLC/ATS/ERS classification in the case of resected disease provides a new framework for pathologists and clinicians to communicate with the aim of improving patient management, predicting patient outcomes and facilitating clinical research. A large number of groups have now presented their results for survival outcomes on the basis of the new classification.

2.1.3.2.1 Survival Outcomes in resected Stage I disease

The number of groups reporting the prognostic impact of the IASLC/ATS/ERS classification for resected stage I pulmonary adenocarcinoma continues to increase. Of the papers reporting on overall survival no differences were seen. This may be a reflection of the available sample sizes in each of the studies, low rates of recurrence for some subtypes, the effect of post relapse therapies, or the duration of follow up. The most important outcomes reported related to relapse free survival. For patients with resected AIS or MIA outcomes were excellent with recurrence free survival at or approaching 100% [199, 261-268].

Three studies found no survival differences based on the IASLC/ATS/ERS classification in stage I disease. One paper was small (n=42) and primarily focused on pathologic-radiologic correlates [269]. A larger study from Yeh et al included 212 patients. Most patients in their study had acinar (34%), papillary (42%) or solid predominant tumours (19%). Five year DFS rates were 70.4%, 69.3% and 61.2% respectively [270]. A further study from Ito et al demonstrated good prognostic outcomes for patients with AIS or MIA, but was less informative in regard to patients with invasive adenocarcinoma subtypes [262]. Interestingly in this Japanese cohort 92 of the 188 patients had AIS or MIA. Only the T stage for patients with invasive disease (T1a vs T1b) was of prognostic significance in multivariate analysis.
The majority of studies found correlations by pathologic subtype with survival outcomes. There were a variety of statistical methods used to arrive at conclusions in each of these papers, but a number of common themes were reported. Ujiie et al reported on the largest cohort of patients to date (n=1120) who underwent treatment at Memorial Sloan Kettering Cancer Centre (MSKCC) [267]. The authors grouped tumours by clinical behaviour into low grade (AIS, MIA or LPA), intermediate grade (acinar or papillary) and high grade (solid, MPA, IMA or colloid). They found that the 5-year cumulative index of recurrence (CIR) was 5.5% (95% CI 2.8-10.8%) for low grade tumours, 16.6% (95% CI 13.9-19.9%) for intermediate grade tumours and 30.7% (95% CI 25.3-37.2%) for high grade tumours. Kadota et al had previously published a paper from MSKCC covering a similar time frame [261].

With regards to “low grade tumours” there was a difference seen for 5-year CIR. The rate for patients following resection of LPA (n=103) was 8% as compared to patients following resection of AIS or MIA (n=36) at 0% (p=0.003).

Most studies pooled pathologic subtypes with similar survival outcomes to improve the statistical power in their cohorts. Song et al compared patients with solid or micropapillary predominant adenocarcinoma to all others. They found worse outcomes for solid/MPA for both DFS (HR 2.18, 1.34 – 3.54, p=0.002) and OS (HR 2.45, 1.19 – 5.07, p=0.015) [263]. These results were similar to those of Yoshizawa et al who found that 5 year DFS rates fell from 100% for AIS/MIA to 84% for LPA, acinar and papillary predominant tumours and 71% for solid, MPA, colloid and IMA tumours (p<0.001) [199]. Woo et al also pooled histologic subtypes with similar outcomes finding a fall in 5 year DFS from 100% in AIS and MIA to 86.1% in LPA, acinar and papillary subtypes to 50.2% in solid, IMA and MPA tumours [264]. They reported that the high grade tumours (solid, IMA and MPA) had significantly higher rates of relapse on multivariate analysis (HR 3.66 [95% CI 1.42 – 9.44], p=0.007) along with vascular invasion (HR 3.72 [95% CI 1.42 – 9.78], p=0.008).

Murakami et al examined outcomes for 347 patients with stage IA tumours following surgical resection [268]. Five year DFS outcomes for invasive adenocarcinomas fell from 99% for LPA, to 82% for acinar, 81% for papillary, 74% for solid and 33% for MPA. Each invasive subtype had significantly worse DFS outcomes on univariate analysis compared to that which was used as the reference subtype (LPA). Using LPA as a comparator was unfortunate given
that all other subtypes were going to be found to be significant given the large difference in relapse rates, and using acinar as the reference subgroup would have been more informative. In the multivariate analysis the lepidic predominant subtype was compared to all other invasive subtypes. Not surprisingly this was statistically significant but otherwise somewhat uninformative. Yanagawa et al presented outcomes for 191 patients [265]. Interestingly they did not find any cases of MPA in their cohort. Five year DFS was lowest for patients with solid predominant tumours at 54%, with the next closest group being papillary predominant at 85.4%. DFS outcomes were significantly worse for solid vs non-solid tumours on multivariate analysis (HR 4.08 [95% CI 1.59 – 10.5, p=0.003]). Yang et al grouped 177 patients with resected T1aN0 cancers into LPA/Acinar/Papillary and Solid/MPA groups [271]. Aggressive subtype was the only significant risk factor for relapse on multivariate analysis (HR 2.81 [95% CI 1.24 – 6.40], p=0.013).

Zhang et al found that patients with papillary predominant tumours had 5-year DFS rates (65.6%) similar to patients with solid predominant tumours (66.7%) and thus their groupings consisted of AIS/MIA, LPA/Acinar and Papillary/Solid/MPA. This led to 5-year DFS rates of 100%, 85.2% and 62.8% respectively (p<0.001) [266]. Sun et al presented results for 136 patients with stage IB disease [272]. They chose to analyse their data by comparing the patients in each predominant subtype to all other patients who were not in that subtype. Using this method, patients with LPA had better 5-year DFS outcomes compared non-LPA patients (75.2% vs 50.8%, p=0.042) while those with MPA (28.4% vs 61.1%, p=0.041) and solid predominant tumours (36.7% vs 57.7%, p=0.049) had significantly worse outcomes. On multivariate analysis only MPA remained significant as a prognostic factor.

In summary, survival outcomes for patients with AIS and MIA are excellent across all studies examining patients with resected stage I adenocarcinoma. The majority of studies have shown differences in disease free survival by histologic subtype with a limited number finding no such difference. Patients with LPA and papillary predominant tumours have showed variable survival outcomes across the reported studies. Solid and micropapillary predominant tumours have the worse outcomes in regard to relapse following surgery for patients with pathologic stage I disease.
2.1.3.2.2 Survival Outcomes in Cohorts Including All Stages of Disease

Eight papers have now reported survival outcomes for patients following resection with all stages of disease, plus a further two smaller studies restricted to patients with stage III disease only. The majority of studies found some association between the IASLC/ATS/ERS classification and survival outcomes, with only two groups not demonstrating any significance for the classification.

The three largest cohorts reported all had over 400 patients each and originated from Taiwan [273], Germany [249] and France [274]. Hung et al analysed a cohort of 573 patients consisting of 6% LPA, 34% acinar, 27% papillary, 20% MPA and 13% solid predominant adenocarcinoma [273]. Rates of recurrence were higher for patients with MPA and solid predominant adenocarcinomas (p<0.01). There was no interaction between the predominant subtype and the location of relapse (local vs distant). The predominant subtype (LPA/acinar/papillary vs MPA/solid) was significant on multivariate analysis for OS (HR 1.6 [1.1 – 2.3], p=0.01), freedom from recurrence (HR 1.5 [1.1 – 2.0], p=0.02) and disease specific survival (HR 2.3 [1.5 – 3.6], p<0.01), with the stage (I vs II/III) and size of invasive tumour also being significant across these three variables.

Warth et al assessed a group of 500 patients with findings that were slightly different to Hung et al [249]. In this cohort the distribution of subtypes was: LPA 8%; acinar 42%; papillary 5%; MPA 7% and solid 38%. In Warth’s paper the predominant pattern was significant for OS (p=0.007), disease specific survival (DSS) (p=0.011) and DFS (p=0.002). However, in this cohort the median OS outcome for patients with papillary predominant tumours (49 months) was similar to those of the solid (58 months) and MPA (45 months) subgroups rather than being like the acinar subgroup (67 months). The LPA subgroup continued to have the best outcomes (median OS 78 months). The authors raised the important question as to whether the papillary subgroup should be assessed as an intermediate prognostic group as seen in other papers or as a poor prognostic subgroup as found in their study. The descriptions in this literature review may imply that distinction of the various proportions of
adenocarcinoma patterns is a simple matter, whereas in pathologic practice it can be very challenging in some tumours. Significant difficulties may be found in distinguishing between subtypes. In this study Warth et al found difficulties arose when trying to distinguish between papillary and micropapillary patterns. Dr Warth and his colleague Dr Weichert examined all specimens at a double-headed microscope and came to a consensus as to the predominant pattern. It is possible that the readers may have influenced each other at times with regards to the predominant pattern they observed. An alternative approach may have been to allow for separate assessment by each reader with subsequent examination together for samples where there were discordant opinions on the predominant subtype.

Mansuet-Lupo et al presented the third largest study from France with 407 patients [274]. In this group there was one case of MIA, with acinar accounting for 47%, solid 27%, and papillary 18%. There were eleven cases of LPA, four of MPA, sixteen with IMA and two with solid with signet ring patterns. LPA, acinar and papillary were considered intermediate grade with all others being high grade (with the exception of MIA). On multivariate analysis the intermediate group had significantly better 5 year OS (58.6% vs 42.4%) and 10 year OS (46.1% vs 21.4%). Age and pathologic stage were also significant in multivariate analysis.

Five smaller studies ranging from 152 patients up to 292 patients have also been reported. Two of these studies did not find a statistical difference in survival by the predominant subtype. In the paper from Westaway et al (n=152) the pathologic stage was significant for survival outcome [275]. Despite pooling the histologic subtypes the OS outcomes did not reach statistical significance (5 year OS for LPA 71%; papillary/acinar 38%; solid/MPA 43%, p=0.16). Urer et al presented a cohort of 226 patients where no survival differences were found [276]. They did not have any cases of AIS/MIA or MPA in their cohort. The survival curves in this paper for intermediate grade (LPA, acinar and papillary) and high grade (solid, MPA, IMA and colloid) overlapped suggesting no prognostic benefit for the adenocarcinoma classification. However, this cohort differs from other presented cohorts with a largely male group (86%) compared to the relatively even distributions of sex in other papers. As the results of the papers from Westaway et al and Urer et al are at odds with all other papers it is prudent to ask if there were difficulties in recognising the predominant patterns, or if these are true results.
Three of the studies did find significant survival outcomes across stages I, II and III disease. Gu et al assessed outcomes for 292 patients with predominantly stage I disease (65%) [201]. In multivariate analysis advanced stage (stage III or IV) was the most significant determinant for both DFS and OS while EGFR wild type status was also a significant factor but for OS outcomes only. Having a solid or MPA tumour as compared to other subtypes was associated with poorer DFS (HR 1.57 [95% CI 1.02 – 2.41], p=0.038) and poorer OS (HR 1.81 [1.04 – 3.16], p=0.037). Von der Thusen et al had similar findings [277]. The stage remained the most significant prognostic survival factor. Better survival outcomes were associated with the LPA subtype while worse outcomes were associated with the solid subtype. The MPA subtype did not reach statistical significant however the number of patients found in this group was small (n=9). It was noted that the frequent nodal involvement for patients with the MPA subtype was consistent with the aggressive nature of this pattern noted in other studies. Russell et al published one of the first papers to associate the IASLC/ATS/ERS classification with survival outcomes [200]. The importance of the histologic subtype was significant in both univariate analysis (p<0.008) and multivariate analysis (p<0.045). Grouping cases into similar survival outcomes with good (AIS/MIA/LPA; 93% 5-year OS), intermediate (acinar and papillary; 68% and 71% respectively) and poor (solid and MPA; 39% and 38% respectively) improved the strength of the statistical association between subtype and overall survival in both univariate (p<0.001) and multivariate (p<0.001) analysis. Those patients whose tumour was IMA (n=9) or colloid predominant (n=10) had a 5-year OS of 51% each.

Two papers have limited their assessment to patients with node positive disease. The larger of these studies from Russell et al looked at a group of 69 patients with involvement of N2 nodal station(s) following surgical resection [278]. In this cohort the median survival was significantly better for patients with acinar predominant tumours compared to the pooled group of solid, MPA, papillary and colloid predominant tumours (HR 0.45 [95% CI 0.22 – 0.91], p=0.026). The poor survival outcomes for papillary predominant tumours may occur to several reasons. It may reflect difficulties with classification (as previously discussed in relation to the study of Warth et al [see page 93]), it may be a function of the small sample size in this subgroup (n=6), or it may be a result significantly heterogeneity in morphology and behaviour of the papillary subtype (as recently suggested by Warth et al in a separate
study [279]). The paper from Suda et al had a smaller cohort of 24 patients with pathologic involvement of N1 or N2 nodes following resection [280]. Again the group of patients with solid or MPA tumours had a worse survival outcome compared to those with papillary or acinar predominant tumours on multivariate analysis (HR 12.7 [95% CI 1.2 – 142.9], p=0.037). A common finding from both papers is that for patients with acinar predominant tumours the lymph node metastases are of solid or micropapillary subtype, while those patients with MPA or solid predominant tumours retain this pattern at the metastatic site [278, 280]. This suggests that the micropapillary and solid components of tumours present the histologic subtypes with the greatest metastatic potential.

The previously mentioned papers in both stage I disease and across all stages were able to assign all patients to a predominant subtype. At odds with this is a study from Rekhtman et al [281]. This study was primarily focused on the association between predominant AC subtypes on oncogenic mutations. As expected, significant heterogeneity was seen in the resection specimens, with 162 / 180 (90%) of tumours showing between two and six patterns (mean three). In attempting to assign a predominant pattern they found that 53 / 180 specimens examined (29%) had two or more patterns present in similar proportions, which they called “co-dominant”, and therefore they were unable to assign a predominant pattern. The reason why these authors had such trouble is unclear, as it has yet to be reported by anyone one else in the breadth of publications discussed in this literature review.

While the data supporting the IASLC/ATS/ERS classification is compelling, there are some limitations to the studies discussed above. The data in all studies has been generated from retrospective analysis, with most papers being generated from single centres. Assessment and reclassification of resection specimens has necessarily not been contemporaneous to their acquisition. The largest study from Ujiie et al (n=1120) has limited itself to stage I disease [267]. However the studies across multiple stages of disease have had smaller numbers, and risk the loss of statistical power to find important differences. In order to maintain such power pooling of predominant subtypes with similar outcomes has occurred during analysis (for example – AIS, MIA and LPA; acinar and papillary; and micropapillary and solid). In comparison, the efforts to update the TNM staging to the eighth edition have been undertaken with significant cooperation [51]. Patients have been included from all continents (excluding
Antarctica), with data being provided on over 94 000 patients globally. The large number of patients subsequently analysed from the cohort allows multiple analyses to be performed and prognostically significant data extracted without compromising statistical power due to multiple analyses. Data on treatments that may have effected outcome (surgery, radiotherapy and chemotherapy) are more reliably collected. A similar effort based on the IASLC/ATS/ERS adenocarcinoma classification would provide more certainty with regards to itself prognostic and predictive utility, and facilitate increased use of the results provided by pathologists to clinicians in their decision making.

In summary, the most recent revision of the WHO classification from 2004 failed to capture the importance of the heterogeneity of resected pulmonary adenocarcinoma [207]. Astute observations from many clinicians involved in the treatment of lung adenocarcinoma led to recognition that some patients had an excellent prognosis following surgery, while others had high rates of recurrence. The result of a culmination of the work from a number of researchers and research groups led to the development of the IASLC/ATS/ERS classification for pulmonary adenocarcinoma [179]. The majority of studies cited in this section have shown that the IASLC/ATS/ERS classification provides prognostic information over and above that of stage alone, hence providing justification for the system to be incorporated into the 2015 edition of the WHO classification [44]. It is important to note, however, that differences exist between the papers discussed above, including the proportion of each subtype observed in the various studies. This raises the question posed in the next section (2.1.3.3).

2.1.3.3 Is the new IASLC/ATS/ERS Classification Reproducible in Resection Specimens?

For a classification system to be adopted it has to reproducible such that the same descriptor is applied to a sample regardless of where a sample is assessed, such that the report can be relied on to inform further decision making with regards to patient care. To date three studies have been published looking at this question with regards to the IASLC/ATS/ERS adenocarcinoma classification in resected specimens. Warth et al undertook a study to investigate the degree of inter- and intra-observer variability amongst specialist pulmonary
pathologists, as well as general and trainee pathologists in applying the IASLC/ATS/ERS criteria [282]. Participants were provided with a copy of the classification article [179]. Slides from 100 cases following surgical resection were selected, and the participants reviewed all diagnostic slides from each case. Participants were not aware of the survival outcomes of the patients whose tumours were involved in the study. Statistically significant agreement was found, with moderate to substantial concordance on the predominant pattern across pathologists (Solid Kappa [K] 0.86; LPA K 0.78; acinar K 0.61; MPA K 0.60; papillary K 0.58; p<0.001). Results for pathologists in training showed lower concordance rates, but these improved after a dedicated training session and repeat review. The authors found that concordance was lower if more slides were available for review for a case. Agreement on papillary compared with micropapillary subtype as the predominant pattern was found to be the most troublesome area for pathologists, given that these subtypes are frequently admixed.

Thunnissen et al investigated interobserver variability amongst a group of 26 specialist pulmonary pathologists across three continents [283]. Participating pathologists provided photomicrographs of six cases both at low magnification and high magnification – one case each representing the five predominant invasive patterns (lepidic, acinar, papillary, micropapillary and solid with mucin) and one case the pathologist considered “difficult”. For typical patterns there was good agreement between participants (K 0.77 +/- 0.07), with lower agreement for “difficult” cases (K 0.38 +/- 0.14). When assigning the predominant pattern rates of agreement was greater than 92% for all subtypes except MPA (62%). The authors commented that difficulties in distinguishing between papillary and micropapillary patterns are “self-evident”, presumably due to some similarities between these patterns. The reasons for the difficulties in distinguishing between lepidic and acinar/papillary patterns are less obvious, but may include the superimposition of tumour on normal tissue, the angle at which slides are cut, underlying distortions in architecture due to other pathologies such as emphysema, and variations in fixation. The authors acknowledge that the use of photomicrographs was a limitation of this study; however this was done to allow timely completion of the research. Furthermore, the use of “classic” examples is not completely reflective of the ambiguities in clinical practice.
Duhig et al have reported a third study which assessed agreement on histologic subtyping in the Australian context [284]. In this work five anatomical pathologists reviewed the slides from 176 cases of resected pulmonary adenocarcinoma. Intraclass correlation coefficients ranged from 0.87 to 0.98 indicating excellent agreement between the reviewers of the slides.

Despite the good reproducibility reported in the previously mentioned papers differences in the proportions of the predominant subtypes differ across the papers mentioned. As mentioned above there is cross over between patterns (papillary, micropapillary, lepidic and acinar) and where the relative proportions are assigned in 5% increments may affect the predominant subtype assigned. In addition regional variations between pathologists in the interpretation and application of the IASLC/ATS/ERS may in part explain some of the differences – this will take on increasing importance as the classification moves from being a prognostic tool to one which is used to decide on subsequent therapies (P Russell, personal communication [285]).

The classification of adenocarcinoma subtypes as now incorporated into the WHO 4th edition marks a significant change in an existing classification system, and as such training of pathologists will be required [44]. Such training could occur through a number of routes including academic or society publications such as journals, training workshops, conferences and meetings. Increasing use of the classification is likely to lead to its refinement over coming years as wide experience in its application is gained and further research is done (P Russell, personal communication [286]).

2.1.4 Adenocarcinoma Classification in Advanced Disease

Until recent years a diagnosis of NSCLC was sufficient for clinical care in advanced lung cancer, with the major point of clinical concern being differentiation from small cell lung cancer. Risks of potential toxicity with bevacizumab for patients with SqCC [191, 192], the reduced of efficacy with pemetrexed in SqCC [100, 190], and the recognition of targetable oncogenic mutations in adenocarcinoma have led to a paradigm shift in the accurate
classification of NSCLC types in advanced disease [287]. As the pulmonary squamous cell carcinoma genome is described, it is possible targetable mutations will also be found in that condition. The need for detailed pathologic classification as it relates to the efficacy of immunotherapeutic drugs such as nivolumab is not yet known. There appears to be a difference between the importance of PD-L1 staining in predicting response to nivolumab for patients with AC and SqCC of the lung as seen in the Checkmate-017 and Checkmate-057 trials [159, 160], however whether this holds true for other PD-1/PD-L1 monoclonal antibodies/IHC companion diagnostic antibodies remains to be seen.

In clinical care the acquisition of tissue is performed to further patient management and guide treatment choices. As such it was traditionally acceptable to make a diagnosis based on a very small sample such as a sputum sample or a fine needle aspiration. The prior WHO classifications (1st to 3rd editions) only address classification of diagnostic entities as it pertained to resection specimens [193-196]. These editions provided no uniform method for reporting NSCLC in small biopsy and cytology specimens. This is despite that fact that the majority of patients present with lung cancer in the advanced setting where surgical resections of disease are rarely performed. The lack of reporting arose due to the lack of a therapeutic need to report such differences.

In the setting of advanced lung cancer a curative outcome (freedom from cancer) has generally not been possible. Therefore, diagnostic specimens are obtained with the aim of obtaining enough tissue to guide treatment choices while picking the least invasive method to safely arrive at a diagnosis. As such, diagnostic samples are usually obtained by methods that aim to preserve quality of life and avoid morbidity prior to treatment while allowing timely assessment and early implementation of treatment, given historically short survival times. Whether the subtype of adenocarcinoma has any bearing on outcomes, as it does in early stage disease, is not yet known.

For the first time the IASLC/ATS/ERS classification makes recommendations for classification of NSCLC in small biopsies and cytology [179]. Where classic morphologic features are present on light microscopy the diagnosis can be made with confidence. If the
morphologic findings are indeterminate, a limited immunohistochemical panel can be used to arrive at a diagnosis of NSCLC-favour adenocarcinoma (due to positive IHC staining with TTF1 or Napsin A and negative staining for p40 or p63), or NSCLC-favour squamous cell carcinoma (due to positive IHC staining with p40 or p63 and negative staining with TTF1 or Napsin A) [288]. If both markers are present in different cell populations within the small biopsy specimen a diagnosis of NSCLC – Not otherwise specified (NOS) – possible adenosquamous carcinoma can be made. If a tumour is found to be negative for lineage-specific immunohistochemical markers of both adenocarcinoma and squamous differentiation it is recommended to make the diagnosis of NSCLC-NOS.

Furthermore the IASLC/ATS/ERS classification system also recommends that the morphologic pattern(s) of adenocarcinoma, if recognised, in small biopsy specimens should be stated where possible [179]. The recognition of morphologic patterns does not require any further new testing beyond the standard haematoxylin and eosin (H&E) slide(s) required for pathologic diagnosis. The disadvantage of providing morphologic subtyping is the significant expertise and time to undertake it properly. Therefore, the authors of the new classification posed the following research question:

In specimens from metastatic sites, is there any clinical significance to recognizing histologic patterns, including the predominant pattern? [179]

This chapter of my thesis aims to address the above question.
2.2 Materials and Methods

2.2.1 Inclusion Criteria

As stated, the aim of this work was to examine the clinical relevance of pathologic subtypes of pulmonary adenocarcinoma at metastatic sites. Further, we also wished to test for mutations in *EGFR* and *KRAS* to see if there were correlations with the histologic patterns observed. Therefore large tissue samples were required in order to allow for accurate assessment the patterns present and to provide adequate samples for molecular testing. Samples obtained from the lung were not included as the predominant subtype from the primary tumour is occasionally discordant with most abundant subtype found in N2 lymph nodes when nodal metastases are assessed or at distant sites at relapse [278, 280, 289].

In routine clinical practice there is no “gold standard” site to biopsy. The choice site for diagnosis is basis on a number of factors including the clinical expertise available, the accessibility of sites to biopsy to obtain tissue, the potential morbidity of the planned procedure, and the likelihood of acquiring sufficient tissue for diagnosis and any further testing required (including molecular testing). Mediastinal nodal disease was accepted as a metastatic site for the purposes of this study given that such tumour cells have to develop sufficient invasive potential to leave the primary tumour and spread to a non-continuous site.

The following were set as the inclusion criteria:

- Tumour tissue obtained sampling of a metastatic site for any purpose (diagnosis, therapeutic procedure [for example resection of brain metastases, fixation of a pathological fracture, biopsy at the time of pleurodesis], or staging). Patients with stage III disease were included if they underwent pathologic sampling via mediastinoscopy but who did not subsequently proceed to surgical resection.

- Samples collected in the years 2000 to 2010 inclusive.

- Histologic diagnosis of adenocarcinoma consistent with a pulmonary origin. Adenocarcinoma was defined as a malignant epithelial tumour with histopathologic
patterns including acinar, papillary, micropapillary and solid with mucin as defined by the IASLC/ATS/ERS classification [179]

- Clinical presentation consistent with primary lung carcinoma following review of the medical records
- Presence of the pathologic specimen within the archives of the Department of Anatomical Pathology at St Vincent’s Hospital, Melbourne

At the time of histological assessment cases were excluded if:

- The histologic diagnosis was not that of adenocarcinoma (in some cases the original diagnosis of adenocarcinoma was found not to be correct, while other cases reviewed with an original recorded diagnosis of “NSCLC” were found not to be adenocarcinoma)
- There was limited available tissue for accurate assessment of pathologic subtype on microscopy
- If there was significant crush artefact present precluding clear recognition of adenocarcinoma subtypes making it impossible to assign a major histologic pattern (see below)
- If there was insufficient tissue to allow for molecular testing

Ethics approval was obtained from the Human Research Ethics Committee (HREC) of St Vincent’s Hospital, Melbourne. A low risk ethics approval was granted that did not require obtaining of consent as the majority of research subjects were expected to have died from their cancer diagnosis, no new procedure or intervention was required and the outcomes of the research work were not anticipated to impact the patients’ relatives.
2.2.2 Identification of Potential Cases

This research work focused on outcomes for patients with lung adenocarcinoma undergoing surgical sampling at metastatic sites – either from regional N2 or N3 lymph nodes or from distant metastatic sites. Surgical samples were acquired either as part of a therapeutic procedure (e.g. resect of a symptomatic metastatic deposit) or as part of a diagnostic or staging procedure (e.g. mediastinoscopy). Samples could be obtained at any time during the course of the patients’ illness – not just from the time of diagnosis. Pathological specimens were required to be within the archives of the Department of Anatomical Pathology at St Vincent’s Hospital Melbourne. As the anticipated number of available cases was small several strategies were used to maximise the likelihood of obtaining a reasonable sample size.

The first search strategy involved use of International Classification of Diseases for Oncology 3rd edition (ICD-O-3) morphology codes to search database of the Department of Anatomical Pathology at St Vincent’s Hospital. This search was performed by Associate Professor Prudence Russell, Anatomical Pathologist with an interest in lung cancer pathology, who was familiar with the database. The following morphology codes were searched for within the database:

- Adenocarcinoma – 8140/3
- Metastatic Adenocarcinoma – 8140/6
- Carcinoma not otherwise specified – 8010/3
- Large Cell Carcinoma – 8012/3

These morphology codes were cross searched against the topography codes for the following organs – lymph node (T08); bone (T1X); pleura (T29); brain (TX2); and meninges and ventricles (TX1). The organ codes for skin (T01 and T02) were added after a case of resected skin metastasis was discovered in the archive. The organ codes are assigned as per the “Systematized Nomenclature of Medicine Clinical Terms” (SNOMED CT) [290]. This system is used to improve the clinical coding of cases across health care institutions in a
standardised manner, allowing the future collection and analysis of data with the aim to improve patient care and outcomes.

The second method involved review of a database of patients with stage IV NSCLC collected for a previous research project by the research candidate. This database was developed by identification of patients in the St Vincent’s Hospital Melbourne multidisciplinary tumour database (iMDT). iMDT is a prospectively collected database of patients with malignancy with input from the Department of Medical Oncology together with other medical and surgical specialties that manage patients with cancer. The data is collected within a Microsoft Access database and is searchable. The iMDT database was searched for the following International Classification of Diseases (ICD)-O-3 morphology codes:

- Adenocarcinoma – 8140/3
- Large Cell Carcinoma – 8012/3
- Non-Small Cell Carcinoma – 8046/3
- Squamous Cell Carcinoma – 8070/3

These terms were cross-referenced with the organ specifier for lung (T28) which is used in the iMDT database to denote the organ of origin of the tumour and the stage variable 4 (that is stage IV disease). The stage IV NSCLC database contained 521 patients who had had collection of clinical data which included histological subtype as recorded at the time of diagnosis and various treatments including surgical procedures. The stage IV NSCLC database was designed to only include patients who were managed under the Medical Oncology Department of St Vincent’s Hospital from the time of diagnosis. Cases were selected for review as part of the current project if they contained any of the collected histologic diagnoses with the exception of SqCC and they had been recorded as having undergone a surgical procedure.

The third strategy used was review of records from multidisciplinary team (MDT – tumour board) meetings conducted at St Vincent’s Hospital Melbourne. The meeting records for the lung MDT and central nervous system MDT had the highest yields for collecting cases. The
records of any patients having been recorded as undergoing mediastinoscopy for lymph node sampling or resection, or any other surgical procedure in the course of lung cancer management were reviewed. As this was the final strategy used the number of additional collected cases was small.

Potential cases identified by the search strategy were unified into a single list. All available slides and blocks for each case were retrieved from storage from the archives of the Department of Anatomical Pathology. The suitability of each case was then further assessed as per the criteria set out in section 2.2.1 and a final decision was made on whether the case could be included in the study. (Data on the number of cases obtained by each strategy were not captured at the time, and unfortunately are not able to be reconstructed retrospectively).

2.2.3 Clinical Data Collection

The medical records of St Vincent’s Hospital, Melbourne were reviewed to confirm that the clinical diagnosis was consistent with pulmonary adenocarcinoma. Data was collected on all included cases and included the following:

- Relevant dates – date of diagnosis; date of the surgical procedure; date of last follow up or date of death
- Demographic information – age at diagnosis; sex; smoking history (current, former or never smoker [as per convention the definition of a never smoker was a lifetime consumption of 100 or fewer cigarettes])
- ECOG PS at diagnosis
- Treatments used including systemic therapy, surgical intervention(s) and radiotherapy

Survival outcomes were of particular relevance to this study, given that we aimed to identify the potential prognostic or predictive significance of major histologic patterns. Patients who
remained under the care of member of the St Vincent’s Hospital Respiratory MDT, or patients under the care of the Department of Medical Oncology had their dates of diagnosis and death contemporaneously entered into the iMDT database. Where patients were treated by specialists outside of St Vincent’s Hospital requests for the above information were made to their treating clinicians. External treating specialists were provided with a copy of the waiver of consent granted by the St Vincent’s Hospital HREC where necessary to obtain clinical information required for completion of this research. Accurate dates of death were obtained for all patients who had died via these two methods, and for the small cohort of patients who remained alive (n = 2) outcomes were censored at the date of last confirmed follow up.

2.2.4 Anatomical Pathology Assessment

All slides and formalin-fixed paraffin embedded (FFPE) blocks from potential cases were retrieved from the archives of the Department of Anatomical Pathology, St Vincents’ Hospital Melbourne. Pathologic assessment was performed by Associate Professor Prudence Russell, Anatomical Pathologist. The H&E slides of all potential cases were reviewed and included or excluded as per the pathologic criteria mentioned previously. In patients who had undergone multiple surgical procedures the largest tumour was chosen as the primary specimen for analysis to allow sufficient tissue for EGFR and KRAS mutation testing. All specimens from patients who had undergone multiple resections were assessed pathologically under the same methods. The size (recorded as the largest dimension in millimetres), location and number of tumours were obtained from the original pathology report.

The presence of the different adenocarcinoma patterns, including acinar, papillary, micropapillary and solid with mucin, as defined by the IASLC/ATS/ERS classification, was recorded as a binary variable. It was possible in each case to identify a major histologic pattern; however, it was not possible to assign percentages to the different histologic patterns present. The term “major” was chosen for the most prominent histologic pattern. This was done to avoid confusion with the recommendation of the new classification to use the term “predominant” for the most prominent histologic pattern identified in a resection specimen.
from the lung [179]. All cases underwent immunostaining with TTF1 (clone SPT24, NovoCastra, Newcastle upon Tyne, United Kingdom).

2.2.5 Molecular Pathology Assessment

Molecular pathologic assessment was conducted by Dr Hongdo Do, postdoctoral research fellow of the Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia (now of the Translational Genomics and Epigenetics Laboratory, Olivia Newton John Cancer and Wellness Centre, Heidelberg, Victoria, Australia).

2.2.5.1 Deparaffinization and DNA Extraction

A formalin-fixed, paraffin-embedded block with adequate tumour for molecular analysis was chosen from each case, and a tumour-rich area was circled on the corresponding glass slide. This region was sampled from each block using two mm diameter dermatology core punches. The punched core tumour tissues were deparaffinized with 800 μl of xylene by incubating for seven minutes, followed by washings with 800 μl of 100 and 70% ethanol. After removal of 70% ethanol, tumour tissues were incubated at 55°C for 15 minutes for removal of residual ethanol. Genomic DNA was extracted using the DNeasy Tissue and Blood kit (Qiagen, Hilden, Germany) following the manufactures instructions with following modifications based on those of Wu et al [291]. After addition of ATL buffer, tissues were heat treated at 98°C for 15 minutes, followed by incubation at 56°C for 3 days after addition of 36 μl proteinase K (Worthington, NJ) at 20 mg/ml concentration.

2.2.5.2 EGFR and KRAS mutation testing

EGFR mutations in exons 18 to 21 and KRAS mutations in codon 12 and 13 were scanned by high resolution melting using the assay conditions previously described [292, 293] with the following modifications. EGFR exon 19 F 5′-TAACGTCTTCTCTCTCTCTCTGTC-3′ and
EGFR exon 19 R 5’-CCCACACAGCAAAGCAGAAACTC-3’ primers were used to amplify a shorter amplicon of 152 base pairs (bp). To reduce sequence artefacts arising from uracil because of cytosine deamination in formalin-fixed, paraffin-embedded DNA[294], 0.5 U of uracil-DNA glycosylase (New England Biolabs) and 0.5X of uracil-DNA glycosylase buffer was added to the high resolution melting reaction that was prepared in a final volume of 20 μl. For uracil-DNA glycosylase treatment, an incubation step at 37°C for 30 minutes was added before the standard polymerase chain reaction amplification. Samples were tested in duplicate, and all high resolution melting positives were sequenced using the conditions previously described [292].

2.2.6 Statistical Analysis

Statistical analysis was undertaken using STATA version 12 (Statacorp, LP, TX). Survival analysis was performed using the Kaplan-Meier method and the log-rank test, with hazard ratios derived using the Cox Proportional Hazards model. Survival times were calculated from the time of diagnosis (not the time of surgical resection). Tests of association were performed using Pearson’s χ² or Fisher’s exact test as appropriate. Median times and hazard ratios are reported with their 95% CI. p less than 0.05 was considered statistically significant.

2.3 Results

2.3.1 Patient Characteristics and Treatment

The data set consisted of 100 patients of whom 66% were male and 34% female. These patients were identified over a ten year time frame from 2000 to 2010. The median age at diagnosis was 64 years (range 36 – 86 years). At the time of analysis 98 patients had died. The ECOG PS as recorded at the time of diagnosis was 0 or 1 in 69 patients and ≥2 in 31 patients. There were 21 never, 35 current and 44 former smokers. Treatment was given to patients according to clinician assessment and in keeping with local management guidelines.
Fifteen of 100 (15%) patients presented with unresectable stage III disease at diagnosis and had tissue acquired from a diagnostic mediastinoscopy. Of these fifteen patients, ten received radiotherapy together with platinum doublet chemotherapy with curative intent (66%). One patient (6%) received palliative chemotherapy with carboplatin/gemcitabine. Three patients (20%) received palliative radiotherapy without systemic therapy, and one patient (7%) received best supportive care only. Of the fifteen patients, fourteen (93%) died as a result of their lung cancer. One patient remains alive at last follow-up of 82 months.

Eighty-five patients presented with stage IV disease at diagnosis. Of these, 44 patients (52%) received systemic therapy. Thirty-eight patients received first-line platinum doublet chemotherapy and three patients received single agent gemcitabine. Two patients had resection of an isolated central nervous system metastasis followed by radiotherapy to the chest and combined concurrent platinum doublet chemotherapy. One patient with an EGFR mutation in exon 18 (G719S) and concomitant de novo exon 20 mutation (T790M) received first line afatinib. One patient with stage IV disease at diagnosis remains alive at last follow-up of 50 months.

For the 55 patients who received systemic anticancer therapy, the median number of lines of treatment was two (range one to five). Thirty-three patients received two or more lines of therapy. Patients with poor performance status were significantly less likely to receive systemic therapy (ECOG 0–1: 43 of 69 [62%] versus ECOG ≥2: 12 of 31 [39%], \( \chi^2 [1] = 4.82, p = 0.028 \)).

2.3.2 Histologic Findings

2.3.2.1 Sample Location and Size

In order of frequency, the site of tissue sampling was: brain (30%), pleura (25%), bone/skeletal muscle (20%), mediastinum (18%), and chest wall or supraclavicular fossa (7%). Fifty-two specimens were acquired at metastatectomy, 43 specimens were acquired through open biopsy either as a diagnostic procedure or as part of a therapeutic procedure,
and five specimens were core biopsies. The breakdown of specimen type by site together with the median size and size range of specimens is included in Table 7.

<table>
<thead>
<tr>
<th>Sample Site</th>
<th>N</th>
<th>Metastectomy (n, mean, range)</th>
<th>Open Biopsy (n, mean, range)</th>
<th>Core Biopsy (n, mean, range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>30</td>
<td>30, 20mm, 7 - 50 mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pleura</td>
<td>25</td>
<td>6, 42mm, 26 - 58 mm</td>
<td>19, 19mm, 2 - 75 mm</td>
<td></td>
</tr>
<tr>
<td>Bone/Muscle</td>
<td>20</td>
<td>14, 40mm, 8 - 80 mm</td>
<td>2, 34mm, 28 - 40 mm</td>
<td>4, 16mm, 11 - 22 mm</td>
</tr>
<tr>
<td>Mediastinum</td>
<td>18</td>
<td>1, 10mm, N/A</td>
<td>17, 12mm, 5 - 30 mm</td>
<td></td>
</tr>
<tr>
<td>Chest Wall*</td>
<td>7</td>
<td>1, 40mm, N/A</td>
<td>5, 13mm, 10 - 16mm</td>
<td>1, 17mm, N/A</td>
</tr>
</tbody>
</table>

**Table 7**: Metastatic Tumour Sample Site and Method of Acquisition, with the largest dimension in millimetres. * - includes supraclavicular fossa

### 2.3.2.2 Pathologic Subtypes

Examples of the major histologic patterns are shown in Figure 1 (page 112). The most frequent major histologic pattern seen in the 100 metastatic lung adenocarcinoma tumours was solid with mucin at 50%, followed by acinar at 29% and micropapillary at 20%. Major papillary pattern was seen in one case only. The 100 tumours showed a range of different histologic patterns, with the number of different patterns observed in each specimen as follows: one pattern seen in 10% of cases; two patterns in 45%; three patterns in 33%; and four patterns in 12% of cases. The frequency of the different patterns present in individual samples was as follows: solid seen in 82% of cases; micropapillary in 68%; acinar in 68%; and papillary in 29% of cases. Major solid pattern was less common in patients who were never smokers when compared with former or current smokers (Table 8, Fisher’s exact test = 0.010). Positive TTF1 immunostaining was present in 84 tumours. No statistical relationship was observed between TTF1 immunostaining and the major histologic pattern.
Figure 1:

Representative photomicrographs of the four histologic patterns seen in the metastatic tumour deposits (H&E, ×200). H&E, hematoxylin and eosin.

Top Left: Papillary Pattern in a Station 7 Lymph Node

Top Right: Micropapillary pattern in a brain metastasis

Bottom Left: Solid with mucin pattern in a parietal pleural metastasis

Bottom Right: Acinar pattern in a brain metastasis
2.3.2.3 Preservation of the Major Pattern

Sixteen patients had two or more samples that met the inclusion criteria for this study (table 9). In these patients the specimens were acquired at different time points during the disease course. In one case only scattered atypical cells were found in the secondary resection specimen (taken from femoral reamings). In the other 15 patients the major pattern was concordant across all the review lesions (major acinar pattern n=3; major micropapillary pattern n=6; major solid pattern n=6). While the major pattern was preserved the relative proportions of additional subtypes seen at each site varied between specimens.

Due to the selection criteria for this study only two patients had a prior resection of their primary lung tumour. In these patients the major subtype seen at the metastatic site examined was concordant with the predominant pattern observed in the primary tumour (one patient with major solid pattern and one patient with major acinar pattern).
<table>
<thead>
<tr>
<th>Patient</th>
<th>Chosen Lesion</th>
<th>Major Type</th>
<th>Secondary Types</th>
<th>Alternative Lesion(s)</th>
<th>Major Type</th>
<th>Secondary Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Femur, 35 mm</td>
<td>Acinar</td>
<td>Solid, MP</td>
<td>Humerus, 20mm</td>
<td>Acinar</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Brain, 24 mm</td>
<td>Acinar</td>
<td>Pap, MP</td>
<td>Brain, 40mm</td>
<td>Acinar</td>
<td>Pap, MP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Skin, surgical scar, 15mm</td>
<td>Acinar</td>
<td>MP, Pap</td>
</tr>
<tr>
<td>3</td>
<td>Femur, 50mm</td>
<td>Acinar</td>
<td>MP, Solid</td>
<td>Femur, 50mm</td>
<td>Acinar</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Brain, 19mm</td>
<td>MP</td>
<td>Acinar</td>
<td>Brain, 15mm</td>
<td>MP</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Femur, 40mm</td>
<td>MP</td>
<td>Acinar</td>
<td>Femur, 30mm</td>
<td>MP</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>L5 mass, 40mm</td>
<td>MP</td>
<td>Pap, Solid</td>
<td>L5 mass, 45mm</td>
<td>MP</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Brain, 25mm</td>
<td>MP</td>
<td>Pap, solid, acinar</td>
<td>Brain, 27mm</td>
<td>MP</td>
<td>Solid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Brain, 12mm</td>
<td>MP</td>
<td>Solid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Brain, 25mm</td>
<td>MP</td>
<td>Solid</td>
</tr>
<tr>
<td>8</td>
<td>Pleura, 50mm</td>
<td>MP</td>
<td>Pap, acinar</td>
<td>Pleura, 25mm</td>
<td>MP</td>
<td>Acinar, Pap</td>
</tr>
<tr>
<td>9</td>
<td>Brain, 25mm</td>
<td>MP</td>
<td>Solid, pap</td>
<td>Brain, 7mm</td>
<td>MP</td>
<td>Solid, pap</td>
</tr>
<tr>
<td>10</td>
<td>Mediastinal LN, 15mm</td>
<td>Pap</td>
<td>Acinar</td>
<td>Femur, 2mm</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Brain, 20mm</td>
<td>Solid</td>
<td>MP</td>
<td>Brain, 8mm</td>
<td>Solid</td>
<td>MP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mediastinal LN, 4mm</td>
<td>Brain, 10mm</td>
<td>Solid</td>
<td>MP</td>
</tr>
<tr>
<td>12</td>
<td>Mediastinal LN, 15mm</td>
<td>Solid</td>
<td>Acinar, MP</td>
<td>Brain, 19mm</td>
<td>Solid</td>
<td>Acinar, MP</td>
</tr>
<tr>
<td>13</td>
<td>Brain, 25mm</td>
<td>Solid</td>
<td>MP</td>
<td>Brain, 12mm</td>
<td>Solid</td>
<td>MP</td>
</tr>
<tr>
<td>14</td>
<td>Brain, 12mm</td>
<td>Solid</td>
<td>MP, Pap</td>
<td>Brain, 9mm</td>
<td>Solid</td>
<td>MP, Pap</td>
</tr>
<tr>
<td>15</td>
<td>Brain, 10mm</td>
<td>Solid</td>
<td>Acinar</td>
<td>Brain, 4mm</td>
<td>Solid</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Cervical LN, 16mm</td>
<td>Solid</td>
<td>MP, Acinar</td>
<td>Brain, 7mm</td>
<td>Solid</td>
<td>Acinar</td>
</tr>
</tbody>
</table>

Table 9: Histologic subtypes in patients with two or more biopsy specimens available.

Pap, papillary; MP, micropapillary; LN, lymph node.

* - scattered atypical cells only
2.3.3 Survival Outcomes

Survival analysis was initially conducted for the 100 patient cohort as a whole. However, it was found that the rules of proportionality were breached due to the effects of systemic therapy on survival outcome. Therefore the cohort was split into two groups on the basis of whether or not systemic therapy had been administered.

2.3.3.1 Patients Who Received No Systemic Therapy

There was no difference in OS when correlated with the major histologic pattern in the metastatic site. OS for patients with different major histologic patterns was as follows: major solid pattern was 4.2 months (95% CI 3.3–7.4 months); major acinar pattern was 4.6 months (95% CI 1.9–16.9 months); and major micropapillary pattern was 4.7 months (95% CI 1.5–11.5 months; Table 10, Figure 2). The only significant factor influencing OS was ECOG status: the median OS for patients of ECOG 0 to 1 was 6.3 months (95% CI 4.0–14.1 months), and patients with ECOG ≥2 was 3.9 months (95% CI 1.8–6.8 months, HR 2.1 [1.1–4.0], p = 0.019; Table 9, Figure 3). Analysis stratified according to stage was not possible because of the small number of patients with stage III disease (n = 4).

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Survival in Months (95% CI)</th>
<th>HR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Patients</td>
<td>45</td>
<td>4.7 (3.4 - 7.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major Pattern</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solid Acinar</td>
<td>22</td>
<td>4.3 (3.3 - 7.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acinar MPA</td>
<td>16</td>
<td>4.6 (1.9 - 16.9)</td>
<td>vs solid 1.2 (0.5 - 2.9)</td>
<td>vs solid 0.675</td>
</tr>
<tr>
<td>Acinar MPA</td>
<td>7</td>
<td>4.7 (1.5 - 11.5)</td>
<td>vs solid 0.9 (0.5 - 1.7)</td>
<td>vs solid 0.737</td>
</tr>
<tr>
<td>ECOG 0 - 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 or more</td>
<td>26</td>
<td>6.3 (4.0 - 14.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 or more</td>
<td>19</td>
<td>3.9 (1.8 - 6.8)</td>
<td>2.1 (1.1 - 4.0)</td>
<td>0.019</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>30</td>
<td>4.2 (3.3 - 6.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>15</td>
<td>11.5 (1.9 - 16.9)</td>
<td>0.5 (0.3 - 1.03)</td>
<td>0.062</td>
</tr>
</tbody>
</table>

Table 10: Overall Survival outcomes for patients who did not receive systemic therapy

N, number; 95% CI, 95% confidence interval; HR, hazard Ratio, ECOG, Eastern Cooperative Oncology Group performance status; MPA, micropapillary adenocarcinoma
**Figure 2:** Overall Survival by Major Subtype for patients not receiving systemic therapy

**Figure 3:** Overall Survival by Performance Status for patients not receiving systemic therapy
2.3.3.2 Patients Who Received Systemic Therapy

Statistically significant differences in OS for patients who received systemic therapy were present according to the major histologic pattern. Patients with major solid pattern tumour had shorter OS at a median of 9.4 months (95% CI 8.6–12.2 months) when compared with patients with major acinar pattern tumour at 15.9 months (95% CI 10.7–24.7 months; HR versus solid 0.32 [0.15–0.68], \( p = 0.003 \)) and patients with major micropapillary pattern tumour at 18.9 months (95% CI 11.6–24.4 months; HR versus solid 0.34 [0.17–0.69], \( p = 0.003 \); table 11, figure 4). No difference in OS was seen between treated patients with major acinar and major micropapillary pattern tumours. No significant differences in OS were identified according to the presence or absence of each histologic pattern (Figures 5 - 8). There was a direction of effect towards longer OS in patients with an absence of the solid pattern (solid present \( n = 46 \) versus absent \( n = 9 \), median OS 11.6 months [95% CI 9.4–14.3 months] versus 17.6 months [95% CI 3.4–44.0 months], HR 2.0 [95% CI 0.98–4.3], \( p = 0.056 \)).
<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Survival in Months (95% CI)</th>
<th>HR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All Patients</strong></td>
<td>55</td>
<td>13 (10.7 - 16.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Major Pattern</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solid</td>
<td>28</td>
<td>9.4 (8.6 - 12.2)</td>
<td>vs solid 0.32 (0.15 - 0.68) vs solid 0.003</td>
<td></td>
</tr>
<tr>
<td>Acinar</td>
<td>13</td>
<td>15.9 (10.7 - 24.7)</td>
<td>vs solid 0.34 (0.17 - 0.69) vs solid 0.003</td>
<td></td>
</tr>
<tr>
<td>Micropapillary</td>
<td>13</td>
<td>18.9 (11.6 - 24.4)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Papillary</td>
<td>1</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td><strong>ECOG</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 or 1</td>
<td>43</td>
<td>13.6 (11.1 - 17.5)</td>
<td>1.66 (0.9 - 3.2)</td>
<td>0.128</td>
</tr>
<tr>
<td>2 or more</td>
<td>12</td>
<td>8.6 (2.8 - 17.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>36</td>
<td>13.0 (9.8 - 17.2)</td>
<td>1.02 (0.6 - 1.8)</td>
<td>0.944</td>
</tr>
<tr>
<td>Female</td>
<td>19</td>
<td>11.9 (8.9 - 20.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>11</td>
<td>13.0 (8.6 - 24.4)</td>
<td>1.56 (0.8 - 3.1)</td>
<td>0.207</td>
</tr>
<tr>
<td>IV</td>
<td>44</td>
<td>12.5 (9.5 - 16.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>EGFR Mutation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>6</td>
<td>17.5 (10.7 - NR)</td>
<td>0.91 (0.4 - 2.1)</td>
<td>0.827</td>
</tr>
<tr>
<td>Wild Type</td>
<td>49</td>
<td>12.5 (9.5 - 15.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>KRAS Mutation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>17</td>
<td>11.6 (6.5 - 17.2)</td>
<td>1.46 (0.8 - 2.6)</td>
<td>0.209</td>
</tr>
<tr>
<td>Wild Type</td>
<td>38</td>
<td>13.0 (10.7 - 17.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 11:** Overall Survival outcomes for patients who received systemic therapy

N, number; 95% CI, 95% confidence interval; HR, hazard Ratio; ECOG, Eastern Cooperative Oncology Group performance status; NR, not reached; *EGFR*, epidermal growth factor receptor; *KRAS* – Kirsten-RAS
Figure 4: Overall Survival by Major Subtype for patients who received systemic therapy
**Figure 5:** Overall survival by the presence or absence of any solid component

**Figure 6:** Overall survival by the presence or absence of any micropapillary component
**Figure 7**: Overall survival by the presence or absence of any acinar component

**Figure 8**: Overall survival by the presence or absence of any papillary component
2.3.4 Correlations between Histology and Molecular Pathology

Mutational analysis for *EGFR* and *KRAS* mutations was successful in all 100 patients. *EGFR* and *KRAS* mutations were identified in 13 of 100 tumours (13%) and 32 of 100 tumours (32%), respectively. *EGFR* and *KRAS* mutations were mutually exclusive.

*EGFR* mutations occurred most often in major micropapillary pattern tumours (6 of 20 [30%]) followed by major acinar pattern tumours (4 of 29 [14%]) and were least frequent in major solid pattern tumours (2 of 50 [4%]; Fisher’s exact = 0.006; Table 12). Both patients with major solid pattern tumours and *EGFR* mutations had uncommon variants (patient 1, exon 18 E709_T710delinsD; patient 9, exon 20 insertion), which are associated with resistance to first-generation *EGFR* inhibitors (Table 14) [295]. The one major papillary pattern tumour harboured a classic exon 21 L858R mutation. All *EGFR* mutations occurred in never or former smokers and were not present in any current smokers (Fisher’s exact *p* < 0.001; Table 13).

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Solid</th>
<th>Acinar</th>
<th>MPA</th>
<th>Papillary</th>
<th>Fisher’s exact</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>Mutated</td>
<td>13</td>
<td>2 (15%)</td>
<td>4 (31%)</td>
<td>5 (38%)</td>
<td>1 (8%)</td>
</tr>
<tr>
<td>Mutation</td>
<td>Wild Type</td>
<td>87</td>
<td>48 (55%)</td>
<td>25 (29%)</td>
<td>15 (17%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>KRAS</td>
<td>Mutated</td>
<td>32</td>
<td>18 (56%)</td>
<td>9 (28%)</td>
<td>5 (16%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Mutation</td>
<td>Wild Type</td>
<td>68</td>
<td>32 (47%)</td>
<td>20 (29%)</td>
<td>15 (22%)</td>
<td>1 (1%)</td>
</tr>
</tbody>
</table>

Table 12: Associations between the major pathologic subtype and mutations in *EGFR* and *KRAS*. N, number; MPA, micropapillary

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Never Smoker</th>
<th>Former Smoker</th>
<th>Current Smoker</th>
<th>Fisher's Exact</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>Mutated</td>
<td>13</td>
<td>9 (69%)</td>
<td>4 (31%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Mutation</td>
<td>Wild Type</td>
<td>87</td>
<td>12 (14%)</td>
<td>31 (36%)</td>
<td>44 (50%)</td>
</tr>
<tr>
<td>KRAS</td>
<td>Mutated</td>
<td>32</td>
<td>1 (3%)</td>
<td>17 (53%)</td>
<td>14 (44%)</td>
</tr>
<tr>
<td>Mutation</td>
<td>Wild Type</td>
<td>68</td>
<td>20 (29%)</td>
<td>27 (40%)</td>
<td>21 (31%)</td>
</tr>
</tbody>
</table>

Table 13: Associations between smoking history and mutations in *EGFR* and *KRAS*
<table>
<thead>
<tr>
<th>No.</th>
<th>Age at Dx</th>
<th>Sex</th>
<th>Tumour Site</th>
<th>Smoking History</th>
<th>Major Pattern</th>
<th>Exon</th>
<th>Mutation</th>
<th>Amino Acid Change</th>
<th>EGFR inhibitor use</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>83</td>
<td>F</td>
<td>Brain</td>
<td>Former</td>
<td>Solid</td>
<td>18</td>
<td>E709_T710delinsD</td>
<td>c.2127_2129del</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>45</td>
<td>F</td>
<td>Pleura</td>
<td>Never</td>
<td>Acinar</td>
<td>18 / 20</td>
<td>G719S / T790M</td>
<td>c.2155G&gt;A and c.2369C&gt;T</td>
<td>Afatinib; 6 cycles; Partial Response; CNS progression</td>
</tr>
<tr>
<td>3</td>
<td>51</td>
<td>F</td>
<td>Pleura</td>
<td>Never</td>
<td>MPA</td>
<td>19</td>
<td>E746_750del</td>
<td>c.2235_2249del15</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>F</td>
<td>Pleura</td>
<td>Former</td>
<td>MPA</td>
<td>19</td>
<td>E746_L747delinsNY</td>
<td>c.2236_2241delinsAATTAT</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>54</td>
<td>F</td>
<td>Pleura</td>
<td>Never</td>
<td>Acinar</td>
<td>19</td>
<td>E746_750del</td>
<td>c.2236_2250del15</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>36</td>
<td>M</td>
<td>Bone/muscle</td>
<td>Never</td>
<td>MPA</td>
<td>19</td>
<td>L747_T751delinsP</td>
<td>c.2239_2251del13insC</td>
<td>Erlotinib; 8 cycles; Partial Response</td>
</tr>
<tr>
<td>7</td>
<td>73</td>
<td>F</td>
<td>Bone/muscle</td>
<td>Never</td>
<td>Acinar</td>
<td>19</td>
<td>L747_P753delinsS</td>
<td>c.2240_2258del18</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>44</td>
<td>M</td>
<td>Pleura</td>
<td>Never</td>
<td>MPA</td>
<td>20</td>
<td>S768_D770dup</td>
<td>c.2303_2311dup</td>
<td>Erlotinib; 1 cycle; unknown</td>
</tr>
<tr>
<td>9</td>
<td>66</td>
<td>M</td>
<td>Pleura</td>
<td>Former</td>
<td>Solid</td>
<td>20</td>
<td>H773_V774insTQPP</td>
<td>c.2318_2319insCACACAAACCCCCC</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>55</td>
<td>M</td>
<td>Brain</td>
<td>Never</td>
<td>MPA</td>
<td>21</td>
<td>L858R</td>
<td>c.2573T&gt;G</td>
<td>Gefitinb; 14 cycles; Partial Response</td>
</tr>
<tr>
<td>11</td>
<td>64</td>
<td>M</td>
<td>Bone/muscle</td>
<td>Never</td>
<td>MPA</td>
<td>21</td>
<td>L858R</td>
<td>c.2573T&gt;G</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>79</td>
<td>M</td>
<td>Pleura</td>
<td>Former</td>
<td>Acinar</td>
<td>21</td>
<td>L858R</td>
<td>c.2573T&gt;G</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>55</td>
<td>M</td>
<td>Mediastinum</td>
<td>Never</td>
<td>Pap</td>
<td>21</td>
<td>L858R</td>
<td>c.2573T&gt;G</td>
<td>Erlotinib; 6 cycles; Partial Response; CNS progression</td>
</tr>
</tbody>
</table>

**Table 14:** Features of patients with *EGFR* mutations. Dx – Diagnosis; MPA – micropapillary; Pap - papillary
KRAS mutations occurred in major solid pattern tumours (18 of 50 [36%]), major acinar pattern (9 of 29 [31%]) and major micropapillary pattern (5 of 20 [25%]) tumours (Table 12), without a significant relationship found between the presence of KRAS mutations and the major histologic pattern (Fisher's exact \( p = 0.789 \)). Thirty-two KRAS mutations were seen with the following frequencies: G12C 20 (63%), G12V 4 (13%), G12D 3 (9%), G12A 2 (6%), G12L 1 (3%), G13C 1 (3%), and G13D 1 (3%). All patients with KRAS-mutant tumours, except one, were former or current smokers (Fisher's exact = 0.006; Table 13).

There was no relationship between oncogenic mutations, the major histologic patterns in tumours and OS, which may reflect the small number of patients in our cohort.

2.4 Discussion

This work is the first to examine the impact of histologic pattern present at metastatic sites in lung adenocarcinoma according to the recommendation in the IASLC/ATS/ERS classification for small biopsy/cytology specimens [179]. The recommendation states that pathologists should list all the histologic pattern(s) present in small biopsy/cytology specimens which may be from the primary tumour or a metastatic site. In addition to identifying the patterns present in this study we also assigned a major pattern on the basis of the tumour subtype that was most abundant in each sample.

2.4.1 Histological Subtyping in Advanced Disease – Survival

2.4.1.1 Systemically Treated Patients

In our cohort of patients treated with systemic therapy (n=55) we were able to demonstrate significant differences in survival outcome on the basis of the major histologic pattern
observed. Those patients with major solid pattern tumour had significantly worse survival outcomes compared to patients with major acinar or major micropapillary patterns. This finding was the only factor that reached statistical significance on survival analysis.

Much attention has been paid to outcomes in resected disease due to the completeness of both clinical and pathologic assessment and the easy availability of the pathologic material. To date there has only been one other study that has examined the importance of pathologic subtyping on the basis of the IASLC/ATS/ERS classification in advanced disease. Campos-Parra et al examined outcomes for a group of 313 patients with metastatic (stage III or IV) pulmonary adenocarcinoma treated with systemic therapy [296]. Samples in this work were assessed for adequacy of tissue to detect the pattern and perform immunohistochemistry so their final analysis was reduced to 257 patients. In their work all patients were diagnosed on the basis of CT guided Tru-Cut biopsy and were treated with platinum based chemotherapy in combination with either paclitaxel or vinorelbine. The distribution of subtypes was different to that in our cohort (lepidic 6.1%; acinar 36.7%; papillary 8.3%; MPA 2.9%; solid 28.1%; excluded 17.9%).

In survival analysis Campos-Parra et al separated their categories into intermediate grade (lepidic and acinar) and high grade (papillary, solid and micropapillary). They found that good performance status (ECOG 0-1), stage III disease and high grade tumours had better progression free survival in multivariate analysis. These same factors, as well as the presence of an activating EGFR mutation, were also associated with better overall survival in multivariate analysis.

Our findings contrast with these of Campos-Parra et al. Where we found that major solid pattern tumours had worse outcomes compared to major acinar pattern tumours their findings were opposite with patients with high grade tumour patients comprising mostly of solid pattern having better outcomes on treatment than intermediate grade patterns comprising mostly of acinar pattern. Survival outcomes as determined according to each individual subtype were not reported by Campos-Parra et al.
There are two potential explanations for these differences. Firstly, it may have occurred due to methodological differences between the studies. Our work used samples from purely metastatic sites while the location of tissue was not reported by Campos-Parra et al – it is assumed though that some patients had their biopsies taken from lung tissue given that patients with lepidic pattern tumours are found in their cohort. Further, the difference may arise due to the size of tissue sample (core biopsy, as compared with surgical specimens used in our study) used in the work of Campos-Parra, and if this is the case then subtyping in metastatic disease would be of minimal clinical utility. The IASLC/ATS/ERS classification notes that small specimens and cytologic findings may not be representative of the tumour as a whole [179]. Few papers have been published in this area. Rudomina et al compared cytologic findings from primary tumours to the final resection specimens and found the presence or absence of micropapillary tufts in diagnostic cytological specimens was not a useful discriminator for finding the presence of the micropapillary pattern the resection specimen (this work predates the IASLC/ATS/ERS classification) [297]. No similar work is available comparing core biopsies to resection specimens. Secondly, both papers represent an early exploration of the use of pathological subtyping according to the IASLC/ATS/ERS classification in advanced disease. Differences may have arisen due to interpretation and application of the classification, or the effect of limited sample sizes. If subtyping in advanced disease is to become part of routine practice then larger studies are required to define the relevance of these subtypes.

Prior to these two contemporary papers very little attention had been dedicated to subtyping pulmonary adenocarcinoma in the advanced setting. It is likely that this neglect stems from a lack of adequate material due to the sampling methods available and the fact that historically very few patients were treated for advanced lung cancer (thus negating the need for a large biopsy). The first work was produced by Sorensen et al in the late 1980s on the basis of the 1981 WHO classification [180, 194, 298]. This work examined samples from patients with advanced lung adenocarcinoma undergoing thoracotomy either for diagnosis or when aborted due to inoperability or with disease at distant sites. Patients were subsequently treated on a clinical trial of vindesine, the combination of lomustine plus cyclophosphamide plus
methotrexate, or the combination of all four drugs. No survival differences were seen by treatment arm in that trial.

The histologic study included 198 patients after exclusion of small samples where subtyping was not possible. According to the WHO criteria the distribution of subtypes was acinar 65%, papillary 12%, solid with mucin 16%, and BAC 7%. No BAC was seen in non-pulmonary samples which is consistent with the IASLC/ATS/ERS definition of the lepidic pattern as being non-invasive. Sorensen et al showed that the median survival time of patients whose tumours had the solid with mucin pattern was shorter at 22 weeks as compared to acinar (29 weeks), papillary (31 weeks) and BAC (40 weeks) patterns. This trend failed to reach statistical significance, but is consistent with what is observed in our work. It is possible that the lack of statistical significance is a reflection of the lack of activity of the cytotoxic drugs available at that time. Patients in the Sorensen study did not receive the more modern platinum based therapies used in the current patient cohort.

Sorensen et al also found that while response rates were similar (complete and partial responses) across the four histologic subtypes the duration of response was shortest in those with solid with mucin subtype. We were not able to explore these findings in our cohort given the variability in treatments used and the timing of follow up scans.

In our study no survival difference was seen for patients with an EGFR mutation. Due to the times of acquisition of samples in our study, a small number of patients were tested for EGFR mutations during their disease course, and only five patients access EGFR TKIs as part of their treatment. Two patients have EGFR mutations associated with resistance to EGFR TKIs, and one patient had a concurrent exon 18 G719S mutation and an exon 20 T790M mutation (associated with de novo resistance to EGFR TKIs). As shown by Kris et al, a survival advantage for a targetable oncogenic mutation is only seen when the patient is treated with the appropriate targeted therapy [155].
2.4.1.2 Survival in Patients Receiving No Systemic Therapy

Patients who did not receive systemic therapy were generally of poorer performance status. The major pattern observed in each sample was predictive of survival only in those treated with systemic therapy, however it was not prognostic if no systemic treatment was given. The only significant predictor of survival was poor performance status (ECOG ≥ 2). There were rare instances of prolonged survival in the absence of systemic therapy (for example one patient with isolated cerebral metastasis and no evidence of extra-cranial disease).

2.4.2 Does Adenocarcinoma Subtype Interact With Treatment Response?

2.4.2.1 Advanced Disease

Our study has shown that the major pattern present in biopsies from patients with advanced disease may be predictive of survival time in the advanced disease setting, while not being prognostic for patients who did not receive systemic treatment. This is different to the setting of early stage resected disease where the predominant subtype clearly carries prognostic relevance and informs about the potential for relapse independent of other known prognostic factors such as stage (as discussed in section 2.1.3.2, page 90) [199, 200, 249, 261, 263-266, 272-274, 299].

Studies from other groups have also found a predictive role for the histological subtype in dictating patients’ outcomes. Yoshida et al examined a group of patients with EGFR mutant lung adenocarcinoma who had developed relapse following surgical resection. All patients were treated with the EGFR tyrosine kinase inhibitors (TKIs) gefitinib or erlotinib following recurrence. Patients whose original resections showed solid predominant adenocarcinoma were significantly less likely to have a radiologic response (61% vs 88%, p=0.03) as well as having the shortest PFS (median 7.7 months; lepidic 9.4 months; papillary 13.3 months; acinar 18.6 months) during treatment of their advanced disease. This study also looked at core biopsies in advanced disease and found that for patients with the solid subtype in their core biopsy the duration of response to EGFR TKI was shorter as compared to those who did not have the solid pattern present (HR 3.4 [95% CI 1.4 – 8.1, p=0.009]).
Taken together the results of our paper and that of Yoshida et al suggest that the solid morphology is associated with short response time (or resistance) to systemic treatments. This has been demonstrated regardless of whether the treatment is cytotoxic chemotherapy as in our work or oncogene specific TKIs in the case of the study from Yoshida et al.

An avenue of future research that has not been covered in prior studies is the relationship between adenocarcinoma subtype and the response to immunotherapy in advanced pulmonary adenocarcinoma. As previously discussed in Section 1.3.3.3 the treatment landscape for advanced NSCLC has recently been dramatically changed by the introduction of immune checkpoint inhibition. To date, this has been achieved with antibodies targeting PD-1 (pembrolizumab [300, 301] and nivolumab [159, 160]) or PD-L1 (atezolizumab [166]). One study by Koh et al has examined the rates of IHC expression of PD-L1 and PD-L2 in resected pulmonary adenocarcinoma [302]. Cases with moderate or strong staining of PD-L1 or PD-L2 in greater than 10% of tumour cells were considered positive. They found no associations for PD-L2 expression and adenocarcinoma subtype. PD-L1 expression differed by subtype, with the lowest rates being seen in patients with lepidic predominant adenocarcinoma (39%), intermediate rates of PD-L1 positive in acinar or papillary predominant adenocarcinomas (58%) and the highest rates being observed in micropapillary or solid predominant adenocarcinomas (76%). In some studies PD-L1 positivity is an enrichment biomarker (that is patients with PD-L1 positive tumours are more likely to respond to immunotherapy against the PD-1/PD-L1 axis). Whether there is an interaction between treatment and response to immunotherapy in advanced adenocarcinoma by histologic subtype is not yet known.

2.4.2.2 Adjuvant Therapy Following Surgical Resection

The responsiveness of the various subtypes to chemotherapeutic agents seen appears to differ from the adjuvant setting to the advanced setting. Tsao et al presented a retrospective analysis of adenocarcinoma subtyping by the IASLC/ATS/ERS for 573 patients who were
included in the adjuvant chemotherapy trials in the LACE cohort [303]. They found that the IASLC/ATS/ERS classification was of prognostic value in the control arms (no adjuvant therapy). Patients with acinar or papillary predominant tumours showed better DFS outcomes when compared to patients with micropapillary or solid predominant tumours. However, analysis according to predominant subtype in the experimental arms (those who received adjuvant chemotherapy) showed predictive value. Benefit was seen for patients with solid and micropapillary predominant tumours receiving adjuvant chemotherapy as compared to observation (HR for DFS 0.58 [0.43 – 0.80], p<0.001) while no benefit was seen for patients with acinar or papillary predominant tumours (HR 1.12 [0.79 – 1.59], p=0.536, p for interaction <0.01).

Similarly Warth et al found in a retrospective analysis of their cohort who had resected stage III and stage IV disease that there was a trend to greater benefit for patients receiving adjuvant therapy for solid predominant tumours as compared to non-solid predominant tumours [249]. These results contrast with our findings from the purely palliative (unresected) setting that patients with major solid pattern tumours had significantly shorter overall survival compared with major acinar and major micropapillary patterns tumours. The reasons for this difference are not apparent, but may relate to the superior efficacy of chemotherapy agents and radiotherapy in the microscopic or minimal residual disease setting as compared to the advanced disease setting.

The effect of the presence or absence of each pattern as a dichotomous variable was examined in our cohort. Survival analysis suggested that the presence of any solid pattern tumour is associated with a trend towards worse overall survival in the metastatic setting. The number of patients lacking solid pattern was small (n=9) so this result requires further exploration in future studies. Conversely Warth et al examined the effect of “minor patterns” (as opposed to the predominant pattern) in resected primary lung adenocarcinoma and found that these patterns had no bearing on outcome in earlier stage disease [249].
**2.4.3 Morphologic Heterogeneity of Pulmonary Adenocarcinoma**

Morphologic heterogeneity is recognised as a hallmark of pulmonary adenocarcinoma. On the basis of increasing observations and evidence this was first formally recognised in the 1999 WHO classification [195], and continues to be observed in resection specimens from resected primary pulmonary adenocarcinomas [179, 197, 249, 304]. Findings from our cohort support prior work demonstrating morphologic heterogeneity of small biopsy specimens at metastatic sites in patients with stage III and IV disease [278, 289]. Ninety percent of cases had two or more patterns seen, which is similar to the rate of “adenocarcinoma of mixed subtypes” seen in resection specimens classified according to the 1999/2004 WHO classification [197-201].

In each case the pathologist was able to subjectively identify (as opposed to quantifying) the most common pattern in each metastatic specimen, which we termed the “major pattern”. This was done to avoid confusion with the use of the term “predominant pattern” which applies to the most abundant morphologic pattern in resection specimens from a primary lung tumour. The major solid pattern was the most frequent, followed by the major acinar and major micropapillary patterns, with only one patient with a major papillary pattern tumour. The relative frequencies of each “major pattern” differ from the frequencies of the predominant patterns observed from cohorts who have undergone curative resection of their primary tumour [199-201, 249, 299]. In the studies of resection specimens the rate of the relative predominant pattern varies with the following ranges: solid predominant 13.0 – 37.6%; acinar predominant 13.8 – 45.1%; micropapillary predominant 2.3 – 10.3%; and papillary predominant 4.7 – 40.7%. In this study in the metastatic setting the major solid pattern was much more frequent, while the major papillary pattern was rare.

The hypothesis that may explain this difference in the observed frequencies is the relative metastatic potential of each subtype. Three groups have recently explored the difference between the predominant pattern in the primary tumour and the most abundant subtype at metastatic sites. Their findings support this hypothesis. Russell et al examined a group of 69 patients with resected primary lung cancer with metastases to N2 nodal station(s) [278]. For
patients with solid predominant (22 of 24; 92%) and micropapillary predominant primary tumours (13 of 13; 100%) the majority of N2 nodes had this same predominant pattern. Conversely for acinar predominant tumours the N2 nodes showed predominant acinar pattern in only 11 of 26 patients (42.3%) with the remainder of cases having solid or micropapillary as the major pattern in the N2 nodes. All papillary predominant patients (n=4) had the micropapillary pattern as the dominant finding in their N2 nodes. Suda et al attempted to replicate the findings of Russell et al in a smaller group from their Japanese centre [280]. Their cohort consisted of 24 patients with papillary predominant (n=9), acinar predominant (n=11) or solid predominant (n=4) adenocarcinoma with involvement of N1 and/or N2 lymph node stations. The main histologic subtype found in the lymph node metastasis was concordant in all cases only for patients with solid predominant primary tumours. The predominant tumour and main pattern in the lymph node metastasis was concordant in only two of the nine patients with papillary predominant primary tumours and seven of the 11 patients with acinar predominant primary tumours. In both papers from Russell et al and Suda et al survival outcomes were driven by the predominant pattern in the primary tumour, and not the most abundant pattern observed in the lymph nodes.

Sica et al examined specimens from 73 patients with stages IIA to IV lung adenocarcinoma with metastases to regional lymph nodes (n=66) and/or to the brain (n=7) [289]. This work predates the IASLC/ATS/ERS classification but a “predominant pattern” was reported in each primary tumour. They found that while “the predominant pattern in the primary tumour is more likely to be seen at the metastatic site”, micropapillary and solid patterns were more likely to metastasize even when only present in small proportions at the primary site.

### 2.4.4 Correlations between Histologic Subtypes and Oncogenic Mutations

The history of histologic subtypes and oncogenic driver mutations in resected pulmonary adenocarcinoma is discussed in detail in the literature review to chapter three. In this section the relationship between pulmonary adenocarcinoma resected from metastatic sites and the \textit{EGFR} and \textit{KRAS} mutation status is discussed.
2.4.4.1 Epidermal Growth Factor Receptor Mutations

The frequency of \textit{EGFR} mutation (13\%) in this cohort of patients with unresectable stage III and IV pulmonary adenocarcinoma is low, but consistent with the mostly Western population. \textit{EGFR} mutations were mostly seen in the major micropapillary and major acinar pattern tumours and in the one major papillary pattern tumour. \textit{EGFR} mutations were infrequent in the major solid subtype. The frequencies of \textit{EGFR} mutations observed by major pathologic subtype in this cohort obtained from metastatic sites are similar to those reported by predominant pattern in resection specimens from other predominantly Western studies [274, 278, 305, 306]. Numerically rates of \textit{EGFR} mutation were higher amongst the major micropapillary subtype, as compared to the major acinar subtype, however the absolute numbers were small (reflective of the available sample size). This may relate to the metastatic potential of each pathologic subtype relative to its proportion in the primary tumour (as discussed in previous section).

The observation that \textit{EGFR} mutations are rare in the major solid pattern at metastatic sites is unlikely to be true in Asian cohorts. In resection specimens from Asian cohorts the predominant solid pattern has a frequency of \textit{EGFR} mutations of approximately 20-30\% in most papers [307, 308] (see Chapter three).

2.4.4.2 Kirsten-RAS Mutations

The frequency of \textit{KRAS} mutations was similar across the major pathologic subtypes at metastatic sites in this cohort. This finding is consistent with the paper from Motoi et al who found no associations between the predominant pathologic subtype in the resection specimen and the frequency of \textit{KRAS} mutations (note: the Motoi paper predates the IASLC/ATS/ERS classification) [197]. Similarly, Mansuet-Lupo et al found no association between the solid subtype and \textit{KRAS} mutation in another cohort who had undergone resection (with the only positive association for \textit{KRAS} mutation being the IMA pattern) [274]. In contrast Russell et
al and Kadota et al both found increased rates of *KRAS* mutation in the solid predominant subtype [278, 305].

2.4.5 Clinical Relevance

2.4.5.1 Cytotoxic Therapy and TKIs

This study is likely to impact future research and treatment for metastatic lung adenocarcinoma. Overall survival outcomes were markedly different for patients on cytotoxic therapy between each of the three major patterns, with the worst outcomes seen for patients with the major solid pattern. This finding may also hold true for patients on *EGFR* TKIs with advanced disease. Yoshi da et al reported a cohort of 61 Japanese patients with *EGFR* mutated (exon 19 deletion or L858R mutation) primary lung adenocarcinomas who subsequently developed systemic recurrence [309]. Those whose originally resected tumour was solid predominant were less likely to respond to gefitinib (61% vs 88%, p=0.03), and they had a worse PFS on multivariate analysis (HR 3.97, 95% CI 1.89 – 8.29, p<0.001). In another 41 patients with advanced disease (no prior surgery) from the Yoshida study, patients with solid subtype tumours were less likely to respond to gefitinib (RR 50% vs 86%, p=0.04). These patients also had a worse PFS (solid subtype 5.4 months [95% CI 1.5 – 10 months] vs non-solid subtype 10.0 months [95% CI 8.8 – 16.9 months], p=0.006). The solid subtype in small biopsy specimens was the only subtype to predict shorter response duration to gefitinib (HR 3.40, 95% CI 1.38 – 8.13, p=0.009). Taken together both our results and those of Yoshida et al suggest that the histologic type of adenocarcinoma in advanced disease could be considered as a stratification factors for future clinical trials given the profound differences in outcomes for patients with major solid subtype tumours and those with non-solid subtypes.

The effect of the predominant subtype in early stage resectable lung cancer is now well documented across multiple studies, including prior work from our group (see section 2.1.3.2 – Evidence supporting the new IASLC/ATS/ERS classification for lung adenocarcinoma in resected disease, page 90). In our study the aggressive behaviour of the major solid pattern
tumours (with shorter survival time) mirrors the poorer recurrence free survival seen following curative intent resection of solid predominant adenocarcinoma. However, patients with resected solid predominant adenocarcinoma get the greatest benefit from adjuvant therapies as demonstrated by Warth et al [249] and in the LACE-Bio analysis on the impact of the IASLC/ATS/ERS predominant subtypes and outcomes with adjuvant chemotherapy [303]. The identification of different outcomes for solid pattern tumours will allow for translational research to understand the biology that drives tumour behaviour. This may allow for better use of existing therapeutic agents, or the development of novel treatments.

2.4.5.2 Relevance of Pathologic Subtypes and Immunotherapy

Immunotherapy has heralded a major change in the management of NSCLC. To date much interest has been placed on the levels of expression of PD-L1 by immunohistochemistry as a predictor of response. The presence of staining for PD-L1 has been shown to enrich for response to PD-1 or PD-L1 inhibition in some studies [160, 161, 166] but this finding is not universal [159]. In the OAK study, improved survival was seen for immunotherapy over chemotherapy in 2nd or 3rd line treatment for patients with PD-L1 negative tumours, despite the absence of any difference in PFS [166]. The field is further confused by the first line studies showing a significant advantage for first line pembrolizumab over chemotherapy in both OS and PFS for patients with PD-L1 IHC staining of greater than 50% [300], while no benefit was seen for the same subgroup of patients in the first line trial of nivolumab versus chemotherapy [163].

The role of histologic subtyping for adenocarcinoma and response to immunotherapy has yet to be reported. Groups are already beginning to investigate relationships between potential correlates of immune activation and response and histologic subtypes. Two groups have investigated rates of PD-L1 staining by IHC in resected pulmonary adenocarcinoma. Cha et al assessed a cohort of 323 patients with resected pulmonary adenocarcinoma [310]. PD-L1 was considered positive in tumour cells if staining was present in greater than 5% of tumour cells, and positive in immune cells if staining was greater than 1% of immune cells. PD-L1 expression in tumour cells was more common in solid predominant adenocarcinoma (25 / 59;
42%) compared to all other subtypes (35 / 264; 13%; p<0.001). The next highest rate of PD-L1 expression was seen in MPA (9 / 30; 30%), with all other subtypes being 14% or less. Infiltrating immune cells were also more likely to be PD-L1 in solid predominant tumours compared to non-solid predominant tumours (OR 3.43; 95% CI 1.49 – 7.89; p=0.004).

Takada et al performed a similar analysis in a cohort of 417 patients [311]. They assessed PD-L1 positive at cut-offs of both 5% and 1%, and pooled solid and micropapillary tumours compared to all others. Solid/MPA tumours were more likely to be PD-L1 positive by either method compared to other histologic subtypes (p<0.0001 for both). For example, at 5% cut-off PD-L1 positive was present in 21 out of 27 solid and MP predominant tumours (78%) compared to 64 out of 390 patients (16%) with other predominant subtypes. A further study from Berhens et al has recently been reported at the World Conference on Lung Cancer, 2016 [312]. Analysis of tumour associated inflammatory cells was performed including assessment of CD3+, CD4+ and CD8+ T-cells. The authors found that CD3+ and CD8+ T-cells were more frequent in solid predominant tumours as compared with non-solid predominant tumours.

Understanding the interaction between immuno-oncologic features of tumours and adenocarcinoma subtypes opens a further avenue of investigation. At present, available immune therapies are expense and only work for a subset of patients – better defining this group may lead to significant cost savings. This may either be in clinical care, or in translational research that unlocks new methods of treatment.

2.4.5.3 Issues with Histologic Subtyping in Small Specimens

In this work we deliberately selected patients with tumours obtained from surgical interventions at metastatic sites. As discussed, the rationale for this choice was to obtain a large tissue sample that was free from crush artefact, was most likely to be representative of the tumour biology, and allowed sufficient tissue for further testing including molecular testing. A limited number of patients in our cohort (sixteen) had surgical samples available from two or more sites, with some samples being separated over time. Spatial and temporal
consistency of subtypes, if replicated in future studies, is a finding of great importance. For histologic patterns to have a bearing on treatment choices clinicians must know that the sample that is obtained is representative of the tumour as a whole and its predicted biological behaviour. Our findings give reassurance that the most clinically appropriate site can be chosen for biopsy, rather than needing to target a particular area.

The majority of work using the IASLC/ATS/ERS classification has been conducted in the setting of resected early stage disease. This work was the first to use pathologic subtyping exclusively at metastatic sites. Campos-Parra et al similarly assessed core biopsy specimens for histologic patterns in advanced disease, however their study also included patients with samples obtained from the lung [296].

Two issues require further clarification and investigation prior to routine application of adenocarcinoma subtyping in advanced disease. Firstly – are patterns seen on small biopsy specimens subject to significant intra- or inter-observer variability? Prior studies have shown concordance rates in resection specimens are good (as measured by Kappa values [283, 313]) and can be improved with dedicated training of pathologists [314]. However, whether this is also true of small biopsy specimens is not yet known.

Secondly – how representative is the major pathologic pattern seen on a core biopsy to the biological behaviour of the tumour in individual patients? This will be somewhat important if information on predicted survival is to be provided to the patient, but will be of greater importance if it influences treatment decisions. Matsuzawa et al have assessed this issue in the setting of a diagnostic core biopsy followed by a definitive surgical resection [315]. They showed that concordance from small biopsy to resection specimen was 66% across all samples, and was affected by the histologic subtypes seen together with the size of the small biopsy specimen (large samples producing higher rates of concordance). Trejo Bittar et al have asked a similar question comparing the accuracy of pathologic subtype determined on intraoperative frozen section specimens in comparison to the final predominant subtype determined on the permanent specimen by H&E staining [316]. The primary histologic pattern on the permanent section was correctly identified in frozen section in 78 out of 112
cases (69.7%). The authors noted that there was ‘moderate’ agreement for the primary pattern, and ‘fair’ agreement for the secondary pattern. They concluded that “subtyping of lung adenocarcinoma on frozen sections is difficult and may not be reliable”.

Further work on these two issues will be required prior to the inclusion of pathologic subtyping in advanced disease as part of routine care.

2.4.6 Limitations

This was the first study to explore the recommendations of IASLC/ATS/ERS pulmonary adenocarcinoma classification in regard to subtyping of tumours in metastatic disease. The findings of this work require validation from other groups.

In this work samples were selected from surgically obtained specimens in order to maximise the amount of tissue available for further testing as well as attempting to limit distortion of the histologic features from issues such as crush artefact. Only a small number of patients had tissue samples of similar size to that which may be usually obtained by percutaneous biopsy. A concern may arise that the morphologic subtype(s) seen in a small specimen may not be representative of major subtype in metastatic tumour. Future studies focusing on core biopsies obtained percutaneously would be required to allow generalisation of these findings as a limited number of patients undergo metastatectomy during the course of their advanced disease.

Due to the nature of the inclusion criteria that we set for this work we had a modest sample size at 100 cases. While we were able to show a difference in survival outcomes for patients treated with systemic therapy, the study was lacking in power. Therefore, there may be other important findings that remain to be discovered in a larger study.
The study demonstrated that for patients receiving systemic therapy the major solid pattern was predictive of a shorter survival time. No difference was observed between the major acinar and major micropapillary subtypes but both of these groups had small numbers (n=13 each). Therefore a larger study is required to demonstrate whether or not there was a difference with statistical confidence.

The study was retrospective in nature. As such the treatment of each patient was done on the basis of local treatment guidelines and at the discretion of the treating clinicians. The differences observed in survival outcomes may have been influenced by differences in the treatment of each patient. Such bias could be mitigated by exploring major pathologic subtypes at metastatic sites as a translation research study. This could be performed as a substudy in the setting of a large well-conducted clinical trial. As such, first line treatment would be standardised across the trial, and differences in progression free survival could be assessed to see if they had any further prognostic impact. By taking a prospective approach, and performing the analysis in the setting of a trial the number of potential sources of bias could be controlled or reduced.

2.5 Conclusion

The IASLC/ATS/ERS classification has added meaningful prognostic information in the setting of resected early stage pulmonary adenocarcinoma. We have used the morphologic subtypes described in this classification and investigated their clinical relevance at metastatic sites. We have demonstrated that the major solid pattern of adenocarcinoma at metastatic sites is associated with worse overall survival outcomes compared to the major acinar and major micropapillary subtypes for patients treated with systemic anticancer therapies. Identification of the major pattern did not affect survival outcomes for those who did not receive systemic treatment.

We have also demonstrated that the major solid pattern had infrequent mutations in EGFR as compared to the major acinar and major micropapillary patterns in this predominantly
Western cohort. No significant association between the major patterns and the frequency of KRAS mutations was seen.

This study was the first study to examine the morphologic adenocarcinoma patterns in the IASLC/ATS/ERS classification at exclusively metastatic sites.
CHAPTER 3

EGFR AND KRAS MUTATIONS IN RESECTED PULMONARY ADENOCARCINOMA
3.1 Literature Review

Anatomical pathology is central to the diagnosis of many conditions in clinical practice and provides important information that allows discussions about prognosis and treatment options. Where clinical outcomes are highly variable astute observations by pathologists and clinicians can allow for the development of new classification systems which may improve prognostication and prediction of treatment effects for individual patients. As discussed in the previous chapter the IASLC/ATS/ERS classification system, now adopted in the 4th edition of the WHO classification of lung tumours, marks a step forward in assessing the aggressiveness of resected lung adenocarcinoma [44, 179]. Such an advance prompts further research to look for other clinical or pathologic associations which may provide new insights in the development or treatment of disease.

This chapter examines the frequency of mutations in EGFR and KRAS in an Australian cohort of patients with resected lung adenocarcinoma. It investigates clinicopathologic associations between the pathologic subtype as per the IASLC/ATS/ERS classification, the presence or absence of mutations in EGFR and KRAS, and survival outcomes in this group.

3.1.1 Epidermal Growth Factor Receptor

EGFR is a member of the ErbB family of transmembrane receptor tyrosine kinases. It takes centre stage as the most common targetable oncogenic pathway in adenocarcinoma of the lung, with increasing access to oral small molecule tyrosine kinase inhibitors.

The ErbB family was found with the initial discovery of the ligand that was subsequently named epidermal growth factor. The seminal work in discovery of epidermal growth factor (EGF) was performed by Stanley Cohen, who shared the Nobel Prize in Physiology or Medicine in 1986 with Rita Levi-Montalcini for his work [317]. EGF was discovered to accelerate incisor eruption and eyelid opening in newborn rats and mice that were treated with a mouse salivary gland extract in 1962 [318]. The role of mouse EGF in stimulation of
skin was subsequently demonstrated in 1965 [319]. In 1975 purification of human EGF from collected urine was performed, and it was demonstrated to have the same effects on human fibroblasts as EGF isolated from mice [320]. Subsequently the receptor for EGF was discovered [321, 322].

*EGFR* is coded from a 26 exon gene on chromosome 7p11-13. It is a transmembrane receptor with an external pocket for ligand binding and an internal tyrosine kinase domain for intracellular signalling on activation [323, 324]. Ligands involved in *EGFR* signalling include transforming growth factor-α, EGF, epiregulin, β-cellulin, heparin binding EGF and amphiregulin [324-326]. Once a ligand binds, *EGFR* forms homodimers or heterodimers with other members of the ErbB receptor family, resulting in phosphorylation of intracellular tyrosine kinase domains leading to downstream signalling in multiple intracellular pathways [325]. *EGFR* is downregulated by protein kinase C dependent mechanisms that result in altered phosphorylation patterns in the intracellular domain leading to reduced affinity for EGF [327-329]. Mouse models resulting in loss of *EGFR* signalling show its importance in normal development. Absence of *EGFR* results in developmental abnormalities in the skin, lung and gastrointestinal tract resembling diseases seen in premature humans [330]. Disruption of *EGFR* pathways in mutant mouse models by two groups also showed the importance of this receptor in multiple other developmental pathways [331, 332].

The role of *EGFR* in tumourigenesis was first suggested in 1984. Ullrich et al sequenced *EGFR* DNA in normal cells and A431 epidermoid carcinoma cells and noted marked amplification of *EGFR* in tumour cells by 15 to 20 fold [333]. Abnormalities in the expression and structure of *EGFR* were subsequently found in multiple human malignancies [334-337], including NSCLC [338-341]. *EGFR* expression by immunohistochemistry was also found to be a poor prognostic marker in NSCLC [342-344]. Given these findings, *EGFR* was selected as a target for therapeutic strategies to treat NSCLC. Successful blockade of the pathway was first achieved in the early 1980s with *EGFR* monoclonal antibodies against the A431 cell line [345-347]. Tyrosine kinase inhibition was also pursued as a development pathway [348].
3.1.2 Early Phase Trials and the Discovery of \textit{EGFR} Mutations

Gefitinib (ZD1839, ‘Iressa’), an oral tyrosine kinase inhibitor of \textit{EGFR}, was first trialled in humans based on activity in cell lines when combined with chemotherapy. Phase one trials showed the drug was well tolerated, and it was noted that a number of patients with NSCLC had objective responses or prolonged stable disease. Toxicity was predictable from animal models, with diarrhoea and skin rashes being the most common events [330, 349-351]. Two concurrent phase two trials (Iressa Dose Evaluation in Advanced Lung Cancer – IDEAL 1 and 2) were run globally (IDEAL 1) and in the USA (IDEAL 2) to assess the optimal dose for gefitinib in NSCLC (250mg daily vs 500mg daily) [352, 353]. The radiographic response rates were 19% and 10% for each trial respectively in a population of patients that were heavily pre-treated. It was noted that for those patients who responded duration of response was long, lasting on average greater than 6 months, and response was also associated with marked symptom improvement. Given the striking improvements noted, the authors of IDEAL 1 undertook a multivariate analysis, showing that response was more frequent in patients with adenocarcinoma histology (OR 3.45, 95% CI 1.29 – 11.02, p=0.021) and female patients (OR 2.65, 95% CI 1.19 – 5.91, p=0.017). Improved response was also noted in Japanese compared to non-Japanese patients, but this did not reach statistical significant in the multivariate model. Despite raised levels of \textit{EGFR} expression, the rationale for choosing \textit{EGFR} as a target, detection of \textit{EGFR} by IHC failed to predict response [341, 354]. It had also been noted that never smokers were more likely to be respond to monotherapy with gefitinib [355]. On the basis of the surrogate endpoint response rate in IDEAL 2, gefitinib received accelerated approval from the United States Food and Drug Administration [356].

The prolonged responses of some patients in the IDEAL trials led to translational research to understand what drove these outcomes. Two groups discovered the underlying mechanism of sensitivity to gefitinib in NSCLC and published their results in 2004 [110, 111]. Both groups hypothesized that mutations in the \textit{EFGR} gene lead to oncogenic activation and tumour progression. It was found that genetic alterations existed in the intracellular activation loop of the tyrosine kinase domain. Point mutations were found in exons 18 and 21, and deletions
in exon 19. Both groups demonstrated that mutations occurred in patients who responded to tyrosine kinase inhibition with gefitinib, but were not present in those who did not respond. Further, mutations were significantly more frequent in the groups that were known to respond clinically (patients with adenocarcinoma, females and never smokers), explaining the beneficial effects of gefitinib seen in these groups. The absence of these mutations in surrounding normal tissue demonstrated that they were somatic in nature. Lynch et al [110] also demonstrated that cell lines with \textit{EGFR} mutation showed a two to three fold increase in activation in the presence of EGF compared to wild type cell lines, that the mutated receptor had a high affinity for gefitinib, and that binding of gefitinib to mutant \textit{EGFR} resulted in its inhibition. Mitsudomi et al confirmed the relationship between \textit{EGFR} mutation and response in a larger cohort of gefitinib treated patients [357].

### 3.1.3 Activating \textit{EGFR} Mutations Predict Response to \textit{EGFR} Inhibitors

The question of whether phenotype or genotype directed response to therapy was first answered by the Iressa Pan Asia Study (IPASS) trial [113]. This well-known phase 3 trial enrolled a clinically enriched population including patients who were non-smokers or light former smokers with no prior treatment and with Stage IIIb or IV adenocarcinoma of the lung. Patients were randomised to first line gefitinib or chemotherapy with carboplatin and paclitaxel (an accepted first line therapy at the time of the trial [358]). 1217 patients were enrolled, and \textit{EGFR} mutation status was successfully determined in 437 (35.9%). 261 samples were positive for a mutation (59.7%). For all patients, progression free survival curves overlapped. Where the mutation status was known a striking benefit in progression free survival for patients who harboured \textit{EGFR} mutations when receiving gefitinib compared to chemotherapy (HR 0.48, 95% CI 0.36 – 0.64, p<0.001), while those known not to have an \textit{EGFR} mutation suffered harm by receiving gefitinib when compared to those on chemotherapy (HR 2.85, 95% CI 2.05 – 3.98, p<0.001).

A number of agents targeting mutated \textit{EGFR} have now been developed. The first generation of reversible TKIs gefitinib and erlotinib (OSI774, ‘Tarceva’) are now part of routine clinical use. Early trials investigated the addition of \textit{EGFR} TKIs to standard chemotherapy
backbones [359-361]. These trials were ran before the knowledge of activating EGFR mutations, and failed to show any survival improvement. Trials in unselected or clinically selected populations have investigated a number of strategies, including 1st line therapy [362], switch maintenance compared with placebo following clinical benefit with first line therapy [363-365], as single agent treatment compared with standard second line chemotherapy options [366-368], or as a last line therapy compared with best supportive care [369]. Of these 10 trials, only the SATURN trial reach its primary endpoint. It demonstrated a statistically significant improvement in PFS for maintenance erlotinib compared to placebo in patients obtaining clinical benefit (defined as complete response, partial response or stable disease) from first line chemotherapy [363]. However, the uptake of EGFR TKIs as an option in maintenance therapy has been low in patients lacking an activating EGFR mutation, in part due to the overall survival advantages demonstrated with pemetrexed chemotherapy as a maintenance option [101]. The majority of these studies did not mandate tissue collection in their protocols. Despite this, several of the studies were able to demonstrate that the presence of an activating EGFR mutation was predictive of an improved response to treatment [363, 364, 370, 371].

The major phase III trials of EGFR TKIs in patients with activating EGFR mutations are listed in table 15. The open label phase IIb trial comparing gefitinib to the second generation pan-HER inhibitor afatinib (LUX-LUNG7) has recently been reported [372]. This trial showed a significant improvement in PFS (HR 0.73 [95% CI 0.57 – 0.95, p=0.017). However, the survival curves were similar with only small delays in the time to progression, and at the expense of increased toxicity. The field has progressed further with the development of the third generation inhibitors rociletinib (CO-1686) [373] and osimertinib (AZD9291) [374] (Note: clinical development of rociletinib has recently been discontinued by its sponsor due to a lower than expected response rate in patients with a secondary EGFR T790M mutation and discontinuations and treatment interruptions for side effects such as hyperglycaemia and QTc prolongation on electrocardiography [375]). The importance of these two new agents will discussed further in chapter four (next chapter).
<table>
<thead>
<tr>
<th>Trial Name</th>
<th>Location</th>
<th>Control Arm</th>
<th>Median PFS</th>
<th>Experimental Arm</th>
<th>Median PFS</th>
<th>Hazard Ratio (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPASS - mutation positive [113]</td>
<td>Asia</td>
<td>Carboplatin / Paclitaxel</td>
<td>N/R</td>
<td>Gefitinib</td>
<td>N/R</td>
<td>0.48 (0.36 - 0.64)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>LUX-LUNG 3 [115]</td>
<td>Worldwide</td>
<td>Cisplatin / Pemetrexed</td>
<td>6.9 months</td>
<td>Afatinib</td>
<td>11.1 months</td>
<td>0.58 (0.43 - 0.78)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>LUX-LUNG 6 [376]</td>
<td>Asia</td>
<td>Cisplatin / Gemcitabine</td>
<td>5.6 months</td>
<td>Afatinib</td>
<td>11.0 months</td>
<td>0.28 (0.20 - 0.39)</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>NEJ002 [116]</td>
<td>Japan</td>
<td>Carboplatin / Paclitaxel</td>
<td>5.5 months</td>
<td>Gefitinib</td>
<td>10.4 months</td>
<td>0.36 (0.25 - 0.51)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>OPTIMAL [117]</td>
<td>China</td>
<td>Carboplatin / Gemcitabine</td>
<td>4.6 months</td>
<td>Erlotinib</td>
<td>13.1 months</td>
<td>0.16 (0.10 - 0.26)</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>EURTAC [118]</td>
<td>Europe</td>
<td>Platinum / Gemcitabine</td>
<td>5.2 months</td>
<td>Erlotinib</td>
<td>9.4 months</td>
<td>0.42 (0.25 - 0.54)</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>WTJOG 3405 [119]</td>
<td>Japan</td>
<td>Cisplatin/Docetaxel</td>
<td>6.3 months</td>
<td>Gefitinib</td>
<td>9.2 months</td>
<td>0.49 (0.34 - 0.71)</td>
<td>p &lt; 0.0001</td>
</tr>
</tbody>
</table>

**Table 15:** Trials comparing standard first line platinum (cisplatin or carboplatin) doublet chemotherapy with *EGFR* tyrosine kinase inhibitors in patients whose lung cancers have activating *EGFR* mutations

PFS, Progression Free Survival; 95% CI, 95% Confidence Interval; N/R, Not Reported
3.1.4 The Discovery of RAS Mutations

The discovery of mutations in the RAS (rat sarcoma virus) gene traces its history back to the 1960s. Harvey reported “an unidentified virus (that) causes the rapid production of tumours in mice” in 1964 [377]. The mice were injected with a leukemic virus and subsequently died of tumours with a sarcomatoid appearance on histology. Similar findings were presented in a paper from Kirsten and Mayer when rats were injected with a different virus [378].

The link from the findings of the 1960s and their relationship to human tumours was reported in 1982. Chang et al showed that the H-RAS and K-RAS genes in the rat were closely related to four similar genes in humans [379]. Studies from multiple groups found that activating mutations could be found in RAS genes in cancer cell lines [380-383]. A further RAS family member, N-RAS, was also described [384].

Santos et al went on to perform further investigations of the role of the K-RAS gene in cell lines and in human samples [385]. They decided to analyse lung carcinoma specimens given the prior frequency with which RAS mutations were discovered in varying cell lines. Only one of eight of the human samples tested was found to have a KRAS mutation. The patient, a 66 year old man with squamous cell carcinoma of the lung, was found to have this mutation in his tumour, however the mutation was absent from surrounding normal tissue. The mutation found in the tumour specimen was also the same as one of those from a previously studied cell line. The DNA from this patient’s tumour was able to transform NIH/3T3 fibroblast cells into malignant cells whilst DNA from other patients’ tumours were not, showing the importance of an activating mutation in the KRAS gene to malignant transformation in normal cells.

3.1.5 KRAS in Lung Adenocarcinoma

With the knowledge of KRAS mutations as described above Rodenhuis et al set about identifying clinicopathologic associations [386]. They performed KRAS mutation testing on
tumours from 39 patients who had undergone surgical resection for NSCLC. \textit{KRAS} mutations were found in five of the ten cases of adenocarcinoma, and none of the 29 other cases of NSCLC. An initial observation of a relationship between \textit{KRAS} mutation and a positive smoking history was made, and then confirmed in subsequent papers from the same group [387, 388]. The prognostic implications of \textit{KRAS} mutations in resected disease are discussed in detail below.

3.1.6 Targeting \textit{KRAS} in Lung Adenocarcinoma

Given the frequency of \textit{KRAS} mutation and its role in oncogenesis multiple attempts have been made to reduce its effects either through direct or downstream inhibition. To date many of these efforts have been unrewarding [389]. So far it has not been possible to successfully inhibit RAS directly (in comparison to other oncogenes such as \textit{EGFR} and \textit{ALK}). While many tyrosine kinase enzymes transition between adenosine diphosphate and adenosine triphosphate (ATP) when active, the RAS enzymes use guanine diphosphate in the inactive state and guanine triphosphate (GTP) in the active state [390]. RAS has high affinity for GTP and thus mutant RAS is able to stay in its active state with relative ease. RAS also lacks “an accessible active site or pocket to which molecules are likely to bind” [390].

Most recently attempts have been made to block mitogen protein activated kinase (\textit{MEK}) with the \textit{MEK} inhibitor selumetinib in combination with docetaxel. \textit{MEK} sits downstream of \textit{KRAS}, so blocking the pathway here has the potential to interfere with continuous signalling from mutated \textit{KRAS}. It is important to note that this method of interference by downstream inhibition of an oncogene (in this case \textit{KRAS} blockade via \textit{MEK} inhibition) is different to inhibition of mutated \textit{EGFR} which occurs directly at the mutated receptor. A phase II trial compared the addition of selumetinib or placebo to docetaxel, with a promising early results [391]. However, the subsequent phase III trial failed to demonstrate any PFS or OS benefit from the combination over single agent therapy [392].
3.1.7 Expansion of Targetable Oncogenes in Lung Adenocarcinoma

Profound response to \textit{EGFR} inhibition, and the subsequent discovery of \textit{EGFR} mutations has led to the pursuit of other “druggable” oncogenes. The increase in effective agents and the ability to sequence therapies against oncogenic targets will increase survival time for a portion of patients. Currently targetable oncogenes and a list of associated therapies is discussed in more detail in Chapter 1.3.3.2 (page 47).

3.1.8 Prognostic Impact of \textit{EGFR} and \textit{KRAS} Mutations in Resected Disease

3.1.8.1 \textit{EGFR} Mutation in Resection Specimens

The discovery of \textit{EGFR} mutations and their importance in advanced lung adenocarcinoma led to further investigation with regards to their prognostic value in resected disease. The earliest study was published by Kosaka et al in 2004 [393]. This paper included 234 patients with resected adenocarcinoma of whom 91 (39\%) had an activating \textit{EGFR} mutation. Patients who were treated with gefitinib at relapse were excluded from the survival analysis with a view to trying to determine the prognostic significance of \textit{EGFR} mutations. The final results showed no survival difference on the basis of \textit{EGFR} mutation. Further studies have delivered conflicting results and the prognostic importance of the \textit{EGFR} mutation status remains uncertain for patients with resected lung adenocarcinoma.

Four studies reported results for patients with stage I disease. Ohba et al limited their study to 256 patients who were found to have stage I disease following resection [394]. In this study the \textit{EGFR} mutation rate was 41\% consistent with the Japanese population and the 5-year DFS and OS rates were high at 75\% and 86\% respectively. No differences in the rates of 5-year DFS or OS were found for patients with \textit{EGFR} mutation as compared to patients who were wild type for both \textit{EGFR} and \textit{KRAS}. Sonobe et al reported subgroup analysis from a study that included only patients with \textit{EGFR} or \textit{KRAS} mutations [395]. In this study no difference in rates of 3-year DFS were seen between patients with each mutation type in stage I disease. An overall survival difference was detected in favour of \textit{EGFR} mutation, however this may
potentially have been driven by smoking related comorbidities for patients with KRAS mutation.

Izar et al presented two papers in stage I disease from US cohorts. In the first paper outcomes for 307 patients were assessed of whom 20% had an EGFR mutation [396]. Rates of recurrence were significantly lower for those with EGFR mutation (10%) as compared to those who were wild type for EGFR (22%, p=0.03). This was also associated with better DFS and OS, with a tumour size of less than 2cm also being important in the assessment of DFS. In the second paper both EGFR and KRAS mutation status were assessed in patients with resected stage I disease [397]. In this paper no survival difference was found between those patients whose tumours had an EGFR mutation and patients whose tumours were wild type for both EGFR and KRAS.

The prognostic effect of EGFR mutation is significantly influenced by stage. Three studies in smaller cohorts (cohort size range 27 – 71) have not demonstrated any prognostic significance for the presence of an EGFR mutation across all stages [398-400]. Larger studies have demonstrated trends for survival outcomes related to the presence of EGFR mutation, however if significance was present in univariate analysis it was lost on subsequent multivariate analysis [401-403].

Only two studies so far have suggested associations between EGFR mutation and favourable outcome. A series from MSKCC was published in 2008 with an expanded cohort subsequently reported in 2012 [404, 405]. The initial report included 296 patients [404]. In this paper patients with EGFR mutations had a better prognosis (3 year OS 90%) while those with KRAS mutations had a worse prognosis (66%) when compared to patients who were wild type at both genes (76%; unadjusted p=0.031). However, on adjusting for stage this finding lost significance (p=0.18). The subsequent update included 1118 patients with mutation rates of 20% for EGFR and 25% for KRAS. Those with EGFR-mutant tumours had significantly better overall survival after adjusting for stage compared to those whose tumours were wild-type for EGFR (HR 0.51, 95% CI 0.34 – 0.76, p<0.001). These results may be confounded by the significant use of EGFR TKIs amongst the patients with EGFR mutations.
A smaller study from Sonobe et al included 180 Japanese patients [395]. This study was limited to those patients with *EGFR* (82%) or *KRAS* mutations (18%). An effect of mutation status was only found in overall survival (not DFS) suggesting that mutation status influenced post-relapse response to therapy. The authors demonstrated this effect with a significant difference in OS on relapse between those patients with *EGFR* (median survival time [MST] 46.7 months) and those with *KRAS* mutation (MST 26 months, p=0.0096). 68% of those with *EGFR* mutation received *EGFR* TKIs at relapse.

3.1.8.2 *KRAS* Mutation in Resection Specimens

The first paper investigating associations between *KRAS* mutation and outcomes in NSCLC was published in 1990. Slebos et al reported outcomes for 69 patients with lung adenocarcinoma of predominantly stage I (69%) and with a *KRAS* exon 2 mutation rate of 27% [406]. In this paper the rates of DFS (p=0.038), OS (p=0.002) and death due to cancer (p<0.001) were all worse for patients whose tumours harboured a *KRAS* mutation. This remained significant after adjustment for other known prognostic factors and stage. As *RAS* mutations were recognised to be common in NSCLC a number of subsequent papers have followed.

Seven studies have been reported from centres in Asian countries. In the earliest paper from 1992 Sugio et al reported on 115 Japanese patients with a *KRAS* mutation rate of 18% in this cohort [407]. The study had some heterogeneity, with only 77% of studied patients able to undergo curative intent resection. In the overall study population survival was not altered by the presence or absence of a *KRAS* mutation. Subgroup analysis in patients with resected node negative disease suggested worse outcomes for patients with a *KRAS* mutation.

*KRAS* status is significant in the Asian population when the analysis is limited to patients with stage I disease. Woo et al studied 190 patients with stage I adenocarcinoma with a *KRAS* mutation rate of 14% in the 168 assessable cases [408]. They found that *KRAS* positivity
(p=0.055), the size of the tumour being >30mm (p<0.001), vascular invasion (p<0.001) and an
elevated Ki 67 (defined by the authors as being >10%, p<0.001) were all independent
predictors of recurrence. Ohba et al assessed outcomes for 354 patients and provided a
subgroup analysis for 256 patients with stage I disease [394]. Outcomes for DFS and OS were
significantly worse for those patients withKRAS mutation in stage I disease (p=0.009 and
p=0.008 respectively) however the rate of mutation was low at 5% (14 out of 256).

Papers reporting across all stages had varying results. The largest studies ranged from 182
patients up to 397 patients in total. These papers failed to find any prognostic significant for
the presence of aKRAS mutation [394, 403, 409]. Results from Sonobe et al (previously
discussed) demonstrated that patients with KRAS mutations had worse outcomes compared to
patients withEGFR mutations for OS but not DFS, likely reflecting the effects of post relapse
therapies [395]. A small study from Kim et al examined outcomes for 71 patients [398]. This
study found that KRAS mutation was significantly associated with poor OS and freedom from
recurrence, however the actual number of patients withKRAS mutation was small (n=5, 7%).

KRAS mutations are more frequent in non-Asian cohorts. Despite the increased proportions of
patients withKRAS mutant tumours the survival findings are variable. A number of small
studies each with approximately 100 patients failed to find any prognostic value for KRAS
mutation [402, 410, 411]. A single study by Izar et al was limited to patients with resected
stage I adenocarcinoma [397]. This cohort included 312 patients who had undergone surgery
only, with aKRAS mutation rate of 41%. KRAS was the only significant prognostic predictor,
with shorter DFS (HR 3.6 [95% CI 2.1 – 6.2, p<0.0001]) and OS (HR 4.36 [95% CI 2.1 – 9.1,
p<0.0001]).

The study from D’Angelo et al included 1118 patients with aKRAS mutation rate of 25% [405].
As previously discussed patients withEGFR mutations had better OS outcomes than those with
KRAS mutations (and those who were wild type forEGFR). However, patients withKRAS
mutations had no OS difference compared to those who were wild type forKRAS (HR 1.17
[95% CI 0.87 – 1.57, p=0.30]). Nadal et al presented an interesting paper examining both the
presence and type ofKRAS mutation and outcomes [412]. In their 179 patients theKRAS
mutation rate was 47%. They were interested in the G→T transversion in DNA which is associated with smoking. They found that \textit{KRAS} mutations were associated with higher rates of recurrence and shorter DFS and OS compared to patients with \textit{KRAS} wild type tumours. Of particular note was the \textit{KRAS} G12C mutation which led to recurrence or death events in 89% as compared to those with non-G12C \textit{KRAS} mutations at 52% (Fisher’s exact p=0.005).

Two groups have published outcomes from large cohorts from multiple centres. The first of these was a meta-analysis from Mascaux et al [413]. They included 28 papers with 3620 patients. Papers included \textit{KRAS} assessment by polymerase chain reaction (PCR) or the use of IHC expression of p21 as a surrogate marker for \textit{KRAS} mutation. The presence of a \textit{KRAS} mutation was a significant factor for OS across the whole cohort, however on further examination this held only for patients with adenocarcinoma histology (HR 1.3 [95% CI 1.2 – 1.5, p=0.01]) and not for those with SqCC histology (HR 1.5 [95% CI 0.9 – 2.5, p=0.48]). Further interrogation of the data found that survival differences were only significant when \textit{KRAS} status was determined by PCR, and not when assessed by IHC for p21.

Shepard et al assessed the prognostic and predictive important of \textit{KRAS} mutation in post hoc pooled analysis of data from patients enrolled in the IALT, ANITA, JBR10 (all cisplatin and vinorelbine) and CALBG9633 (carboplatin and paclitaxel) trials of adjuvant chemotherapy vs observation following surgical resection [414]. They found both in the overall analysis and in the subgroup of patients with adenocarcinoma only that there was no significant prognostic difference between \textit{KRAS} mutation and \textit{KRAS} wildtype for DFS or OS. The results of Shepard et al and Mascaux et al are at odds with each other, however those of Shepard et al have the strength of controlling for a number of other variables given that the data has been acquired from randomised clinical trials.
3.1.9 Adenocarcinoma Subtypes and Oncogenic Mutations – Historical Associations

3.1.9.1 Bronchioalveolar Carcinoma

*EGFR* mutations were first associated with the now outdated term BAC not long after their discovery. Hsieh et al examined a cohort of 35 Taiwanese patients who had undergone surgical resection for primary lung adenocarcinoma [415]. In their cohort 17 patients (48%) harboured exon 19 deletion mutations or the L858R point mutation or exon 18 missense mutations. Fourteen of 21 patients (66%) with any BAC pattern present had an *EGFR* mutation compared to only 3 of 14 patients with pure invasive patterns (21%; p=0.009). These findings from patients with resected pulmonary adenocarcinoma were subsequently replicated by a number of groups, showing that at various cut-offs the increasing presence of BAC pattern was associated with *EGFR* mutations [416-421].

Further work by multiple groups examined the differences between mucinous BAC (mBAC) and non-mucinous BAC (nmBAC). Prior to the discovery of *EGFR* mutations it was known that mBAC tumours were more likely to harbour *KRAS* mutations than nmBAC and invasive adenocarcinomas [422, 423]. Following the discovery of *EGFR* mutations subsequent papers confirmed the link between mBAC and *KRAS* mutations, and showed that *EGFR* mutations were rarely found in these tumours. Conversely, nmBAC pattern tumours commonly had *EGFR* mutations but infrequently had *KRAS* mutations [251, 424-428]. In the IASLC/ATS/ERS classification mBAC has been superseded by the term invasive mucinous adenocarcinoma, and BAC has been replaced by the lepidic growth pattern which can appear in a number of histologic types [179].

3.1.9.2 The Acinar Pattern

With the focus on BAC, very little attention was paid to other adenocarcinoma subtypes with respect to *EGFR* mutations. Sonobe et al dichotomised 153 patients with adenocarcinoma by the presence or absence of each subtype. The presence or absence of an acinar component
had similar *EGFR* mutation rates (46/96, 48% and 34/57, 59.6%) [429]. Chantranuwat et al found that in their samples from 58 Thai patients the rate of *EGFR* mutation in acinar dominant tumours was similar to the background rate (15/24, 62.5%; background rate 56.9%) [421].

### 3.1.9.3 The Papillary Pattern

There were conflicting papers on the presence of *EGFR* mutations in the papillary pattern. In four Thai patients with primarily papillary tumours Chantranuwat et al found no *EGFR* mutations [421]. Conversely Ding et al [430] and Sonobe et al [429] showed that the presence of an *EGFR* mutation was more frequent in tumours with the papillary pattern. Kim et al provided some interesting indirect evidence of *EGFR* mutations and the papillary subtype [431]. They examined the outcomes for 36 Japanese patients who developed relapsed disease following surgical resection at their hospital, and were subsequently treated with gefitinib in the initial clinical trials. They found that patients with a “dominant papillary subtype” were more likely to respond to gefitinib compared to patients with other subtypes (it should be noted that the numbers in each subtype were small).

### 3.1.9.4 The Solid Pattern

Three groups had examined the solid pattern and its relationship with *EGFR* mutations prior to the IASLC/ATS/ERS classification. In two of these papers the solid pattern was found to be less likely to harbour an *EGFR* mutation compared to other patterns [429, 430]. In the paper by Chantranuwat et al *EGFR* mutations were seen in six of the 14 solid pattern tumours (42.9%) compared to the background rate of 56.9% [421].

### 3.1.9.5 The Micropapillary Pattern

Archar et al undertook analysis of MPA tumours for 15 patients in the US [432]. They used a strict definition of MPA (as per Silver and Askin [232]) where >75% had to consist of a
micropapillary pattern. All their patients had an active or prior smoking history. They found mutation rates of 33% for *KRAS* (n=5) and 20% each for *EGFR* and *BRAF* (n=3 for each). In a larger cohort, Ninomiya et al found that in their Japanese cohort the presence of greater than a 5% MPA pattern was associated with increased likelihood of *EGFR* mutation (41/61, 67% vs 22/46, 48%; p=0.043) [420].

3.1.9.6 Forerunners to the IASLC/ATS/ERS Classification

Prior to the release of the IASLC/ATS/ERS Classification two groups compared mutation frequencies to histologic patterns. The first paper, by Motoi et al, was published in 2008 [197]. They employed a modification of the 2004 WHO classification of lung tumours in assessing pathologic subtypes. The study included 100 American patients with resected lung adenocarcinoma. They documented each histologic subtype (acinar, papillary, BAC and solid) in 10% increments and also included the micropapillary pattern for the first time. They picked the most prominent pattern in the sample to assign each tumour into a group. This lead to the following proportions – acinar 31%; papillary 37% (of which 4 of these cases were micropapillary); BAC 7%; and solid 25%. The underlying mutation rates were *EGFR* 17% and *KRAS* 14%. These mutations were mutually exclusive. There were strong correlations between the major papillary pattern and the presence of *EGFR* mutations (p<0.001) as well as the major solid subtype and a significantly lower frequency of *EGFR* mutations (p=0.01). No correlation was found between the major histologic subtype and the presence of *KRAS* mutations.

Dacic et al reported on a separate American cohort of 345 patients with resected adenocarcinoma and *EGFR* and *KRAS* mutation rates of 11% and 30% respectively [433]. They also used modifications of the 2004 WHO classification in assessing tumour samples. The absence of the solid pattern was a strong predictor for *EGFR* mutations (p=0.0103), while the mucinous pattern was a predictor of the presence of *KRAS* mutations (p=0.0034).
3.1.10 The IASLC/ATS/ERS Classification and Oncogenic Mutations

The publication of the IASLC/ATS/ERS classification (and its addition to the 4th edition of the WHO classification) has provided a reliable and reproducible method for distinguishing the subtypes of pulmonary adenocarcinoma [44, 179]. At the time of commencement of this study there were few papers looking for associations between pathologic subtype and oncogenic mutations. As a natural extension of this classification multiple groups have begun to explore various clinical and pathologic correlates, including the association of oncogenic mutations with predominant adenocarcinoma subtypes in resection specimens. Given the differences in the underlying mutation rates these papers are discussed on the basis of Asian and non-Asian cohorts.

3.1.10.1 EGFR Mutations in Asian Cohorts

To date, papers from eleven groups have been published looking at associations by histologic subtypes and oncogenic mutations [272, 299, 307, 308, 434-440] (Table 16). All have covered EGFR mutations, with smaller numbers covering KRAS and other less common mutations. Most cohorts had high proportions of never smokers. The rate of EGFR mutations ranged from 43.5% to 76.6%. Where reported statistical methods varied across the papers. Several common themes emerged from the report studies despite the differences in statistical methods. For patients with solid predominant adenocarcinoma the rates of EGFR mutation were reported as being statistically significantly lower than the comparison group described in eight papers (the composition of the comparison group varied across papers) [299, 307, 308, 434, 435, 437, 438, 440]. In the remaining three papers the rates of EGFR mutation for solid predominant tumours were numerically lower compared to other groups [272, 436, 439]. It should be noted that EGFR mutations in Asian patients with solid predominant adenocarcinoma remain a relatively frequent occurrence despite these statistical findings, with reported rates ranging from 8.6% up to as high as 60.8% depending on the baseline mutation rate and the cohort studied. EGFR mutations were also occasionally reported in patients with invasive mucinous adenocarcinoma (range 0 – 33%), however this was an uncommon event [308, 435-440].
Associations between predominant subtypes and increased frequency of EGFR mutation over the reference group used (which varied across papers) were made, most commonly for MPA [272, 307, 308, 434], and also with LPA [308, 434], papillary [435] and acinar [437] predominant subtypes. There was no consistent signal to suggest that mutation rates for one predominant subtype were a more frequent event than the background mutation rate seen in these cohorts. Several groups also investigated the importance of the presence of particular patterns when observed within a tumour specimen as a minor subtype. It has been suggested that the presence of a lepidic component [299, 307], a papillary component [299, 307] or a micropapillary component [307] increases the likelihood of an EGFR mutation being found. Conversely the presence of either a solid or mucinous component makes EGFR mutation less likely [299, 438].
**Table 16:** Papers Reporting *EGFR* mutation rate by IASLC/ATS/ERS predominant subtype in Asian Cohorts

Abbreviations – AIS – Adenocarcinoma in Situ; ARMS – Amplification Refractory Mutation System; *EGFR* – Epidermal Growth Factor Receptor; HRM – high resolution melt; MIA – Minimally Invasive Adenocarcinoma; MPA – Micropapillary Adenocarcinoma; NR – not reported; PCR – Polymerase Chain Reaction; RT – reverse transcriptase.

<table>
<thead>
<tr>
<th>First Author</th>
<th>Test Method</th>
<th>Spectrum Covered</th>
<th>Number; <em>EGFR</em> Mutation Rate</th>
<th>AIS / MIA</th>
<th>LPA</th>
<th>Papillary</th>
<th>Acinar</th>
<th>MPA</th>
<th>Solid</th>
<th>Invasive</th>
<th>Mucinous</th>
<th>Variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shim [307]</td>
<td>PCR</td>
<td>Exons 18 – 21</td>
<td>n = 107, 50.5%</td>
<td>5 / 8</td>
<td>16 / 32</td>
<td>17 / 34</td>
<td>10 / 12</td>
<td>6 / 21</td>
<td>17 / 23</td>
<td>3 / 18</td>
<td>0 / 2</td>
<td></td>
</tr>
<tr>
<td>Song [434]</td>
<td>PCR</td>
<td>Exons 19 and 21</td>
<td>n = 161, 41.6%</td>
<td>13 / 18</td>
<td>15 / 37</td>
<td>24 / 57</td>
<td>17 / 32</td>
<td>17 / 23</td>
<td>3 / 18</td>
<td>16.7%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Tsuta [435]</td>
<td>HRM</td>
<td>Exon 19 and L858R</td>
<td>n = 880, 40.5%</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCR</td>
<td>Exons 18 – 21</td>
<td>n = 249, 55.4%</td>
<td>9 / 21</td>
<td>21 / 30</td>
<td>98 / 173</td>
<td>56.6%</td>
<td>7 / 18</td>
<td>3 / 18</td>
<td>13.3%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Sun 2014</td>
<td>ARMS</td>
<td>NR</td>
<td>n = 102, 38.2%</td>
<td>8 / 16</td>
<td>8 / 24</td>
<td>9 / 30</td>
<td>12 / 17</td>
<td>1 / 12</td>
<td>2 / 3</td>
<td>8.3%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Yoshizawa</td>
<td>PCR</td>
<td>Exons 18 – 21</td>
<td>n = 167, 53.9%</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Zhang [437]</td>
<td>RT-PCR</td>
<td>Exons 18 – 22</td>
<td>n = 349, 76.6%</td>
<td>28 / 34</td>
<td>39 / 54</td>
<td>152 / 183</td>
<td>83.3%</td>
<td>5 / 6</td>
<td>28 / 46</td>
<td>38.6%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Hu [438]</td>
<td>PCR</td>
<td>Exons 18 – 22</td>
<td>n = 981, 64.7%</td>
<td>58 / 71</td>
<td>118 / 155</td>
<td>356 / 488</td>
<td>72.9%</td>
<td>17 / 24</td>
<td>60 / 163</td>
<td>36.8%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yanagawa</td>
<td>PCR-invader</td>
<td>Known (note)</td>
<td>n = 241, 54.3%</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Jie [440]</td>
<td>Scorpion ARMS</td>
<td>Exon 19 and L858R</td>
<td>n = 122, 48.4%</td>
<td>12 / 25</td>
<td>8 / 12</td>
<td>25 / 55</td>
<td>41.8%</td>
<td>2 / 5</td>
<td>1 / 4</td>
<td>25%</td>
<td>3 / 11</td>
<td></td>
</tr>
<tr>
<td>Li [308]</td>
<td>RT-PCR</td>
<td>Exons 18 – 22</td>
<td>n = 230, 43.5%</td>
<td>8 / 9</td>
<td>22 / 38</td>
<td>41 / 92</td>
<td>44.6%</td>
<td>11 / 13</td>
<td>14 / 62</td>
<td>8.3%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ranges</td>
<td></td>
<td></td>
<td>n = 122, 48.4%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0%</td>
<td></td>
</tr>
</tbody>
</table>

(PCR invader is able to test for exon 18 G719A/C/S; exon 19 deletion; exon 20 S768I and T790M; exon 21 L858R and L861Q.)
3.1.10.2 EGFR Mutations in non-Asian Cohorts

The reported frequency of EGFR mutations in non-Asian cohorts is lower than that from studies reported from Asian centres, ranging from between 9.6 and 28.8% [274, 278, 305, 306] (table 17). Amongst the non-Asian cohorts the proportion of patients with a current or former smoking history was high, ranging from 72% to 83%. Again, the common theme across these four studies is of a reduced frequency of EGFR mutations in solid predominant adenocarcinoma. Likewise, of the papers reporting patients with IMA only one EGFR mutation was found among the 62 patients reported (1.6%) [274, 305, 306]. Across these papers there was no predominant subtype which was found to have an increased frequency of EGFR compared to the reference group used. Kadota et al found that LPA tumours were statistically more likely to have activating EGFR mutations compared to other subtypes (although the rates of mutation in MIA specimens in their cohort were low, and no mutations were observed in AIS specimens) [305]. In two papers the micropapillary subtype was rare (n=4/407, 1.0%) [274] or not seen [306].

A fifth study from a non-Asian cohort was reported by Rekhtman et al [281]. In this cohort of 180 patients the EGFR and KRAS mutation rates were 19% and 35% respectively. In their paper the heterogeneity of morphologic adenocarcinoma patterns was again recognised. While they did follow the IASLC/ATS/ERS classification system they reported that 53 cases (29%) had two or more patterns with a similar “co-dominant” amount, and as such they were unable to allocate a predominant subtype. As of the current time this issue has yet to be raised in other papers using the new classification. Similar to other groups they found no evidence of EGFR mutations in IMA while KRAS mutations were seen in 41% (7/17) of cases. In addition, KRAS mutations were more common in tumours with a solid component of ≥20% of the tumour. They reported inverse relationships between the portion of tumour that was solid or lepidic and mutation status, such that as the lepidic component rose, the rate of EGFR mutations rose while the rate of KRAS mutations fell. The opposite was true as the amount of the solid component rose.
Table 17: Papers Reporting EGFR mutation rate by IASLC/ATS/ERS predominant subtype in non-Asian Cohorts

Abbreviations – AIS – Adenocarcinoma in Situ; EGFR – Epidermal Growth Factor Receptor; MIA – Minimally Invasive Adenocarcinoma; MPA – Micropapillary Adenocarcinoma; PCR – Polymerase Chain Reaction

<table>
<thead>
<tr>
<th>First Author</th>
<th>Test Method</th>
<th>Spectrum Covered</th>
<th>Number; EGFR Mutation Rate</th>
<th>AIS / MIA</th>
<th>LPA</th>
<th>Papillary</th>
<th>Acinar</th>
<th>MPA</th>
<th>Solid</th>
<th>Invasive Mucinous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Russell [278]</td>
<td>Sequenom Oncocarta Panel 1.0</td>
<td>Multiple known mutations</td>
<td>n = 59</td>
<td>28.8%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kadota [305]</td>
<td>PCR</td>
<td>Exon 19 and L858R</td>
<td>n = 846</td>
<td>14.7%</td>
<td>4 / 33</td>
<td>12.1%</td>
<td>27 / 97</td>
<td>27.8%</td>
<td>29 / 151</td>
<td>19.2%</td>
</tr>
<tr>
<td>Mansuet-Lupo [274]</td>
<td>Fragment Analysis (FA) and TaqMan</td>
<td>Insertions /deletions (FA); Known Point Mutations (TaqMan)</td>
<td>n = 397</td>
<td>9.6%</td>
<td>2 / 11</td>
<td>18.1%</td>
<td>10 / 72</td>
<td>13.9%</td>
<td>21 / 185</td>
<td>11.3%</td>
</tr>
<tr>
<td>Villa [306]</td>
<td>PCR and Pyrosequencing</td>
<td>Known – exon 19, codons 719, 768, 790 and 858 – 861</td>
<td>n = 200</td>
<td>20.5%</td>
<td>8 / 22</td>
<td>36.4%</td>
<td>18 / 41</td>
<td>43.9%</td>
<td>2 / 7</td>
<td>28.6%</td>
</tr>
<tr>
<td>Ranges</td>
<td></td>
<td></td>
<td></td>
<td>9.6 – 28.8%</td>
<td>12.1 – 36.4%</td>
<td>18.1 – 43.9%</td>
<td>13.9 – 28.6%</td>
<td>11.3 – 44.0%</td>
<td>9.0 – 50.0%</td>
<td>3.1 – 13%</td>
</tr>
</tbody>
</table>
Associations between the new classification and KRAS mutation status have now been studied in nine cohorts [274, 278, 299, 305, 308, 435, 437, 438]. Eight of the nine studies reported patients with IMA. In these papers the rate of KRAS mutation for patients with IMA was higher, with the majority of papers demonstrating a statistically significant association. Three studies also found that KRAS mutations were statistically more frequent in solid predominant adenocarcinomas as compared to other subtypes [278, 305, 438]. Yoshizawa et al were the only group to examine the influence of minor subtypes and KRAS mutations, reporting that KRAS mutations were less frequent if a minor non-mucinous lepidic component was present while being more frequent if a minor mucinous lepidic component was seen [299] (Table 18).
Table 18: Papers Reporting KRAS mutation rate by IASLC/ATS/ERS predominant subtype

Abbreviations – AIS – Adenocarcinoma in Situ; HRM – high resolution melt; KRAS – Kirsten RAS; MIA – Minimally Invasive Adenocarcinoma; MPA – Micropapillary Adenocarcinoma; NR – not reported; PCR – Polymerase Chain Reaction; RT – reverse transcriptase.

<table>
<thead>
<tr>
<th>First Author</th>
<th>Test Method</th>
<th>Number; KRAS Mutation Rate</th>
<th>AIS / MIA</th>
<th>LPA</th>
<th>Papillary</th>
<th>Acinar</th>
<th>MPA</th>
<th>Solid</th>
<th>Invasive Mucinous</th>
<th>Variants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Asian Cohorts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tsuta [435]</td>
<td>HRM</td>
<td>n = 862 11.1%</td>
<td>NR 4.0%</td>
<td>NR 9.2%</td>
<td>NR 15.6%</td>
<td>NR 3.7%</td>
<td>NR 16.2%</td>
<td>NR 5.3%</td>
<td>NR 74.4%</td>
<td></td>
</tr>
<tr>
<td>Yoshizawa [299]</td>
<td>PCR</td>
<td>n = 158 13.3%</td>
<td>AIS 0% MIA 8.3%</td>
<td>NR 0%</td>
<td>NR 4.5%</td>
<td>NR 23.1%</td>
<td>NR 0%</td>
<td>NR 25%</td>
<td>4 / 4 100%</td>
<td></td>
</tr>
<tr>
<td>Zhang [437]</td>
<td>PCR</td>
<td>n = 266 2%</td>
<td>0 / 10 0%</td>
<td>0 / 34 0%</td>
<td>1 / 54 1.8%</td>
<td>3 / 183 1.6%</td>
<td>0 / 6 0%</td>
<td>1 / 46 2.2%</td>
<td>2 / 14 14.3%</td>
<td>0 / 2 0%</td>
</tr>
<tr>
<td>Hu [441]</td>
<td>RT – PCR</td>
<td>n = 981 7.1%</td>
<td>0 / 33 0%</td>
<td>1 / 71 1.4%</td>
<td>5 / 155 3.2%</td>
<td>26 / 488 5.7%</td>
<td>2 / 24 8.3%</td>
<td>19 / 163 11.6%</td>
<td>16 / 44 36.4%</td>
<td></td>
</tr>
<tr>
<td>Li [308]</td>
<td>RT – PCR</td>
<td>n = 230 16.5%</td>
<td>2 / 7 28.6%</td>
<td>1 / 9 11.1%</td>
<td>5 / 38 13.1%</td>
<td>13 / 92 14.1%</td>
<td>0 / 13 0%</td>
<td>13 / 62 21.0%</td>
<td>4 / 9 44.4%</td>
<td></td>
</tr>
<tr>
<td><strong>Ranges</strong></td>
<td></td>
<td></td>
<td>2 – 16.5%</td>
<td>0 – 28.6%</td>
<td>0 – 11.1%</td>
<td>1.8 – 15.6%</td>
<td>1.6 – 23.1%</td>
<td>0 – 16.2%</td>
<td>2.2 – 25%</td>
<td>14.3 – 100%</td>
</tr>
<tr>
<td><strong>Non-Asian Cohorts</strong></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Russell [278]</td>
<td>Sequenom Oncocarta Panel 1.0</td>
<td>n = 59 22.0%</td>
<td></td>
<td></td>
<td></td>
<td>3 / 25 12.0%</td>
<td>1 / 13 7.7%</td>
<td>9 / 21 42.8%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kadota [305]</td>
<td>Sanger Sequencing</td>
<td>n = 864 26.4%</td>
<td>6 / 33 18.2%</td>
<td>21 / 97 21.6%</td>
<td>46 / 151 30.5%</td>
<td>66 / 300 22.0%</td>
<td>22 / 78 28.2%</td>
<td>43 / 163 26.4%</td>
<td>24 / 42 57.1%</td>
<td></td>
</tr>
<tr>
<td>Mansuet-Lupo [274]</td>
<td>TaqMan</td>
<td>n = 397 34.0%</td>
<td>3 / 11 27.2%</td>
<td>24 / 72 33.3%</td>
<td>60 / 185 32.4%</td>
<td>1 / 4 25%</td>
<td>36 / 106 34.0%</td>
<td>12 / 16 75%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ranges</strong></td>
<td></td>
<td></td>
<td>22.0 – 34.0%</td>
<td>12.5 – 18.2%</td>
<td>21.6 – 40%</td>
<td>30.5 – 34.6%</td>
<td>12.0 – 32.4%</td>
<td>7.7 – 28.2%</td>
<td>26.4 – 42.8%</td>
<td>57.1 – 75%</td>
</tr>
</tbody>
</table>

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3.1.10.4 Impact of *EGFR* and *KRAS* Mutation on Survival

Several groups have investigated if an interaction exists between the presence of an oncogenic mutation in *EGFR* or *KRAS* and the IASLC/ATS/ERS classification in resected disease. Mansuet-Lupo et al did not report a significant effect on OS for patients with *EGFR* mutation as compared to those with wild type *EGFR* in their entire cohort or in the subset with stage I disease [274].

Hu et al found no impact on overall survival (OS) outcomes in multivariate analysis when the presence or absence of an *EGFR* mutation was included [438]. A trend to short recurrence free survival (RFS) and longer OS was seen in patients with *EGFR* mutation. Further, in comparing between good and poor prognostic driver mutations, an OS advantage was seen for patients with *EGFR* mutations in comparison to patients with *KRAS* or *HER2* mutations. In a subset analysis of patients with resected stage IIIA disease the presence of mutations in any of *KRAS*, *BRAF*, or *HER2* conferred worse OS for patients with acinar or papillary predominant tumours than that seen for solid or micropapillary predominant tumours.

In a smaller study Russell et al were able to perform molecular analysis together with survival outcomes for 59 patients who had had surgical resection of lung adenocarcinoma with N2 nodal involvement [278]. Patients with acinar predominant adenocarcinoma had significantly better survival than those with micropapillary or solid predominant adenocarcinoma. They found a trend that suggested that those patients with resected micropapillary tumours harbouring an activating *EGFR* mutation had a similar survival outcome to patients with acinar predominant tumours, whereas micropapillary predominant tumours with wild type *EGFR* maintained a poorer outcome.

Yoshizawa et al examined outcomes for a subset of patients in their paper [299]. Mutation testing results were available for 167 patients for *EGFR* mutation status and 158 patients for *KRAS* mutation status. As the cohort was Japanese the rate of *EGFR* mutation was high at 54% while *KRAS* mutations were infrequent at 13%. A statistically and clinically significant
improved rate of 5 year OS was seen for those patients with *EGFR* mutant tumours, but with no difference in DFS at 5 years. Inclusion of this result in a multivariate analysis was not reported in their paper.

### 3.1.11 Conclusion

The development of *EGFR* inhibitors, and the subsequent discovery that these medications were highly efficacious for patients with sensitising *EGFR* mutations heralded a major shift in the treatment of advanced lung adenocarcinoma. *KRAS* mutations remain prevalent in patients with lung adenocarcinoma and a history of smoking. To date there has been no therapy that successfully targets advanced *KRAS* mutant lung adenocarcinoma specifically. Neither *EGFR* mutation nor *KRAS* mutation has been found to be reliably prognostic following resection of early stage lung adenocarcinoma with curative intent.

The IASLC / ATS / ERS classification of lung adenocarcinoma, as subsequently incorporated into the 4th edition of the WHO Classification of lung tumours, marks a major advance in pathologic assessment of lung cancers [44, 179]. The classification has already been shown to be of prognostic (Chapter 2.1.3.2, page 90) and predictive significance [303]. Such findings have spurred further research to understand clinicopathologic correlates. At the time of inception of this study the relationship between oncogenic drivers and the IASLC / ATS / ERS classification was not known. However, subsequently a number of studies have been published (section 3.1.10, page 158) exploring this connection.

This study aims to assess the rates of *EGFR* and *KRAS* mutation by the IASLC / ATS / ERS adenocarcinoma subtype within a predominantly Caucasian cohort. We also explore whether there is interaction between the predominant adenocarcinoma subtype, the oncogene mutation status and survival outcomes.
3.2 Materials and Methods

3.2.1 Inclusion Criteria

Patients with resected pulmonary adenocarcinoma were identified by reviewing a prospectively maintained database at St Vincent’s Hospital, Melbourne. The database is maintained by members of the Lung Cancer MDT. It includes patients who have undergone treatment at St Vincent’s Hospital, Melbourne (public hospital) or the adjoining St Vincent’s Private Hospital, Melbourne for management of thoracic malignancies. Data is prospectively entered by the treating clinicians and nurse coordinators from respiratory medicine, thoracic surgery or medical oncology. Data captured includes age, sex, smoking history, ECOG performance status at diagnosis, clinical and pathologic stage at diagnosis, date of recurrence for curatively treated patients, last known date alive as recorded from their last clinic visit date or date of death if this event is known. The establishment and maintenance of the database received prospective ethics approval from the HREC of St Vincent’s Hospital Melbourne, and patients provide informed consent for collection of their data.

Patients were included in this study if they had undergone surgery between November 2000 and December 2011. The starting time for patient inclusion coincided with the development of the database for cases of thoracic malignancy (discussed below) and the commencement of a thoracic surgeon with an academic interest (A/Prof Gavin Wright). Inclusion of patients for this study ceased in December 2011, at the time of commencement of this research project. Patients enrolled in this study had prospectively consented to use of tissue from their surgical resection for translational research. Samples were included from patients who had undergone standard resections (such as lobectomy or pneumonectomy) or limited resections (wedge resection or anatomical resection) where the patient wasn’t able to tolerate a more extensive operation. Operations were performed with curative intent, and patients were only included if preoperative imaging and operative findings demonstrated no evidence of metastatic disease (distant metastases, pleural metastases or malignant effusions).

All patients had pathologically confirmed adenocarcinoma defined as a malignant epithelial tumour with histologic patterns including lepidic, acinar, papillary, micropapillary and solid...
with mucin as defined according to the IASLC/ATS/ERS classification [179]. Representative images of each histological subtype are demonstrated in a prior publication from our group [200]. Clinical information was obtained from the prospectively maintained surgical database and comprehensive chart review. The definition of a never smoker was a patient with a lifetime equivalent consumption of 100 cigarettes or less. Survival time was calculated from the date of surgery.

3.2.2 Clinical Data Collection

The medical records of St Vincent’s Hospital, Melbourne were reviewed to confirm that the clinical diagnosis was consistent with pulmonary adenocarcinoma. Data was collected from the following sources:

- The medical records of St Vincent’s Hospital, Melbourne
- The prospectively maintained database of patients with thoracic malignancy at St Vincent’s Hospital, Melbourne (updated by the Departments of Respiratory Medicine, Thoracic Surgery and Medical Oncology)
- The private medical records of clinicians who are part of the Lung Cancer Multidisciplinary Team at St Vincent’s Hospital Melbourne
  - A/Prof Gavin Wright – Thoracic Surgeon
  - Mr Naveed Alam – Thoracic Surgeon
  - A/Prof Matthew Conron – Respiratory Physician

In the event that a patient was no longer under the care of a member of the St Vincent’s Hospital Lung MDT unit information was requested by a research officer (Timothy Clay) from a clinician known to be involved in the patients’ most recent care or through direct contact with the patient or a listed relative.

Data was collected collated on all included cases from the previously listed sources. The following pieces of data were collected:
• Relevant dates – date of the surgical procedure (used as start point for analysis of DFS and OS); date of confirmation of recurrence where applicable (either by imaging consistent with recurrence disease or pathologic confirmation of recurrence); date of last follow up or date of death

• Demographic information – age at diagnosis; sex; smoking history (current, former or never smoker [as per convention the definition of a never smoker was a life time consumption of 100 or fewer cigarettes])

• ECOG PS at diagnosis as listed in the prospectively maintained thoracic oncology database at St Vincent’s Hospital Melbourne

• Treatments used including systemic therapy, surgical intervention(s) and radiotherapy. This data was collected as available from the previously listed sources.

Consideration was given to collecting data about patient ethnicity, given the known associations with rates of EGFR mutation by Asian as compared to non-Asian ethnicity. The only potential method to do this was to use country of birth as a surrogate for ethnicity. During data collection it became clear that the recorded country of birth in the medical record did not necessary represent the ethnic heritage of some of the study participants, and therefore this could not be assessed further in the current study.

3.2.3 Anatomical Pathology Assessment

The location, number and size of tumours were retrieved from the pathology report. Pathologic staging as determined by the AJCC staging manual was collected (as per the 5th, 6th or 7th edition) as reported in the contemporaneous pathology report [45, 442, 443]. Data on the presence or absence of each of the following features were also collected from the original pathology report:

• Pleural invasion – visceral or parietal

• Lymphatic or vascular invasion

• Perineural or intraneural invasion
A pathologist (Associate Professor Prudence Russell), blinded to patient outcome, reviewed all H&E slides. The histologic patterns (lepidic, acinar, papillary, micropapillary and solid with mucin) were semi quantitatively assessed in 5% increments. The most abundant pattern compromising of at least 30% of the tumour was labelled the “predominant” pattern as per the IASLC / ATS / ERS classification [179].

Tumours with a predominant lepidic component were classified as either adenocarcinoma-in-situ (AIS – a tumour less than 30mm in maximum size with no evidence of invasion), minimally invasive adenocarcinoma (MIA – a tumour less than 30mm in maximum size with an invasive component measuring less than 5mm), or lepidic predominant adenocarcinoma (LPA – for lepidic predominant tumours where the size was greater than 30mm and/or the invasive component was greater than 5mm in size). In addition to LPA the other invasive subtypes were acinar predominant, papillary predominant, solid predominant and micropapillary predominant. Variants of adenocarcinoma (invasive mucinous adenocarcinoma [IMA] and colloid predominant adenocarcinoma) were also identified when present.

3.2.4 Molecular Pathology Assessment

Molecular pathologic assessment was conducted by Dr Hongdo Do, postdoctoral research fellow of the Peter MacCallum Cancer Centre, East Melbourne, Victoria, Australia (now of the Translational Genomics and Epigenetics Laboratory, Olivia Newton John Cancer and Wellness Centre, Heidelberg, Victoria, Australia). The methods for $EGFR$ and $KRAS$ mutation testing are previously described in Chapter two (Section 2.2.5, page 108).
3.2.5 Statistical Analysis

Statistical analysis was undertaken using STATA version 12 (Statacorp, LP, TX). Survival analysis was performed using the Kaplan-Meier method and the log-rank test, with hazard ratios derived using the Cox Proportional Hazards model. Survival analysis was performed from the date of surgery until a survival event occurred (death or recurrence as appropriate). Where a patient was lost to follow up data survival analysis was censored at their last known time of review. Tests of association were performed using Pearson’s χ² or Fisher’s exact test as appropriate. Median times and hazard ratios are reported with their 95% CI. p less than 0.05 was considered statistically significant.

3.3 Results

3.3.1 Patient Characteristics

Between November 2000 and December 2011 there were 178 patients who underwent surgical resection and were eligible for inclusion in the analysis. Eighty-five patients were male (48%) and 93 were female (52%). The median age was 68 years (range: 20 – 87 years). The ECOG PS preoperatively was 0 in 124 patients (70%), 1 in 50 patients (28%) and 2 in four patients (2%). There were 38 current smokers (21%), 100 former smokers (56%) and 40 never smokers (23%).

The surgical management of the primary tumour was as follows – lobectomy in 151 patients (85%); bilobectomy in six patients (3%); pneumonectomy in six patients (3%); wedge resection in ten patients (6%); and segmentectomy in five patients (3%). Pathologic assessment of the nodal status was possible in 174 patients (98%). The pathologic features are presented in table 19.
Table 1: Pathologic Characteristics for resection specimens from 178 patients.

AIS – Adenocarcinoma in Situ; MIA – minimally invasive adenocarcinoma; LPA – Lepidic Predominant Adenocarcinoma; IMA – Invasive Mucinous Adenocarcinoma

3.3.2 EGFR and KRAS Mutation

EGFR mutations were detected in 53 patients (30%) of whom 27 patients had an exon 19 deletion mutation; 19 patients an exon 21 L858R mutation and one patient an exon 21 L861Q mutation. Two patients had mutations of uncertain clinical significance in exon 18 – one with a deletion/insertion mutation (E709_T710 del_ins D) and one with multiple point mutations
(E709K, K714N, V717E and G719C). There were four patients with exon 20 insertions or duplications. As expected *EGFR* mutations were associated with female sex (38/93 [41%] vs 15/85 [18%], $\chi^2 (1) = 11.4, p=0.001$) and smoking status (never 26/40 [65%]; current 5/38 [13%]; former 22/100 [22%], $\chi^2 (2) = 31.6, p<0.001$).

*KRAS* mutations were detected in 50 patients (28%) at the following locations – G12C 20; G12V 14; G12D 4; G12S 4; G12R 3; and one each at G12F, G12A, G13C, G13D, and G13V. Thirty-seven of these mutations were due to guanine (G) to thymidine (T) transversions, consistent with a smoking related aetiology [444]. *KRAS* mutations were strongly associated with smoking history (former 34/100 [34%] current 15/38 [40%]; never smokers 1/40 [3%]; $\chi^2 (2) = 17.1, p<0.001$).

### 3.3.3 The IASLC/ATS/ERS Classification and Mutation Status

The rates of *EGFR* and *KRAS* mutations by each subtype were compared to the group with the largest number of patients (acinar predominant). Similar *EGFR* mutation rates to acinar (37%) were seen across lepidic pattern tumours (AIS, MIA and LPA – 50%), papillary (27%), and MPA (35%) predominant subtypes with no statistical differences found. *EGFR* mutations were less frequent in the solid predominant subtype (n=3 [9%), odds ratio 0.17 [95% CI 0.05 – 0.61], p=0.007). Further, of the patients with solid predominant subtype only one patient had a mutation that was sensitive to *EGFR* tyrosine kinase inhibitors (TKIs) (L858R point mutation) whilst the two other patients’ tumours harboured exon 20 insertion mutations (associated with primary resistance to *EGFR* TKIs). No *EGFR* mutations were found in the adenocarcinoma variants, IMA or colloid predominant tumours. No associations between the predominant subtype and *KRAS* mutation status were found (Table 20).
<table>
<thead>
<tr>
<th>Pattern</th>
<th>N</th>
<th>EGFR Mutation Rate</th>
<th>Odds Ratio</th>
<th>p</th>
<th>KRAS Mutation Rate</th>
<th>Odds Ratio</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIS/MIA/LPA</td>
<td>18</td>
<td>9 (50%)</td>
<td>1.71 (0.61 - 4.82)</td>
<td>p=0.307</td>
<td>5 (27.8%)</td>
<td>1.33 (0.41 - 4.27)</td>
<td>p=0.627</td>
</tr>
<tr>
<td>Colloid / IMA</td>
<td>8</td>
<td>0 (0%)</td>
<td>-</td>
<td>-</td>
<td>3 (37.5%)*</td>
<td>2.08 (0.45 - 9.61)</td>
<td>p=0.347</td>
</tr>
<tr>
<td>Acinar</td>
<td>76</td>
<td>28 (36.8%)</td>
<td>Referent</td>
<td></td>
<td>17 (22.4%)</td>
<td>Referent</td>
<td></td>
</tr>
<tr>
<td>Papillary</td>
<td>26</td>
<td>7 (26.9%)</td>
<td>0.63 (0.24 - 1.69)</td>
<td>p=0.360</td>
<td>9 (34.6%)</td>
<td>1.84 (0.69 - 4.85)</td>
<td>p=0.220</td>
</tr>
<tr>
<td>Solid</td>
<td>33</td>
<td>3 (9.1%)</td>
<td>0.17 (0.05 - 0.61)</td>
<td>p=0.007</td>
<td>12 (36.4%)</td>
<td>1.98 (0.81 - 4.83)</td>
<td>p=0.132</td>
</tr>
<tr>
<td>MPA</td>
<td>17</td>
<td>6 (35.3%)</td>
<td>0.93 (0.31 - 2.80)</td>
<td>p=0.905</td>
<td>4 (23.5%)</td>
<td>1.07 (0.31 - 3.70)</td>
<td>p=0.918</td>
</tr>
<tr>
<td>Total</td>
<td>178</td>
<td>53 (29.8%)</td>
<td></td>
<td></td>
<td>50 (28.1%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 20: *EGFR* and *KRAS* mutation rates by predominant histologic subtype.

AIS – Adenocarcinoma in situ; MIA – Minimally Invasive Adenocarcinoma; LPA – Lepidic Predominant Adenocarcinoma; IMA – Invasive Mucinous Adenocarcinoma; MPA – Micropapillary Adenocarcinoma.

* *KRAS* rates – IMA 2/3 (66%); Colloid 1/5 (20%).
3.3.4 Survival Outcomes

The median follow up time was 3.6 years (range 1 month – 11.5 years). Seventy patients (39%) had died at most recent follow up. In univariate analysis the stage, predominant subtype and the presence of visceral or parietal pleural invasion were significant for overall survival. EGFR and KRAS mutation status did not have a significant effect on overall survival.

As we wished to investigate whether EGFR or KRAS mutation modified survival outcomes, these factors were included in the multivariate analysis. In multivariate analysis increasing stage and the solid predominant subtype were significant negative prognostic factors, while no significant influence on survival outcomes was observed by the presence of either EGFR or KRAS mutations (Table 21).

Seventy-three patients (41%) were recorded as having a recurrence event. Of these 24 patients (33%) had an EGFR mutation, 15 patients (20%) had a KRAS mutation and 34 patients (47%) were wild type for both EGFR and KRAS. Twenty-nine patients in total had information available on post recurrence systemic therapy. Of the 24 patients with EGFR mutations ten patients received EGFR TKIs, with disease control (stable disease or better) in nine patients.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Factor</th>
<th>Median (months)</th>
<th>Hazard Ratio (univariate)</th>
<th>Univariate p</th>
<th>Hazard Ratio (multivariate)</th>
<th>Multivariate p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>1</td>
<td>122.1 (98.4 - NR)</td>
<td>1 (Referent)</td>
<td>p=0.001</td>
<td>1 (Referent)</td>
<td>p=0.024</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>45.7 (25.5 - NR)</td>
<td>2.78 (1.53 - 5.05)</td>
<td>p=0.001</td>
<td>2.11 (1.10 - 4.03)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>24.7 (16.7 - 58.4)</td>
<td>6.85 (3.80 - 12.33)</td>
<td>p&lt;0.001</td>
<td>5.11 (2.72 - 9.64)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Pattern</td>
<td>AIS/MIA</td>
<td>NR (60.6 - NR)</td>
<td>0.31 (0.04 - 2.29)</td>
<td>p=0.251</td>
<td>0.55 (0.07 - 4.27)</td>
<td>p=0.574</td>
</tr>
<tr>
<td></td>
<td>LPA</td>
<td>NR (25.7 - NR)</td>
<td>0.54 (0.13 - 2.29)</td>
<td>p=0.406</td>
<td>1.05 (0.24 - 4.66)</td>
<td>p=0.943</td>
</tr>
<tr>
<td></td>
<td>Colloid</td>
<td>NR (9.8 - NR)</td>
<td>1.21 (0.28 - 5.13)</td>
<td>p=0.797</td>
<td>1.70 (0.39 - 7.45)</td>
<td>p=0.476</td>
</tr>
<tr>
<td></td>
<td>Acinar</td>
<td>110.0 (59.8 - NR)</td>
<td>1 (Referent)</td>
<td></td>
<td>1 (Referent)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Papillary</td>
<td>63.8 (46.5 - NR)</td>
<td>1.32 (0.65 - 2.71)</td>
<td>p=0.441</td>
<td>1.80 (0.84 - 3.87)</td>
<td>p=0.133</td>
</tr>
<tr>
<td></td>
<td>Solid</td>
<td>40.3 (23.0 - 78.9)</td>
<td>2.40 (1.34 - 4.28)</td>
<td>p=0.003</td>
<td>2.86 (1.54 - 5.36)</td>
<td>p=0.001</td>
</tr>
<tr>
<td></td>
<td>MPA</td>
<td>42.2 (20.9 - NR)</td>
<td>1.81 (0.81 - 4.04)</td>
<td>p=0.147</td>
<td>1.95 (0.85 - 4.45)</td>
<td>p=0.113</td>
</tr>
<tr>
<td></td>
<td>IMA</td>
<td>No events</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pleural Invasion</td>
<td>Absent</td>
<td>110.0 (68.4 - NR)</td>
<td>1 (Referent)</td>
<td>p=0.012</td>
<td>1.16 (0.69 - 1.95)</td>
<td>p=0.573</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>45.7 (34.7 - 88.1)</td>
<td>1.82 (1.14 - 2.92)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR Mutation</td>
<td>Present</td>
<td>98.4 (58.4 - NR)</td>
<td>1 (Referent)</td>
<td></td>
<td>1 (Referent)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>68.4 (42.2 - NR)</td>
<td>1.07 (0.63 - 1.81)</td>
<td>p=0.809</td>
<td>1.25 (0.68 - 1.95)</td>
<td>p=0.475</td>
</tr>
<tr>
<td>KRAS Mutation</td>
<td>Present</td>
<td>98.4 (49.2 - NR)</td>
<td>1 (Referent)</td>
<td></td>
<td>1 (Referent)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>78.9 (42.2 - NR)</td>
<td>0.96 (0.57 - 1.63)</td>
<td>p=0.886</td>
<td>1.12 (0.62 - 2.01)</td>
<td>p=0.712</td>
</tr>
</tbody>
</table>

Table 21: Survival Outcomes.

(NR – not reached; AIS – Adenocarcinoma in situ; MIA – Minimally Invasive Adenocarcinoma; MPA – Micropapillary adenocarcinoma; Invasive Mucinous Adenocarcinoma). 95% confidence intervals shown in brackets.
3.4 Discussion

3.4.1 *EGFR* mutation, *KRAS* mutation and IASLC/ATS/ERS Subtype

We investigated the rates of *EGFR* mutation and *KRAS* mutation in our single centre Australian cohort according to the IASLC/ATS/ERS classification for resected pulmonary adenocarcinoma [179]. *EGFR* mutation was statistically less frequent in solid predominant tumours in comparison to the reference group (acinar predominant tumours). Our findings were generally consistent with the findings from studies conducted in other non-Asian cohorts [274, 278, 305, 306]. At the time of the inception of the work in this chapter the IASLC/ATS/ERS classification had only recently been released and it was not known what, if any, correlations would be found.

We did not find that any particular predominant subtype was enriched for *EGFR* mutation, with rates across remaining subtypes being statistically similar. Numerically *EGFR* mutations were more common in tumours from the lepidic family (AIS, MIA and LPA). Clinically, however, the finding of an increased *EGFR* mutation rates in tumours from the lepidic family is of little relevance as tumours of these subtypes rarely recur and would very seldom need adjuvant therapy on the basis of current medical knowledge. No *EGFR* mutations were found in patients with adenocarcinoma variants (IMA and colloid predominant) however the total number of samples was small.

The rate of *EGFR* mutations in our cohort, at 30%, was higher than seen in other comparable Western cohorts. When classical activating mutations only are considered (exon 19 deletions, the L858R mutation and one patient with L861Q mutation) this rate drops to 26%. Data on the ethnicity of our patient cohort is not reliably collected in the available clinical records. Anecdotally there is a higher proportion of patients of Asian ethnicity treated at St Vincent’s Hospital Melbourne as compared with many other Australian hospitals, and this may potentially explain the rate of *EGFR* mutation seen in the current study cohort.
In our cohort the rates of KRAS mutation across predominant subtypes were similar. We did not find any predominant subtype where KRAS mutations were more or less common. Previously some groups have found an association between KRAS mutation and the solid predominant subtype [278, 305, 438]. Tumours from two of the three patients with resected IMA from our cohort had KRAS mutations, again consistent with the known associations for this subtype [299].

The predominant subtypes do not associate strongly enough with the presence or absence of an oncogenic mutation. As such, they cannot be used to exclude a patient from molecular testing for a specific mutation in the event of an appropriate adjuvant therapy becoming available or on the occurrence of systemic relapse. With the increase availability of multiplexed testing on small samples the need to tirage samples for the order of molecular testing will become less of an issue in clinical practice.

### 3.4.2 Interaction between Mutations, Subtype and Survival

The survival outcomes in this cohort were highly dependent on the pathologic stage, with increasing stage being associated with worse OS outcomes. On multivariate analysis the solid predominant subtype was also associated with inferior OS outcomes. The hazard ratios suggested a trend towards worse outcomes with micropapillary adenocarcinoma and papillary adenocarcinoma on multivariate analysis. Statistical significance was not reached potentially due to small sample sizes and the overriding importance of stage. The presence of pleural invasion was significant only in univariate analysis.

EGFR and KRAS mutation status were not prognostic in univariate analysis. Further, their inclusion in multivariate analysis did not influence survival outcomes. A previous paper from Russell et al had examined patients with resected stage III pulmonary adenocarcinoma and suggested an interaction between micropapillary predominant tumours and differential outcomes based on the presence or absence of an EGFR mutation [278]. Unfortunately we were unable to explore this finding further in our cohort due to small numbers of patients in
each subgroup. In our cohort 43% of patients had acinar predominant tumours, with lepidic pattern tumours (AIS, MIA and LPA) accounting for 10% of patients, papillary 15% and MPA 9%. On average each of these groups had an \textit{EGFR} mutation rate of 30%, and a similar \textit{KRAS} mutation rate. This leaves a small number of subjects within each domain in which to perform a survival analysis. While it is possible to do such an analysis, the likelihood of finding a true positive effect is low, and if such an effect were to exist then the actual result we have would be a false negative result due to a lack of power [445]. If mutation status does affect survival outcomes in non-solid predominant tumours (see below) then the effect size is likely to be small and overwhelmed by the importance of stage. An estimate of the power required to determine a true result with more confidence was not performed as part of this work as we lacked the ability to include more cases into the study.

The question of interaction between the tumour subtype by the IASLC/ATS/ERS classification and oncogenic mutations is one worthy of further exploration. Lee et al examined survival outcomes for 118 Korean patients with surgically resected lung adenocarcinoma [446]. Of these 55 patients had tumours that carried an \textit{EGFR} mutation. In multivariate OS analysis of patients with an \textit{EGFR} mutation the solid predominant subtype had a significantly detrimental effect on OS (HR 7.2 [95% CI 1.9 – 27.0, \textit{p}=0.003]) along with the presence of \textit{EGFR} amplification or TTF-1 amplification. Yoshida et al examined outcomes for a group of 134 Japanese patients with exon 19 or 21 \textit{EGFR} mutations who had relapsed following surgical resection [309]. \textit{EGFR} mutations were detected in 61 patients, of whom 18 patients (29%) had solid predominant tumours on their original pathology. All patients were treated with gefitinib. Those patients with an original diagnosis solid predominant adenocarcinoma had a lower response rate by Response Evaluation Criteria in Solid Tumours (RECIST) criteria (66% vs 88%, \textit{p}=0.03), a shorter PFS on multivariate analysis (HR 3.97 [95% CI 1.9 – 8.3, \textit{p}<0.001]) and a shorter overall survival when treated with gefitinib (\textit{p}=0.028). These two studies indicate that there is an interplay between mutation status (inherent to the tumour) and the solid predominant adenocarcinoma subtype. Translational research may help to identify whether these effects are driven by the tumour cell or by a difference in the stromal response to differing adenocarcinoma subtypes.
In this study we were able to detect known features of pathologic significance in resected pulmonary adenocarcinoma – most notably the worsening overall survival with increasing stage and with the solid predominant pattern. However, the micropapillary pattern failed to reach significance on univariate or multivariate analysis (the hazard ratio and median overall survival were similar to solid predominant adenocarcinoma, although the confidence intervals were wide, reflecting the available sample size). In light of this, it is possible that other potentially significant associations have not been uncovered due to insufficient statistical power.

3.5 Conclusion

We have found that in our cohort *EGFR* mutations are less common in patients with resected solid predominant adenocarcinoma as compared to other predominant subtypes according to the new IASLC/ATS/ERS classification of resected pulmonary adenocarcinoma. Furthermore, *EGFR* mutations were not observed in invasive mucinous adenocarcinoma. These results are similar to the common theme across other studies of *EGFR* mutation frequency and the IASLC/ATS/ERS classification. No statistical difference was seen by predominant subtype and *KRAS* mutation status. The predominant subtype is not sufficiently predictive (positively or negatively) for the presence of a particular oncogenic mutation. Therefore, in the clinical setting, testing of a tumour for oncogenic mutations should occur on the basis of the utility of the individual result to a patient. The presence of *EGFR* or *KRAS* mutation had no effect on survival outcomes, and there was no interaction found between the mutation status, the predominant subtype and survival outcomes. The advent of the IASLC/ATS/ERS classification for resected pulmonary adenocarcinoma together with the research findings generated to date present a fruitful ground for translational research in order to discover new approaches for the treatment of lung adenocarcinoma.
CHAPTER 4

JAK / STAT AND INTERLEUKIN-6
EXPRESSION IN PULMONARY
ADENOCARCINOMA
4.1 Literature Review

The discovery of targetable oncogenic mutations in pulmonary adenocarcinoma has introduced a major paradigm shift in the management of advanced disease (together with recent advances in immuno-oncology). As discussed in chapter three, first and second generation inhibitors of mutant *EGFR* have higher response rates, longer durations of activity and better toxicity profiles than standard platinum doublet chemotherapy. Despite this resistance to therapy develops at a median of nine to 13 months. In a small number of patients there is no response to *EGFR* blockade despite the presence of an activating mutation – a situation known as de novo resistance. Dual blockade of a second signalling pathway in an oncogene addicted tumour may potentially offer the ability to overcome de novo resistance for some patients and a longer duration on a line therapy for others by delaying progression of cancer and the need to switch treatment. One such example already exists in clinical practice. The dual blockade of *BRAF* mutant melanoma with both a *BRAF* inhibitor and a *MEK* inhibitor leads to improved PFS and OS compared to single agent *BRAF* inhibition [447, 448].

This chapter examines the rates of staining by immunohistochemistry for IL6, JAK1 and pSTAT3 in resected lung adenocarcinoma specimens. The study aims to see whether staining for these markers is enriched in patients whose tumours harbour *EGFR* mutations compared to those with *KRAS* mutations and those who are wild type for both oncogenes.

4.1.1 *EGFR* Downstream Signalling

In the previous chapter I briefly discussed the history of the discovery of *EGFR*, and its importance in embryogenesis. *EGFR* (ErbB1) is part of the ErbB family of receptors which also include ErbB2 (HER2), ErbB3 and ErbB4. A number of ligands bind to *EGFR* including transforming growth factor-α, EGF, epiregulin, β-cellulin, heparin binding EGF and amphiregulin [324-326]. The outcomes of ligand binding depend on the dimerization partner for *EGFR* (homodimerisation or heterodimerisation with another member of the ErbB
family) and the cellular context [323, 325]. The interaction between EGFR and a ligand ends when the receptor dimer is internalised by endocytosis and either recycled to the cell surface or degraded in a lysosome [449]. A number of potential pathways can be activated by EGFR including phospholipase-Cγ Src, Ras/Raf, PI3K/Akt/mTOR and Janus Kinase (JAK)/Signal Transducers and Activators of Transcription (STAT) (Figure 9) [323, 325, 450, 451].

In addition to EGFR related activation, JAK and STAT also respond to IL6 signalling through the IL6 receptor and gp130 (discussed further below). On phosphorylation STATs form dimers which subsequently translocate to the nucleus to regulate gene expression and downstream effects such as cellular proliferation and survival, immune evasion and angiogenesis [452, 453]. Negative feedback loops also exist with suppression of STATs by proteins such as Protein Inhibitors of Activated STATs (PIAS; these bind to STATs inhibiting ability of STATs to bind to DNA) and Suppressors Of Cytokine Signalling [454-456].

Each pathway targets specific gene products, and the resulting effects may alter a number of cellular properties including adhesion, migration, growth, apoptosis or differentiation [325, 457]. Studies using EGFR inhibitors in patients whose tumours express a sensitising EGFR mutation have demonstrated the effectiveness of this therapeutic approach (discussed in Chapter three). As with many anticancer therapies resistance to EGFR inhibition develops after prolonged treatment. Therefore, effort is being directed to prolonging the time on first line therapy or finding ways to overcome established resistance by examining alteration of other signalling pathways. This chapter focuses on examining one of these signalling pathways (IL-6 / JAK1 / STAT3) and, in particular, whether this pathway is more prominent in tumours with activating EGFR mutations compared to tumours that are wild-type for EGFR.
Figure 9: Signalling through \textit{EGFR} and the relationship to IL6, JAK and STAT3 signalling (see overleaf)
**Figure 9 (continued):** EGF (and other ligands) bind to *EGFR* which forms a homodimer or heterodimer with another member of the HER family. This leads to phosphorylation of the tyrosine kinase domain. Intracellular signalling cascades are activated including the RAS / RAF / MEK / ERK pathway and the PI3K / Akt / mTOR pathway. Phosphorylation and activation of JAK can occur either directly through the *EGFR* receptor or via IL6 binding to its receptor. This leads to phosphorylation and dimerization of STAT3. pSTAT3 localises to the nucleus binding to DNA and leading to gene transcription. The JAK / STAT pathway can be negatively regulated via SOCS and PIAS3.

AKT – AKT oncogene (also known as protein kinase B); DNA – deoxyribonucleic acid *EGF* – Epidermal Growth Factor; *EGFR* – Epidermal Growth Factor Receptor; ERK – extracellular signalling-related kinase; gp130 – Glycoprotein 130; IL6 – Interleukin 6; IL6R – IL6 receptor; JAK – Janus Kinase; MEK – MAPK/ERK Kinase; mTOR – Mammalian Target of Rapamycin; P – phosphorylated; PI3K – Phosphoinositide 3 Kinase; PIAS – Protein inhibitor of activated STAT3 RAF – Rapidly Accelerated Fibrosarcoma; RAS – Rat Sarcoma; SOCS – Suppressors of Cytokine Signalling; STAT – Signal Transducers and Activators of Transcription
4.1.2 Mechanisms of Resistance to EGFR Inhibitors in Clinical Practice – de novo and acquired resistance

Resistance to EGFR TKIs can happen in one of two settings. De novo resistance occurs when patients with sensitising mutations fail to respond or derive sustained clinical benefit from EGFR inhibition. Fortunately this situation is not common, but remains frustrating to patients and clinicians. Acquired resistance occurs after a period of response or clinical benefit on treatment with EGFR TKIs. Jackman et al proposed a clinical definition of acquired resistance to EGFR inhibition in 2010 [458]. The definition included patients who were treated with single agent EGFR TKIs and either had a mutation associated with sensitivity to these drugs, or objective response as evidenced by either partial or complete response, or prolonged disease control without a reduction in disease burden radiologically (>6 months). In this section the review concentrates on demonstrated resistance mechanisms in clinical practice.

4.1.2.1 De Novo Resistance

De novo resistance to EGFR TKIs in patients with EGFR mutations is uncommon, but is known to occur. Several mechanisms are found by which this may occur.

4.1.2.1.1 EGFR Exon 20 Mutations – Insertions and Uncommon Mutations

The predominant oncogenic transformations in exon 20 involve in-frame insertions of between three and 21 base pairs, the T790M mutation where threonine is replaced by methionine, and other uncommon point mutations. Arcila et al examined exon 20 insertion mutations in a sample of 1500 adenocarcinoma specimens obtained from patients at MSKCC over a two-year period. Three hundred and sixty seven cases with EGFR mutations were found, of which exon 20 insertion mutations accounted for 33 positives (9%). The authors, however, suggest the rate may be as high as 11% due to lack of availability of some specimens for testing due to attrition of available tissue, with an estimated total rate of exon 20 insertions mutations of 3% in “all comers”. In this cohort only five patients received
treatment with EGFR inhibitors. It was noted that the effects of each mutation on the structure of the receptor may result in different affinities for EGFR TKIs [138].

Earlier work from Wu et al looked at mutation rates for insertion and uncommon point mutations in a group of 515 patients from Taiwan, of whom 441 patients had pulmonary adenocarcinoma [459]. Uncommon point mutations were found in 7 patients, exon 20 insertions in 12 patients, and one patient had both an uncommon point mutation and an insertion mutation. Six of the eight patients with uncommon point mutations had another known activating mutation such as exon 18 G719A or exon 21 L858R. Sixteen patients (including two with T790M mutations discussed further below) were treated with gefitinib, with only four partial responses seen and 12 patients with non-response.

4.1.2.1.2 T790M in de novo Resistance

The T790M point mutation is a well-known resistance mechanism in both de novo and acquired resistance. Methionine is a larger molecule than threonine, and the resulting structural change leads to steric hindrance interfering with the binding of erlotinib and gefitinib [460]. T790M mutations have been occasionally reported as germline mutations which may raise the susceptibility to the development of lung cancer. Bell et al reported a family with six members affected by lung cancer over three generations [461]. Multiple tumours were acquired from the proband showing T790M either as an isolated mutation, or in combination with classical activating mutations in exon 19 or 21. Further, the authors demonstrated that somatic mutations tended to occur in the same allele as the underlying germline mutation. Tumour from a second family member showed T790M mutation combined with the exon 18 mutation G719A. One further affected and one unaffected family member had germline T790M mutations demonstrated in EGFR sequenced from peripheral blood mononuclear cells.

Shih et al provided one of the first reports of non-response to first line gefitinib due to T790M mutation. They reported a patient whose tumour harbouried a combined L858R/T790M mutation detected by direct sequencing on a specimen obtained at diagnosis. Initial stable
disease was demonstrated at four weeks with first progression documented at eight weeks [462]. Two groups have looked at the effect of the presence of a T790M mutation at initial diagnosis on response to EGFR inhibitors. Maheswaran et al used the highly sensitive Scorpion Amplification Refractors Mutation System (SARMS) technology and direct sequencing to test for EGFR mutations in circulating tumour cells, free plasma DNA or archived paraffin embedded tumour tissue [463]. In 10 of 26 patients treated with gefitinib or erlotinib a T790M mutation was found in addition to their sensitising mutation, often at levels below the threshold of direct sequencing. For these 10 patients the PFS on an EGFR inhibitor was significantly shorter (7.7 months vs 16.5 months, HR 11.5, 95% confidence interval 11.5 [2.94 – 45.1], p<0.001).

Rosell et al examined the effect of T790M mutation in the pre-treatment samples of 129 patients with known activating EGFR mutations who were treated with erlotinib [464]. Treatment was either first line (n=65) or second line (n=64). Using the TaqMan assay they found 45 patients (35%) whose tumours harboured a T790M mutation, occurring more frequently in patients with an L858R mutation (22 out of 48) as compared to those with exon 19 deletion mutations (23 out of 81, p=0.05). The presence of a T790M mutation was associated with shorter PFS (12 months [95% CI 7.6 to 16.4] vs 18 months [95% CI 14.1 to 21.9], p=0.05). This remained significant on multivariate analysis (HR for presence of a T790M mutation 4.35 [95% CI 1.85 – 10.17], p=0.001).

Yano et al examined 97 samples from 93 patients with classical activating EGFR mutations [465]. They included 44 patients whom they considered to have intrinsic resistance to EGFR TKIs, which they defined as stable or progressive disease as per RECIST criteria. They used a cycleave rtPCR technique which can detect the presence of T790M mutations if present in as little as 5% of tumour cells. They did not find any T790M mutations at baseline in patients who failed to response to EGFR TKIs. Further discussion of T790M mutation as an acquired resistance mechanism is discussed in the acquired resistance section.
4.1.2.1.3 BIM Polymorphism

Two groups have examined the effect of the BIM polymorphism in patients with activating EGFR mutations treated with EGFR TKIs. BIM stands for Bcl2-like 11. It is a member of the B-cell CLL/lymphoma 2 (Bcl2) family of proteins. BIM functions to activate cell death and apoptosis when appropriate growth factors are absent [466]. Three groups have demonstrated that BIM is upregulated when EGFR mutated cell lines are treated with EGFR TKIs and EGFR inhibition is less effective in these cell lines when BIM activation is blocked [467-469].

Ng et al hypothesised that loss of BIM function may be responsible for decreased response for patients with EGFR mutated tumours [470]. Using massive parallel sequencing they found a deletion polymorphism in BIM in blood samples from patients with resistant chronic myeloid leukaemia, and demonstrated that this was also found in the HCC2279 cell line. This cell line harbours an exon 19 deletion mutation (del E746A750) but fails to respond to EGFR inhibition. They demonstrated that in a group of 141 patients with EGFR mutated tumours, 26 patients (18.4%) had a BIM deletion polymorphism. These patients had a significantly shorter PFS when treated with EGFR TKIs (6.6 months vs 11.9 months, HR = 2.08 [95% CI 1.29 – 3.38], p=0.0028 on multivariate analysis). The other significant predictor of short PFS in their study was the presence of a resistance exon 20 mutation.

Lee et al were unable to confirm the findings reported above [471]. They examined BIM deletion polymorphism and found 21 cases (10.6%) out of 197 patients treated with EGFR TKIs for cancers with activating mutations. They found no difference in PFS (11.9 months for patients with BIM deletion vs 11.3 months for those without) with the survival curves superimposed on each other. Further studies will be required to therefore confirm whether BIM polymorphism has a substantial role in de novo resistance to EGFR TKIs.
4.1.2.2. Acquired Resistance

4.1.2.2.1 T790M in Acquired Resistance

The drive to search for secondary resistance mutations in *EGFR* mutated lung cancer came from the knowledge that secondary resistance mutations occur in patients with CML treated with imatinib. On this basis Pao et al sequenced *EGFR* exons 18 to 24 in patients with prior response to gefitinib or erlotinib, and in three of six patients they identified the T790M mutation from samples obtained following treatment with gefitinib or erlotinib [472]. To support the clinical findings they conducted cell line studies to demonstrate the effect of the T790M mutation. Pao et al transfected 293T cells with classical exon 19 or exon 21 L858R mutations and demonstrated response to gefitinib or erlotinib as evidenced by a reduction in phosphor-EGFR (pEGFR). Treatment of cell lines with these mutations and a concurrent T790M mutation or those with wild type *EGFR* failed to show a reduction of pEGFR.

T790M mutations were subsequently confirmed by multiple other groups as an acquired resistance mechanism, with a prevalence of approximately 50% in specimens obtained from patients progressing on gefitinib or erlotinib [465, 473-478]. As mentioned before, one possible explanation for clinical resistance is steric hindrance preventing the binding of first generation *EGFR* TKIs [479]. Yun et al argue against this as the likely mechanism [480]. They found through crystallographic modelling that T790M mutation does not sterically block the binding of reversible *EGFR* inhibitors. Their data extends the research of Carey et al who demonstrated that the presence of classical activating *EGFR* mutations led to an increased affinity for reversible *EGFR* inhibitors (erlotinib and gefitinib) and a decreased affinity for ATP in cells harbouring these mutations compared with cells with wild type *EGFR* [481]. Yun et al showed that L858R (Dissociation constant [Kd] in nano-moles [nM] = 2.4) and T790M (Kd nM=4.6) mutated cells have a similar affinity for gefitinib, while wild type *EGFR* (Kd nM = 35.3) has a much lower affinity for gefitinib. The combined L858R/T790M has a lower affinity for gefitinib than either mutation on its own, but it is still three times higher than wild type *EGFR* (Kd nM = 10.9). Further, they demonstrated that the combined L858R/T790M mutation restores the affinity of *EGFR* for ATP to similar levels of wild type *EGFR*, while L858R and T790M have reduced affinity. Drugs such as gefitinib and erlotinib compete for the same site as ATP and thus the authors conclude that their
effectiveness in combined L858R/T790M mutant cells is reduced leading to the clinically observed resistance.

One question that is raised is whether the T790M mutation represents an acquired resistance mechanism, or if T790M mutations are present in a small subpopulation of tumour cells prior to TKI treatment. The discovery of a small subpopulation of T790M mutated cells prior to EGFR inhibitor therapy is dependent both on the quality of the DNA obtained from the tumour sample and the sensitivity of the assay. Maheswaran et al analysed three sources of material from patients with lung adenocarcinoma – circulating tumours cells, plasma free DNA and tumour in archived paraffin embedded tissue [463]. They used SARMS, direct sequencing or both techniques. Assessing the tumour tissues of 26 patients with EGFR mutations the more sensitive SARMS technique was able to detect the presence of T790M mutations in the pre-treatment samples of 10 patients (38%) not detected by standard sequencing. Further, the presence of pre-treatment T790M was associated with worse PFS (p<0.001). Patients with low level T790M mutation still showed responses to EGFR inhibition. Conversely, Arcila et al used the sensitive locked nucleic acid (LNA)-PCR technique in the pre-treatment samples of 26 patients, 15 with T790M at progression and nine without, and found no evidence of T790M mutation in the pre-treatment samples [475]. Fujita et al assessed samples from 38 patients who had undergone surgical resection for early stage disease and had an EGFR mutation. Using the SARMS method they found no detectable T790M mutations from these resection specimens, while the highly sensitive colony hybridisation (CH) assay was able to detect T790M mutations in 30 out of 38 samples to colony levels which in some cases were less than 0.01% of the total colonies tested [482]. The highest level of colonies seen was 1.8% of the total number of colonies tested in a single sample.

The effect of T790M on patient outcomes when acquired at progression is different to its detection prior to treatment with 1st generation TKI therapy. In cell line studies Chmielecki et al found that cell lines developed to generate resistance to EGFR inhibitors grew at a slower rate than the parent cell line [483]. Oxnard et al examined the implications of these findings in a patient group [484]. From a group of 125 patients 107 (84%) had adequate tissue available for analysis at progression. The analysis was limited to 93 of these patients
after excluding patients who had investigational agents in the first line setting (n=6), who had adjuvant TKI following resection of early stage disease (n=4) and patients with isolated CNS progression (n=6). The rate of T790M mutation was 62%. In univariate analysis patients with a T790M mutation at progression had a longer post progression survival (19 months) compared with those who did not (12 months, p=0.036). This effect was lost in multivariate analysis when performance status and the presence of new mutations at progression were taken into account. The research also showed that patients with T790M mutation were more likely to progress in a known disease site rather than a new metastasis (p=0.010) while patients without acquired T790M mutation had lower performance status (p=0.007). These results are supported by similar findings from a Japanese patient cohort [485]. Fujita et al [482] argue that their results previously mentioned may support the findings of Oxnard et al [484]. No patients in the Fujita study had a T790M mutation detected by SARMS in their pre-treatment specimens. The presence or absence of a T790M mutation as detected by SARMS made no difference in the time to treatment failure on \textit{EGFR} TKI. Using the super-sensitive CH assay they divided their cohort into high level (T790M in >0.5% of colonies, n=8), low level (0 – 0.5% of colonies, n=23) and absent (no T790M in colonies, n=7). Patients with high levels of T790M by CH assay had significantly longer median time to treatment failure (41 months), compared to those with low level expression (7 months, log-rank p=0.0019) and no expression (7 months, log-rank p=0.0097). They hypothesize that those with higher levels of T790M on CH assay develop this as their resistance mechanism while those with little or no T790M via CH assay have other resistance mechanisms and shorter post progression survival. Unfortunately the study was not able to report on resistance mechanisms found at the time of progression.

4.1.2.2.2 MET Amplification and Hepatocyte Growth Factor

MET amplification offers an alternate pathway through which resistance to \textit{EGFR} TKIs develops while on treatment. This mechanism offers resistance through a “kinase switch” where the mutated gene remains inhibited by a TKI, but growth and survival signals are restored through signalling via an alternate receptor tyrosine kinase [486]. MET amplification was first identified in cell lines in work by Engelman et al [487] with resistance mediated via restoration of MAPK/ERK or PI3K/AKT pathways [488]. They developed resistance in the exon 19 mutated HCC827 cell line and in the absence of T790M mutation
showed that amplification of MET was responsible for resistance. A number of papers showed MET amplification as a resistance mechanism in post progression biopsies from patients treated with EGFR TKIs against sensitive mutations [475, 487, 489], with concurrent T790M mutation in up to 40% of patients [489]. While MET amplification can be seen prior to treatment it is more common in the setting of progression [393, 489].

Hepatocyte growth factor (HGF) is a ligand for the MET receptor. Yano et al demonstrated that in cell line studies the addition of HGF led to resistance to EGFR inhibition [488]. They examined samples from patients with no evidence of T790M mutation or MET amplification. In pre-treatment clinical samples HGF expression was seen by IHC in one out of eight patients who were subsequently sensitive to EGFR inhibition with gefitinib. Three patients with intrinsic resistance to EGFR TKIs had high expression of HGF. A further three patients who developed resistance to EGFR TKIs showed an increase in HGF detected on IHC between pre- and post-gefitinib treatment biopsies.

To confirm these results Yano et al examined resistance mechanisms in a larger group of 97 patients [465]. In 45 samples from patients with intrinsic resistance, 13 (29%) had high-level HGF expression, while there were no T790M mutations and only 2 patients with MET amplification (4%). In 23 samples from patients with acquired resistance high-level HGF expression (n=14; 61%) was common as was T790M mutation (n=12; 52%) while MET amplification was rare (n=2; 9%). High level HGF expression was seen concurrently with T790M mutation in 6 of 12 patients and with MET amplification in 1 of 2 patients, demonstrating that several potential resistance mechanisms may be active at the same time. In 29 samples from tumours sensitive to EGFR TKIs high-level HGF expression was rare (n=3; 10%) while there were no T790M mutations or MET amplification found. Two small reports (patient samples of n=10 and n=11 respectively) suggested that HGF over-expression tended to occur in patients with T790M mutation rather than as a separate event [477, 490].
**Figure 10**: MET amplification and HGF amplification as bypass resistance mechanisms to *EGFR* inhibition (adapted from [153, 491, 492])
Figure 10: MET amplification and HGF amplification as bypass resistance mechanisms to EGFR inhibition (continued)

Blockade of mutant EGFR by first and second generation TKIs leads to decreased cell proliferation and survival. Amplification of the MET gene leads to restoration of cell signalling via ErbB3 [487]. Amplification of the ligand for MET, hepatocyte growth factor (HGF) can also contribute to resistance to EGFR inhibition [465, 488].

AKT – AKT oncogene (also known as protein kinase B); EGFR – Epidermal Growth Factor Receptor; ERBB3 – Human epidermal growth factor receptor 3; ERK – extracellular signalling-related kinase; HGF – hepatocyte growth factor; MEK – MAPK/ERK Kinase; MET – MET gene; mTOR – Mammalian Target of Rapamycin; P – phosphorylated; PI3K – Phosphoinositide 3 Kinase; RAF – Rapidly Accelerated Fibrosarcoma; RAS – Rat Sarcoma; TKI – tyrosine kinase inhibitor
4.1.2.2.3 Transformation to Small Cell Lung Cancer

Transformation to SCLC is an uncommon but clinically recognised phenomenon. Sequist et al set out to look at molecular changes in a cohort of 37 patients with progression following EGFR TKIs for sensitive EGFR mutations [493]. Tumour tissue acquired at progression underwent routine H&E staining, and the authors were surprised to find that five patients (14%) had tumours with phenotypic transformation to SCLC while retaining the underlying sensitising EGFR mutation. Three of the five patients had clinical progression consistent with the usual features seen in patients with SCLC at diagnosis. In clinical practice such patients respond to the usual small cell carcinoma regimen of platinum/etoposide. This resistance mechanism is uncommon with only sporadic case reports in the literature [475, 494].

4.1.2.2.4 Epithelial to Mesenchymal Transition (EMT)

Cells need to be able to transition between epithelial and mesenchymal states at various parts of normal development [495]. During EMT epithelial markers that are involved in cell-cell adhesion, such as e-cadherin, are lost and mesenchymal markers such as fibronectin and vimentin are gained. There may be associated morphological changes in the histopathologic appearances of the tumour cells [493]. Uramoto et al looked at the expression of EMT markers in the samples of nine patients pre- and post-gefitinib treatment [496]. In their sample six patients had the T790M mutation at progression. Four patients had either down-regulation of epithelial markers or up-regulation of mesenchymal markers, however the changes reported were not of a great magnitude.

Chung et al provided the first report of histologic changes consistent with EMT following EGFR inhibition [497]. Their 50 year old patient had adenocarcinoma with acinar and lepidic patterns on surgical resection. Biopsy of a pancreatic lesion at progression showed a TTF1+ tumour with the same activating EGFR mutation confirming lung as the source of the primary tumour. Microscopically the pancreatic lesion had spindle cell morphology with loss of staining for e-cadherin and positive staining for vimentin. Further evidence came from
Sequist et al [493]. They analysed 12 patients from their cohort of 37 – seven with no resistance mechanism found and five with T790M mutation. Three of these 12 specimens had phenotypic changes of EMT all occurring in patients without T790M mutation. Two of the three specimens had positive of vimentin staining and loss of e-cadherin staining and both also maintained the same EGFR mutation.

In their discussion, Sequist et al noted that “EMT and a histological change to SCLC may be enriched specifically in EGFR-mutant tumours acquiring resistance to TKI therapy”. This statement was made on the basis that similar transformations were not observed in their cases treated with chemotherapy or chemoradiotherapy [493]. More time may be required to see if similar resistance phenomena develop with selective targeting of other mutated pathways in lung cancer such as the ALK translocation [498].

4.1.3 Pathway Components

This section describes the discovery of IL6, JAKs and STAT3, their normal function and the role of STAT3 as an oncogene.

4.1.3.1 Interleukin 6 (IL 6) and Glycoprotein 130 (gp130)

Interleukin-6 was first cloned by Hirano et al in 1986 [499]. Its role was first described in the process of maturation of B-cells into antigen secreting plasma cells with many early studies using plasmocytoma models (reviewed in [500]). It carried various names dependent on the context of discovery. It was eventually realised that multiple groups were describing the same molecule, and a decision was made to name the molecule IL-6 [501]. IL-6 is a multifunctional cytokine that is important in a number of roles including regulation of the immune system, haematopoiesis and as part of an appropriately activated acute inflammatory response [502, 503]. IL-6 is also implicated in a number of disease states including inflammatory arthropathies, inflammatory bowel disease, sepsis, insulin resistance and malignancy [503, 504].
IL-6 initially binds IL-6 receptor (IL6R, glycoprotein 80 [gp80]) [505]. Gp80 expression is predominantly limited to IL6 responsive cells, however soluble gp80 is also found in human serum [506]. Gp80 has no intrinsic tyrosine kinase domain and therefore requires a further mechanism by which to activate effector mechanisms. This signalling occurs through association with gp130. Gp130 was first described in context of activated T-lymphocytes in 1978 [507], however its role in IL6 signalling was not recognised until the work of Taga et al in 1989 [505]. The importance of gp130 in IL6 signalling is demonstrated by embryonal lethality in mice engineered to lack gp130 [508], and mouse models with disruption of the pathway showing increased susceptibility to bacterial and viral infection (reviewed in [509]). Gp130 is widely expressed in both normal and malignant tissue [510, 511]. Taga et al were able to show that IL6/gp80 complex could activate gp130 by two mechanisms. First, IL6 could bind to cellular gp80 and then activate gp130. Secondly, IL6 could bind to soluble gp80 in the serum and then associate with cellular gp130 [505].

Tyrosine phosphorylation of gp130 can occur at several sites and lead to activation of different pathways. At one of these sites the Mitogen Activated Protein Kinase (MAPK) pathway is activated leading to mitogenesis. At other sites the pathway activated is JAK/STAT [512].

4.1.3.2 Janus Kinases (JAKs)

JAKs represent a key step in signalling pathways for a number of ligands. The family consists of four members all discovered in the early 1990s – JAK1 [513], JAK2 [514, 515], JAK3 [516, 517] and tyrosine kinase 2 (TYK2) [518]. Absence of JAK1 results in perinatal death while absence of JAK2 leads to death in utero [519-521]. IL6 and members of the IL6 family bind to gp130 and signal through activation of JAK1, JAK2 or TYK2 [522, 523]. Phosphorylation of JAK leads to activation of STAT.
4.1.3.3 Signal Transducers and Activators of Transcription (STAT)

The STAT family was first described from work by the group led by James Darnell [524]. It had been demonstrated that a member of the STAT family could be activated and phosphorylated at the same tyrosine residue by different upstream signals from interferon-alpha, interferon-gamma and epidermal growth factor. Further, it was noted that phosphorylation of STATs was dependent on activation of JAKs. The STAT family consists of seven members named STAT1 to STAT6 (with there being a STAT5a and a STAT5b). The protein of interest in this work is STAT3.

STAT3 was originally identified by multiple groups in 1994 [522, 525-528]. It is critical in normal development. Absence of STAT3 in utero leads to embryonic lethality [529], while this does not occur with loss of other members of the STAT family [530]. However, in normal adult tissue loss of STAT3 seems to be less of an issue [530]. Multiple cellular functions are modulated by STATs in normal cells including proliferation, differentiation and apoptosis [531, 532]. Activation of STATs is transient, and following phosphorylation STATs form homo- or hetero-dimers with other members of the STAT family [533-535]. STAT3 has roles in normal function in multiple sites, including the skin, thymus, T-cells and myeloid cells, the nervous system and in acute phase responses mediated through IL-6 [530]. STATs remain active for up to 30 minutes at a time until they are subsequently deactivated through either dephosphorylation or proteolysis [531, 534]. Negative feedback is also exerted through SOCS and PIAS (as previously discussed) [454-456].

4.1.3.4 STAT3 as an Oncogene

The role of STAT3 in oncogenesis was first recognised in 1995. Yu et al examined STAT3 in v-Src transformed cell lines. They found that STAT3 was phosphorylated in three v-Src transformed rodent fibroblast cell lines while STAT3 was in its resting non-phosphorylated state in non-transformed cell lines [536]. Subsequently Bromberg et al showed that STAT3 was required for malignant transformation by activated v-Src. They took the rodent fibroblast cell line NIH 3T3 and transfected it with both v-Src, and different types of
dominant-negative STAT3 or vehicle. Cell lines transfected with the dominant-negative STAT3 suppressed v-Src transformation [537]. Further work demonstrated that constitutively activated STAT3 was capable of driving malignant transformation in the absence of upstream activation of tyrosine kinase (with absence of tumour formation with wild type STAT3) [538].

The first paper to demonstrate the effects of blockade of the IL6/JAK/STAT3 pathway was conducted in the U266 multiple myeloma cell line by Catlett-Falcone et al [539]. They showed high levels of activation of STAT3 in human multiple myeloma samples. Further, they demonstrated that Bcl-X was a target gene and that activation of this gene resulted in cell survival through resistance to apoptosis. Interference with either IL6 or JAK resulted in decreased STAT3 activation. The use of a dominant negative STAT3, resulting in blockade of STAT3 activity, reduced transcription of the Bcl-X gene.

The downstream target genes of STAT3 regulate several processes including an increase in proliferation and survival of cells, angiogenesis and immune suppression [532, 535, 540, 541]. Gao et al noted that “STAT3 target genes are implicated in all processes of tumourigenesis including proliferation, apoptosis, angiogenesis, invasion and migration.” [542]

4.1.4 Linking IL6/JAK/STAT and EGFR

4.1.4.1 In Vitro – Cell Line Studies

A large number of cell line studies have been carried out to examine the roles of IL6, JAK and STAT in lung adenocarcinoma, as well as examination of the effects of manipulation of each of these factors and other substances that have effects on this signalling pathway. A link has been suggested between activating mutations in EGFR and activation of JAK/STAT. This section examines the evidence from cell line work supporting such a link.
4.1.4.1.1 EGFR mutation, KRAS mutation and STAT3

The presence of EGFR mutation or KRAS mutation has variable effects on the expression of activated STAT3 in cell line studies. Studies conducted by a group at the Dana Farber Cancer Institute found that the addition of an EGFR mutation to fibroblast cell lines increased the levels of pSTAT3. [543]. In the subsequent study, the addition of gefitinib to two of the cell lines (L858R and exon 20 mutated cell lines) was able to suppress STAT3 activity [544]. Gao et al used a similar technique, and found that the addition of an exon 19 deletion mutation to an epithelial cell line increased pSTAT3, but in this case the effect was not able to be reversed with gefitinib [545]. Akca et al showed that the addition of an exon 19 mutation to a lung cancer cell line improved cell survival in conditions where the cells were starved of energy and in the absence of EGF, and that STAT3 remained activated in this situation. Only one group (Song et al [546]) found in opposition to these studies – that pSTAT3 levels were not influenced by the addition of an EGFR mutations.

A number of groups have examined the basal levels of STAT3 activation in both EGFR mutant, KRAS mutant and wild type cell lines. In these studies activation was determined either by looking for the presence of pSTAT3 or looking for evidence of STAT3 DNA binding. In the majority of cell lines with EGFR mutations, activation of STAT3 is seen and is usually strong. Six of the seven cell lines with pre-existing EGFR mutations showed increased STAT3 activity [544-549], with only one cell line (PC9) showing no activation of STAT3 in resting conditions [546, 547]. Similarly, six cell lines with known KRAS mutation showed increase STAT3 activity [452, 546, 548, 549] with only one cell line showing low or absent activation at rest (NCI-H460 cell line) [452, 545, 546, 548, 549]. STAT3 activation was also demonstrated in cell lines that were wild type for both EGFR and KRAS mutations [452, 546, 549].

Taken together these studies suggest that the introduction of an EGFR mutation to a cell line increases STAT3 activity, and the ability to block STAT3 activity with EGFR TKIs is variable. The upregulation of STAT3 can be seen in many cell lines regardless of the presence or absence of a driver oncogene.
4.1.4.1.2 Effects of EGF stimulation and EGFR and KRAS inhibition on STAT3

Several studies have examined the effects of EGF stimulation in adenocarcinoma cell lines. Cells lines tested included those with EGFR and KRAS mutations as well as those which were wild-type for both. The addition of EGF, even in the presence of an EGFR mutation, increased STAT3 activity in some cell lines but not all [544, 545, 550]. These papers suggest that the downstream response to EGF stimulation is not uniform, however the number of cell lines tested was small.

Treatment with EGFR TKIs in EGFR mutated cell lines led to similar results regardless of the drug or cell line used. In the majority of cell lines treated with either gefitinib or erlotinib, no reduction in STAT3 activation was seen [544, 546-548, 551-554]. This suggests that STAT3 activation is maintained through an alternate pathway and may contribute to survival of a portion of cells. Interestingly treatment of cell lines harbouring resistance to first generation EGFR TKIs led to increased STAT3 activation when erlotinib [555] or afatinib [556] were used.

In both cell lines and clinical practice EGF inhibition is not effective against cells harbouring a KRAS mutation. MEK is an enzyme downstream of KRAS and inhibition has been attempted of the pathway at this point. Inhibition of MEK in such cell lines leads to increased STAT3 activation [551, 554, 557].

4.14.1.3 Direct Inhibition of STAT3

Relatively few authors have looked at the effects of modulation of STAT3 activation directly. This effect is usually achieved with inhibition of activating kinases upstream of STAT3. EGFR inhibition in cell lines doesn’t change levels of STAT3 expression as discussed in the
last paragraph. Two studies have blocked STAT3 directly. One of these studies showed that 
STAT3 blockade led to apoptosis of EGFR mutant cell lines [548]. A second study used 
cells with both concurrent activation EGFR and T790M mutations. Afatinib alone produced 
cell cycle arrest but not cell death, and lead to hyperactivation of STAT3. STAT3 blockade 
alone was not successful. The combination of STAT3 blockade and afatinib produced the 
highest rates of apoptosis, suggesting that STAT3 contributed to de novo resistance to 
therapy. [556].

4.1.4.1.4 Effects of JAK Blockade in EGFR mutated cell lines

Much attention has been paid to the effects of JAK blockade in lung adenocarcinoma cell lines 
given the frequency of STAT3 activation observed together with the difficulties of translating 
direct STAT3 blockade into a clinical therapy. Attention has been focused on global blockade 
of JAK as well as targeted blockade of JAK1 and JAK2. Most testing has been performed in 
EGFR mutated cell lines.

Pan JAK inhibition (blocking both JAK1 and JAK2) can be achieved. Studies using the agent 
pyridone 6 (P6) have shown its ability to reduce the levels of STAT3 activation in cells with 
avivating EGFR mutations as well as the T790M resistance mutation [545, 546, 556]. A 
graduated increase in the concentration of P6 was associated with decreased pSTAT3 and 
increasing levels of PARP cleavage, a marker of apoptosis [556]. P6 was also able to block 
STAT3 activation and restore sensitivity to erlotinib in EGFR mutated cell lines stimulated 
with IL6 [558]. Small molecule inhibitors of JAK (such as ruxolitinib) have also been able to 
reduce pSTAT3 in EGFR and KRAS mutated cell lines [551, 554].

Having demonstrated that JAK signalling was important in STAT3 activation, studies then 
tried to determine which molecule was important in the signalling pathway, JAK1 or 
JAK2. JAK2 blockade is possible with several different pharmacological inhibitors. In EGFR 
mutated cell line studies results are conflicting with seven groups supporting a role for JAK2 
in STAT3 activation and three groups finding it was not responsible. Studies supporting a role 
JAK2 inhibitors demonstrated a decrease in STAT3 activation [549, 552, 553, 555, 559-561]
and sensitivity to JAK2 inhibition [559, 561, 562]. In three studies acquired resistance to EGFR TKIs was able to be reversed with JAK2 inhibition [555, 561, 563]. A further three studies found no link between blockade of JAK2 and levels of STAT3 activation in EGFR mutated cells [544, 546, 556].

Two studies confirmed the role of JAK blockade with P6 [546, 556]. Both studies confirmed the role of JAK in EGFR mutant cell lines with pan-JAK blockade, and then showed that blockade of JAK2 alone had no effect on STAT3 levels. As a result both studies concluded that activation of STAT3 is likely to come through JAK1 activation.

4.1.4.1.5 Effects of JAK Blockade in KRAS mutated cell lines

Studies examining JAK blockade in the presence of KRAS mutation have conflicting results. One study found that JAK2 blockade was unable to stop STAT3 activation (in the presence of EGF stimulation), while four groups were able to successfully reduce STAT3 activation by JAK2 blockade [452, 552, 553, 564, 565]. No studies explored the effects of direct JAK1 blockade alone.

Yoon et al examined the effects of MEK and JAK inhibition in KRAS mutant cell lines [557]. Only one cell line was sensitive to MEK inhibition alone, while all cell lines were responsive to dual inhibition of MEK and EGFR (with gefitinib). Interestingly MEK inhibition resulted in resistance via STAT3 activation in the KRAS/PTEN co-mutant cell lines only.

4.1.4.1.6 Interleukin 6 and Adenocarcinoma Cell Lines

STAT3 activation may occur via interleukin 6, its receptor gp130, and the Janus Kinases. The production of IL6 in adenocarcinoma cell lines has been detected although the levels detected vary between cell lines. Further, some cell lines, including some EGFR mutated cell lines, do not produce IL6 [551, 566-568]. Looyenga et al demonstrated the importance of the
IL6 in the activation of the JAK/STAT pathway in seven adenocarcinoma cell lines with different oncogene drivers through the blockade of the gp130. They showed that gp130 blockade lead to a reduction of STAT3 activation in all cell lines tested regardless of the oncogenic driver [551]. These results were replicated by three other groups [545, 546, 549].

Studies of the effects of modulation of IL6 levels in adenocarcinoma cell lines had variable results. An early study suggested that the addition of IL6 resulted in growth inhibition, while its’ removal led to cell growth. [566]. The next study from Bihl et al found that blocking the action of IL6 led to reduced proliferation, but this effect was limited only to cell lines that produced their own IL6 [567]. More recently Yamaji et al found no differences in the rates of cellular proliferation between cell lines that did and did not produce IL6 [568]. Further, forced production of IL6 in a cell line that doesn’t usually make IL6 had no effect on cellular proliferation.

A link between IL6 activation and downstream activation of STAT3 has been demonstrated in numerous studies. Huang et al showed that IL6 was produced in an autocrine fashion, with replenishment of IL6 levels in cell culture after removal of all IL6 present. [560]. Knockdown of STAT3 via several methods reduced secretion of IL6, while an activating mutation in STAT3 increased IL6 levels. Yeh et al conducted a similar experiment using the EGFR and KRAS mutated cell lines and showed that pSTAT3 could also by replenished by an autocrine loop [549].

An elegant study which ties many of the points about IL6, STAT3 and EGFR mutation was performed by Gao et al [545]. They were able to transform an immortalised cell line to a malignant cell line with the addition of cultured medium obtained from a separate EGFR mutated cell line via induction of STAT3. They were able to inhibit this change via anti-gp130 and anti-IL6 antibodies, but not by antibodies against other members of the IL6 family (anti-OSM or anti-LIF). The introduction of the EGFR mutation in to the immortalised cell line led to production of IL6 and subsequently STAT3 phosphorylation. Cultured medium from mutated cells could induce pSTAT3 in the existing immortalised cell line. Blockade of mutated cells with gefitinib did not reduce pSTAT3, whereas blockade of gp130 or IL6 did.
Gao et al hypothesised that the EGFR inhibition with gefitinib could not overcome the production of IL6 and pSTAT3 as the levels of IL6 produced were high, and an autocrine loop had already been established between IL6 and STAT3. However, when the surrounding medium was removed and replaced with fresh medium lacking IL6, gefitinib was able to stop de novo production of IL6, breaking the autocrine loop.

A clinically relevant outcome of activation of STAT3 via IL6 upregulation is the resistance to cancer therapeutics, as demonstrated in three studies. Huang et al used an adenocarcinoma cell line and generated daughter cell lines with active and inactive STAT3 [560]. STAT3 activation led to paclitaxel resistance, while reduction led to paclitaxel sensitivity. Similar effects were noted with increasing or lowering IL6 levels.

Two groups investigated EGFR mutated cell lines with resistant features. Yao et al treated a multiple colonies of a cell line with an EGFR exon 19 deletion mutation until it was highly resistant to erlotinib. Some resistant colonies had features of epithelial-to-mesenchymal transition (EMT) in the absence of T790M mutation or increased c-MET. They showed this was due to upregulation of Transforming Growth Factor-β (TGF-β) leading to the activation of IL6 and the JAK/STAT pathway. In these cell lines blockade of TGF-β reversed EMT features and restored sensitivity to erlotinib [558]. Kim et al treated cell lines with activating and T790M mutations with afatinib. Afatinib treatment increased IL6 and STAT3 activation, while STAT3 activation could be stopped through IL6 blockade. Further, it was demonstrated that hyperactivation of STAT3 may be reinforced by conditioned media produced by lung fibroblasts, which contain high levels of IL6, highlighting the importance of crosstalk between cells in a tumour environment [556].

4.1.4.1.7 Summary of Cell Line Studies

Activation of STAT3 is seen frequently in adenocarcinoma cell line studies and is often, but not always, found in cell lines with oncogenic drivers. The level of expression of STAT3 is
variable, with higher levels not being attributable to one particular oncogene driver over another. Similarly, the results of EGF stimulation were also variable, with some cell lines showing increased STAT3 activation and others showing no change. Inhibition of STAT3 is achievable via blockade of the pathway proximally at either IL6, gp130 or JAK. While global blockade of JAK is clearly effective, there is conflicting evidence as to whether JAK1 or JAK2 is the responsible enzyme. Studies that have looked at cellular survival have shown that where blockade of a part of the pathway way was successful, either alone or with another targeted inhibitor, apoptosis resulted. Interleukin 6 is one of a number of molecules which targets STAT3 through its receptor gp130. The production of IL6 by adenocarcinoma cell lines is again variable and not linked to one specific oncogene driver over another. Where IL6 is produced it partakes in an autocrine loop with activation of STAT3 that cannot be broken by upstream inhibition of the driver oncogene, suggesting that any attempts to downregulate the pathway need to come via blockade somewhere within this autocrine loop.

4.1.4.2 In Vivo – Animal Models

A small number of studies have examined the effects of altering the IL6/JAK/STAT pathway in animal models either alone or in combination with small molecule inhibitors. These studies are important to investigate possible strategies to advance in clinical trials.

To date STAT3 has proven difficult to inhibit via pharmacological methods. As such, there has only been one in vivo study conducted altering this molecule in lung adenocarcinoma models. Yeh et al used a form of the PC14 cell line (PC14PE6) that is known to form pleural effusions, and further developed an ascites-producing cell line (PC14PE6/AS2) via injection of the parent cell line intraperitoneally in nude mice [549]. Subsequent intraperitoneal injection lead to ascites while intravenous injection formed lung metastases and malignant pleural effusions. They altered the PC14PE6/AS2 cell line to silence expression of STAT3. Mice intraperitoneally injected with these cells were unable to form malignant ascites (0/15; peritoneal deposits being found after animal sacrifice) compared to 9/10 mice injected with treated with cells with functional STAT3. For intravenously injected mice no difference was seen in the proportions of mice developing lung metastases, however the number of lesions
seen for mice injected with functional STAT3 cells was significantly higher. No mice injected with silenced STAT3 cell developed pleural effusions (0/15) compared with 3/5 mice injected with functional STAT3 cells. The authors concluded that STAT3 had an important role in metastasis and effusion development.

4.1.4.2.1 JAK Blockade

JAK blockade in in vivo models has predominantly focused on cells harbouring EGFR mutations. The earliest study was conducted by Blaskovich et al using the A549 KRAS mutant cell line [565]. Tumours were established to a volume of approximately 150mm$^3$ in nude mice. The animals were then treated with JSI-124, a JAK2 inhibitor, or vehicle (inactive placebo) administered intraperitoneally. Tumours in vehicle treated animals continued to grow to a volume of 500mm$^3$ at 26 days, where animals treated with 1mg/kg/day had and 0.5mg/kg/day had tumours that were 76% and 52% smaller respectively. Of note, while JSI-124 treatment did slow the rate of growth, it did not stop it (that is to say the tumours kept increasing, but at a slower rate).

JAK blockade in EGFR mutated cell lines has similar effects. Gao et al pre-treated two EGFR mutated cells lines in vitro with pan-JAK blockade via P6 or control prior to implantation into the flanks of nude mice. P6-preteated tumours were shown to be of smaller size and mass after 14 days of growth compared to tumours developed from control treated cells [545]. Looyenga et al implanted nude mice with an EGFR mutated cell line and were able to slow tumour growth via JAK blockade using oral administration of ruxolitinib when compared to control (placebo) [551].

Murakami et al tested two animal models [559]. They developed a xenograft mouse model in which tumours were established to a volume of 100mm$^3$ followed by treatment with vehicle or two dose levels of the JAK2 blocker AZD1480. Tumours in treated mice were smaller and showed a significantly fewer tumour blood vessels. Murakami et al also developed a transgenic mouse model, with mice expressing an EGFR exon 19 deletion mutation in type II alveolar cells from birth. From 7 weeks of age the mice were treated with vehicle or
AZD1480 at increasing doses. At higher doses of AZD1480 fewer tumours were seen and with fewer CD31+ cells when the mice were sacrificed and examined at 10 weeks of age. AZD1480 led to an initial decrease in STAT3 activation at 2 and 6 hours, followed by a rebound increase at 12 and 16 hours. In a separate cohort, transgenic mice treated with AZD1480 vs vehicle from 7 weeks of age survived significantly longer (217 days vs 106 days, p<0.0001).

Cao et al used homoharringtonine (HHT) to block signalling from IL6 via JAK1 to STAT3. They inoculated groups of nude mice with the H1975 cell line which is resistant to first generation TKIs through T790M mutation. They found that HHT significantly repressed tumours in xenograft mice in comparison to those mice treated with gefitinib or vehicle (p<0.05). Further, in tumours from the HHT treated mice pSTAT3 was suppressed where it was not in the other two groups. The findings of these studies highlight the importance of JAK/STAT3 in the angiogenesis and metastasis pathways [549, 551, 559, 569].

JAK blockade has also been examined in combination with EGFR inhibition. Kim et al established exon 19 deletion/T790M co-mutated tumours in nude mice [556]. Mice were treated with pan-JAK blockade with P6 and afatinib. Both significantly slowed (but didn’t stop) growth as single agents compared to control treated animals. The combination of both agents was synergistic, and lead to a decrease in tumour volume over the course of the experiment. Harada et al had similar findings using JSI-124 and erlotinib in an erlotinib resistant cell line [555]. JSI-124 had minimal effect with a similar growth curve to control treatment. Erlotinib slowed the rate of growth, while the combination of JSI-124 and erlotinib resulted in minimal increase in tumour volume, of statistical significant compared to the three other groups of mice. Gao et al treated mice with several xenograft models. [563]. Erlotinib treated the EGFR mutated xenograft but had no effect in the resistant on xenografts with T790M mutation. JAK blockade with AZD1840 was partially effective all xenograft models. The strongest suppression of tumour growth was seen in the xenografted mice treated with both agents together.
4.1.4.2 Interleukin 6 Blockade

Two groups have examined the effects of manipulation of interleukin 6 levels using *in vivo* models. Yamaji et al used three AC cell lines and three SqCC cell lines in their *in vivo* work [568]. Interestingly the tumour doubling time was significantly shorter in the IL6 producing cell lines compared to the non-producing cell lines. This study also used a xenograft with no expression and with forced expression of IL6. This experiment showed a statistically significantly shorter tumour doubling time for mice inoculated IL6 producing cell line compared to mice inoculated with the parent cell line.

Yao et al conducted an elegant experiment based on the principle of the tumour microenvironment inducing tumour resistance to therapy [558]. NSCLC lung cancer cells harbouring an *EGFR* mutation were implanted in nude mice and allowed to develop tumours to the size of approximately 100mm$^3$. Inflammation was then induced locally near the tumour with various chemical compounds. The induction of inflammation was associated with decreased sensitivity to erlotinib, and the authors were able to demonstrate that this was not due to increased cellular proliferation but rather due to decreased apoptosis. The injection of an IL-6 antibody restored sensitivity to erlotinib in one cohort of mice, demonstrating the importance of the pathway in resistance to *EGFR* inhibitors.

4.1.4.3 Clinical Studies

4.1.4.3.1 STAT3 Expression and Relationship to *EGFR* Mutation

Many papers have examined expression of STAT3 in NSCLC. A number of these studies have significant heterogeneity with reported outcomes not separated by histologic subtypes. Several papers in which the proportion of patients with AC was low or not reported are not included in this section of the review [548, 570-575]. Qu et al examined the upregulation of STAT3 and downstream genes in NSCLC and COPD [576]. They demonstrated that the highest levels of STAT3 expression were seen in patients with NSCLC (SqCC > AC),
followed by COPD and then normal tissue. Jiang et al also found that STAT3 expression was more common in malignant than non-malignant tissue [577].

The assessment of pSTAT3 status has been considered by several groups. Across these studies IHC has been used to quantify pSTAT3, however all studies used different methods to assigned positivity or negativity, or to give a semi quantitative assessment [545, 551, 552, 577, 578]. Some studies were able to find associations between clinical features and pSTAT3. Gao et al found the presence of pSTAT3 was associated with non-solid histology tumours (p=0.015), TTF1+ positivity (0.015) and a small primary tumour (≤3cm; p=0.003) [545]. Kim et al found an association between pSTAT3 expression and tumour differentiation, however the method used to assess tumour differentiation was not described. They were able to assess survival outcomes and found that pSTAT3 status had no influence on overall survival in univariate analysis [578]. Jiang et al found that pSTAT3 was significantly correlated with a more advanced stage (stage III and IV, p=0.034), female sex (p=0.004), a non-smoking history (p=0.006), and the presence of lymph node metastases (p=0.009) [577].

Four groups have assessed the relationship between pSTAT3 status and the presence or absence of an EGFR mutation. Gao et al were able to show a strong correlation between pSTAT3 and the presence of an activating EGFR mutation (exon 19 deletion or L858R; p=0.002) [545]. Jiang et al also found a relationship between EGFR mutation and pSTAT3 status (p=0.003) [577]. Looyenga et al found no statistical association [551], however this study limited its EGFR mutation testing to exon 19 only, and had a portion of patients with non-adenocarcinoma histology [551, 579]. In a study of 50 patients with stage I adenocarcinoma, Takata et al found no association between EGFR mutation and pSTAT3 expression [552].

In summary, differing results were found across these studies with regards to the presence of EGFR mutation and pSTAT3 detected by IHC. There were no consistent findings with regards to clinical associations and pSTAT3 status. Difference in methods of assessment of pSTAT3 between papers limit the comparisons of the outcomes discussed above. This
research seeks to clarify whether EGFR mutation enriches for pSTAT3 (as well as IL6 and JAK1) by using two common scoring methods.

4.1.4.3.2 Influence of EGFR inhibition on pSTAT3

Given the inherent difficulties of translational research there are only two studies examining the effects of EGFR inhibition on STAT3 activation. The first of these was conducted by Albanell et al [580]. Their work examined changes in pSTAT3 expression in skin biopsies from patients enrolled in a sub-study as part of the phase I clinical trials of gefitinib (ZD1839). One hundred and four punch biopsies of skin were available from 65 patients, with biopsies taken pre-treatment and again at 2 weeks after the commencement of treatment. Changes in the skin were seen consistent with the inhibition of the normal trophic effects of EGF as mediated by EGFR. Staining for pEGFR fell from 44 ± 3.4% (range 0 – 90%) pre-therapy to 2.1 ± 0.8% (range 0 – 27%) on gefitinib. The decrease when comparing between paired samples was statistically significant (p<0.001). Twenty four paired samples were available for analysis of pSTAT3. pSTAT3 was mainly seen in the suprabasal skin layers prior to treatment with gefitinib. In the paired post-therapy biopsies a significant increase in pSTAT3 was seen in the basal layer of the epidermis (p<0.001).

Haura et al conducted a small study looking at the effect of EGFR inhibition with a four week period of gefitinib treatment prior to definitive surgical resection in early stage NSCLC [581]. Forty two patients were screened, allowing recruitment of 23 patients to the eventual study. Of these, 12 (52%) had AC histology. EGFR mutation status was not reported. Twenty two patients had stable disease and one patient had progressive disease following the treatment period, and as such the study was terminated given the lack of response. In the postoperative specimens minimal or absent pEGFR was found consistent with the expected effects of gefitinib. Significant expression of pSTAT3 was demonstrated. Unfortunately the study did not present assessment of staining in paired samples, likely due to the size of the available pre-treatment biopsies. The results of both of these papers suggest that EGFR inhibition leads to increased levels of STAT3 activation as evidenced by increased pSTAT3 in both tumour cells and normal cells.
4.1.4.3.3 JAK Immunohistochemistry in Tissue

Two groups to date have explored JAK staining via immunohistochemistry in NSCLC. Liu et al examined staining for JAK1 and phosphorylated JAK1 (pJAK1) in 100 patients with NSCLC (the rates of adenocarcinoma and SqCC were not presented in the manuscript) [582]. A semi-quantitative scoring method was used. The absence of expression or mild intensity staining in 1-50% of cells was considered negative. Positive staining was considered as mild intensity staining in 51-100% of cells or moderate/strong intensity staining in any proportion of cells. By this method the rates of positivity were 63% for JAK1 and 44% for pJAK1 respectively. Further, they also showed that expression of JAK1 or pJAK1 was rare in normal lung tissue.

Looyenga et al assessed staining for JAK2 in a cohort of 245 patients with stage I or II NSCLC, of whom 66% had adenocarcinoma (including ‘BAC’), 29% had SqCC, 3% had large cell carcinoma and 2% were not otherwise specified [551]. Semi-quantitative expression for JAK2 was found to be present and considered as positive in 79% of samples.

4.1.4.3.4 Interleukin 6 and gp130 Immunohistochemistry in Tissue

A study from Haura et al is the only study to date to explore expression of the IL6 receptor gp130 in NSCLC [583]. The breakdown of histologic subtypes is not reported in the paper. Tumour tissue and surrounding normal lung from 10 patients was examined. Nine tumours stained positive for both IL6 and gp130, with one tumour showing absence of staining with both markers. Diffuse cytoplasmic and nuclear staining for IL6 was seen in the positive nine cases. The high frequency of gp130 expression is consistent with its constitutive presence in normal cells.

Koh et al examined IL6 staining by IHC in a mixed population of 90 patients [584]. This population consisted of 50 patients with AC, 31 patients with SqCC, five patients with large
cell carcinoma and four patients with carcinoid tumours. A scoring system was used summing scores by assessment of the number of cells expressing IL6 by IHC (0 = negative, 1 <25%, 2 = 26 – 50%, 3 > 50%) and the intensity of staining (0 negative, 1 weak, 2 moderate, 3 high). A sum of scores of 3 or more was considered positive. Positive staining was numerically more common in SqCC compared to the other subtypes pooled however this did not reach statistical significance. IL6 staining was more common in tumours of higher T stage (T2 or more), any node positivity, or the total stage being II or III vs I. The authors conducted two multivariate analyses including either the total stage (model 1) or both the T and N stage separately (model 2) to avoid the interaction between these factors. Positive IL6 staining by IHC was associated with a worse overall survival (model 1 – HR 4.981 [1.195 – 20.767, p=0.0275]; model 2 – HR 6.111 [1.162 – 32.129, p=0.0326]) with staging factors also being statistically significant. To date no other report has been produced that replicates these findings.

A group from Taiwan looked at IL6 levels in malignant pleural effusion (MPE) in two studies. In the first paper Yeh et al compared the levels of IL6 in the effusions from patients with AC (n=32) and chronic heart failure (n=44). IL6 levels detected in MPE were 10-fold higher than those detected in effusions from patients with chronic heart failure. The authors also examined nuclear pSTAT3 in 114 patients with adenocarcinoma (stage I/II – 42, stage III – 35, stage IV with MPE – 37) and found that pSTAT3 was more frequent with increasing stage. Further clinical information was not provided. In the second study Huang et al collected samples of MPE from 20 patients with lung cancer (histologic types not specified) and treated isolated cultured cells with inhibitors of Jak2/STAT3, PI3K/Akt, MEK/ERK and NF-kB [560]. Significant inhibition of IL6 levels was seen with all four inhibitors used individually in comparison to control treated cells.

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**4.1.4.3.5 Serum Levels of IL6**

A number of groups have examined the clinical relevance of IL6 levels in serum and patients with lung carcinoma across the disease spectrum. Please note all of papers discussed under this heading had significant heterogeneity in the histologic subtypes included with no one
paper having a purely adenocarcinoma cohort. Furthermore, the levels of serum IL6 used to
delineate risk groups differ in each paper also.

An association appears to be present from an early stage. Pine et al conducted a nested case-
control study as part of the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening
Trial that examined levels of IL6, IL8 and C Reactive Protein (CRP) prior to a lung cancer
diagnosis [585]. Their interest stemmed from the roles of IL6 and IL8 as inflammatory
cytokines, the reported link between chronic inflammation and lung carcinogenesis, and the
association between high IL6 and IL8 in the development of malignancy. For cases
compared with controls IL6 was significantly elevated within 2 years prior to diagnosis of
lung cancer, independent of a number of clinical and laboratory markers. Interestingly the
elevation of IL6 or IL8 did not predict the histological subtype of lung cancer (AC, SqCC or
SCLC) subsequently diagnosed.

Serum IL6 has been investigated in patients with early stage disease, advanced disease and in
relationship to cancer cachexia. Four groups have investigated serum IL6 in early stage
disease [584, 586-588]. Two groups showed that serum IL6 levels were higher in patients
with earlier stages of resected NSCLC [584, 586]. Kita et al found that increased serum IL6
was associated with recurrence in stage IA and IB patients [586]. Ujiie et al demonstrated
worse OS in multivariate analysis in the setting of raised serum IL6 (along with T and N
stage )[587], while Koh et al did not demonstrate this association in a cohort of a similar size
[584]. Wojciechowska-Lacka et al examined 61 patients undergoing curative radiotherapy
for stage I or II disease with 60 Gy in a 6 week course [588]. Eighty percent of patients had
IL6 levels above the lower limit of detection of their assay (5pg/mL) prior to treatment. They
found that IL6 decreased in patients who had a clinical response to treatment (stable disease
or better) compared to those patients who didn’t respond.

A great deal of attention has been paid to serum IL6 levels in the setting of advanced
malignancies, particularly with the possible role of anti-IL6 therapies to treat cancer and
cancer cachexia [589, 590]. With regards to papers in NSCLC, the aims and methods
reported in each paper were substantially different, allowing for an overview only. Generally
IL6 levels were elevated in patients with advanced disease compared with healthy controls [591-594] with only one group not demonstrating this finding [595]. Several groups also reported a link between raised serum IL6 and cancer cachexia and/or weight loss [593, 595, 596] with one paper not finding this outcome [592]. Martin et al [593] and Songur et al [596] both found that raised IL6 levels were associated with worse performance status.

Wang et al examined a number of inflammatory markers in patients with stage III disease undergoing radical chemoradiotherapy [597]. The main finding of note was an association with higher IL6 levels and a greater symptom burden during treatment. Two groups looked at the relationship of IL6 to systemic therapy. De Vita et al found that patients who responded to cisplatin based chemotherapy were likely to have lower levels of IL6, while disease progression was associated with higher levels of IL6 [591]. Tas et al were able to show that IL6 levels fell following two cycles of cisplatin/vinorelbine [598]. Findings with regards to IL6 levels and survival outcomes are variable. This is likely a reflection of the small sample sizes of studies, the number of variables examined and their relative influence of survival. Two groups found no difference in survival outcomes based on IL6 levels [595, 598], two groups found significant differences on univariate survival analysis that were lost at multivariate analysis [594, 599], and two groups found survival differences that were maintained in multivariate analysis [593, 596].

4.1.5 Conclusion

Resistance to EGFR inhibition in the setting of advanced EGFR mutated lung adenocarcinoma remains a day to day problem in clinical practice. Improving the duration of response to first line treatment is a key aim of researchers and clinicians. Examining pathways that are frequently up- or down-regulated may find potential areas to target with therapeutic effect. Significant interest has been paid to the IL6 / JAK-1 / STAT3 pathway, however much of the work focuses of its activity in in vitro and in vivo studies. The number of studies examining this pathway in clinical specimens is limited.
This chapter examines activation of IL6 / JAK-1 / STAT3 pathway in lung adenocarcinoma specimens from patients who have undergone surgical resection. The study examines two scoring systems for IHC for IL-6, JAK1 and pSTAT3 expression. The main aim is to examine the rates of positivity for these markers compared to oncogenic mutations in EGFR and KRAS (or wild type for both) to see whether staining levels for activation of IL6, JAK-1 or pSTAT3 are enriched in the EGFR mutant cohort.

4.2 Materials and Methods

Cases for this work were collected from the cohort previously described in chapter three. The methods for inclusion criteria, clinical data collection, anatomical pathology assessment and molecular pathology assessment are as previously described (pages 167 – 170).

4.2.1 Preparation of the Tissue MicroArray (TMA)

Tissue was acquired at the time of routine surgical resection, and following prior consent as previously discussed. The tissue was handled in a uniform manner on arrival at the anatomical lab at St Vincent’s Hospital, Melbourne. Briefly, the operative lung samples were inflated and fixed in 10% buffered neutral formalin overnight. Slides were cut for routine anatomical pathology, and excess tissue was stored in FFPE blocks [600].

A significant collaboration had been established between members of the Lung Cancer Multidisciplinary Team at St Vincent’s Hospital, Melbourne and clinicians and scientists at the Peter MacCallum Cancer Centre (PMCC), Melbourne. FFPE tissue blocks had previously been retrieved for 168 patients described in chapter three. Representative 1mm tumour cores were obtained from a FFPE tumour block for each patient. Two tissue microarrays were constructed by Mr Richard Young and Mr David Byrne, research assistants.
in the laboratory of Associate Professor Ben Solomon at Peter MacCallum Cancer Centre. Due to tissue attrition a smaller number of cases (as outlined in results) had remaining tissue in the TMA compared to the total number of patients discussed in Chapter 3.

4.2.2 Immunohistochemical Assessment

4.2.2.1 Preparation of Slides

The following antibodies were acquired.

- IL6 (R&D systems, Minneapolis MN, USA)
- Gp130 (Santa Cruz Biotechnology, Dallas TX, USA)
- JAK1 (Cell Signalling Technology, Danvers MA, USA)
- Tyrosine phosphorylated STAT3 (Cell Signalling Technology, Danvers MA, USA).

To reduce the chance of artefact due to specimen age fresh sections were cut from the TMA once the antibodies had been optimised for use on whole tissue sections [601]. Sections were cut at a thickness of three microns and placed on AAS slides. The sections then underwent staining for IHC at the Department of Anatomical Pathology, St Vincent’s Hospital Melbourne. Staining was performed on the Ventana Benchmark XT automated immunostainer (Ventana Medical Systems, Tuscon, Arizona) as per the manufacturer’s instructions.

The following patterns of staining were observed when assessing presence and intensity of staining:

- IL6 and gp130 IHC – even cytoplasmic staining
- JAK1 – cytoplasmic staining that was granular in nature
- pSTAT3 showed even nuclear staining (consistent with the activated state and translation to the nucleus).
4.2.2.2 Scoring of Slides

Assessment of slides was performed by Associate Professor Prudence Russell, anatomical pathologist. As there is no standard cut off for considering positivity of negativity of each marker two methods were used. The methods were chosen on the basis of prior papers published on this subject. In the first method the presence of any staining was considered positive while the absence of any staining was considered negative, as per the criteria used by Gao et al [545].

In the second method the intensity of staining together with the proportion of cells staining positive was used as per the HSCORE method. This method involves assessing the intensity of staining (with a value of 0 [no staining], 1, 2 or 3 [strong staining]) and multiplying by the percentage of cells staining positive (from 0-100%) to give a score in the range of 0 – 300. As per the study of Looyenga et al, a score of greater than 40 was considered positive [551].

Representative photomicrographs of the intensity of staining for each of IL6, gp130, JAK1 and pSTAT3 are shown in Figures 11 to 14 (pages 220 – 223).
Figure 11: Examples of Immunohistochemistry Staining for Interleukin 6 (IL6 antibody: R&D systems, Minneapolis, MN, USA). Even Cytoplasmic Staining was observed

- Top Left – 0
- Top Right – 1+
- Bottom Left – 2+
Figure 12: Examples of Immunohistochemistry Staining for gp130 (gp130 antibody: Santa Cruz Biotechnology, Dallas TX, USA). Even Cytoplasmic Staining was observed

- Top Left – 0
- Top Right – 1+
- Bottom Left – 2+
- Bottom Right – 3+
Figure 13: Examples of Immunohistochemistry Staining for JAK1 (JAK1 antibody: Cell Signalling Technology, Danvers MA, USA). Granular Cytoplasmic Staining was observed

- Top Left – 0
- Top Right – 1+
- Bottom Left – 2+
- Bottom Right – 3+
Figure 14: Examples of Immunohistochemistry Staining for tyrosine phosphorylated STAT3 (pSTAT3 antibody: Cell Signalling Technology, Danvers MA, USA). Nuclear staining was observed:

- Top Left – 0
- Top Right – 1+
- Bottom Left – 2+
- Bottom Right – 3+
4.2.3 Statistical Analysis

Statistical analysis was undertaken using STATA version 12 (Statacorp, LP, TX). Tests of association were performed using Pearson’s $\chi^2$ or Fisher’s exact test as appropriate. Survival analysis was performed using the Kaplan-Meier method with hazard ratios derived using the Cox Proportional Hazards model. Survival analysis was performed from the date of surgery until a survival event occurred (death or recurrence as appropriate). Where a patient who had not reached a survival event their survival analysis was censored at the last known time of review. Median times and hazard ratios are reported with their 95% confidence interval (CI). $p$ less than 0.05 was considered statistically significant.

4.3 Results

4.3.1 Patient Characteristics

Between November 2000 and December 2011, 143 patients who underwent surgical resection were eligible for inclusion in the analysis and had sufficient tissue available in the TMA for IHC staining. Seventy-one patients were male (49%) and 72 patients were female (51%). The median age was 69 years (range 20 – 87). The ECOG performance status preoperatively was zero in 98 patients (69%), one in 42 patients (29%) and two in three patients (2%). There were 30 current smokers (21%), 81 former smokers (57%) and 32 never smokers (22%).

The surgical management of the primary tumour was as follows – lobectomy in 123 patients (86%); bilobectomy in three patients (2%); pneumonectomy in five patients (4%); wedge resection in nine patients (6%); and segmentectomy in three patients (2%). Pathologic assessment of the nodal status was possible in 139 patients (97%). The pathologic features are presented in table 22.
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<td>1</td>
<td>1</td>
<td>Absent</td>
<td>33</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lymphatic Only</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Vascular Only</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Both</td>
<td>85</td>
<td>59</td>
</tr>
<tr>
<td>N stage</td>
<td></td>
<td></td>
<td><strong>Neural Invasion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>94</td>
<td>66</td>
<td>Absent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>12</td>
<td>Perineural</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>20</td>
<td>Intraneural</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Not evaluable</td>
<td>4</td>
<td>2</td>
<td></td>
<td>137</td>
<td>96</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td><strong>Predominant Subtype</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IA</td>
<td>52</td>
<td>36</td>
<td>AIS</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>IB</td>
<td>35</td>
<td>24</td>
<td>MIA</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>IIA</td>
<td>17</td>
<td>12</td>
<td>LPA</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>IIB</td>
<td>8</td>
<td>6</td>
<td>Acinar</td>
<td>65</td>
<td>45</td>
</tr>
<tr>
<td>IIIA</td>
<td>30</td>
<td>21</td>
<td>Papillary</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>IIIB</td>
<td>1</td>
<td>1</td>
<td>Solid</td>
<td>26</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Micropapillary</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IMA</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Colloid</td>
<td>2</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Table 22:** Pathologic Characteristics for resection specimens from 143 patients.

AIS – Adenocarcinoma in Situ; MIA – minimally invasive adenocarcinoma; LPA – Lepidic Predominant Adenocarcinoma; IMA – Invasive Mucinous Adenocarcinoma
4.3.2 EGFR and KRAS Mutation

EGFR mutations were detected in 43 patients (30%) of whom 21 patients had an exon 19 deletion mutations; 15 patients an exon 21 L858R mutation and one patient an exon 21 L861Q mutation. Two patients had mutations of uncertain clinical significance in exon 18 – one with a deletion/insertion mutation (E709_T710 del_ins D) and one with multiple point mutations (E709K, K714N, V717E and G719C). There were four patients with exon 20 insertions or duplications. As expected EGFR mutations were associated with female sex (30/72 [42%] vs 13/71 [18%], $\chi^2$ (1) = 9.2, p=0.002) and history of smoking (never 22/32 [69%]; current 4/30 [13%]; former 17/81 [21%], $\chi^2$ (2) = 29.9, p<0.001).

KRAS mutations were detected in 42 patients (29%) at the following locations – G12C 18; G12V 13; G12D 4; G12S 3; G12R 2; and one each at G13C and G13V. Thirty-two of these mutations were due to guanine (G) to thymidine (T) transversions, consistent with a smoking related aetiology [444]. KRAS mutations were strongly associated with smoking history (former 27/81 [33%], current 14/30 [47%]; never smokers 1/32 [3%]; $\chi^2$ (2) = 15.6, p<0.001).

4.3.3 JAK / STAT Pathway Staining

The positivity rates for each method of scoring were as follows (table 23):

<table>
<thead>
<tr>
<th></th>
<th>Method 1 (Any Staining Positive)</th>
<th>Method 2 (HScore &gt;40 positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL6</td>
<td>142</td>
<td>45 (32%)</td>
</tr>
<tr>
<td>gp130</td>
<td>142</td>
<td>140 (96%)</td>
</tr>
<tr>
<td>JAK1</td>
<td>140</td>
<td>109 (78%)</td>
</tr>
<tr>
<td>pSTAT3</td>
<td>134</td>
<td>59 (44%)</td>
</tr>
</tbody>
</table>

Table 23: Rates of positive staining for each antibody using both methods.

IL6 – interleukin 6; gp130 – glycoprotein 130; JAK1 – Janus Kinase 1; pSTAT3 – phosphorylated Signal Transducers and Activators of Transcription 3
As positive staining was found for gp130 in all but two examined specimens no further analysis was performed on the basis of staining for this protein. The rate of positivity for IL6 did not change between either method, while using the HSCORE method reduced the number of samples considered positive for JAK1 and pSTAT3. With regards to pSTAT3, 22% of samples remained positive using method 2, while the other 22% of samples dropped from positive to negative, suggesting that in many tumours the level of activation was fairly low. Using both scoring methods, statistically significant associations were detected between IL6, JAK1 and pSTAT3, consistent with activation of the pathway in clinical specimens (table 24). If a Bonferroni correction for multiple comparisons is applied, all but one of these associations maintain statistical significance at a level of p=0.00833.
<table>
<thead>
<tr>
<th>Method 1</th>
<th>Method 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSTAT3 Negative</td>
<td>pSTAT3 Positive</td>
</tr>
<tr>
<td><strong>IL6 Negative</strong></td>
<td></td>
</tr>
<tr>
<td>58 (78%)</td>
<td>32 (54%)</td>
</tr>
<tr>
<td><strong>IL6 Positive</strong></td>
<td></td>
</tr>
<tr>
<td>18 (22%)</td>
<td>27 (46%)</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>59</td>
</tr>
<tr>
<td>$\chi^2 = 8.7$, $p = 0.003$</td>
<td>$\chi^2 = 15.0$, $p &lt; 0.001$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>JAK1 Negative</th>
<th>JAK1 Positive</th>
<th>JAK1 Negative</th>
<th>JAK1 Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL6 Negative</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28 (90%)</td>
<td>68 (62%)</td>
<td>96</td>
<td>44 (90%)</td>
</tr>
<tr>
<td><strong>IL6 Positive</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 (10%)</td>
<td>41 (38%)</td>
<td>44</td>
<td>5 (10%)</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>109</td>
<td>140</td>
<td>49</td>
</tr>
<tr>
<td>$\chi^2 = 8.7$, $p = 0.003$</td>
<td></td>
<td>$\chi^2 = 15.8$, $p &lt; 0.001$</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pSTAT3 Negative</th>
<th>pSTAT3 Positive</th>
<th>n</th>
<th>pSTAT3 Negative</th>
<th>pSTAT3 Positive</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>JAK1 Negative</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 (32%)</td>
<td>6 (10%)</td>
<td>30</td>
<td>40 (38%)</td>
<td>5 (18%)</td>
<td>45</td>
</tr>
<tr>
<td><strong>JAK1 Positive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 (68%)</td>
<td>52 (90%)</td>
<td>102</td>
<td>64 (62%)</td>
<td>23 (82%)</td>
<td>87</td>
</tr>
<tr>
<td><strong>N</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>74</td>
<td>58</td>
<td>132</td>
<td>104</td>
<td>28</td>
<td>132</td>
</tr>
<tr>
<td>$\chi^2 = 9.0$, $p = 0.003$</td>
<td></td>
<td>$\chi^2 = 4.2$, $p = 0.041^*$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 24:** Associations between rates of positive staining for IL6, JAK1 and pSTAT3. * - not statistically significant following a Bonferroni correction for multiple comparisons (significance at the level of $p = 0.00833$)

IL6 – interleukin 6; JAK1 – Janus Kinase 1; pSTAT3 – phosphorylated Signal Transducers and Activators of Transcription 3
4.3.4 Is There an Association Between Staining for IL6 / JAK1 / pSTAT3 and EGFR or KRAS mutations in our Cohort?

The frequency of positivity for IL6, JAK1 and pSTAT3 was compared using both scoring methods for IHC staining to the presence or absence of mutations in EGFR and KRAS. The rates of expression as detected by immunohistochemistry were similar across samples from patients those tumours had EGFR mutations, those with KRAS mutations and tumours that were wild type for both oncogenes (table 25). Therefore, the presence of an EGFR mutation did not enrich for IL6, JAK1 or pSTAT3 activity when measured by immunohistochemistry, as compared to samples from tumours with KRAS mutations or those samples that were wild type for both EGFR and KRAS genes.
<table>
<thead>
<tr>
<th>Method 1</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EGFR</strong></td>
<td><strong>KRAS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild Type</td>
<td>Mutated</td>
<td>n</td>
<td>Wild Type</td>
<td>Mutated</td>
</tr>
<tr>
<td><strong>IL6 Negative</strong></td>
<td>69 (70%)</td>
<td>28 (65%)</td>
<td>97</td>
<td>69 (68%)</td>
</tr>
<tr>
<td><strong>IL6 Positive</strong></td>
<td>30 (30%)</td>
<td>15 (35%)</td>
<td>45</td>
<td>32 (32%)</td>
</tr>
<tr>
<td>N</td>
<td>99</td>
<td>43</td>
<td>142</td>
<td>101</td>
</tr>
<tr>
<td>χ² =0.3, p=0.59</td>
<td></td>
<td></td>
<td></td>
<td>χ² =0.0, p=0.998</td>
</tr>
<tr>
<td><strong>JAK1 Negative</strong></td>
<td>23 (24%)</td>
<td>8 (18%)</td>
<td>31</td>
<td>24 (24%)</td>
</tr>
<tr>
<td><strong>JAK1 Positive</strong></td>
<td>74 (76%)</td>
<td>35 (82%)</td>
<td>109</td>
<td>77 (76%)</td>
</tr>
<tr>
<td>N</td>
<td>97</td>
<td>43</td>
<td>140</td>
<td>101</td>
</tr>
<tr>
<td>χ² =0.45, p=0.50</td>
<td></td>
<td></td>
<td></td>
<td>χ² =0.55, p=0.46</td>
</tr>
<tr>
<td><strong>pSTAT3 Negative</strong></td>
<td>55 (59%)</td>
<td>20 (50%)</td>
<td>75</td>
<td>49 (52%)</td>
</tr>
<tr>
<td><strong>pSTAT3 Positive</strong></td>
<td>39 (41%)</td>
<td>20 (50%)</td>
<td>59</td>
<td>45 (48%)</td>
</tr>
<tr>
<td>N</td>
<td>94</td>
<td>40</td>
<td>134</td>
<td>94</td>
</tr>
<tr>
<td>χ² =0.82, p=0.36</td>
<td></td>
<td></td>
<td></td>
<td>χ² =1.89, p=0.17</td>
</tr>
</tbody>
</table>

**Table 25:** Relationship between staining for IL6, JAK1 and pSTAT3 by EGFR and KRAS mutation status
<table>
<thead>
<tr>
<th>Method 2</th>
<th>EGFR</th>
<th></th>
<th></th>
<th></th>
<th>KRAS</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild Type</td>
<td>Mutated</td>
<td>n</td>
<td></td>
<td>Wild Type</td>
<td>Mutated</td>
<td>n</td>
<td></td>
</tr>
<tr>
<td>JAK1 Negative</td>
<td>36 (37%)</td>
<td>13 (30%)</td>
<td>49</td>
<td></td>
<td>37 (37%)</td>
<td>12 (31%)</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>JAK1 Positive</td>
<td>61 (63%)</td>
<td>30 (70%)</td>
<td>91</td>
<td></td>
<td>64 (63%)</td>
<td>27 (69%)</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>97</td>
<td>43</td>
<td>140</td>
<td></td>
<td>101</td>
<td>39</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\chi^2$ =0.62, $p$=0.43</td>
<td></td>
<td></td>
<td></td>
<td>$\chi^2$ =0.42, $p$=0.51</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JAK1 Negative</td>
<td>76 (81%)</td>
<td>29 (72%)</td>
<td>105</td>
<td></td>
<td>70 (74%)</td>
<td>35 (87%)</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>JAK1 Positive</td>
<td>18 (19%)</td>
<td>11 (18%)</td>
<td>29</td>
<td></td>
<td>24 (26%)</td>
<td>5 (13%)</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>94</td>
<td>40</td>
<td>134</td>
<td></td>
<td>94</td>
<td>40</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\chi^2$ =1.15, $p$=0.28</td>
<td></td>
<td></td>
<td></td>
<td>$\chi^2$ =2.81, $p$=0.09</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 25 continued:** relationship between staining for IL6, JAK1 and pSTAT3 by *EGFR* and *KRAS* mutation status

Note: Rates of positive staining for IL6 did not change when applying method 1 or method 2. Therefore, as the data is the same it has only been shown once
4.3.5 Is There an Association Between Staining for IL6 / JAK1 / pSTAT3 and Clinicopathologic Features?

Staining for IL6, JAK1 and pSTAT3 were compared with the following clinical and pathologic features – patient sex; smoking status; T stage; N stage; overall stage; predominant histologic pattern; presence of pleural invasion; and presence of lymphovascular invasion. Limited statistical associations were found, potentially as a result of multiple comparisons being made (table 26, pages 233 – 234):

- Smaller primary tumours (T stage 1) were associated with increased positive staining for JAK1 measured by either method of assessment
- An N stage of 0 (no lymph node metastases) was statistically associated with higher rates of pSTAT3 when any staining was considered positive, but no statistical significant was found when the second method of assessing for pSTAT3 positivity (H score > 40) was applied
- Similarly, a lower stage (stage 1) was associated with higher rates of pSTAT3 positivity when any staining was considered positive, but not when using the H score method
- Tumour patterns with a predominant lepidic component (AIS, MIA or LPA) were statistically more likely to be positive for JAK1 via method 1, and pSTAT3 via method 2, using Fisher’s exact test.

If a Bonferroni correction is applied for multiple statistical comparisons then no clinicopathologic factors retained statistical significance. No pathologic or clinical factor was found to have statistical associations with each of IL6, JAK1 and pSTAT3 at the same time or by the same method, suggesting that there is no correlation between activation IL6 / JAK1 / pSTAT3 pathway with these factors.
<table>
<thead>
<tr>
<th>Variable</th>
<th>IL6</th>
<th></th>
<th>p</th>
<th>JAK Method 1</th>
<th></th>
<th>p</th>
<th>JAK Method 2</th>
<th></th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td></td>
<td>Negative</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>21</td>
<td>50</td>
<td>χ² =0.30</td>
<td>p=0.59</td>
<td>15</td>
<td>56</td>
<td>χ² =0.09</td>
<td>p=0.77</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>24</td>
<td>47</td>
<td></td>
<td></td>
<td>16</td>
<td>53</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>Never</td>
<td>22</td>
<td>10</td>
<td>χ² =0.30</td>
<td>p=0.59</td>
<td>8</td>
<td>24</td>
<td>χ² =0.36</td>
<td>p=0.84</td>
</tr>
<tr>
<td></td>
<td>Current</td>
<td>24</td>
<td>5</td>
<td></td>
<td></td>
<td>5</td>
<td>22</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Former</td>
<td>51</td>
<td>30</td>
<td></td>
<td></td>
<td>18</td>
<td>63</td>
<td></td>
<td>28</td>
</tr>
<tr>
<td>T stage</td>
<td>1</td>
<td>40</td>
<td>17</td>
<td>χ² =0.49</td>
<td>p=0.78</td>
<td>6</td>
<td>51</td>
<td>χ² =12.5</td>
<td>p=0.002</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>44</td>
<td>20</td>
<td></td>
<td></td>
<td>15</td>
<td>47</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>3 and 4</td>
<td>13</td>
<td>8</td>
<td></td>
<td></td>
<td>10</td>
<td>11</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>N stage</td>
<td>0</td>
<td>62</td>
<td>31</td>
<td>χ² =1.57</td>
<td>p=0.45</td>
<td>17</td>
<td>74</td>
<td>χ² =3.83</td>
<td>p=0.15</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>11</td>
<td>6</td>
<td></td>
<td></td>
<td>3</td>
<td>14</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>22</td>
<td>6</td>
<td></td>
<td></td>
<td>10</td>
<td>18</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>Stage</td>
<td>1</td>
<td>58</td>
<td>28</td>
<td>χ² =0.74</td>
<td>p=0.69</td>
<td>13</td>
<td>71</td>
<td>χ² =5.86</td>
<td>p=0.053</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>16</td>
<td>9</td>
<td></td>
<td></td>
<td>7</td>
<td>18</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>23</td>
<td>8</td>
<td></td>
<td></td>
<td>11</td>
<td>20</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>Predominant Pattern</td>
<td>AIS/MIA/LPA Acinar</td>
<td>6</td>
<td>8</td>
<td>Fisher's exact</td>
<td>p=0.071</td>
<td>1</td>
<td>13</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Papillary Solid</td>
<td>48</td>
<td>17</td>
<td></td>
<td></td>
<td>13</td>
<td>52</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>MPA</td>
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<td>p=0.94</td>
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<td></td>
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**Table 26:** Clinicopathologic correlations of positivity for IL6, JAK1 and pSTAT3 (see overleaf)
<table>
<thead>
<tr>
<th>Variable</th>
<th>Method 1 Negative</th>
<th>Positive</th>
<th>Method 2 Negative</th>
<th>Positive</th>
<th>p</th>
<th>Method 2 Negative</th>
<th>Positive</th>
<th>p</th>
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<td>p = 0.21</td>
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<td>p = 0.97</td>
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<td>p = 0.56</td>
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<td>Fisher’s exact</td>
<td>p = 0.12</td>
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<td>16</td>
<td>41</td>
<td>7</td>
<td>Fisher’s exact</td>
<td>p = 0.27</td>
<td></td>
</tr>
<tr>
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<td>p = 0.73</td>
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<td>13</td>
<td>5</td>
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</tr>
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<td>32</td>
<td>63</td>
<td>16</td>
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</tr>
</tbody>
</table>

Table 26 continued:

Clinicopathologic correlations of positivity for IL6, JAK1 and pSTAT3

AIS – adenocarcinoma in situ; LPA – lepidic predominant adenocarcinoma; MIA – minimally invasive adenocarcinoma

Note: Rates of positive staining for IL6 did not change when applying method 1 or method 2. Therefore, as the data is the same it has only been shown once.
4.3.6 Assessment of Survival Outcomes by Staining for IL6, JAK1 and pSTAT3

The presence or absence of IHC staining for IL6, JAK1 and pSTAT3 was assessed for its prognostic significance. No overall survival difference was seen on the basis of staining for IL6. Longer survival on univariate analysis was seen for patients whose tumours stained positively for JAK1 or pSTAT3 by method 1 (the presence of any staining). However when the presence or absence of staining by method 2 (the H-SCORE method) this survival difference was lost (Table 27, Figures 15 - 19).

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th>Median Survival (months)</th>
<th>Hazard Ratio (95% CI)</th>
<th>Univariate p</th>
</tr>
</thead>
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<td><strong>IL6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>97</td>
<td>98.4 (63.8 - NR)</td>
<td>0.77 (0.39 - 1.51)</td>
<td>0.45</td>
</tr>
<tr>
<td>Positive</td>
<td>45</td>
<td>NR (NR - NR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Method 1 pStat3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>75</td>
<td>122 (49.1 - NR)</td>
<td>0.44 (0.22 - 0.90)</td>
<td>0.024</td>
</tr>
<tr>
<td>Positive</td>
<td>59</td>
<td>NR (98.4 - NR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>JAK1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>31</td>
<td>63.8 (23.0 - NR)</td>
<td>0.49 (0.25 - 0.97)</td>
<td>0.042</td>
</tr>
<tr>
<td>Positive</td>
<td>109</td>
<td>122.1 (88.1 - NR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Method 2 pStat3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>105</td>
<td>122.1 (68.4 - NR)</td>
<td>0.84 (0.38 - 1.84)</td>
<td>0.67</td>
</tr>
<tr>
<td>Positive</td>
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<td>NR (46.5 - NR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>JAK1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>49</td>
<td>NR (42.2 - NR)</td>
<td>0.71 (0.38 - 1.34)</td>
<td>0.29</td>
</tr>
<tr>
<td>Positive</td>
<td>91</td>
<td>122.1 (88.1 - NR)</td>
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</tbody>
</table>

**Table 27:** Survival by the presence or absence of immunohistochemical staining for IL6, JAK1 and pSTAT3

95% confidence intervals shown in brackets. NR = not reached.
Figure 15: Overall Survival by presence or absence of staining for IL6 (HR 0.77 (95% CI 0.39 - 1.51), p=0.45)
Figure 16: Overall Survival by presence or absence of staining for JAK1 (HR 0.49 (95% CI 0.25 - 0.97), p=0.042)

Figure 17: Overall Survival by low or high staining for JAK1 as defined by the H Score (HR 0.71 (95% CI 0.38 - 1.34), p=0.29)
Figure 18: Overall Survival by presence or absence of staining for pSTAT3 (HR 0.44 (95% CI 0.22 - 0.90), p=0.024)

Figure 19: Overall Survival by low or high staining for pSTAT3 as defined by the H Score (HR 0.71 (95% CI 0.38 - 1.34), p=0.29)
Increasing stage, predominant pattern and the presence of visceral or parietal pleural invasion all had statistically significant effects on survival outcomes in this cohort of patients (this was expected as these patients represent a subset of patients in chapter three; see table 28 below). As such, stage, predominant pattern and the presence of pleural invasion were included in a multivariate analysis of survival outcomes together with the presence of staining for pSTAT3 or JAK1 (via method one). Stage and the solid predominant histologic pattern retained statistical significance with regards to survival outcomes on multivariate analysis, while pleural invasion and the absence of staining for pSTAT3 and JAK1 were no longer significant (table 28).

An exploratory multivariate analysis was performed using stage, predominant histology pattern and pSTAT3 expression via method one or JAK1 expression via method 1. This was done in the event that there were potential interactions between variables in the larger multivariate analysis. In both models, pSTAT3 and JAK1 failed to reach statistical significance for overall survival. Survival analysis was also performed by pairs of either stage or predominant subtype and either pSTAT3 or JAK1. Again, no significant survival outcomes were found on limitation of the multivariate model to two variables.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Factor</th>
<th>n</th>
<th>Median (Months)</th>
<th>HR (Univariate)</th>
<th>Univariate p</th>
<th>HR (Multivariate)</th>
<th>Multivariate p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage</td>
<td>1</td>
<td>87</td>
<td>122 (122.1 - NR)</td>
<td>1 (Referent)</td>
<td></td>
<td>1 (Referent)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>25</td>
<td>49.1 (25.5 - NR)</td>
<td>4.1 (1.8 - 9.2)</td>
<td>0.001</td>
<td>3.04 (1.17 - 7.88)</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>31</td>
<td>24.7 (18.1 - 58.4)</td>
<td>11.3 (5.2 - 24.3)</td>
<td>&lt;0.001</td>
<td>10.2 (3.90 - 26.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Predominant Pattern</td>
<td>AIS/MIA/LPA</td>
<td>14</td>
<td>NR (NR - NR)</td>
<td>0.22 (0.03 - 1.69)</td>
<td>0.15</td>
<td>0.60 (0.07 - 5.00)</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>Acinar</td>
<td>65</td>
<td>122.1 (68.4 - NR)</td>
<td>1 (Referent)</td>
<td></td>
<td>1 (Referent)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Papillary</td>
<td>22</td>
<td>NR (48.7 - NR)</td>
<td>0.91 (0.33 - 2.48)</td>
<td>0.85</td>
<td>1.62 (0.49 - 5.40)</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>Solid</td>
<td>26</td>
<td>45.7 (23.0 - NR)</td>
<td>2.31 (1.12 - 4.75)</td>
<td>0.023</td>
<td>3.46 (1.43 - 8.41)</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>MPA</td>
<td>11</td>
<td>42.2 (21.0 - NR)</td>
<td>1.68 (0.56 - 5.05)</td>
<td>0.35</td>
<td>1.50 (0.43 - 5.22)</td>
<td>0.53</td>
</tr>
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<td>122.1 (98.4 - NR)</td>
<td>1 (Referent)</td>
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<td>1 (Referent)</td>
<td></td>
</tr>
<tr>
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<td>Present</td>
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<td>49.1 (34.0 - NR)</td>
<td>2.43 (1.32 - 4.50)</td>
<td>0.004</td>
<td>1.10 (0.54 - 2.24)</td>
<td>0.79</td>
</tr>
<tr>
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<td>63.8 (23.0 - NR)</td>
<td>1 (Referent)</td>
<td></td>
<td>1 (Referent)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>109</td>
<td>122.1 (88.1 - NR)</td>
<td>0.50 (0.25 - 0.97)</td>
<td>0.042</td>
<td>0.75 (0.34 - 1.65)</td>
<td>0.47</td>
</tr>
<tr>
<td>pSTAT3</td>
<td>Absent</td>
<td>75</td>
<td>122.1 (49.1 - NR)</td>
<td>1 (Referent)</td>
<td></td>
<td>1 (Referent)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>59</td>
<td>NR (98.4 - NR)</td>
<td>0.44 (0.22 - 0.90)</td>
<td>0.024</td>
<td>1.18 (0.51 - 2.76)</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Table 28: Univariate and multivariate analysis of survival outcomes by stage, predominant subtype, pleural invasion and presence of staining for JAK1 and pSTAT3 (as assessed by method 1 – any positive staining).

(NR – not reached; AIS – Adenocarcinoma in situ; MIA – Minimally Invasive Adenocarcinoma; MPA – Micropapillary adenocarcinoma). 95% confidence intervals shown in brackets.
4.4 Discussion

4.4.1 Expression Rates for IL6, JAK1 and pSTAT3 by Immunohistochemistry

The rates of expression of IL6 by IHC in our cohort were modest at 32%. This study represents only the fifth such work to examine IL6 staining in surgical resection specimens. Koh et al reported on a cohort of Japanese patients with NSCLC [584]. In their non-squamous NSCLC cohort (that included a total of nine patients who had a diagnosis of large cell carcinoma or carcinoid), the rate of IL6 staining was 22% (thirteen out of 59 samples). The median age and high rates of prior smoking were similar to our cohort. Our study had similar numbers of males and females, where Koh et al had more males than females as is usually seen. The two studies also used different antibodies for IL6 assessment.

Two prior studies showed much higher rates of IL6 staining. Haura et al found nine out of ten patients whose tumours expressed IL6 in a mixed cohort of patients with NSCLC [583]. The high rate of positivity may be related to the small sample size, and any other interpretation of the data is limited by the absence of information on patient demographics and disease characteristics.

Gao et al found high rates also, with 61 out of 82 patients (74%) being considered positive for IL6 by IHC (defined at 2+ or 3+ staining in their study) [545]. In comparison, only six of our 45 positive patients stained with 2+ intensity, the rest having 1+ staining intensity. The results may potentially be explained by technical factors. Both studies (Gao et al and our study) used the same antibody to assess IL-6 staining, however different platforms were used to perform the tests (Gao et al used a Dako based system, while this study used the Ventana Benchmark XT automated immunostainer [Ventana Medical Systems, Tuscon, Arizona]). Differences in
interpretation of the intensity of staining across studies may also have contributed to different rates of expression. It is not possible to compare demographics between studies as this data was not presented in the manuscript from Gao et al (however both studies were conducted in cohorts of predominantly Western ethnicity).

Ninety six percent of patients in our study had positive staining for the gp130. Haura et al have been the only other group to examine this, and they similarly found positive expression by IHC in nine out of 10 cases [583]. Both studies are also consistent with the known widespread expression of gp130 in both normal and malignant tissues [510, 511].

We found that rates of staining for JAK1 were fairly high in our cohort, with 78% of samples showing positive staining, or 65% of specimens positive when an H-Score of >40 was used to determine positivity. Our rates of staining for JAK1 are very similar to those previously reported in mixed cohorts of NSCLC, for JAK1 (by Liu et al [582]) and JAK2 (by Looyenga et al [551]).

There is no consistent method for quantification of pSTAT3 expression across studies. As such, this work adopted two scoring systems, assessing for the presence of any staining as per the work of Gao et al [545], and using a semi-quantitative method (H score >40) as used in the study from Looyenga et al [551]. The expression of any staining for pSTAT3 was fairly common at 44%. The range for any staining in our study was numerically similar to that reported from three other groups [545, 548, 573].

Assessing pSTAT3 above a higher level of expression (H score >40) reduced the rate of positivity to 22%. Therefore, in half of the cases of pSTAT3 positivity the actual level of expression was low. This is consistent with the lower rate of IL6 positivity that we observed (see further comments below). Our findings are similar to those of Gao et al who found that 50% of their cohort of 92 patients had some staining for pSTAT3 [545]. However, the rates of patients with more intense levels of staining (2+ or 3+) were only
21%, with most positive staining being fairly weak (1+) in the other 29% of positive patients. However, other authors set the cut off level for pSTAT3 positivity at a much higher level, and still had high rates of pSTAT3 positivity [574, 578, 602]. Sources of heterogeneity are discussed further below. The study from Takata et al is a notable outlier [552]. The cohort was small (n=50) and pSTAT3 positivity was assigned at staining levels of greater than 10%. By this definition 84% of samples were positive for pSTAT3. Unlike other studies, all except for one patient in the Takata cohort had a lepidic pattern tumour (described as BAC at the time the paper was written).

Across the reported studies there are multiple sources of heterogeneity that may have contributed to differences in the rate of pSTAT3 positivity results. While all but two studies used the pSTAT3 antibody manufactured by Cell Signalling Technology (Danvers MA, USA), the cut-offs of staining used to ascribe positivity differed between studies (see table 29, page 244). A number of studies had a mixture of NSCLC histologic types included, where the pSTAT3 status was not subsequently reported by individual histologic subtypes [551, 571, 573, 574, 577]. Three studies, all from Asian centres, had high rates of never smoking, consistent with the demographics of patients diagnosed with lung cancer in their country [577, 578, 602]. In our cohort, and that of Cortas et al, the number of women was similar to the number of men, which is not in keeping with the usual gender ratios in a predominantly Caucasian population where more men are usually seen than women in an NSCLC cohort [573].
<table>
<thead>
<tr>
<th>First Author</th>
<th>Country</th>
<th>Histology</th>
<th>% AC</th>
<th>N</th>
<th>Antibody Manufacturer</th>
<th>Cut Off for Positivity</th>
<th>pSTAT3 Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gao [545]</td>
<td>USA</td>
<td>AC</td>
<td>100%</td>
<td>92</td>
<td>Cell Signalling Technology</td>
<td>Any Staining</td>
<td>50%</td>
</tr>
<tr>
<td>Cortas [573]</td>
<td>USA</td>
<td>NSCLC</td>
<td>53%</td>
<td>134</td>
<td>Santa Cruz Biotechnology</td>
<td>Any Staining</td>
<td>37%</td>
</tr>
<tr>
<td>Haura* [548]</td>
<td>USA</td>
<td>AC</td>
<td>100%</td>
<td>95</td>
<td>Cell Signalling Technology</td>
<td>Any Staining</td>
<td>61%</td>
</tr>
<tr>
<td>Mukohara [571]</td>
<td>Japan</td>
<td>NSCLC</td>
<td>50%</td>
<td>60</td>
<td>Cell Signalling Technology</td>
<td>&gt;5%</td>
<td>59%</td>
</tr>
<tr>
<td>Jiang [577]</td>
<td>China</td>
<td>NSCLC</td>
<td>79%</td>
<td>127</td>
<td>Cell Signalling Technology</td>
<td>&gt;5%</td>
<td>64%</td>
</tr>
<tr>
<td>Takata [552]</td>
<td>Japan</td>
<td>AC**</td>
<td>100%</td>
<td>50</td>
<td>Cell Signalling Technology</td>
<td>&gt;10%</td>
<td>84%</td>
</tr>
<tr>
<td>Yang* [603]</td>
<td>China</td>
<td>AC</td>
<td>100%</td>
<td>54</td>
<td>Cell Signalling Technology</td>
<td>&gt;10%</td>
<td>59%</td>
</tr>
<tr>
<td>Looyenga [551]</td>
<td>USA</td>
<td>NSCLC</td>
<td>66%</td>
<td>245</td>
<td>Cell Signalling Technology</td>
<td>H Score &gt; 40</td>
<td>22%</td>
</tr>
<tr>
<td>Kim [578]</td>
<td>Korea</td>
<td>AC</td>
<td>100%</td>
<td>162</td>
<td>Cell Signalling Technology</td>
<td>&gt;25% staining, at least moderate intensity</td>
<td>51%</td>
</tr>
<tr>
<td>Zimmer [574]</td>
<td>Germany</td>
<td>NSCLC</td>
<td>78%</td>
<td>67</td>
<td>New England Biolabs</td>
<td>2+ or 3+ intensity</td>
<td>61%</td>
</tr>
<tr>
<td>Zhao* [602]</td>
<td>China</td>
<td>AC</td>
<td>100%</td>
<td>33</td>
<td>Cell Signalling Technology</td>
<td>Score &gt;200 ***</td>
<td>57%</td>
</tr>
<tr>
<td>Our Cohort</td>
<td>Australia</td>
<td>AC</td>
<td>100%</td>
<td>134</td>
<td>Cell Signalling Technology</td>
<td>Any Staining</td>
<td>44%</td>
</tr>
</tbody>
</table>

Table 29: Rates of positive staining for pSTAT3 in previously reported studies. AC – adenocarcinoma; NR – not reported; NSCLC – non-small cell lung cancer

* Patients with adenocarcinoma only from the study cohort reported in this table (full cohort included all NSCLC).

** Cohort predominantly consisted of patients with “BAC”.

*** Scoring system = intensity (0 – 4) x % staining
4.4.2 Relationship Between IL6, JAK1 and pSTAT3 Staining

We assessed the activation of the IL6 / JAK1 / pSTAT3 pathway by comparing the frequency of positive staining on immunohistochemistry for each of these markers. Positive statistical correlations were found for cross comparison of each of the three markers. This held whether the staining expression was measured as present or absent and when the staining expression was counted as positive via an H score of greater than 40. JAK1 expression in the absence of IL6 expression was uncommon. Similarly, positivity for pSTAT3 was uncommon in the absence of JAK1 expression. While there was statistically significant correlation between staining for IL6 and pSTAT3 there were still a number of samples where pSTAT3 was positive in the absence of IL6. This is consistent with the knowledge that STAT3 can be activated by other signalling pathways or directly by *EGFR*. This study is the only one to consider three parts of the pathway at the same time – IL6, JAK1 and pSTAT3.

Our findings are consistent with the only other groups to publish on this association. Gao et al found that any expression of pSTAT3 was correlated with moderate to strong staining for IL6 (counted as 2+ or 3+ on a scale of 0 to 3+, p=0.001) [545]. In their study of 92 patients with pulmonary adenocarcinoma pSTAT3 was found to be positive in 46 patients, and of these only four patients had positive pSTAT3 in the absence of expression of IL6. Looyenga et al assessed staining for JAK2 and pSTAT3 via the H score method [551]. They considered a score of >100 positive for JAK2 and >40 positive for pSTAT3. In their cohort of 245 patients with NSCLC, 53 were positive for pSTAT3 and all except one of these were also positive for JAK2.

The statistical relationships between expression of IL6, JAK (either JAK1 as in our study or JAK2 in the work of Looyenga et al), and pSTAT3 is consistent with the knowledge that these molecules form a pathway, with upstream activation leading to downstream signalling [545, 551].
4.4.3 Activation of the IL6 / JAK1 / pSTAT3 Pathway and Oncogenic Mutations in EGFR or KRAS

In this study the major aim was to assess the presence of activation of the IL6 / JAK1 / pSTAT3 pathway in clinical samples from patients with pulmonary adenocarcinoma and examine this in relationship to the presence of oncogenic mutations in EGFR and KRAS. We hypothesised that oncogenic EGFR mutations may enrich for IL6 / JAK1 / pSTAT3 staining. Assessment of the pathway was more comprehensive than prior studies of clinical specimens as three components were concurrently assessed. Modest activation of the pathway was found with similar frequencies across patients with EGFR mutation, KRAS mutation and those who were wild type for both oncogenes. We did not find any statistical relationship between staining for IL6, JAK1 or pSTAT3 (by both scoring methods) and the presence of activating mutations in either EGFR or KRAS. EGFR mutation was not an enrichment factor for IL6 / JAK1 / pSTAT3 activation as measured by IHC.

Gao et al was the first to suggest that there was strong association in clinical specimens [545]. Of the patients in their cohort 14 out of 16 with EGFR mutations had positive IHC staining for pSTAT3, as compared with 32 out of 76 with wild type EGFR (p<0.002). Jiang et al also found this association in their patients with adenocarcinoma (EGFR mutation 40 / 49 positive for pSTAT3 as compared with EGFR wild type 28 / 51) [577].

Takata et al did not find an association between pSTAT3 and EGFR mutation, however their rates of positive expression for pSTAT3 were quite high (42 out of 50 patients) [552]. This is potentially related to the underlying anatomical pathology features of the tumours they studied, with almost all being early stage and with a predominant or significant proportion of lepidic growth in the specimens examined.
Looyenga et al did not find a link between $EGFR$ mutation status and positive staining for pSTAT3 [551]. The study also did not find a link between $KRAS$ mutation and staining for pSTAT3 either. Interpretation of these results is somewhat tempered by the mixed histologic subtypes studied (34% of cases enrolled were of non-adenocarcinoma NSCLC subtypes), and the fact that $EGFR$ mutation testing was limited to exon 19 only [551, 579].

Observations and comments from a paper by Song et al are supportive of our findings of a lack of association between $EGFR$ mutations and pSTAT3 staining [546]. Song et al discussed that activation of STAT3 is not a universal finding amongst cell lines that have been found to carrying common activating $EGFR$ mutations. Further, there are many cells lines that do not carry $EGFR$ mutations but still have activation of STAT3. Finally, they took cell lines that normally have wild type $EGFR$ and developed them to stably express $EGFR$ with an exon 19 deletion. In these cell lines no activation of STAT3 was seen despite the presence of an $EGFR$ mutation. It is not known whether the presence or absence of pSTAT3 in patients with $EGFR$ mutant tumour prior to $EGFR$ inhibition effects responses rates or duration of response.

In this current work the relationship between the mutation status for $EGFR$ and $KRAS$ and the state of activation of IL6, JAK1 and pSTAT3 have each been compared, and thus the pathway has been interrogated in clinical specimens in greater detail than has previously been reported by other groups. As noted, no statistical associations have been found between any of these features.

**4.4.4 Prognostic Significance of Expression of IL6, JAK1 and pSTAT3 in Clinical Samples**

As our cohort had detailed survival data available we explored the prognostic relevance of expression of IL6, JAK1 and pSTAT3 in relation to known prognostic factors. We found no prognostic impact for any of these variables when staining was assessed as either
present or absent. Assessment of the presence of JAK1 and pSTAT3 measured by method 1 (any staining counted as positivity) showed that the presence of positive staining was associated with better overall survival in univariate analysis only (along with stage, IASLC/ATS/ERS adenocarcinoma subtype and the presence of lymphovascular invasion). In multivariate analysis the solid predominant adenocarcinoma subtype and increasing stage were the only factors to maintain statistical significance, with presence of either feature being associated with worse prognosis.

This is the first study to examine the prognostic value of IL6 staining in a purely adenocarcinoma cohort. IL6 staining did not have any prognostic impact on survival outcomes in univariate analysis. Koh et al had previously reported that the presence of a moderate level of IL6 staining or higher was strongly associated with worse outcomes, however this finding was made in a small cohort (n = 90) and with mixed histologic subtypes [584]. They reported an effective size for IL6 staining of a greater magnitude than stage (stage I vs stage II and III) by statistical methods, which does not seem intuitive.

This study is also the first to examine the prognostic significance of JAK1 staining following surgical resection in adenocarcinoma. No prior work on this topic in NSCLC is reported. The presence of any JAK1 staining (method 1) was associated with better survival in univariate analysis, however this effect was lost in multivariate analysis. No statistical difference in survival outcomes was observed in univariate analysis when higher levels of activation of JAK1 were assessed by method 2.

Our results are consistent with most prior publications with regards to pSTAT3. Two groups have examined the role of pSTAT3 in prognosis in resected lung adenocarcinoma [573, 578], and four studies in groups with NSCLC [548, 551, 575, 604]. Five papers showed no survival difference by the presence or absence of staining for pSTAT3. Galleges-Ruiz et al suggested that pSTAT3 may be a good prognostic factor using the backwards selection statistical technique on a Cox model [570]. Xu and Lu reported a meta-analysis of survival outcome by pSTAT3 status which included six
studies of patients with NSCLC [605]. They found that the presence of pSTAT3 staining was associated with worse prognosis. These results were heavily weighted by two papers with the largest cohort sizes. Data from one paper was extracted from the Kaplan-Meier curve to estimate a hazard ratio, however the p-value reported by the authors in the original paper did not support statistical significance (log rank p=0.29), and a hazard ratio was not reported [575]. In the second study pSTAT3 was prognostic in univariate analysis but not on subsequent multiple variate analysis [604]. The report of a significant effect on prognosis found in the meta-analysis is difficult to interpret given that tumour stage did not reach statistical significance.

4.4.5 The IL6 / JAK / STAT Pathway Remains a Relevant Target in EGFR Mutated Lung Adenocarcinoma

As previously noted, the presence of an EGFR mutation did not enrich for staining of IL6, JAK1 or pSTAT3. Despite this, a portion of samples from patients with EGFR mutations did show activation of the pathway. IL6 positivity was observed in 35% of EGFR mutant specimens, JAK1 was present in 82% of specimens by method 1 and 70% of specimens by method 2, and pSTAT3 was present in 50% of specimens by method 1 and 18% of specimens by method 2. This activation status is present in the absence of any systemic therapy, and it is important to note that pSTAT3 levels increase on EGFR inhibition as demonstrated in clinical specimens of skin (wild type) and tumour (wild type or EGFR mutant) [580, 581].

Current trials are focused on the addition of a JAK inhibitor following the development of resistance to first generation EGFR inhibitors [606, 607]. Phase I studies of a STAT3 inhibitor OPB-51602 have shown clinical activity with some prolonged responses (the longest being seven months) in patients with prior TKI therapy for EGFR mutated lung cancer [608]. Similarly, a STAT3 antisense oligonucleotide, AZD9150, has shown activity in an EGFR mutant tumour xenograft model and in a patient with highly refractory NSCLC (EGFR mutation status not reported) [609]. Therefore opportunities
exist to test the safety and efficacy of dual blockade of the EGFR pathway and IL6/JAK/pSTAT3 in the clinical setting.

4.5 Conclusion

This work made a detailed assessment of the activation of the inflammatory pathway IL6 / JAK1 / pSTAT3 as assessed by immunohistochemistry in resected pulmonary adenocarcinoma specimens. While there has been much interest in this pathway in preclinical work, there have been limited studies on clinical samples, and often these have been conducted in heterogeneous NSCLC populations rather than histologically defined subgroups. As there is no standard criteria for assessing “positivity”, we chose to use two methods previously used on other papers on this subject area: assessment of any staining as positive [545]; and assessment of positivity at an H-score of >40 (H-score = intensity of staining [scale 0 – 3] multiplied by percentage of cells positive [0 – 100], range 0 – 300) [551].

Expression of IL6 was present in 32% of samples studied regardless of the method used. Gp130, the molecule required for IL6 signal transduction, was nearly universal (96% of samples), and therefore not studied further. The presence of JAK1 was common (78% by method 1 and 65% by method 2). pSTAT3 expression was 44% by method 1, dropping to 22% by method 2, suggesting that in half of the “positive” cases the actual level of pSTAT3 was low. The comprehensive assessment of IL6, JAK1 and pSTAT3 allows comparisons between these markers to assess activation of the pathway as a whole. All but one of the six cross comparisons retained statistical significance after correction for multiple comparisons, consistent with activation of the pathway as a whole in clinical specimens.

The major aim of this study was to assess the rates of activation of IL6, JAK1 and pSTAT3 in comparison to oncogenic mutations in EGFR, given that increased STAT3 activation had been linked to EGFR mutation by some groups. We found that EGFR
mutation did not enrich for activation of IL6, JAK1 or pSTAT3 by either method of IHC assessment when compared to tumours from patients with KRAS mutation or wild type for both mutations. Despite this, a number of patients with EGFR mutations showed activation of the IL6 / JAK1 / pSTAT3 pathway in the absence of any systemic therapy (specifically EGFR inhibition).

As in Chapter 3, the prognostic importance of stage and the solid predominant adenocarcinoma subtype were demonstrated in this cohort. The presence of oncogene mutations did not alter prognosis. The presence of positive staining for JAK1 or pSTAT3, when assessed by method 1 (any staining positive), was associated with better prognosis in univariate analysis, however this effect was lost on multivariate analysis. No consistent clinicopathologic associations were found with staining for IL6, JAK1 or pSTAT3.

The blockade of the IL6 / JAK1 / pSTAT3 pathway remains a relevant potential pathway to target in advanced EGFR mutant lung cancer, despite our findings of lack of enrichment of the pathway in EGFR mutant tumours. Prior preclinical research and the demonstration of changes in pSTAT3 as a result of EGFR inhibition in clinical specimens mean that this may present an opportunity to extend the response to first line EGFR inhibition.
CHAPTER 5

CONCLUDING REMARKS
5.1 Concluding Remarks

Lung cancer remains the most common cause of cancer related mortality in Australia and around the world, with poor 5-year overall survival outcomes [1-3]. The cornerstone of management remains detection at the earliest possible stage, accurate clinical and pathologic assessment, and treatment in accordance with best evidence available. This thesis contributes to the understanding of one of the most common forms of lung cancer, pulmonary adenocarcinoma. A detailed assessment of pathologic features according to the IASLC / ATS / ERS classification of pulmonary adenocarcinoma (incorporated in the 4th edition of the WHO Classification of Tumours of the Lung, Pleura, Thymus and Heart) has been performed in patients with early stage resected disease and patients with advanced metastatic pulmonary adenocarcinoma. Correlations are examined between clinical features and oncogenic mutations in EGFR and KRAS, as well as an assessment of the IL6 / JAK1 / pSTAT3 pathway in resected pulmonary adenocarcinoma [44, 179].

5.2 Major Findings

5.2.1 Pulmonary Adenocarcinoma Subtyping in Resected and Advanced Disease

For the first time this thesis outlines a detailed pathologic assessment of patterns of pulmonary adenocarcinoma at purely metastatic sites according to a research question directly posed in the IASLC / ATS / ERS classification manuscript [179]. A number of new clinicopathologic findings were made. Chief amongst these was the finding that in patients who received systemic therapy (cytotoxic chemotherapy), the major solid pattern was associated with the worse survival outcomes with similar and longer survival seen in patients with major acinar or major micropapillary patterns. If replicated this major discovery has implications for discussions about survival times and prognosis with patients. Other studies support the findings of differential response to treatment by adenocarcinoma pattern including:
• Differences in the benefit of adjuvant chemotherapy following surgical resection by predominant adenocarcinoma subtype as seen in the LACE-Bio analysis [303]

• The finding that patients with solid predominant adenocarcinoma may derive greater benefit from adjuvant postoperative radiotherapy [249]

• Lower response rates and shorter PFS on EGFR TKIs for patients for patients with EGFR mutant lung cancer at the time of recurrence if the prior pattern at surgical resection was solid predominant as compared with others [309]

To date there is one other contemporaneous publication has been made on subtyping in metastatic disease [296]. Differences in survival were observed with better survival outcomes for ‘high-grade’ tumours (papillary, micropapillary and solid). It is probable that the differences in findings are related to significant methodological differences between their study and this current study.

In the metastatic patient cohort significant morphologic heterogeneity of pulmonary adenocarcinoma subtypes was seen. This is in keeping with the known morphologic heterogeneity of adenocarcinoma found in resected primary tumours. Two interesting observations arose about metastatic patterns at distant sites. Firstly, the major solid pattern was the most common seen, accounting for 50% of the samples assessed, followed by the acinar pattern (29%), MPA (20%) and the papillary pattern (1%). These findings are congruent with work from other groups examining patterns metastasising from the primary tumour to nodal or distant metastatic sites as compared to the predominant pattern of the tumour [278, 280, 610]. The second novel finding of this thesis is the preservation of the major pattern across several metastatic sites in the same patient. Sixteen patients in the cohort had two or more surgical specimens available for histologic review. In all cases the relative portions of adenocarcinoma patterns seen may have varied, but the major adenocarcinoma pattern remained consistent. It was also observed that the major solid pattern was less common in patients with a never smoking history compared to patients with acinar or MPA major pattern tumours.
In both the metastatic and early stage resected cohorts the frequency of oncogene driver mutations was examined for \textit{EGFR} and \textit{KRAS} mutations. The associations with these mutations and smoking history were statistically significant as expected. In both cohorts the predominant (early stage) or major (metastatic) solid pattern was statistically significantly less likely to have an \textit{EGFR} mutation. For patients in the resection cohort, this finding was consistent with low rates of \textit{EGFR} mutation for solid predominant patients reported in other non-Asian cohorts \cite{274, 278, 305, 306}. However, \textit{EGFR} mutations are still observed in predominant/major solid pattern tumours and as such the histologic subtype of adenocarcinoma seen should not exclude a sample from being tested for oncogenic mutations of clinical relevance.

No interactions were observed between the pathologic subtype (predominant or major as appropriate to each study) and mutation status for \textit{EGFR} or \textit{KRAS} with regards to survival outcomes. If such an effect were to exist a larger cohort of patients would be required to allow sufficient statistical power to answer the question with confidence.

5.2.2 The IL6 / JAK1 / pSTAT3 pathway and resected pulmonary adenocarcinoma

A detailed assessment of the inflammatory signalling pathway linking IL6, JAK1 and pSTAT3 was undertaken in resected pulmonary adenocarcinoma. This work adds to the significant volume of prior cell line and xenograft derived data, and adds knowledge to the limited prior studies that have been performed in clinical samples. This work was unique in that it studied multiple components of the pathway in the same specimens, and used a homogeneous population of samples from pulmonary adenocarcinoma (rather than a mixed NSCLC group). The lack of a standardised definition of IHC positivity for IL6, JAK1 and pSTAT3 led to use two methods to assess clinicopathologic outcomes in this thesis – the presence of any IHC staining as being positive \cite{545}; or the presence of staining at an H-score of >40, where the H-score is defined as the intensity of staining on a scale of 0 to 3 multiplied by the portion of cells staining positively (0 – 100%) to give a range of 0 to 300 \cite{551}.
The study particularly aimed to assess whether the presence of an activating mutation in *EGFR* enriched for staining of IL6, JAK1 or pSTAT3 as has been suggested in other works. The interest in the intersection from these two factors arose from the suggestion that upregulation of pSTAT3 may contribute to resistance to *EGFR* inhibition in advanced disease. We found that the portion of samples deemed positive for IL6, JAK1 and pSTAT3 was similar across patients with *EGFR* mutations, *KRAS* mutations or wild type for both. This was true whether either of the two cut-off points for defining IHC positivity were used. While *EGFR* mutations did not enrich for inflammatory signalling via this pathway, there were still a number of patients who had positive inflammatory signalling in the presence of an *EGFR* mutation.

It was demonstrated that there were statistically significant relationships between IL6, JAK1 and pSTAT3 via either method of staining used to assess positivity, consistent with activation of the pathway. Some patients could be positive for pSTAT3 in the absence of IL6 or JAK1 staining, consistent with the knowledge that phosphorylation of STAT3 may occur through more than one signalling pathway.

Clinical annotation of the specimens also allowed for assessment of survival outcomes and clinicopathologic correlations. Staining for IL6, JAK1 and pSTAT3 had no prognostic bearing on overall survival outcomes in multivariate analysis. The presence of positive staining did not have any strong associations with other clinical or pathologic factors. No clinicopathologic feature was statistically associated with positive staining for each of IL6, JAK1 and pSTAT3 by the same variable.
5.3 Future Directions

5.3.1 Adenocarcinoma Classification in Metastatic Disease

The IASLC / ATS / ERS Classification of pulmonary adenocarcinoma arose from astute clinical and pathologic observations in the setting of resected primary lung adenocarcinoma [179]. Multiple papers supporting the prognostic utility of classification lead to its inclusion in the 4th Edition of the WHO classification [44]. This work is one of the first explorations of pulmonary adenocarcinoma subtypes at distant metastatic sites. The available sample size for assessment was modest at 100 patients. The findings in this work require replication by other groups to determine their generalisability. A potential way to eliminate bias would be to perform a prospective cohort study where sizeable tumour specimens are obtained at diagnosis, and all patients are treated with the same systemic therapy. Alternatively, validation of our results would be possible in the setting of a randomised clinical trial in the first line treatment of advanced pulmonary adenocarcinoma in which samples are taken as part of a translational component. This will allow for reduction of some of the inherent biases which can occur in a retrospective study such as ours.

Understanding whether our findings extend to samples obtained by smaller biopsies at metastatic sites and from samples taken from primary tumours in the setting of metastatic disease will also be important. We deliberately targeted surgical sampling of metastatic disease at distant sites to allow sufficient tissue for examination, with preservation of architectural patterns, and representative of the metastasising patterns rather than what was found in the primary tumour. The majority of samples obtained in routine care are either small, acquired from the lung, or of a nature such as a cytology sample where architecture cannot be described. If subtyping at metastatic sites is prognostically relevant, assessment of the utility of subtype determination in samples that are more reflective of every day practice is of importance.
5.3.2 Adenocarcinoma Subtyping – Bedside to Bench

It is becoming clear that the predominant or major pathologic subtype of pulmonary adenocarcinoma is predictive for response to systemic therapy. In the metastatic setting I have shown that the major solid pattern has a worse survival compared to others on cytotoxic therapy, and Yoshida et al have shown that advanced EGFR mutant disease has lower response rates and duration of response in the setting of the solid pattern compared to patients with non-solid tumours [309]. Tsao et al have shown that predominant subtype predicts benefit (for solid or MPA tumours) or lack of benefit (acinar and papillary tumours) from adjuvant chemotherapy [303]. The effects of subtype on response and duration of response to immunotherapy remains to be studied and will be of great interest.

The prognostic effects of adenocarcinoma subtype on survival, and the predictive effects on response to therapy open a new pathway for translational research from bedside back to bench. There will be factors inherent to the tumour, whether environmental or genetic (or both), that drive differentiation down a pathway to a particular adenocarcinoma phenotype. These factors may also determine response to therapy, or they may unlock novel ways to target and treat lung adenocarcinoma systemically beyond those modalities that are already available.

5.3.3 IL6 / JAK1 / pSTAT3 and EGFR mutant lung adenocarcinoma

In this thesis assessment of the IL6 / JAK1 / pSTAT3 pathway had hoped to find enrichment of expression in the setting of EGFR mutant lung adenocarcinoma, a finding that wasn’t able to be made. However, a portion of patients with EGFR mutant lung adenocarcinoma had expression of this pathway in the absence of the effects of systemic therapy. Demonstration of the changes in this pathway in response to EGFR inhibition over time would be useful in understanding if the changes seen in cell line and xenograft studies are replicated in clinical behaviour. Finding agents that can be safely and conveniently combined with EGFR inhibition to block this pathway may allow for extension of time on a line of therapy that is otherwise highly effective and well tolerated,
and may reduce the small portion of patients who have de novo resistance to such therapies.

**Conclusion**

This thesis adds to the growing body of knowledge about the pathology of lung adenocarcinoma, and its impact on clinical outcomes.

Several new and novel findings in the setting of metastatic disease have been made, requiring validation in other cohorts. A contribution to the understanding of the relationship between *EGFR* and *KRAS* mutations and pathologic subtyping in early stage disease has been made. A detailed assessment of the IL6 / JAK1 / pSTAT3 pathway in clinical adenocarcinoma specimens has been made for the first time.

Ongoing clinical and translational studies in lung adenocarcinoma remain vital given the frequency of this disease and the morbidity and mortality that it causes. Anything that can be done to improve outcomes will be welcomed by patients, their families and the clinicians who assess and treat them on a day to day basis.
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