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Clinicopathological and Inflammatory Prognostic Factors in Non-Small Cell Lung Cancer

A thesis presented for the degree of

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at

Trinity College Dublin

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Declaration

I hereby declare that this thesis is the work of my own hand. It is a record of work planned and carried out by myself excepting those instances specifically referred to and gratefully acknowledged. All previous work has been fully accredited and referenced. This work was carried in Dublin between 2005 and 2009.

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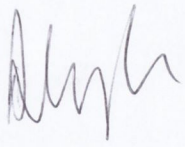

Dermot Stephen O'Callaghan

Dublin

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Summary

Lung cancer is the most common cause of cancer death worldwide. Despite improvements in diagnosis and treatments, the overall prognosis for patients with lung cancer remains poor and novel therapeutic approaches are required. Molecular assessments of tumour material and blood from non-small cell lung cancer (NSCLC) patients may help identify different prognostic and predictive subgroups. Recent publications suggest that biomarker profiles constructed from patient series with long and accurate follow-up outperform standard pathologic TNM staging in estimating risk of disease recurrence. The impact of neoadjuvant treatments on host immune response remains poorly characterised.

A review of all operative cases of lung cancer at St. James's Hospital was first performed to identify the clinicopathological features that conferred a survival advantage in patients with surgicopathological-confirmed (*p*)-stages I to IIIA NSCLC. There were 566 patients operated on from 1998 - 2008 (median age 65 years, 62% males). Stage distribution was: IA: 19.2%; stage IB: 34.8%; stage IIA: 4.4%; stage IIB: 20.8%; stage IIIA: 20.8%). Distribution of histological subtypes was: squamous cell, 51.2%; adenocarcinoma, 40.9%; mixed cellularity carcinomas, 4.2%; and the large cell neuroendocrine variant, 3.0%. Lobectomies were performed in 81.7% of cases, while pneumonectomy was necessary in 18.3% of patients. Sleeve resections were undertaken in 7% of cases. Median overall survival in our population was 37 months, comparable to that of other international thoracic oncology centres. Clinicopathological characteristics in our population associated with improved survival by univariate analysis included: younger age at time of surgery, female sex, smaller tumour size, history of smoking, surgeries other than pneumonectomy, clear resection margins, lower T and N stages and overall pathological stage. Age, gender, smoking status, tumour size and pathological stage emerged as independent prognostic factors by multivariate analysis.

The local adaptive immune response was analysed in 196 NSCLC cases with stage I-IIIA disease from the initial study population. Tumour- and stroma-

infiltrating $CD3^+$, $CD8^+$ and $Foxp3^+$ cells were identified by immunohistochemistry in order to assess the relative proportions of total, cytotoxic and regulatory T-lymphocytes (Tregs), respectively. Enumeration of immune subsets was performed using a novel automated image analysis algorithm which was compared to the traditional method of manual counting. Patients were divided into two groups, based on the median value of the ratio of intratumoral to intrastromal lymphocyte count. The correlation of each lymphocyte subtype with survival was assessed using the log-rank statistic. High $CD3^+$ lymphocyte tumour islet/stroma (TI/S) infiltration ratio conferred a prognostic advantage ($P=0.03$). Patients with a high $CD8^+$ lymphocyte TI/S infiltration ratio also had improved survival compared to those with a low TI/S ratio ($P<0.001$). Conversely, there was an inverse association between survival and TI/S $Foxp3^+$ Treg density ($P<0.001$). By multivariate analysis, TI/S ratio of $Foxp3^+$ (hazard ratio (HR) = 4.73, 95% confidence intervals (CI) 2.86 to 7.85, $P<0.001$) and $CD8^+$ (HR=0.40, 95% CI 0.25 to 0.63, $P<0.001$) emerged as independent predictors of survival.

Circulating lymphocyte cytokine mRNA levels from 21 patients were measured using quantitative polymerase chain reaction (Q-PCR) to assess the impact of neoadjuvant combination chemotherapy on the inflammatory profile in NSCLC. Changes in levels of INF-γ, TGF-β, IL-10 in response to induction treatment with cisplatin, gemcitabine and cetuximab were observed. However, there was no correlation between cytokine expression and response to treatment as assessed by CT imaging.

These results confirm the critical role played by the host immune system in outcome for lung cancer patients. Microlocalization of infiltrating T-lymphocytes is a powerful predictor of outcome in surgically resected NSCLC. Assessment of regional lymphocyte distribution could be used to identify poor prognostic subgroups that may benefit from additional therapy. Future immunotherapeutic strategies capable of downregulating intratumoral immunosuppressive responses and enhancing effector cytotoxic activity may be worthy of pursuit. Circulating lymphocyte cytokine mRNA expression does not appear to help predict which patients will respond to neoadjuvant combination chemotherapy.

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Abbreviations used in the text

ABC	avidin-biotin peroxidase
AFE	Automated Feature Extraction
AIDS	Acquired Immunodeficiency Syndrome
AJCC	American Joint Committee on Cancer
Ang	angiopoietin
CD	cluster of differentiation
cDNA	complimentary deoxyribonucleic acid
CI	confidence interval
CIBD	chronic inflammatory bowel disease
CIN	cervical intraepithelial neoplasia
cm	centimetre
CMI	cell mediated immunity
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
CRC	colorectal carcinoma
CRP	C-reactive protein
CT	computer tomography
C_T	Crossing threshold
CTLA	cytotoxic lymphocyte-associated antigen
° C	degree centigrade
DAB	3,3'-diaminobenzidine tetrahydrochloride
DNA	deoxyribonucleic acid
dATP	deoxyadenosine Triphosphate
dCTP	deoxycytidine Triphosphate
DTT	dithiothreitol
dGTP	deoxyguanosine Triphosphate
dNTP	deoxyribonucleotide triphosphate
dTTP	deoxythymidine Triphosphate
DPX	distyrene/tricresyl phosphate/xylene
DSS	digital slide scanning

EBV	Epstein Barr virus
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ER	oestrogen receptor
ETS	environmental tobacco smoke
FAM	carboxyfluorescein
FasL	fas ligand
FEV_1	forced expiratory volume in one second
FISH	fluorescence in situ hybridisation
Foxp3	Forkhead box P3
FRET	fluorescence resonance energy transfer
g	gram
GENIE	GENetic Imagery Exploitation
H & E	haematoxylin and Eosin
HBV	hepatitis B virus
HCl	hydrochloric acid
HCV	hepatitis C virus
HHV	human herpes virus
HI	humoral immunity
HIF	hypoxia-inducible factors
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPF	high powered field
HPV	human papilloma virus
HR	hazard ratio
IASLC	International Association for the Study of Lung Cancer
I-ELCAP	International Early Lung Cancer Action Program
IHC	immunohistochemistry
IL	interleukin
IFN	interferon
IPF	idiopathic pulmonary fibrosis
L	litre
KCl	potassium chloride

LAD	linear array device
mg	milligram
MIF	macrophage migration inhibitory factor
MHC	major histocompatibility complex
mL	millilitre
mm	millimetre
mM	millimolar
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
µl	microlitre
NaCl	sodium chloride
NF-κB	nuclear factor-κB
nM	nanomolar
NK	natural killer
NSCLC	non-small cell lung cancer
NPCR	National Program of Cancer Registries
NSCLC	non-small cell lung cancer
PET	positron emission tomography
PBMC	peripheral blood mononuclear cell
PBS	phosphate buttered saline
PCR	polymerase chain reaction
PBMC	peripheral blood mononuclear cell
PR	progesterone receptor
Q-PCR	quantitative real-time polymerase chain reaction
R^2	correlation coefficient
ROS	reactive oxygen species
RNOS	reactive nitrogen oxide species
RPM	revolutions per minute
SCLC	small cell lung cancer
SD	standard deviation
SEER	Surveillance, Epidemiology and End Results
SI	International System (of Units)
SVS	ScanScope virtual slide

TAM	tumour associate macrophage
TAMRA	tetramethyl-6-Carboxyrhodamine
TCR	T-cell receptor
TE	tris-EDTA
TGF	transforming growth factor
T_H	helper T-lymphocyte
TI/S	tumour islet/stromal
TiBALT	tumour-induced bronchus-associated lymphoid tissue
TIL	tumour infiltrating lymphocyte
TKI	tyrosine kinase inhibitor
TNF	tumour necrosis factor
TNM	Tumour, Node and Metastasis
TRAIL	TNF-related apoptosis-inducing ligand
UICC	International Union Against Cancer
UK	United Kingdom
UNG	uracil N-glycosylase
USA	United States of America
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 NON-SMALL CELL LUNG CANCER

1.1.1 The epidemiology of lung cancer

Lung cancer is the most commonly occurring form of cancer in most western countries and is the leading cancer-related cause of death (Parkin et al., 2005). Approximately 1.35 million people are diagnosed with lung cancer worldwide each year and the incidence is steadily rising (Youlden et al., 2008). From 1985 to 2005, the number of cases diagnosed annually increased by more than 50% globally. There are nearly 1.2 million lung cancer related deaths every year, accounting for 29% of total cancer-related deaths (Parkin et al., 2005).

In the United States and parts of Europe, lung cancer is responsible for as many deaths in men as the next three most common cancers (prostate, colorectal and stomach) combined (Jemal et al., 2006). Although incidence rates in women throughout the world are lower, lung is now the third commonest cancer site after breast and cervix. In certain regions lung cancer has overtaken breast cancer as the commonest cause of cancer-associated mortality among women (Patel et al., 2004).

Lung cancer can occur at any age but is most commonly diagnosed in late middle age and in the elderly. There is significant geographical variation in the incidence and mortality rates of the disease, with highest rates reported in industrialized nations. Whereas a peak in incidence appears to have been observed in Western and Northern Europe, rates continue to increase in Southern and Eastern Europe with overall age-adjusted incidence in males of more than 100/100,000 cases per year in some countries (Ferlay et al., 2007, Janssen-Heijnen and Coebergh, 2003).

1.1.2 Risk factors for the development of lung cancer

In order for bronchial cells to acquire a malignant phenotype, disruption of intranuclear genetic material and cell regulatory systems must occur. The triggers for this process may be genetic or take the form of environmental insults as a result of ionizing radiation or viral, chemical, or physical triggers or a combination thereof.

1.1.2.1 Cigarette smoking

Prior to the dramatic increase in the prevalence of cigarette smoking, lung cancer was an extremely rare disease. It is noteworthy that in 1912 there were only 374 documented cases in the worldwide medical literature (Adler, 1912). Convincing evidence for the causal association between tobacco smoke inhalation and lung cancer was first demonstrated in a landmark epidemiologic study published by British researchers (Doll and Hill, 1950). This led to the U.S. Surgeon General issuing a report in 1964 that highlighted the link between smoking and lung cancer and emphasized the deleterious health consequences of tobacco use in general.

Smoking is now recognized as the principal etiological factor in the majority of cases; in developed countries, smoking is estimated to cause approximately 90% of lung cancer deaths (Tyczynski et al., 2003). Thus, lung cancer is an almost entirely preventable disease. The indisputable role of smoking in the pathogenesis of lung cancer is among the most extensively studied causal relationships in biomolecular research. Each of the histopathological variants shows an association with smoking, with the link strongest between tobacco exposure and the squamous and small cell subtypes (Alberg et al., 2007).

The dramatic increase in the rates of lung cancer during the latter half of the last century closely mirrors patterns of tobacco consumption, with a lag time of approximately two decades. There has also been a notable decrease in smoking prevalence among men in many industrialized countries. This has had a significant impact on the numbers of lung cancers diagnosed since at least the mid-1990s, implying that trends in tobacco exposure in young men have been favourable since at least the early 1980s (Didkowska et al., 2005). By contrast, there has been a consistent rise in the numbers of new cases in women, reflecting an increase in smoking prevalence among women worldwide. Worryingly, even though the peak of lung cancer appears to have passed in males in the United States and parts of Europe in line with changing smoking trends, data indicate that no such peak has been reached in females (Devesa et al., 2005).

1.1.2.2 Environmental tobacco smoke

Environmental tobacco smoke (ETS), also referred to as 'passive' or 'involuntary' smoking, has been extensively investigated as a potential cause of lung cancer. Data from the European Prospective Investigation into Cancer and Nutrition study on ETS estimate the proportion of lung cancer in never- and ex-smokers attributable to ETS to be between 16 and 24%, mainly due to the contribution of work-related exposure (Vineis et al., 2007).

Much of epidemiologic research on ETS has examined the relative risk increase among non-smoking women according to level of smoking by their husbands. Evidence of an elevated risk among non-smoking wives of smoking husbands when compared with non-smoking wives of non-smoking husbands was first published nearly 30 years ago (Hirayama, 1981). Prolonged exposure to ETS in the workplace also confers increased risk (Reynolds, 1999) and forms the basis for smoking-ban legislation in a number of countries.

1.1.2.3 Non-smoking related factors

Tobacco use is implicated as causative in most cases of lung cancers. However, approximately 10% of cases occur in people with no or minimal smoking history. Therefore, although the proportion of lung cancer unrelated to tobacco smoke is small, the absolute number of patients affected is significant. Numerous other environmental and occupational exposures are known or suspected to contribute to the development of lung cancer. Genetic factors have also been implicated as aetiologically relevant when the disease develops in those without significant tobacco smoke exposure (Gorlova et al., 2007).

Data from investigations of occupational groups (often with prolonged exposure to physical or chemical agents at the workplace) estimate that more than 10,000 men and women in the US develop cancer due to exposure to occupational carcinogens (Steenland et al., 1996). Furthermore, there is clear evidence that many carcinogens act synergistically with cigarette smoking to further augment risk.

One of the most important naturally occurring lung carcinogens is radon, a ubiquitous inert radioactive gas. Produced in the decay series of uranium, high

levels of radon have long been linked to increased lung cancer risk. Underground miners exposed to elevated radon levels have particularly high rates of the disease. Radon present in indoor air may be responsible for 1% of all lung cancers and account for 2000 cases per year throughout Europe (Boffetta and Nyberg, 2003).

The link between asbestos and lung cancer was first made over 70 years ago (Wood and Gloyne, 1934). This association was strengthened by epidemiologic data from the 1950s showing that textile workers in Britain exposed to high levels of asbestos dust had a 10-fold increased incidence (Doll, 1955). The risk conferred by asbestos exposure is increased in a multiplicative manner in smokers, due to a synergistic effect with several carcinogens found in cigarette smoke. Other proven or suspected occupational risk factors include nickel, chromates, beryllium, arsenic, chloromethyl ethers and silica (Coultas and Samet, 1992).

Both indoor and outdoor pollution have been implicated as aetiologically relevant in the development of lung cancer. Inhabitants of urbanized areas appear to be at increased risk, although there are considerable difficulties in obtaining reliable estimates of exposures. Inhalation of atmospheric contaminants may account for approximately 1 to 2% of total cases (Cohen et al., 2005). The widespread use of solid fuels such as unprocessed coal for cooking and domestic heating is believed to account for a significant proportion of cases among non-smokers, particularly in developing countries.

1.1.3 Histopathological subtypes of lung cancer

In 2004, the World Health Organization (WHO) introduced an updated classification system of lung cancer (Travis et al., 2004). Four main cell types are recognised on the basis of light microscopy appearance: squamous cell carcinoma, adenocarcinoma, large cell (or undifferentiated) carcinoma and small cell carcinoma. Each of these histopathological entities arises from transformed bronchial epithelium. As squamous cell, large cell and adenocarcinoma demonstrate overlapping clinical characteristics and anticipated treatment responses, they are grouped together under the umbrella term non-small cell lung cancer (NSCLC).

Overall, NSCLC accounts for approximately 80% of all lung cancers, whereas small cell carcinoma (SCLC) accounts for approximately 15% of cases (Travis et al., 1995). Less common histological subtypes include carcinoid tumours and carcinomas with pleiomorphic, sarcomatoid or sarcomatous elements. The relative proportion of each of the four main histological types shows considerable variation between different countries. In Northern America for example, adenocarcinoma has become the most prevalent type seen whereas squamous cell carcinoma is more common in Europe. The biological reasons for this regional variation are incompletely understood and are likely multifactorial but in part may represent an effect of different methods and rates of tissue diagnosis.

It has been hypothesised that changing smoking patterns, resulting in relatively increased deposition of carcinogenic particulate matter to peripheral regions of the bronchial tree, may account for some of the relative increase in the numbers of adenocarcinomas diagnosed, as this subtype of cancer most often arises in the peripheral airways and alveolar tissue (Hoffmann and Hoffmann, 1997). However, among adult never-smokers, adenocarcinoma is the most common histopathological variant of lung cancer observed (Subramanian et al., 2007).

There is a lack of standardization of histological grading in lung cancer. A four-tiered classification system to describe NSCLC as well-differentiated, moderately differentiated, poorly differentiated, and undifferentiated has been proposed, with final reported grade reflecting the least differentiated component. Determination of histologic grade also incorporates assessment of other pathological features, such as specific growth patterns, cytologic atypia, and mitotic rates. However, no specific criteria have been developed for a universal assessment and there is no 'gold standard' method (Travis et al., 2004). This lack of standardization has likely contributed, at least in part, to the discrepancy between different studies reporting the prognostic relevance of tumour grade (Sun et al., 2006, Ichinose et al., 1995),.

1.1.4 Staging of non-small cell lung cancer

1.1.4.1 Current TNM classification system

Patients with NSCLC are currently staged according to the sixth edition of the international TNM classification system (Table 1.1) on the basis of primary tumour characteristics (T), the presence or absence of regional lymph node involvement (N) and the presence or absence of distant metastases (M). The TNM system is a powerful prognostic tool that correlates strongly with outcome and is used to determine the most appropriate therapeutic strategy. This classification was published in 1997 by Clifton Mountain, a cardiothoracic surgeon in Houston Texas, and is based on data from a relatively small U.S. database of 5319 surgical patients (Mountain, 1997).

1.1.4.2 Proposed updates to the lung cancer staging system

Recently, a revision of the current recommendations for the stage classification has been proposed, since the 1997 edition was based on a selected patient population treated as a single institution. The various descriptors had not undergone rigorous validation and the patients studies all emanated from a single geographic region. As a result, the International Association for the Study of Lung Cancer (IASLC) established a committee in 1998 with the specific aim of updating the TNM classification of lung cancer. This objective was to be accomplished by the collection of data worldwide from lung cancer patients. Information from nearly 68,000 NSCLC patients who fulfilled inclusion criteria was studied and cases of NSCLC treated with all modalities of care between 1990 and 2000 were entered into a single database. Following analysis and validation of the database, several recommendations were made by the revision committee (Goldstraw et al., 2007).

Findings that could be used to refine the definitions of the T component in order to more clearly delineate prognosis and survival by T category were observed. Tumour size was confirmed as a crucial determinant of outcome and led the IASLC committee to propose subclassification of T1 tumours into T1a ($\leq$2 cm) and T1b (>2 to $\leq$3 cm) and T2 tumours into T2a (>3 to $\leq$5 cm) and T2b (>5 to $\leq$7 cm). It was also suggested that T2 tumours larger than 7cm be reclassified as T3 and that T4 disease due to malignant pleural effusion be moved to M1 classification. An additional recommendation was that satellite lesions in the same lobe as the primary

Primary Tumour (T)

TX The primary tumour cannot be assessed, or there are malignant cells in the sputum or bronchoalveolar lavage but not seen on imaging or bronchoscopy.

Tis Carcinoma *in situ*

T0 No evidence of primary tumour

T1 Tumour that is 3 cm or less in its greatest dimension, does not invade the visceral pleura, and is without bronchoscopic evidence of invasion more proximal than a lobar bronchus

T2 Tumour that has any of the following features:

- Size more than 3 cm in its greatest dimension
- Involvement of a mainstem bronchus, with a proximal extent at least 2 cm away from the carina
- Invasion of the visceral pleura
- Association with atelectasis or obstructive pneumonitis that extends to the hilar region, but does not involve the entire lung

T3 Tumour of any size with any of the following features:

- Invasion of the chest wall (including superior sulcus tumours), diaphragm, mediastinal pleura, or parietal pericardium
- Involvement of a mainstem bronchus within 2 cm of the carina, but without invasion of the carina
- Association with atelectasis or obstructive pneumonitis of the entire lung

T4 Tumour of any size with any of the following features:

- Invasion of the mediastinum, heart, great vessels, trachea, oesophagus, vertebral body, or carina
- Association with a malignant pleural or pericardial effusion
- Satellite tumour nodule(s) within the same lobe of lung that contains the primary tumour

Table 1.1 Staging of non-small cell lung cancer (TNM classification).
(From: Mountain, Chest, 1997;111:1710-17.)

Lymph Nodes (N)

N0	No regional lymph node involvement
N1	Involvement of ipsilateral intrapulmonary, peribronchial, or hilar lymph nodes
N2	Involvement of ipsilateral mediastinal or subcarinal lymph nodes
N3	Involvement of contralateral mediastinal or hilar lymph nodes. Alternatively, involvement of either ipsilateral or contralateral scalene or supraclavicular lymph nodes

Distant Metastasis (M)

M0	No distant metastasis
M1	Distant metastasis or pulmonary nodules in separate lobe from primary

OVERALL STAGE

Grouping	TNM Staging
Occult carcinoma	TX N0 M0
Stage 0	Tis N0 M0
Stage IA	T1 N0 M0
Stage IB	T2 N0 M0
Stage IIA	T1 N1 M0
Stage IIB	T2 N1 M0
	T3 N0 M0
Stage IIIA	T1 N2 M0
	T2 N2 M0
	T3 N1 M0
	T3 N2 M0
Stage IIIB	Any T N3 M0
	T4 Any N M0
Stage IV	Any T Any N M1

Table 1.1 (contd.) Staging of non-small cell lung cancer (TNM classification) (From: Mountain, *Chest,* 1997;111:1710-17.)

tumour, currently designated as T4 (stage IIIB) be changed to a T3 designation, reflecting the improved prognosis of this population compared to stage IIIB patients overall. For similar reasons, nodule(s) in a different lobe in the ipsilateral lung would become T4 instead of M1. These various designations were validated for each of the histopathological subtypes (Rami-Porta et al., 2007).

As the current staging system for lymph node involvement was validated from the IASLC database, it was recommended that the N descriptors be maintained as they currently exist. The committee however recommended changing the current M staging system into M1a and MIb subgroups, with M1a reflecting patients with metastatic disease confined to nodules in the contralateral lung and M1b indicating extrapulmonary tumour spread.

It is expected that the various changes proposed will be accepted by the International Union Against Cancer (UICC) and the American Joint Committee on Cancer (AJCC) without revision and incorporated into the seventh edition of the TNM staging system.

1.1.5 Natural history of lung cancer

The generally accepted natural history model of lung cancer assumes a direct progression from a cluster of malignant cells, through small tumours (that are generally not clinically apparent but may be detectable by screening techniques), to large tumours that produce symptoms, to death (Geddes, 1979). This had led to the evaluation of population-based screening strategies, mostly using a variety of radiological techniques, to detect pre-symptomatic, early stage lung cancer. However, results from a number of studies published over the past four decades investigating the usefulness of serial plain chest radiography have been disappointing (Kubik and Polak, 1986, Fontana et al., 1986). Encouraging projected survival rates have also been reported with screening of at risk populations using computer tomography (CT) in the International Early Lung Cancer Action Program (I-ELCAP) study (Henschke et al., 2006), although this was a non-randomised trial that had no control arm. A number of large phase III randomised controlled studies are already underway that should help establish whether this intervention can impact on lung cancer mortality.

The aim of intervention in those with early stage disease is to prevent progression to advanced disease. In the case of NSCLC, this most commonly involves surgical resection in suitable patients. Interestingly, data from recent screening studies suggest that not all patients with pre-advanced lung cancer have disease that will inevitably progress to advanced (i.e. incurable) disease with time as had been widely assumed. This had led some to challenge the currently accepted natural history model (Bach, 2008).

1.1.6 Clinical management of NSCLC

1.1.6.1 Clinical features

Most patients with lung cancer initially present with symptoms, although approximately 5% of lung cancers are discovered incidentally. Symptoms may be due to the primary tumour (e.g. haemoptysis or unexplained cough), regional invasion (e.g. chest wall pain) or distant metastatic spread (e.g. unexplained bone pain or new neurological symptoms).

1.1.6.2 Diagnostic investigation

Clinical investigation of suspected lung cancer has two principal aims: to obtain tissue samples to confirm diagnosis and to determine the extent of disease dissemination (*i.e.* stage) in order to plan an appropriate therapeutic strategy. In patients being considered for radical treatment (e.g. lung resection or chemoradiotherapy), thorough assessment is warranted to exclude the presence of metastatic disease.

Tissue diagnosis and information on staging is usually obtained by flexible bronchoscopy. To complete staging, CT scanning of thorax and upper abdomen is performed as a minimum, complimented by additional diagnostic modalities where necessary. Approximately 75% of patients who present with lung cancer will have unresectable disease, either due to detection of bulky locally advanced or metastatic disease or adverse patient factors such as impairment of lung function or other comorbidities (Vrdoljak et al., 1994).

1.1.6.3 Therapeutic approaches in non-small cell lung cancer

The treatment of the patient with NSCLC is dependent on the final clinicopathological stage and patient status. For stage I and II disease, surgical resection with systematic mediastinal lymph node dissection at time of surgery is the treatment of choice (Scott et al., 2007). In those with excessive surgical risk, stereotactic radiotherapy or radiofrequency ablation are emerging as potential therapeutic options for node-negative tumors.

Most experts advocate combination chemoradiotherapy for propsectively recognised stage IIIA NSCLC in suitable patients (Robinson et al., 2007). Routine adjuvant chemotherapy has been recommended after complete resection of stage IIIA lung cancer encountered unexpectedly at surgery. The optimal treatment for pre-operatively confirmed stage IIIA disease and minimal N2 involvement remains controversial though neoadjuvant treatment has been advocated by some (Spira and Ettinger, 2004).

There is now compelling data indicating that adjuvant chemotherapy improves outcome in stage II and IIIA disease (Arriagada et al., 2004, Winton et al., 2005). A series of randomized studies have shown both overall- and disease-free survival advantages using a platinum-based combination of agents in this setting. However, the benefit of chemotherapy for fully-resected stage I patients remains unproven.

Patients with locally advanced inoperable thoracic disease and adequate performance status should be considered for combination chemotherapy and radiotherapy. For those with advanced local disease or lung cancer with distant metastasis, systemic chemotherapy may be appropriate (Socinski et al., 2007, Jett et al., 2007). There is now ample evidence that treatment with palliative chemotherapy using a platinum-based doublet regimen is superior to best supportive care in those without significant weight-loss or co-morbidity (Stinchcombe and Socinski, 2009). This strategy has been shown to offer meaningful improvements in quality of life and modestly increased median survival.

Recently, novel targeted therapies such as erlotinib, bevacizumab and cetuximab have emerged as promising additions to the therapeutic armamentarium for advanced lung cancer (Kelly and Huang, 2008). The role of these agents in the adjuvant, neoadjuvant and palliative setting has been the focus of a number of studies with others ongoing or planned. Although their precise role in NSCLC has yet to be clearly defined in many areas, the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) have become an established standard of care in advanced NSCLC following initial platinum based chemotherapy (Shepherd et al., 2005).

1.1.7 Survival

The establishment of large population-based cancer registries such as the EUROCARE program in Europe and the Surveillance, Epidemiology and End Results (SEER) and National Program of Cancer Registries (NPCR) projects in the US has facilitated international comparisons of cancer management practises and of survival estimates. This in turn provides information that allows governmental agencies to plan and implement effective national cancer strategies and to improve delivery of health care for cancer patients.

Investigators from the EUROCARE-4 project analysed the outcomes of more than three million adult cancer cases from 82 cancer registries in 23 European countries diagnosed during the period 1995-1999 (Sant et al., 2009). Results showed that the mean European 5-year age-adjusted relative survival for lung cancer was 12%, essentially unchanged since the EUROCARE-3 period (1990-1994). This is despite the advent of novel methods of clinical investigation, refinements in surgical techniques, the intense investigation of various forms of chemotherapy and the advent of several novel targeted therapies.

There were also considerable differences in survival rates between and within European countries for lung cancer, with Northern and Western European countries faring better than Eastern European countries. Age-standardized 1- and 5-year survival rates in Ireland were 26.5% and 9.8%, respectively. Both of these estimates were below the European average. EUROCARE-4 data also confirmed

that survival for younger patients was better. Survival rates for women were also better than those of their male counterparts (12.3% vs. 9.4%, respectively).

Published data from the US show an overall 5-year survival rate of 15%, which is marginally better than the European average reported in the EUROCARE-3 study (Jemal et al., 2006). However, similar to Europe, there has been only a marginal increase in survival rates for lung cancer patients in the last three decades.

1.2 INLAMMATION AND LUNG CANCER

1.2.1 Introduction

There is now a general consensus that chronic inflammation plays a critical role in tumorigenesis (O'Byrne and Dalgleish, 2001). In response to injury, there is activation of a complex integrated system of mechanisms designed to eliminate the stimulus, repair damaged tissue and promote wound healing through measured and organized cellular proliferation. Under normal circumstances, this host physiological response subsides once repair is completed. However, when this usually tightly regulated self-limiting mechanism becomes disturbed, a state of chronic inflammation may ensue.

Virchow first proposed the link between inflammation and neoplasia when he observed that cancer appeared to develop at sites of previous chronic inflammation (Virchow, 1863). The initial suggestion that tumour-infiltrating lymphocytes (TIL) may represent an expression of host antitumour activity was supported by evidence linking TIL with improved prognosis in solid cancers (Evans and Alexander, 1972, Underwood, 1974). Forty years later, there is now a wealth of accumulated data to support the hypothesis that organs affected by chronic inflammation provide the perfect milieu in which an abnormal clone or clones of cells are capable of evading detection by the host immune system and progress to invasive carcinoma.

1.2.2 Chronic immune activation and carcinogenesis

The adaptive immune response is known to comprise two essential and complementary components, namely cell-mediated immunity (CMI) and humoral immunity (HI). The $CD4^+$ T-helper (T_H) lymphocyte is an essential element of both systems and is responsible for orchestrating two different but overlapping cytokine patterns that influence other effector cells and in turn shape the pattern of the inflammatory response (Mosmann et al., 1986).

The molecular mechanisms that underlie the evolution of matured naïve (T_H0) $CD4^+$ cell into distinct populations that are characterised by either a CMI or

HI phenotype are still not completely understood. However, the nature of the cytokine milieu in which antigen presentation occurs influences the differentiation of T_H0 cells (**Figure 1.1**). Early production of interleukin (IL)-12 by mature dendritic cells stimulates upregulation of cell-mediated immunity by effector/memory cells and is characterized by the production of the T_H1 cytokines IL-2, interferon (IFN)-γ and tumour necrosis factor (TNF)-α (Finn, 2008). These cytokines participate in initiation and maintenance of inflammation and, in general, possess anti-angiogenic and pro-apoptotic effects. By contrast, humoral immunity is characterized by the production of pro-angiogenic, anti-apoptotic T_H2 cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13 (Becker, 2006). Immune deviation toward a dominant T_H1 response facilitates cancer cell killing and tumour rejection (Knutson and Disis, 2005). Conversely, a dominant T_H2 response may be regarded as favourable for tumour progression (Terabe et al., 2004).

Under prevailing physiological conditions, a delicate balance exists between cell-mediated and humoral immune responses. In response to an inflammatory stimulus (e.g. exposure to a viral pathogen) there is activation of both systems in order to clear the injurious stimulus. Once the offending pathogen is successfully cleared, and in order for effective wound healing to occur, there is a relative reduction in CMI and upregulation of HI (O'Byrne et al., 2000). Thereafter, the normal balance between CMI and HI is re-established. However, in circumstances where the host is unable to effectively remove the inciting stimulus, the inflammatory cellular components persist and a chronic inflammatory response may develop. Such a pattern is characteristic of a number of human infectious diseases such as Epstein Barr virus (EBV), human papilloma virus (HPV), hepatitis B and C, human immunodeficiency virus (HIV)/ Acquired Immunodeficiency Syndrome (AIDS), *Helicobacter pylori* and schistosomiasis (Dalgleish and O'Byrne, 2006). A failure of host infection-clearing mechanisms is typical of each of these chronic infections.

Several lines of evidence indicate that many cancers develop in the setting of such chronic immune activation. In these circumstances, there is not only an overall upregulation of immune responses but a shift in the immune response to one that is characterised by a wound healing, pro-angiogenic, anti-apoptotic cytokine pattern

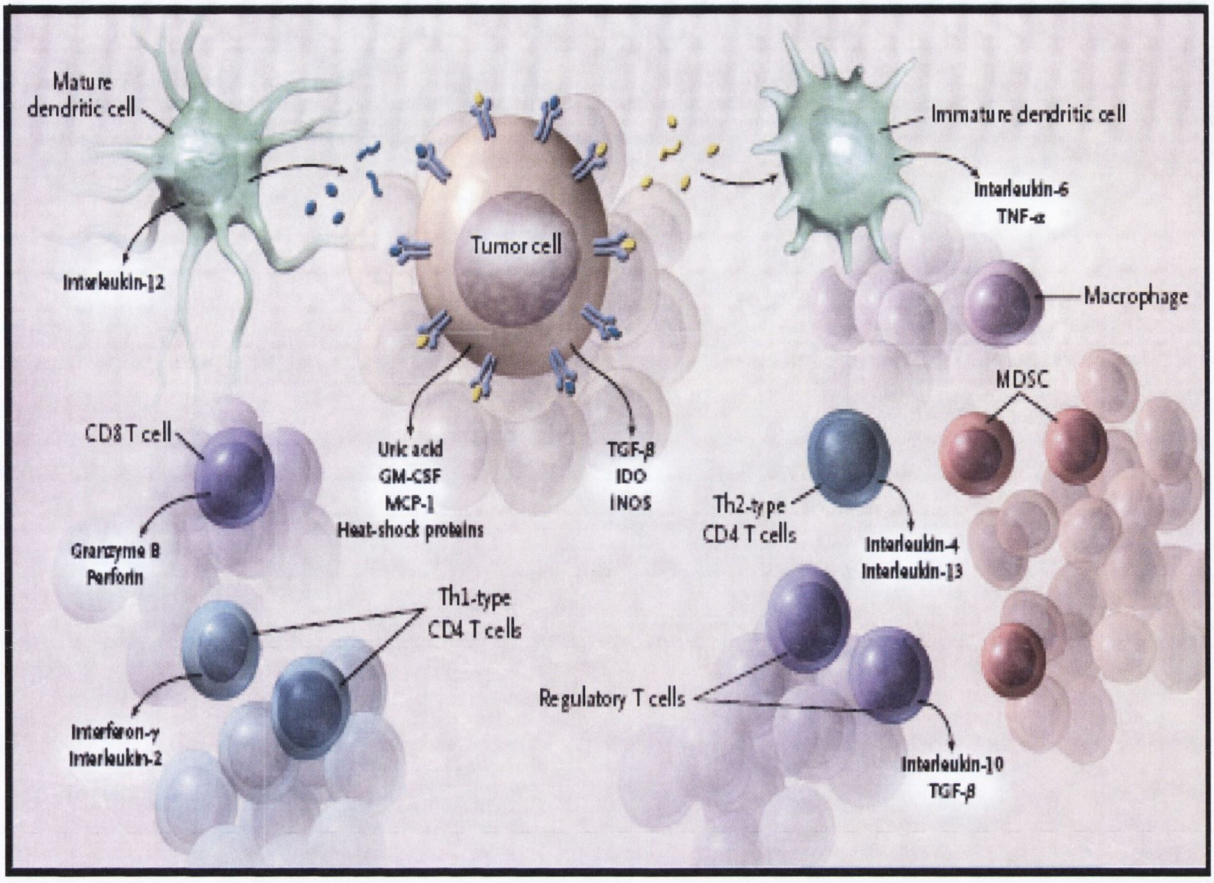

Figure 1.1 Immunoregulatory forces in the tumour microenvironment.

Tumour antigens and soluble tumour products attract dendritic cells to the tumour site which take up tumour antigens, mature into IL-12–producing cells, and stimulate IFNγ-producing T_H1 cells. These cells help expand the population of $CD8^+$ cytotoxic T-cells that can destroy tumour cells through effector molecules granzyme B and perforin. Another set of tumour antigens promote maturation of a different type of dendritic cell that makes proinflammatory cytokines IL-6 and TNF-α and give rise T_H2 cells that make IL-4 and IL-13 and are not effective in tumour rejection. This immunosuppressive environment also promotes generation of regulatory T cells and accumulation of macrophages and myeloid-derived suppressor cells (MDSC).

Key: GM-CSF=granulocyte–macrophage colony-stimulating factor; IDO=indolamine-2,3-dioxygenase; iNOS=inducible nitric oxide synthase; MCP-1=monocyte chemotactic protein 1; and TGF-β=transforming growth factor β.

(From: Finn, *N Engl J Med*, 2008;358:2704-2715.)

often. As the malignant process develops cancer cells evolve to subvert the CMI response. Taken together, these findings highlight the critical role played by the immune system in determining outcome with respect to tumorigenesis (O'Byrne et al., 2000, Dalgleish and O'Byrne, 2006).

1.2.3 Inflammatory disorders, chronic infection and cancer risk

Initial evidence from epidemiological studies exposed a link between diseases characterised by chronic immune activation and increased cancer risk. There are approximately 2 million infection-attributable cancer cases diagnosed worldwide every year, with the majority caused by viral agents (Parkin, 2006). This constitutes approximately 18% of the global cancer burden. Infection-associated oncogenesis is postulated to arise through persistent local and systemic immune activation, characterised by upregulation of host HI responses often with a corresponding relative suppression of CMI (O'Byrne and Dalgleish, 2001). The cycle of epithelial regeneration and proliferation is perpetuated and is postulated to lead to genomic alterations (point mutations, deletions or rearrangements) (Ohshima et al., 2003, Coussens and Werb, 2001). Other immune-mediated processes, such as overproduction of reactive oxygen species (ROS) and reactive nitrogen oxide species (RNOS), activation of growth factors and alteration of signal-transduction pathways to promote cell survival and proliferation pathways are considered to be additional components of risk toward tumour development (Yang et al., 2009).

Previous experiments have demonstrated the capacity of viral agents to antagonize binding of T-lymphocyte populations to antigen-presenting cells, thereby preventing cytotoxic effector cell-mediated killing of infected cells (Yuan et al., 2006). Thus, in creating a HI-predominant environment with relative suppression of CMI, persistent infection predisposes to an environment in which damaged cells can survive undetected by the host immune system. Any subsequent genotoxic insult that affords such a transformed cell replicative potential might precede the onset of frank carcinogenesis. Disorders characterised by chronic inflammation that are linked with the development of cancer are listed in **Table 1.2**.

Disorder	Associated Cancer(s)	Aetiology Agent
Gastritis	Gastric adenocarcinoma, MALToma	*Helicobacter pylori*
Schistosomiasis	Bladder, liver carcinoma	Schistosomes
Cholangitis	Cholangiocarcinoma	Liver fluke, bile acids (?)
Hepatitis	Hepatocellular carcinoma	Hepatitis B and C viruses
Various	Burkitt's Lymphoma, Nasopharyngeal carcinoma, Post transplant lymphoma Non Hodgkin's lymphoma	Epstein Barr virus
AIDS	Kaposi's sarcoma Lymphoma (HHV-8 driven)	HIV virus
Chronic cervicitis	Cervical carcinoma, Anal carcinoma Upper aerodigestive tract (?)	Human papilloma virus
Inflammatory bowel disease	Colorectal carcinoma	
Reflux oesophagitis	Oesophageal carcinoma	Gastric Acid
Asbestos, silicosis	Lung carcinoma, mesothelioma	Asbestos fibres, Silica particles
Chronic bronchitis	Lung carcinoma	Tobacco smoke, Environmental pollutants
Lichen Planus	Oral squamous cell carcinoma	
Chronic prostatitis (?)	Prostate carcinoma	? Infectious cause

Table 1.2 Chronic inflammatory disorders that predispose to malignancy

1.2.3.1 Colorectal cancer

The chronic inflammatory bowel diseases (CIBD) ulcerative colitis and Crohn's disease perhaps exemplify the strongest evidence linking persistent inflammation and malignant transformation (Gillen et al., 1994). Patients with these disorders have an approximately 2-3 fold greater lifetime risk of developing colorectal carcinoma (CRC) compared to the general population, and cancer risk increases with duration and anatomic extent of colitis (Xie and Itzkowitz, 2008). In CIBD, the intestine is exposed to a continuous cycle of injury and epithelial regeneration. Not only does this pattern serve as a potent stimulus to the host immune system, but it also increases the potential for regenerating epithelium to sustain somatic mutations. Moreover, these changes develop in an altered immune environment, wherein survival and proliferation of cells with neoplastic potential is facilitated.

The development of CRC through malignant transformation of colonic polyps is also linked to dysregulated inflammation. Early polyps have been shown by histopathological assessment to be inflammatory in nature (Higaki et al., 1999), while the development of colorectal cancer through the adenoma-carcinoma sequence is accompanied by reduced infiltration of T_H1 cytokine expressing cells, implying a functional switch of host anti-tumour immunity (Cui et al., 2007). By contrast, the reduced CMI response that is characteristic in CRC patients is reversed following curative surgery, providing additional evidence that CRC can suppress the systemic immune response (Heriot et al., 2000).

1.2.3.2 Gastric cancer and lymphoma

Persistent infection with the *Helicobacter pylori (H. pylori)* bacterium is now recognised as the commonest cause of gastric carcinoma worldwide, with the development of malignancy invariably preceded by chronic atrophic gastritis (Parsonnet et al., 1991). Persistent colonization by *H. pylori* confers an approximately 4-fold increased risk of developing stomach cancer. Both gastric carcinoma and Mucosa-associated lymphatic tissue lymphoma (MALToma) are characterised by IL-8 induced recruitment of chronic inflammatory cells (Calam et al., 1997) that results in DNA damage to gastric epithelium mediated by ROS and RNOS (Davies et al., 1994). Wang et al showed that patients infected with CagA+

H. pylori strains exhibit a shift from a CMI to a dominant HI response during the progression of gastric carcinogenesis (Wang et al., 2007). These authors also demonstrated that whereas T_H1 cellular immunity was associated with earlier stages of gastric carcinogenesis, T_H2 responses dominated in advanced stages of disease.

1.2.3.3 Hepatoma

In addition to strong geographical correlation, numerous cohort and case-control studies have established both the hepatitis B (HBV) and hepatitis C (HCV) viruses as pathogenic in liver cancer (Baffis et al., 1999, Kuper et al., 2000). Together, HBV and HCV are estimated as causative of more than 75% of all cases of hepatocellular carcinoma worldwide. A chronic inflammatory response directed against viral proteins, coupled with a perturbation in the balance between pro- and anti-apoptotic mediators in hepatocytes infected with HBV and/or HCV is proposed as central to liver carcinogenesis. Host cytotoxic T-lymphocytes and cytokines interact with infected hepatocytes, resulting in recurring cycles of cellular injury, apoptosis, necrosis, and regeneration (Schottenfeld and Beebe-Dimmer, 2006). A significant correlation between duration and intensity of inflammation and tumorigenesis with respect to hepatocellular carcinoma has been shown.

Whereas an effectual T_H1 response is necessary to clear the viruses after acute infection, chronically infected persons often display a switch to a dominant systemic HI-cytokine pattern. Patients chronically infected with HCV have suppressed T_H1 responses, whereas downregulation of T_H2 responses coincides with successful treatment of HCV infection (Yang et al., 2006). Evidence from mice models also indicates a central role for the nuclear factor-κB (NF-κB), a key component of inflammatory, oncogenic and apoptotic pathways. In response to an acute inflammatory stimulus, activation of this gene-transcription factor within myeloid cells results in augmented production of pro-inflammatory and pro-apoptotic mediators (Pikarsky et al., 2004). However, premalignant hepatocytes are capable of adapting NF-κB in order to promote their own endurance through the induction of cell-survival genes rendering them resistant to death-promoting machinery.

1.2.3.4 Cancers of the cervix, anus, and head and neck

The human papilloma virus (HPV) is a sexually transmitted DNA virus linked with cancers of the cervix, anus, and head and neck; a pathogenic role in development of lung cancer has also been suggested (Markham, 1996). Strains 16 and 18 are most strongly associated with malignant transformation, though several other subtypes of HPV are of suspected human carcinogenic potential. Epidemiological studies have suggested that 90% of cases of cervical carcinoma are attributable to viral infection. The disease is extremely uncommon among non-sexually active female populations (e.g. religious nuns). Chronic cervicitis is found in the pre-malignant stages of cervical cancer; the onset of malignancy is invariably preceded by a progression through several pre-malignant phases referred to as cervical intraepithelial neoplasia (CIN) and forms the rationale for cervical screening approaches.

Lymphoproliferative responses associated with regression of chronic HPV infection (and therefore reduced risk of progression to cancer) are characterised by a predominant CMI cytokine pattern (Dillon et al., 2007). Moreover, patients with clinical and cytological evidence of resolution of HPV-induced CMV have skin test evidence of specific cellular immunity to HPV-16 E7 oncoprotein (Hopfl et al., 2000). In contrast, persistence of CIN is associated with an elevated IL-10/IL-2 ratio in whole blood, indicating a predominant T_H2 response.

1.2.3.5 Kaposi's sarcoma

Kaposi's sarcoma (KS) is an angioproliferative disease that occurs predominantly in immunocompromised individuals, and is one of the Acquired Immunodeficiency Syndrome (AIDS)-defining illnesses. The disorder is associated with the sexually transmitted human herpes virus-8 (HHV8) that incites a predominantly T-cell and macrophage inflammatory infiltrate. HIV infected individuals typically exhibit a predominant HI response, with co-existing suppression of CMI (Dalgleish and O'Byrne, 2002). This pattern of immunoactivation induces the production of an array of pro-angiogenic cytokines and growth factors that mediate neovascularization and upregulate KS spindle cell proliferation prior to the onset of frank carcinogenesis (Ensoli and Sturzl, 1998).

1.2.4 The role of chronic inflammation in lung cancer pathogenesis

1.2.4.1 Cigarette smoking

Cigarette smoking is established as the leading risk factor for lung cancer, and the overwhelming majority of patients diagnosed with the disease are current or former smokers. There is increasing evidence that tobacco smoke exposure promotes widespread inflammatory and mutagenic effects in the lungs that promote a pro-cancer immune response. Indeed, the initial pathological hallmark of smoking is a widespread inflammatory infiltrate throughout the lung parenchyma (Bracke et al., 2006). Repeated injury to lung epithelial cells caused by inhalation of noxious particulate matter from cigarette smoke drives ongoing recruitment of host inflammatory cells to lung airways and alveolar tissue. Increased numbers and enhanced activation of macrophage, dendritic cells, activated lymphocytes and granulocytes characterise the lungs of smokers, thereby producing an environment in which transformed cells can proliferate (Smith et al., 2006).

1.2.4.2 Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is a disorder characterised by an abnormal local and systemic inflammatory response and is strongly associated with lung cancer (Skillrud et al., 1986, Nomura et al., 1991). Within the lung, cigarette smoke incites a potent inflammatory reaction in the airways and alveoli of susceptible smokers, a process aetiologically important in driving both proteolytic alveolar destruction and airway remodelling, the pathological hallmarks of COPD (Saetta et al., 2001). The inflammatory responses that characterise COPD drive a repetitive cycle of injury and repair throughout the lungs. These persistent biological effects also increase the risk of transformation of normal bronchial epithelium to a malignant phenotype.

There are data confirming that reduction in lung function, the physiologic hallmark of COPD, is associated with increased markers of systemic inflammation. In particular, levels of forced expiratory volume in one second (FEV_1) show an inverse correlation with levels of C-reactive protein (CRP), a sensitive marker of inflammation (Vestbo, 2007). Numerous epidemiological studies have evaluated the relationship between lung cancer and abnormalities of pulmonary function, with moderate to severe airflow obstruction now recognized as an independent predictor

of incident lung cancer (Mannino et al., 2003). This risk increases with worsening severity of lung function impairment as measured by FEV_1 and appears particularly strong in women (Loganathan et al., 2006).

1.2.4.3 Non-smoking related factors

Exposure to tobacco smoke is indisputably the most important risk factor for the development of both COPD and lung cancer in susceptible hosts. However, approximately 10% of lung cancers occur in lifelong non-smokers, indicating that other factors must be aetiologically relevant in lung carcinogenesis. In this regard, a number of other disorders of the respiratory system in which inflammation is pathogenic, both infectious and non-infectious, have been linked to the development of lung cancer.

Much of the evidence for the increased risk of lung cancer in the setting of prior infection due to *Mycobacterium tuberculosis* has been derived from large epidemiological studies. A large population-based case-control study conducted in China among individuals with evidence of previous tuberculous disease showed a positive correlation with both adenocarcinoma and squamous cell cancer subtypes that was independent of smoking status and socio-economic group (Brenner et al., 2001). Zheng et al reported that a prior diagnosis of tuberculosis increased the risk of lung cancer by 50% with the effect most apparent among those with recent infection (Zheng et al., 1987).

A causal association between lung cancer and *Chlamydia pneumonia* has been the subject of investigation (Littman et al., 2005), using serologic testing as evidence of prior infection. The precise mechanisms to explain this epidemiological link remain poorly understood. Chronic infection with *Chlamydia pneumonia* has been shown to result in impaired apoptosis of infected cells by induction of the key immunosuppressive cytokine IL-10 resulting in reduced antitumour CMI responses (Geng et al., 2000). Another postulated mechanism by which *Chlamydia pneumonia* may increase risk of malignancy is through upregulation of IL-8 and promotion of angiogenesis and cellular proliferation (Arenberg et al., 1996).

Accumulated epidemiological data have suggested that fibrotic lung diseases may also predispose to lung tumorigenesis. Initial evidence came from the finding of co-existing interstitial lung disease and lung carcinoma in autopsy studies (Haddad and Massaro, 1968). The observed increased incidence found in follow-up studies of workers exposed to asbestos and silica appears to support this hypothesis (Doll, 1955, Smith et al., 1995). Results from a European collaborative study confirmed the association between occupational crystalline silica dust exposure and lung cancer, with an two-fold increased risk observed among individuals with greatest exposure (Cassidy et al., 2007). Moreover, established pneumoconiosis from either silica or asbestos confers an even greater risk.

A study examining a cohort of patients with idiopathic pulmonary fibrosis (IPF) found the incidence of lung cancer was increased seven-fold compared with controls (Hubbard et al., 2000). This increased risk was independent of cigarette smoking history. It has been suggested that overexpression and subsequent mutation of the p53 gene that occur with inflammation/fibrosis-associated oxidative DNA damage and repair may contribute to the emergence of a pro-tumour environment in IPF patients (Kuwano et al., 1996).

Animal experiments have provided additional important evidence of the potential contribution of immune activation to lung carcinogenesis in the context of exposure to inorganic dusts. Using a mouse model, Saffiotti and co-workers showed that intratracheal instillation of chemical particles evokes a potent inflammatory response that eventually leads to the development of NSCLC (Saffiotti, 1996). Insufflation of particulate silica, a potent lung irritant, causes a brisk granulomatous reaction in the murine bronchial tree. Recruitment of inflammatory cells to affected airways predictably leads to hyperplasia of affected bronchiolar epithelium and type 2 pneumocytes. Subsequently, the mouse lung characteristically undergoes progressive changes through hyperplasia to adenoma formation and, ultimately, carcinoma.

1.2.4.4 Cyclooxygenase

In recent years, attention has been drawn to the pathogenic role played by the prostaglandins (PG) and their upstream regulators of production, the

cyclooxygenases (COX), in the progression of malignant disease (Masferrer et al., 2000). COX consists of two distinct isoforms, COX-1 and COX-2. Whereas COX-1 is constitutively expressed and regulates a number of physiological functions, production of the early response gene COX-2 is induced in response to inflammatory stimuli through the action of various cytokines and growth factors. However tumour promoters also upregulate COX-2 synthesis and many of the fundamental components of carcinogenesis and cancer development are known to be mediated via its actions. These include impairment of CMI, apoptosis resistance, cellular proliferation, enhanced angiogenesis, increased invasion and metastasis (Richardson et al., 2003).

Increased levels of COX has been demonstrated in the initiation stage of a variety of cancers (Williams et al., 1999). Epidemiological evidence indicates that long-term users of non-steroidal anti-inflammatory drugs (agents which block the deleterious pro-inflammatory effects of COX) have an approximate 50% reduction in risk of colon cancer, and may confer reduced risk of lung, oesophagus and gastric cancer (Wall et al., 2007, Hernandez-Diaz and Garcia Rodriguez, 2007). Several investigators have confirmed that both NSCLC and associated precursor lesions (adenomatous hyperplasia and carcinoma *in situ)* are associated with COX-2 overexpression (Hida et al., 1998, Hosomi et al., 2000, Wolff et al., 1998). Quantification of COX-2 expression may add useful independent prognostic information in resected NSCLC patients (Lu et al., 2004).

1.2.5 The impact of host immune response on outcome in cancer

From the earliest stages of cancer development, both a systemic and locoregional inflammatory response is induced in the host. The nature of this response is a critical determinant of whether tumour growth is facilitated or inhibited. Experimental evidence indicates that there is a proliferation of host immune effector populations in response to nascent tumours (Finn, 2008). Both circulating and tissue tumour-specific cytotoxic T-lymphocytes and B lymphocyte-derived antibodies specific for tumour antigens have been demonstrated in cancer patients using tetramer technology and flow cytometry (Altman et al., 1996, Meidenbauer et al., 2003). A variety of other cells including macrophages, mast cells and dendritic cells are also known to microscopically infiltrate tumours.

Previously believed to represent a frustrated host defence to burgeoning neoplastic growth, there is now evidence that the developing tumour is capable of adapting this host immune response to create a microenvironment conducive to its own survival and progression (Whiteside, 2006).

There are conflicting data with regard to the inflammatory response and outcome in the setting of cancer affecting different organs, with evidence to support both beneficial and negative effects. Whether an immune response augments or impedes cancer growth is likely determined by several factors. The specific pattern of this response appears crucial in determining tumour progression and recent evidence suggests that precise localization of immune cells within different parts of the tumour influences phenotype (Welsh et al., 2005).

1.2.5.1 T-lymphocytes

Abundant intratumoral infiltration by T-lymphocytes is a frequent observation. This pathological finding has been linked to improved patient survival in numerous studies (Zhang et al., 2003, Galon et al., 2006, Piersma et al., 2007, Kondratiev et al., 2004). By contrast, other reports have suggested that increased lymphocytic invasion in cancers predicts worse outcome (Oudejans et al., 2002, Grabenbauer et al., 2006, Oudejans et al., 1997). However, it is clear that a failure of host immune surveillance mechanisms, in which lymphocytes play a pivotal role, is a key step in the early stages of tumour development.

Prominent lymphocytic infiltration is a characteristic finding in many forms of malignancy. However, functional aberrancy of these T-cell populations is often apparent. Several processes may account for this anergy. Alterations of the T-cell receptor (TCR) prevent effective recognition of the major histocompatibility complex (MHC)-antigen complex when this population encounters malignant cells. In addition, decreased expression of signal transduction proteins, blunted proliferative capabilities, and a change in cytokine expression profile have also been observed (Rodriguez et al., 2004). As distinct from normally functioning circulating lymphocytes, TILs respond inappropriately or not at all to conventional activating stimuli (Whiteside, 2003, Sogn, 1998). Together, these effects result in impairment of effective cancer cell killing mechanisms.

Tumour cell surface antigens and soluble products are recognised and processed by host antigen-presenting cells and result in the activation of specific $CD4^+$ helper T-lymphocyte populations. The nature of the cytokine profile elaborated by tumour cells influences whether the T-cell population that is expanded and recruited to the site of the tumour is a dominant T_H1 or T_H2 pattern. For example, abundant expression of the immunosuppressive cytokine transforming growth factor (TGF)-β promotes maturation of T_H2 $CD4^+$ cells (O'Byrne et al., 2000) and also directly inhibits cytotoxic $CD8^+$ T-lymphocyte-mediated cancer killing mechanisms (Chen et al., 2005a). Whether the predominant prevailing immune pattern is the result of tumour effects, driven by surrounding stromal constituents or develop as a consequence of interplay between both elements remains uncertain (Whiteside, 2006).

Tumours are adept at evading recognition by infiltrating immune components by rendering themselves functionally compromised with regard to antigen presentation through downregulated expression of classical human leukocyte antigen (HLA) class I surface molecules (Marincola et al., 2000). In effect, tumours elude detection by being poor stimulators of host surveillance mechanisms. Recent data has also confirmed enhanced apoptosis in circulating and tissue cytotoxic $CD8^+$ cells among cancer patients, mediated through dysregulation of pro- and anti-apoptotic mitochondrial proteins, effects that appear to further facilitate tumour progression (Saito et al., 2000, Kim et al., 2004).

There is mounting evidence to suggest that anti-tumour immune function is further compromised by cancer-mediated inhibition of T-cell cytotoxic activity and by induction of pro-apoptotic mechanisms targeted against specific subpopulations of effector cells. For example, the key tumour suppressor gene p53 is repressed under conditions of immune activation, specifically by the macrophage migration inhibitory factor (MIF) (Cordon-Cardo and Prives, 1999). MIF is a multifunctional cytokine that mediates pro-inflammatory effects under certain conditions. Yet it is also capable of downregulating activated T-cells within the tumour microenvironment. Yan et al showed that tumour-derived MIF, produced in supraphysiological concentrations, inhibits rather than augments T-cell activation in murine neuroblastoma cell lines (Yan et al., 2006).

Apoptosis of T-lymphocytes is controlled principally via the death-inducing Fas ligand (FasL/CD95L) pathway which is expressed in a variety of human tumour types (Andreola et al., 2002, Okada et al., 2000). Not only does expression of FasL on tumour cells render them resistant to Fas-induced lysis by cytotoxic T-lymphocytes, but evidence suggests that cancer cells themselves are capable of actively killing TILs. Using in vitro models, FasL-positive tumour cells have been shown to kill FasL-sensitive target cells, while apoptosis of TILs has been demonstrated in situ within FasL-expressing human tumours (Bennett et al., 1998, Hahne et al., 1996). Other pro-apoptotic mediators such as TNF-related apoptosis-inducing ligand (TRAIL) are believed to represent mechanisms by which tumours are capable of orchestrating a 'counterattack' against the host immune system (Giovarelli et al., 1999). Experimental work has also provided evidence that several crucial anti-apoptotic pathways are upregulated, enabling enhanced cancer cell survival. Examples include upregulation of COX-2, activation of the AKT and NF-κB pathways and enhanced induction of membrane EGFR (Royds et al., 1998, Shishodia et al., 2004, Ciardiello and Tortora, 2001).

Both positive and negative prognostic significance has been attached to the finding of TILs in different solid organ cancers. Failure to fully characterise and identify specific T-cell phenotypes may contribute to the seeming incongruity with respect to the relevance of lymphocytic infiltration in some early studies. Subsequent experimental work identified a subset of $CD4^+$ T-cells known as regulatory T-cells (Tregs) that maintain peripheral tolerance via suppression of self-antigen reactive T-cells. Human Tregs co-express the CD25 antigen and are identified by the presence of the intracellular marker forkhead box P3 (Foxp3), a transcriptional repressor required for maturation and immunosuppressive functionality (Fontenot and Rudensky, 2005).

Tumours express a variety of antigens, including self-antigens. It has been suggested that intratumoral accumulation of Tregs may result in downregulation of T-cells reactive against tumour antigens thereby creating a more favourable microenvironment for clonal tumour cell expansion. Using an experimental model of spontaneous tumour development, Willimsky et al showed that Tregs suppress specific cytotoxic anti-tumour elements (Willimsky and Blankenstein, 2005).

In human ovarian cancer, recruitment of Tregs dampens effective T-cell anti-tumour immunity *in vivo* and predicts markedly reduced survival after debulking surgery (Curiel et al., 2004). Significant numbers of Tregs have also been demonstrated in biopsies of patients with mesothelioma, a malignancy with a dismal prognosis (Hegmans et al., 2006). In contrast, depletion of Tregs from implanted tumours in a murine model of mesothelioma resulted in a significantly extended survival in treated animals. Thus, while enhanced intratumoral effector $CD8^+$ and/or non-regulatory $CD4^+$ lymphocyte infiltration may confer a prognostic advantage, increased numbers of $CD4^+CD25^+Foxp3^+$ Tregs appears to be detrimental.

In gastric adenocarcinoma, high intratumoral Treg counts correlates significantly with invasion into vascular, lymphatic and perineural compartments and increased risk of disease relapse (Perrone et al., 2008). By contrast, breast cancer patients who demonstrate a complete pathological response to neoadjuvant chemotherapy demonstrate an intratumoral lymphoid profile that is devoid of $Foxp3^+$ cells but extensively populated by cytotoxic cells (Ladoire et al., 2008). In epithelial ovarian cancer, determination of intratumoral $CD8^+$/Treg cell ratio predicted survival better than estimation of Treg distribution alone (Sato et al., 2005). Thus, strategies incorporating simultaneous quantification of different T-cell population interrelationships may further enhance predictive capacity of histopathological evaluation and identify patient groups likely to benefit from additional therapeutic intervention.

Increased numbers of Tregs have also been described in the peripheral blood of patients with a number of solid organ and haematological malignancies (Woo et al., 2001, Liyanage et al., 2002, Wolf et al., 2003). Circulating Tregs appear equally capable of downregulating T-cell activation *in vitro* suggesting that this population exerts a systemic tolerance with respect to tumour associated antigens. Malignant pleural effusions from patients with lung cancer demonstrate increased numbers of $CD4^+CD25^+Foxp3^+$ T-cells in comparison to pleural lavage specimens from patients with lung cancer but no pleural effusion (Chen et al., 2005b). A high constitutive expression of cytotoxic lymphocyte-associated antigen-4 (CTLA-4) characterises these cells. CTLA-4 is expressed on activated T-cells and serves as a potent block to cellular activation of all T-cell populations (Zou, 2006).

While many investigators have shown that different patterns of cellular immune infiltrates appear to facilitate tumour growth, there is also accumulating evidence to suggest that this pattern of immune activation may also limit neoplastic progression. As such, numerous studies have shown tumour-infiltrating lymphocytes may be a good prognostic marker. The presence of intratumoral T-lymphocytic infiltration in surgically resected solid organ cancers frequently portends a more favourable outcome, regardless of response to chemotherapy. With respect to ovarian carcinoma, the presence of tumour-infiltrating $CD3^+$ T-lymphocytes confers a significantly better outcome irrespective of response to adjuvant cytotoxic chemotherapy (Zhang et al., 2003). Increased numbers of intratumoral T-cells were also shown to be associated with enhanced expression of several characteristic CMI associated pro-inflammatory cytokines including IL-2, IFN-γ and lymphocyte-attracting chemokines such as CXCL9, CCL21 and CCL22. Moreover, the absence of intratumoral T-cells is characterised by upregulation of vascular endothelial growth factor (VEGF), itself a suppressor of CMI responses (Dalgleish and O'Byrne, 2006).

1.2.5.2 Natural killer cells

Although the anti-viral functions of natural killer (NK) cells have long been known, the significance of intratumoral infiltration by NK populations has not been firmly established. NK cells display spontaneous cytotoxic activities in the presence of cancer cells, indicating a central role in host defence against cancer progression.

In an experimental murine model, NK cells appear capable of eradicating lung tumours (Chapoval et al., 1998). There is some evidence to suggest that increased NK cell numbers also predict survival in human carcinoma (Coca et al., 1997, Whiteside et al., 1996) with extent of infiltration related to stage (Ishigami et al., 2000). Moreover, reduced circulating NK cell cytotoxic activity (and hence functional CMI) is characteristic of and confers a worsened outlook in several solid organ cancers. This association is particularly strong among malignancies in which chronic viral infection is pathogenically important (Kaklamani et al., 1991). However, analysis of activation status of NK cells in cancer islets shows increased expression of the inhibitory receptor NKG2A, indicating an inactivated phenotype, whereas their stromal counterparts are characterised by an immune activated status

(Katou et al., 2007). Thus, evaluation of the numbers of tumour-infiltrating NK cells in isolation may be insufficient to generate prognostic information. Of greater relevance may the precise localization within the different tumour compartments (infiltration into tumour islets or surrounding stroma) of these cells.

Recently, human invariant natural killer T (NKT) cells, a distinct lymphocyte population with a markedly restricted T-cell repertoire, have also demonstrated potent anti-tumour activity through mechanisms distinct from T-lymphocytes and NK cells. Detection and quantification of these cells may provide additional prognostic information in human cancers. In colorectal carcinoma for example, high NKT-cell infiltration correlates with fewer lymph node metastases as well as improved overall and disease-free survival (Tachibana et al., 2005).

1.2.5.3 Macrophages

Macrophages are ubiquitous immune cells that direct a number of vital physiological and host defence processes, including pathogen phagocytosis, regulation of inflammation and tissue repair. Tumour-associated macrophages (TAM) comprise a major constituent of the leukocyte infiltrate characteristic of malignant disease and are present in abundance in both primary and metastatic lesions. Within tumours, TAMs interact with cancer cells to produce a rich source of cytokines and growth factors that shape the local microenvironment (Bingle et al., 2002).

Functional plasticity is a well-described characteristic of macrophages, of which there are at least two major classes, referred to as M1 ('classically activated') and M2 ('alternatively activated') macrophages (Lewis and Pollard, 2006). Whereas M1 macrophages elaborate pro-inflammatory cytokines and are regarded as having an anti-tumour phenotype, M2 macrophages are recruited into tumours and facilitate malignant progression through impairment of adaptive immunity. Although the precise mechanisms that regulate this functional polarization during tumour development have yet to be fully elucidated, the switch toward M2 differentiation appears to be regulated by an increase in immunosuppressive cytokines such as PGE_2, TGF-β and IL-10 that are both tumour-derived and produced by TAMs themselves (Sica et al., 2008).

There is evidence that macrophages are capable of exerting effects that either impede or assist tumour development, depending on the nature of local activation signals. Under certain circumstances, TAMs are responsible for non-specific direct cancer cell destruction via production of a host of cytotoxic molecules (e.g. IL-1, IL-2, IFN-γ and reactive oxygen intermediates) and through recruitment of other immune effector populations, including cytotoxic $CD8^+$ T-lymphocytes and NK cells. However, TAMs may also create an environment conducive to enhanced tumorigenesis via production of pro-angiogenic, pro-lymphogenic factors (Brigati et al., 2002, Tsung et al., 2002). The overproduction of the immunosuppressive cytokine IL-10, which effectively blocks cytotoxic responses of these effector populations, is characteristic of TAMs that behave in a pro-tumour fashion and is associated with a worse prognosis. Increased IL-10 effectively blunts tumouricidal capabilities of phagocytes by suppressing the generation of pro-inflammatory cytokines (Zeni et al., 2007).

In recent years, a wealth of evidence has emerged supporting the contention that macrophages play a key role in tumour progression by facilitating cancer cell proliferation, migration and metastasis, stimulating angiogenesis and suppressing host anti-tumour immunity. Numerous studies have also shown that increased numbers of TAMs correlate with adverse patient outcome and reduced survival (Pollard, 2004, Lewis and Pollard, 2006).

The phenotype of macrophages in the tumour microenvironment appears quite distinct to that observed in non-malignant disorders, with a number of proliferative and survival factors overexpressed by TAMs. In response to a variety of locally elaborated cytokines, chemokines, trophic factors and enzyme products, TAMs may exert pleiotropic pro-tumour effects. M2 cells are identified by a wound-healing phenotype and display poor antigen-presenting functions (Sica et al., 2008). Under the influence of IL-10, there is reduced expression by TAMs of several immunostimulatory cytokines, including IL-1, IL-12 and TNF-α. Enhanced levels of T_H2 cytokines promote an increase in the numbers of T-cells that have deficient cytotoxic capacity, resulting in relative lymphocyte anergy. The net effect of these changes is an impairment of host cytotoxicity and an environment conducive to cancer progression.

It has been proposed that different subpopulations of TAMs perform distinct roles depending on the precise location of this population within tumours. For example, TAMs at areas of basement membrane upregulate proteolytic enzyme production and enhance cancer cell motility, in turn augmenting invasive potential (Lin et al., 2001). Matrix degradation is facilitated by the release of a number of matrix-metalloproteases (MMPs), including MMP2 and MMP9, that enable neoplastic spread (Schmalfeldt et al., 2001). The enlarging tumour eventually outgrows its blood supply, resulting in regions that are characterised by significantly reduced oxygen concentrations (hypoxia). Within these hypoxic parts of the tumours, there is up-regulation of hypoxia-inducible factors (HIFs) that promote pro-invasive and pro-angiogenic activity (Pugh and Ratcliffe, 2003). MMP9 may also help orchestrate these activities (Schmalfeldt et al., 2001).

Increased numbers of TAMs also correlates positively with both microvessel density and expression of the vascular epidermal growth factor (VEGF), a potent endothelial cell mitogen (Valkovic et al., 2002). This observation has led to the suggestion that VEGF may exert a chemotactic action on TAMs to poorly vascularized tumour regions (Lewis et al., 2000). The net effect is the establishment of a network of new blood vessels that provide an ongoing supply of oxygen and nutrients to established tumours.

TAMs attracted to and immobilized in hypoxic areas secrete reduced amounts of key pro-inflammatory mediators required for recruitment of host anti-tumour effector cells. There is also evidence to suggest that TAMs are involved in the regulation of metastasis, via release of pro-invasive macrophage migration inhibitory factors (MIF) and increased synthesis of MMP-7 (Lewis and Murdoch, 2005, Wang et al., 2005).

Most studies have reported that increased macrophage density correlates with poor outcome. However, there are data indicating TAM infiltration may also predict a favourable prognosis. Studies in melanoma (Piras et al., 2005), stomach (Ohno et al., 2003) and colorectal carcinoma (Funada et al., 2003) have reported that high TAM numbers confers a survival advantage, whereas reduced infiltration is associated with prostate cancer progression (Shimura et al., 2000).

The precise pathobiological mechanisms that account for the pleiotropic effects of TAMs remain to be fully elucidated. A possible explanation for why assessment of macrophage infiltration has been shown to confer a prognostic advantage in some studies but not others is that the TAM activity varies depending on the precise microlocalization within tumours. Cytokines and growth factors released from and directed at TAMs unquestionably alter the local tumour environment. Whether the resultant changes are for or against tumour growth appears dependent on which regions of the tumour the infiltrating macrophages are principally located. For example, stromal macrophages are known to express matrix-degrading factors as well as pro-angiogenic factors (Bingle et al., 2002). These effects facilitate stroma re-modelling and promote neovascularization, thus supporting tumour growth. For example, in prostate cancer, tumour cell islet but not stromal macrophages express nitric oxide synthase and TNF-α, both factors involved in tumour cell killing mechanisms (Shimura et al., 2000).

Macrophages can also serve as antigen-presenting cells to activate cytotoxic T-cells. In gastric carcinoma Ohno et al showed that the extent of $CD8^+$ T-cell infiltration directly correlates with macrophage infiltration suggesting that macrophages play an important part in the activation of T-cell-mediated tumour cell lysis (Ohno et al., 2002). When these same investigators examined the spatial distribution of TAMs in gastric cancer, they found that increased infiltration of macrophages into tumour cell nests strongly correlates with tumour cell apoptosis and $CD8^+$ lymphocyte infiltration (Ohno et al., 2003). Patients with a high-nest TAM pattern had a significantly better 5-year survival rate compared to those with the low-nest TAM group (87% *vs.* 44%, respectively) suggesting that aggregation of TAMs into cancer cell islets facilitates augmented antigen presentation and enhanced cytotoxicity.

Increasing macrophage infiltration along the tumour front in colon cancer patients has also been linked to favourable prognosis. In their study, Forssell et al found a strong correlation between intensity of lymphocyte and macrophage infiltrates, suggesting not only that a vigorous macrophage response at sites of tumour invasion may constitute a protective effect but that interplay between these cell types is necessary for effective antitumour defence (Forssell et al., 2007). These

investigators also used an *in vitro* co-culture model to show that a high macrophage to colon cancer cell ratio resulted in inhibition of cancer cell growth, implying that extent of cell-to-cell contact may also influence TAM behaviour.

1.2.5.4 Mast cells

Accumulation of mast cells is characteristic of a number of angiogenesis-dependent conditions including arthritis (Dean et al., 1993), wound repair (Trautmann et al., 2000) and cancer (Aaltomaa et al., 1993, Elpek et al., 2001, Lachter et al., 1995). Mast cells have been shown to be a significant source of the pro-angiogenic molecules VEGF, TGF-β and basic fibroblast growth factor (bFGF) and there is evidence to suggest that mast cell infiltration may mediate angiogenesis in different tumour types (Meininger and Zetter, 1992, Blair et al., 1997). The two major secretory products of mast cells are chymase and tryptase.

Although increased numbers of mast cells have been demonstrated in virtually all malignancies, the functional significance of this finding remains unclear. Many studies show strong evidence that tumour-infiltrating mast cells are negative prognostic factors in cancer patients, including those with skin, cervical, lung cancer and several haematological malignancies (Ribatti et al., 2001). Mast cell invasion appears to confer an increased risk of metastasis in breast cancer (Hartveit et al., 1984) and patients with Hodgkin's lymphoma characterised by higher infiltration rates have worse relapse-free survival (Molin et al., 2002).

Kondo et al showed that the number of chymase-positive mast cells correlated significantly with angiogenesis (as assessed by microvessel density) in gastric cancer (Kondo et al., 2006). Mast cell counts increased as the depth and histological stage increased and levels were more abundant in poorly-differentiated compared to well-differentiated cancers. Furthermore, postoperative survival rates for patients with tumours demonstrating high numbers of chymase-positive cells were worse compared to patients with low tumour counts.

Other investigators have found mast cell invasion to be a good prognostic marker. Using microarray technology, Rajput et al evaluated for the presence of mast cells using the c-Kit immunohistochemical marker in 4,444 invasive breast

cancer cases (Rajput et al., 2008). These authors showed that stromal mast cell invasion in breast cancer was an independent prognostic marker for improved survival. Ali et al examined samples from 60 patients with malignant pleural mesothelioma treated with neoadjuvant intrapleural IL-2 immunotherapy for the presence of tryptase-positive and chymase-positive mast cells (Ali et al., 2009). High tryptase mast cells counts were found to be significantly associated with both overall survival and time to progression. By multivariate analysis, high tryptase mast cells count emerged as an independent favourable prognostic factor in this study.

1.2.6 Inflammatory cell infiltrate patterns in NSCLC

There is conflicting evidence with regard to inflammatory response patterns in lung cancer and their relationship to survival. As with other solid tumours, accumulating data indicate that the nature of the both the innate and adaptive immune responses to neoplastic challenge in the lung is of critical importance.

Early investigation suggested that an enhanced lymphoreticular response conferred a survival advantage in lung cancer (Lipford et al., 1984, Lee et al., 1989). However, variations in the numbers and clinical characteristics of patients analysed in subsequent studies, together with considerable heterogeneity in the methods employed to categorise and examine the inflammatory cell infiltrate has meant that a less than clear-cut relationship with prognosis has emerged.

1.2.6.1 T-lymphocytes

There is increasing evidence that the immune system recognises and mounts a specific, albeit relatively ineffective anti-tumour response by recognising cell-surface specific antigens in NSCLC (O'Byrne and Dalgleish, 2001). The adaptive immune response, comprising T-cells, B-cells and antibodies, has been the focus of extensive research in lung cancer. Flow cytometric analyses have demonstrated that the lymphocyte population within NSCLC is characterised by a predominantly anti-tumour T_H1-cytokine phenotype (Ortegel et al., 2000). Furthermore, increased infiltration by $CD3^+$, $CD8^+$ and B-type TILs shows a positive correlation with the extent of tumour cell apoptosis using TUNEL techniques to quantify fragmented DNA segments. This relationship appears to be strongest with higher-grade

tumours and is maintained even in patients with advanced disease (Tormanen-Napankangas et al., 2001).

Data from initial investigation of the prognostic implication of TILs assessed overall counts irrespective of location rather than classifying various infiltrating cell types into different compartments with the tumour mass (Riemann et al., 1997). Support for the notion that immune cell 'microlocalization' within lung tumours may be of greater relevance than absolute numbers *per se* was provided by a study by Johnson and colleagues (Johnson et al., 2000). These investigators found that grouping patients defined by different levels of semi-quantitatively assessed chronic inflammatory cell infiltrate did not provide useful prognostic information. However, the identification of high numbers of $CD3^+$ T-lymphocytes within cancer islets as opposed to supporting peritumoral stroma predicted a more favourable prognosis. Another study found that determination of microlocalization infiltrative patterns of $CD8^+$ lymphocytes did not appear to provide additional prognostic information, although numbers evaluated in this study were small and immune populations were assessed in a semi-quantitative fashion (Trojan et al., 2004).

A principal antitumour host immunomechanism is direct cytotoxicity of $CD8^+$ T-lymphocytes, via the recognition of tumour-associated antigens presented on MHC class I molecules. The degree of $CD8^+$ T-lymphocyte infiltration within cancer nests alone has variously emerged as a positive (Kawai et al., 2008, Al-Shibli et al., 2008), neutral (Johnson et al., 2000, Hiraoka et al., 2006) or negative (Wakabayashi et al., 2003) prognostic marker in previous studies. However, different methods have been used to quantify the lymphoid infiltrates in NSCLC specimens. Whereas some studies have used randomly selected regions for analysing cell counts, others have focused on those regions that have the highest counts on lymphocytes within regions of tumour epithelium and stroma. This introduces a potential regional analysis bias that might account for some of the discrepancy of published results. Furthermore, tumour specimens from some of the previous studies were collected prior to the advent of newer, more sophisticated staging technology such as CT and positron emission tomography (PET) imaging modalities. Thus, while patients from previous studies may have been regarded as having early-stage disease that was amenable to surgical resection, undetected

metastatic disease may have accounted for some of the differences in outcome and acted as a potential confounder.

Aggregation of different cell types into tumour islets may play an important role in the activation of cytotoxic pathways. Concomitant infiltration by $CD8^+$ and $CD4^+$ TILs appears an indicator of favourable prognosis, suggesting that cellular cooperation between different lymphocyte subsets may be necessary for effective tumour cell killing (Hiraoka et al., 2006). In this regard, $CD4^+$ T-cells appear to augment tumour cell antigenicity and susceptibility to lysis by $CD8^+$ TILs via local release of TNF-α and IFN-γ (Weynants et al., 1988). Similarly, synergy between TILs and TAMs may be required for optimum anti-tumour activity, since a combined high tumour/islet $CD8^+$ count ratio and high tumour/islet macrophage count ratio confer a prognostic advantage (Kawai et al., 2008).

Several factors have been implicated in the relative lymphocyte anergy that results in impaired T-cell mediated cytotoxicity in lung cancer patients with high numbers of TILs and poor outcomes. Although there is frequently extensive infiltration of $CD8^+$ TILs within tumour nests, it appears that it is mainly the peritumorally-located $CD8^+$ cells that are immunologically active as determined by measurement of IFN-γ expression (Trojan et al., 2004). By comparison, intratumoral effector TILs are inadequately activated and display blunted cytotoxic activity, in turn facilitating cancer progression.

There is increasing evidence to support the hypothesis that NSCLC cells themselves induce a local and systemic HI environment, with a T_H2 cytokine predominant pattern (O'Byrne et al., 2000). A host of human tumours, including NSCLC, are characterised by overexpression of several forms of COX which control production of different prostaglandin isoforms. The enzyme PGE_2 stimulates production of $CD4^+$ T_H2 cells but is a potent inhibitor of T_H1 $CD4^+$ T-cells. Experiments using both cell lines and resected tissue specimens have confirmed that lung cancer cells fail to express the T_H1 cytokines IL-2 and IFN-γ whereas elaboration of IL-10 is significantly enhanced in response to stimulation with IL-4 and TNF-α (Huang et al., 1995). Furthermore, elevated serum concentrations of IL-10 predict reduced survival in advanced NSCLC (De Vita et al., 2000).

The local elaboration of various immunosuppressive cytokines, including IL-10, either from tumour cells themselves or from bordering stromal constituents, may hamper effective cytotoxic mechanisms (Urosevic et al., 2001). Alterations in the TCR, loss of signal transducing proteins and/or attenuated co-stimulatory signals from antigen-presenting cells have all been suggested as contributory factors to account for this anergy (Woo et al., 2001). Infiltrating TILs are known to demonstrate significantly reduced expression of MHC class II molecules such as HLA-DR 39 (Foukas et al., 2001), while cancer cells display reduced expression of class I MHC molecules (Kataki et al., 2002). Effective cytotoxicity may also be further inhibited by overproduction of various immunosuppressive factors such as TGF-β by lung cancer cells despite a predominant CMI response (Ortegel et al., 2002).

Further contributing to a milieu of immune tolerance, lung tumours promote accumulation of $CD4^+CD25^+$ Tregs into the tumour bed. As is the case in other cancers, elevated levels of Tregs in NSCLC correlate with tumour invasiveness and metastatic potential. These cells express constitutively high-levels of cell surface CTLA-4 suggesting ongoing activation of neighbouring cells (Woo et al., 2002). Levels of intratumoral TGF-β are contributed to by infiltrating Tregs, although directed inhibitory effects by this population are maintained even in the absence of this cytokine. Effective T-cell activation appears to be specifically suppressed by Tregs through inhibition of IL-2 which results in a selective inhibition of local host immune responses (Thornton and Shevach, 1998).

The expression of the immunosuppressive mediator Foxp3 is, at least in part, regulated by tumour-derived COX-2, which is constitutively overexpressed in lung cancer. Furthermore, COX-2 inhibition *in vivo* leads to impairment of Treg function and reductions in the numbers of Foxp3-expressing cells function, resulting in decreased tumour size through restoration of effectual host antitumour responses (Sharma et al., 2005).

To date, there have been relatively few studies that have examined the clinical impact of intratumoral infiltration of T-lymphocyte characterised by a regulatory phenotype in the setting of lung cancer. Woo et al found increased

amounts of tumour-associated $CD4^+CD25^+$ cells in NSCLC patients with early stage disease (Woo et al., 2001) compared to normals. An immunosuppressive phenotype was also highlighted by the finding of spontaneous TGF-β secretion among TILs. Among patients with stage I NSCLC, increased intratumoral accumulation of $Foxp3^+$ Tregs relative to total TIL infiltration is associated with increased risk of disease recurrence (Petersen et al., 2006). However, assessment of Treg counts in isolation did not yield prognostic information. These data suggest that anticancer strategies that specifically target intratumoral Tregs, either through a reduction in numbers or via abrogation of immunosuppressive activity, may prove worthy of future investigation.

1.2.6.2 Natural killer cells

Cytotoxicity mediated by tumour-infiltrating NK cells may be an important determinant of outcome in NSCLC. In common with other innate immune effectors, the anti-tumour activity of NK cells is mediated independent of antigen-presentation mechanisms. Direct lysis of malignant cells that have altered surface expression of MHC class I molecules occurs in the context of release of perforin, a cytolytic protein that inserts itself into the plasma membrane of the target cell (Young et al., 1986).

There is evidence that patients exposed to cigarette smoke, the commonest risk factor for the development of NSCLC, have attenuated circulating NK cell activity (Phillips et al., 1985). Similarly, bronchoalveolar lavage sampling of smokers reveals reduced NK functioning in comparison to non-smokers (Takeuchi et al., 1988). Although capable of direct tumour cell lysis in *in vitro* models, there is emerging data that NK recognition and destruction of tumour cells in NSCLC is impaired. Esendagli et al recently showed reduced numbers of NK cells within cancer tissue compared to adjoining non-malignant lung regions (Esendagli et al., 2008). Downregulated expression of several markers of NK activation (e.g. NKG2D, NKp46, NKp30 and NKp44) may account for the apparent reduced cytotoxicity within tumours. This finding may help explain the attenuation of NK-mediated direct cancer killing mechanisms. There is also emerging evidence that dampening of NK cell associated innate immune surveillance is mediated in a TGF-β-dependent manner via cross-talk with local Tregs (Wahl et al., 2006).

The prognostic relevance of host NK responses in NSCLC has been the focus of attention in a number of studies. Kerr et al showed a benefit among post-operative cases with a high prevalence of NK tumour infiltration (Kerr et al., 1998). This association was confirmed in two subsequent studies examining different histological NSCLC subtypes. Takanami et al demonstrated a significant relationship between NK cell count and outcome in resected adenocarcinoma (Takanami et al., 2001). Vellegas et al demonstrated a significant positive correlation between post-operative survival and numbers of intratumoral $CD57^+$ NK cells in patients after resection of squamous cell cancer, particularly in stage IB disease (Villegas et al., 2002). Overall, the impact of NK cell infiltration appears greatest in early stage disease, suggesting that as tumours progress NK anti-tumour effector mechanisms become overwhelmed. Interestingly, an earlier study examining a more heterogeneous patient cohort did not identify any link between extent of NK cell infiltration and outcome (Riemann et al., 1997). However, there were considerable methodological differences among the various studies that may account for the divergence of results.

Co-operation between infiltrating NK and alveolar macrophages may be necessary for effective killing of transformed cells (Siziopikou et al., 1997), yet simultaneous assessment for both cell types has infrequently been carried out. The number of NK cells in direct contact with cancer cells may also be an important consideration. With respect to anatomical microlocalization, most NK cells reside within the peritumoral stroma and are not in direct contact with cancer cells (Carrega et al., 2008). This finding further highlights the importance of detailed characterisation of immune effector populations and may help explain the functional aberrancy of NK cells in the malignant environment (Brittenden et al., 1996).

1.2.6.3 Macrophages

Relatively few investigators have examined the link between tumour infiltration by macrophages and patient survival in NSCLC. Furthermore, some authors have shown that increased numbers of TAMs confer a survival advantage whereas others have identified this pattern as a marker of poor outcome. This has led to considerable debate regarding their prognostic relevance (Bingle et al., 2002) (Dimitriadou and Koutsilieris, 1997). However, this discrepancy is almost certainly

related to differences in numbers evaluated, tumour stage, histology, size and grade as well as the wide variation in techniques employed to analyse patterns of macrophage infiltration. Perhaps crucially, researchers have often not taken the microanatomical localization into consideration (Chen et al., 2003, Toomey et al., 2003) .

The importance of accurate assessment of regional infiltrative patterns was highlighted in a study which demonstrated that microanatomical distribution of macrophages in resected NSCLC specimens exerts a powerful impact on survival (Welsh et al., 2005). Specifically, macrophage infiltration of tumour cell islets was shown to confer a marked survival advantage, whereas stromal macrophages were associated with a significantly worse prognosis. Both increasing tumour cell islet macrophage density and islet/stromal macrophage ratio also emerged as favourable independent prognostic indicators. Stromal macrophage density was established as an independent predictor of poor survival. An increased ratio of tumour islet/stromal mast cells was also associated with a survival advantage. Notably, 5-year survival for patients with islet macrophage density above the median was 52.9% versus 7.7% with those below the median ($p<0.0001$). Moreover, similar results were evident when patients were divided into those with a complete or incomplete surgical resection.

Recently published data appears to support the hypothesis that macrophages populating tumour islets represents a population that is characterized by a predominant M1 phenotype. Using immunohistochemistry to quantify expression of the cytotoxic markers TNF-α, HLA-DR, inducible nitric oxide synthase (iNOS) and myeloid related protein (MRP) 8/14, Ohri et al showed that patients with prolonged survival post surgical resection show a predominance of tumour islet macrophages characterised by an M1 phenotype compared to those with poor survival (Ohri et al., 2009).

Two subsequently published studies have reported consistent findings regarding the role of inflammatory infiltrate microlocalization, further supporting the notion that lung cancers are invaded by at least two distinct macrophage types. Using tissue microarray techniques, Kim et al showed that patients with a high

tumour islet macrophage density survived longer after surgical resection compared to those with low tumour islet macrophage density, irrespective of stage (5-year survival rates, 63.9% versus 38.9%, respectively, p=0.0002). By comparison, assessment of total macrophage count alone was not an independent predictor of outcome. High numbers of stromal macrophage were also associated with worse outcome within the high or low tumour islet macrophage groups. Patients with high tumour/low stromal macrophage counts survived longest while those with low tumour islet/high stromal macrophage counts had shortest survival (Kim et al., 2008).

Kawai et al evaluated the prognostic impact of immune infiltrate patterns in patients with stage IV NSCLC treated with standard platinum-doublet based chemotherapy (Kawai et al., 2008). These investigators similarly focused on the role of inflammatory cell microlocalization, using tumour biopsy specimen to study the relative distribution of macrophages, mast cells and $CD8^+$ T-cells in the different tumour compartments. Results showed that individuals with higher tumour islet/stroma macrophage ratio had a median survival time more than double that of those with more macrophages in stroma than islets. The one-year survival rates in the two groups were 60.8% versus 21.4%, respectively. Those with combined high tumour/islet macrophage ratio and high tumour/islet ratio $CD8^+$ counts had an even greater survival advantage and regional distribution of both cell types emerged as favourable independent prognostic factors. By contrast, a high macrophage infiltrate in cancer stroma conferred significantly worse outcome than those with low stroma macrophage counts. There was no correlation between infiltrating immunocytes and chemotherapy-associated disease response.

It is likely that macrophages located in tumour islets exhibit a predominantly anti-tumour phenotype whereas those in adjacent stroma are characterised by pro-angiogenic, anti-apoptotic effects that support cancer growth and dissemination. Indeed, TAMs populating tumour islets appear to be distinguished by a distinct 'cytotoxic' phenotype that elaborate cytotoxic cytokines such as IL-1α, IL-1β, IL-6 and TNF-α (Kataki et al., 2002). By contrast, populations within the peritumoral stroma secrete mitogenic factors favouring tumour progression. Such a hypothesis would help account for apparent contradictory results showing an interrelationship

between high TAM density, different angiogenic factors such as microvessel count and IL-8 levels, and worse outcome in NSCLC (Chen et al., 2003, Takanami et al., 1999).

The phenotype of macrophages is critically dependent on their differentiation and activation status. Using immunostaining, Zeni et al showed that the proportion of TAMs (but not tumour cells) expressing IL-10 is increased in late stage NSCLC, was more frequently observed in those with lymph node metastases and predicts worse overall survival (Zeni et al., 2007). This suggests that these phagocytes behave as M2 polarised TAMs and are complicit in neoplastic extension by promoting angiogenesis, tissue remodelling and suppression of protective CMI responses.

1.2.6.4 Mast cells

There are also conflicting data regarding the relationship between mast cell infiltration and outcome in NSCLC. In adenocarcinoma, but not squamous cancer, VEGF-positive tumours appear to induce intratumoral migration of mast cells. A strong association has been demonstrated between increased tryptase positive mast cell (MC_T) and tryptase- and chymase-positive mast cell (MC_{TC}) density and microvessel count at the advancing tumour edge in adenocarcinoma (Ibaraki et al., 2005).

Using immunohistochemical techniques, Imada et al demonstrated VEGF expression in stromal mast cells and showed that enhanced stromal mast cell infiltrate correlates with microvessel density in moderate-to-well differentiated adenocarcinoma (Imada et al., 2000). Mast cell counts were noted to increase as tumour stage increased. These authors suggested that new microvessel growth may be promoted in regions of mast cell accumulation, accounting for the worse prognosis observed in patients after surgical resection for stage I disease. However, Tataroglu et al found that the mast cell count and microvessel numbers were higher in early stage (Tataroglu et al., 2004).

Mast cell density was shown to be an independent predictor of poor survival among 180 patients who underwent curative-intent surgical resection for pulmonary

adenocarcinoma and correlated with microvessel density (Takanami et al., 2000). Tomita et al also showed a strong correlation between mast cell infiltration and angiogenesis in both adenocarcinoma and squamous cell carcinoma (Tomita et al., 2000). However, the same authors also found that increased mast cell numbers correlated with improved survival among patients that underwent surgery for primary lung adenocarcinoma. Patients with increased high mast cell infiltration from this study had a higher proportion of well differentiated tumours. The authors did not report on the precise intratumoral distribution of mast cells in this study.

A range of methodologies have been employed to quantify mast cells and their relationship to angiogenesis in NSCLC and various criteria have been used to define high *vs.* low inflammatory population infiltrates. Moreover, simple quantification of the absolute numbers of tumour-associated mast cells is not necessarily a reflection on the proportion of this population that are functionally active (Tataroglu et al., 2004). However, patterns of mast cell microlocalization may be useful as a prognostic indicator in NSCLC. Welsh et al studied 175 patients post surgical resection and showed that high islet/stromal ratio independently predicts favourable outcome (Welsh et al., 2005). These researchers found that the overall 5-year survival for patients with total islet mast cell counts above the median was 40% compared to 22% with those below the median value.

The apparently contradictory roles suggest that mast cells may orchestrate pro- or anti-tumour actions depending on several factors. These likely include their location within the tumour, pattern of protease elaboration (i.e. MC_T *vs.* MC_{TC}) and interactions with surrounding tumour cells and other host immune cells.

1.2.7 Summary

The precise mechanisms that underlie these different biological observations remain to be more clearly elucidated. Moreover, no standardized scheme exists to assess and quantify lymphoreticular infiltrate. This has led to difficulty in comparisons between studies and the generalizability of results. Nonetheless, it is likely that any future immunomodulatory strategies capable of discriminating between different phenotypes of a given immune population are more likely to be translated into successful therapies in the clinical setting.

1.3 QUANTIFICATION OF IMMUNE CELL INFILTRATE

1.3.1 Immunohistochemistry

Infiltration by inflammatory cells into malignant tumours is a frequently observed histopathological phenomenon. Estimation of the numbers of infiltrating lymphocytes into different tumour compartments is crucial to the understanding of the processes that govern tumour-host interaction and patient outcome. In order to better quantify this process, immunohistochemical staining of target cells is generally performed.

Immunohistochemistry (IHC) is a routinely used research and clinical technique to detect specific molecular markers antigens and their pattern of distribution within a tissue cell type. Coons and Jones are credited with the initial development of IHC in the early 1940s (Coons and Jones, 1941). However, it is only during the past 30 years that the method has become established as a major component of diagnostic surgical pathology, particularly in the field of cancer biology.

IHC exploits the principle of antibodies binding specifically to antigens in biological tissue. The immunologic reaction is then visualized by microscopic examination. Thus, IHC represents an ideal tool to assess the precise location of host immune components in different microanatomical regions of lung cancer specimens. In the case of detection of lymphocytes, individual antibodies have been rigorously evaluated and are widely employed by both diagnostic and research institutions, including those at the St. James's Hospital Diagnostic Pathology Laboratory and the Institute of Molecular Medicine at Trinity College Dublin.

1.3.2 Evaluation of immunohistochemical staining

There is no universally accepted method to quantify numbers of TILs in IHC specimens. A variety of scoring systems is used, with the choice of method determined by the immunohistochemical properties of marker of interest. Interpretation of IHC has traditionally been done manually, an approach considered to be the gold standard. Semiquantitative assessment is frequently employed to

estimate different cell populations in tissue specimens. Arbitrary cut-off levels for assessing the extent of cellular infiltration are commonly used though results from quantification of different IHC markers frequently show considerable inter- and intraobserver variation (Belien et al., 1999, Cregger et al., 2006).

Examination of tissue sections stained even by routine haematoxylin and eosin (H&E) implicitly necessitates interpretation. Evaluation of different cellular components is made subjectively, with past experience used as a reference point. Therefore, even standard H&E examination makes use of a 'semiquantitative' covert reference standard, against which judgements of what constitutes normal are made. Furthermore, it has been demonstrated that different observers will interpret the same H&E section differently (Sirota, 2005).

This applies to an even greater degree when interpretation of IHC is considered, as has been shown in proficiency-testing exercises carried out by the College of American Pathologists and the UK National External Quality Assessment Immunocytochemistry. In a multicentre trial to more closely examine this issue, Rudiger et al showed that nearly one quarter of expert pathologists falsely interpreted as negative IHC staining for the oestrogen receptor, a common diagnostic marker used in the evaluation of breast cancer histological specimens (Rudiger et al., 2002). A recurring problem identified is failure of consistently accurate enumeration of cells where intensity of staining varies and stating with certainty that a cell is 'positive' or 'negative'. This diagnostic dilemma may also, at least in part, have contributed to discrepancies in the published literature on the relevance of lymphocytic infiltration in lung cancer and hamper comparisons between different studies (Seidal et al., 2001).

To overcome the inherent variations in assessment techniques and assist in therapeutic decision making a number of scoring systems have been introduced and subsequently adapted. For example, the H-score was devised for assessment of the oestrogen receptor but has gained wide applicability (McCarty et al., 1985, Abd El-Rehim et al., 2005). This method is based on the extent of reactivity and intensity and is the sum of the percentage of cells with weak, moderate and strong staining with a range of 0 (100% negative cells) to 300 (100% strong staining).

Many investigators determine IHC immune infiltrate scores based on arbitrary semiquantitative cutoff points. This approach was initially developed in the 1950s and was based on crude grading of infiltration (Black et al., 1956). In this system, grade 0 corresponds to absence of lymphocytes and grades 1 to 3 correspond to increasing lymphocyte infiltration from a few scattered cells (grade 1) to marked infiltrate that mimics a lymphoid organ (grade 3). More recent modifications to this system employ subjectively determined cell density percentages. There is, however, no consensus as to what percentage values to use to segregate different groups, or even how many groups should be used (Al-Shibli et al., 2008, Petersen et al., 2006). Precise determination of numbers of immune cells in IHC sections represents a more accurate method of inflammation quantification and may have relevance in assessing response to treatment (Youssef et al., 1998). However, it is a much more laborious and time-consuming approach. In order to be validated, each of these various methods generally requires independent assessment of samples by at least two pathologists, further increasing the workload necessary.

In the case of TIL evaluation, identification of positively stained cells is somewhat facilitated by the distinct morphological appearance of lymphocytes. Even so, partial or suboptimal staining of individual cells within tissue section can influence whether they are classified as positive or not. Thus, subjective interpretation of staining will influence results of lymphocyte counts.

It is important to stress that reliable quantification of cellular staining is no less critical to immunohistochemical testing than is sample preparation (specimen fixation, paraffin embedding and subsequent deparaffinization) or tissue analysis (antigen retrieval, staining technique, antibody specificity and operator competence) (Taylor, 1992). Nevertheless, an unambiguous method that permits accurate and consistent quantification of the analyte of interest has the potential to overcome many of the inherent deficiencies of observer-based, subjective visual manual cell count methods. In this regard, the use of image analysis offers a number of advantages.

1.3.3 Automated image analysis of immunohistochemistry specimens

Evaluation of image analysis systems as a molecular oncology diagnostic

tool was initially undertaken in the field of cytology automation in the 1970s (Green, 1979, Taylor et al., 1975). These efforts focused on the analysis of blood cells and cervical smears to develop automated cytodiagnostic equipment. However, early success was restricted due to the simplicity of the images generated which usually contained a few isolated cells against a plain background.

Interest in automated immunohistochemical analysis has increased since the first computerised systems were introduced in the 1980s. Significant advances have been made in both software and hardware capabilities of image analysis systems. The last decade has witnessed continuous refinements in image acquisition technologies and data management applications of associated large computer files, since modern equipment allows the generation of extremely high resolution images of histological slides. Accumulated data now indicate that these techniques provide effective qualitative and quantitative means of evaluating different molecular markers of interest that is equivalent to that of expert pathologist interpretation (Bacus et al., 1988, Turbin et al., 2008). Indeed, results from comparative studies suggest that image analysis may even be superior to manual techniques for certain markers (Cross, 2001, Umemura et al., 2004). Moreover, modern image analysis systems can eliminate regions from histological preparations containing histological noise, such as cellular debris, necrosis and synthetic materials. These and other factors contribute to the difficulties faced even by experienced pathologists in performing manual examination of IHC specimens.

While human analysis outperforms automated systems in terms of feature recognition and object classification (such as distinguishing tumour from non-tumour), it is dependent on the experience and alertness of the interpreter (Walker, 2006) and is no better than computerized techniques at quantification of staining extent and intensity.

Automated image analysis offers a number of advantages over manual assessment. As opposed to human interpretation, a computer is completely objective in its analysis. Every time a computer is instructed to perform a task, it will do so the same way every time. Once a set of definitions is provided, such as defining what parameters are to be employed to identify positive staining for a T-lymphocyte

population, the computer will select all cells that match this inputted description and omit all those that do not. In this fashion much of the subjectivity that is associated with manual cell counting is overcome.

A degree of subjectivity remains, however, in establishing how the computer performs the analysis. The strict rules governing what constitutes a positive stain must be refined by the user in the first instance. Furthermore, the decision relating to which areas of tissue in which to perform the analysis remains subjective, since these regions are selected by the operator. However, once these rules are created the image analysis system will make objective choices. As a result, any concomitant errors that might arise from environmental factors such as operator fatigue or changes in ambient light in making a manual count are eliminated (Turbin et al., 2008). The automated counter is therefore perfectly consistent, and will generate absolutely identical counts for repeated analyses performed in the same digitized microscopy field, something that is not true for its human counterpart.

A second major advantage of image analysis is time saving. Computers are capable of performing complex analytical tasks in fractions of a second. When multiple measurements from large of amounts are data are required, the benefits are even greater. Lymphocyte counting is often an extremely laborious task, particularly in cases of abundant infiltration with hundreds of positive and negative cells in a single image. Moreover, the degree of human error in counting is a function of the number of the objects, such that the more cells analysed, the less precise the results are (Yoo, 2004). Thus, automated analysis has the potential to greatly reduce the amount of valuable research time required for this process.

The speed of the analysis is determined by the complexity of rules governing the analysis (such as computer algorithms) in addition to the degree of automation. An algorithm is a computer-based specific set of instructions for performing a task or solving a problem. Input algorithms that use multiple parameters to characterise lymphocytes will have increased accuracy but will slow the counting process. Creation of the computerized algorithm itself can also be time-consuming and requires validation to ensure its accuracy. The extent of human input required to

select the regions in which to perform cell counts will also determine the speed of the analysis (Turbin et al., 2008).

1.3.4 Cell detection by image analysis

Digital images consist of two-dimensional arrays of dots. On a computer screen these are referred to as pixels, the colour of which determines the picture of a whole. Measurements made using image analysis software depend on the distribution, number and individual value assigned to each pixel in the image. In an object of varying colour or shade, its size and shape are dependent upon the definition of where its edge lies. In coloured objects such as immunohistochemically stained lymphocytes this is determined by thresholding, a process by which an image analysis program determines what constitutes an object.

In order for the computed algorithm to accurately identify IHC stained elements within a region of tissue, a number of parameters must be established. In the case of lymphocytes, the key input parameters are positive staining colour threshold and morphologic criteria such as cell size, roundness and elongation. In assessment of lymphocyte counts, the setting of these thresholds is of critical importance. If the different threshold values are set too low, objects that would be easily recognized by the human eye as not being lymphocytes will be erroneously included. If thresholds are set too high, some lymphocytes will fail to be correctly identified and the resultant count will be too low.

The task of setting thresholds to identify lymphocytes based on their size, shape and colour is helped by the fact that the majority of these cells infiltrating into tumours show relatively little heterogeneity. Lymphocytes are in general fairly circular with uniform nuclear, cytoplasmic and/or membranous staining patterns. If immunohistochemical staining is uniform, the intensity of the antigen-antibody product (e.g. 3,3'-diaminobenzidine tetrahydrochloride) should also be similarly uniform. In order to calculate the number of cells per unit area, the number of pixels per μm^2 is calculated. The area of interest, tumour or stroma, is then calculated as a number of pixels and converted into International System (SI) of units.

Since cells are discrete three-dimensional entities, the ideal method of measurement of lymphocytes in tumour and stroma regions is cells per mm^3. However, such quantification would necessitate complex stereological tools such as 'optical dissector' or 'unbiased Brick' which are impractical for large projects and are themselves subject to bias (Guillery and August, 2002, Marrone et al., 2003) . As histological sections are quasi-two dimensional, a suitable measure is cells per mm^2. This is the most frequently employed method to quantify cell counts.

Several authors have combined manual counting methods and image analysis techniques. Statistical comparisons between the two measures, however, have been infrequently correlated. Norazmi et al used computer-assisted image analysis to quantify the number of $CD3^+$ and $CD45^+$ lymphocytes in frozen tissue sections of colonic tumours using the avidin-biotin peroxidase (ABC) technique (Norazmi et al., 1990). A computer with a video digitizer card mounted on top of a light microscope was used for the automated count assessment in this study. Detection thresholds were set to discriminate between brown, positively-stained cells and surrounding stromal regions. Visual analysis was classified according to whether lymphocyte infiltration was low, moderate, dense or very dense. A good correlation between cell counts obtained by visual analysis and that generated by image analysis was shown, prompting the authors to conclude that the two methods were compatible.

Loughlin et al studied ductal invasive breast cancer immunostained for CD3, CD4, CD8, and CD20 in order to detect different TIL populations and used the number of pixels in each image matching a partition of Lab Colour space to calculate the number of immunostained cells (Loughlin et al., 2007). 'Colour range' and 'Histogram' tools from Adobe Photoshop7 (Adobe Systems Incorporated, San Jose, CA) software were employed to perform the analysis. TIL counts for the different markers in this study showed up to a 100-fold between case variation, confirming the marked heterogeneity of infiltrative patterns between cases. There was, however, no comparison between the method described and manual counting.

Rexhepaj et al compared results obtained using automated quantification of nuclear staining of oestrogen receptor (ER) in breast cancer tissue from 743 patients

with those from manual assessment by an expert pathologist (Rexhepaj et al., 2008). A high correlation between automated and manual count methods was observed (Spearman's ρ =0.9, P <0.001) confirming the accuracy of the image analysis algorithm used in this study.

The various techniques described have yet to be routinely incorporated into diagnostic labs and have principally been employed as basic research adjuncts. Alternative methods of validating the accuracy of image analysis continue to be developed in order that they might gain more widespread acceptance. Such investigational approaches include the comparison of protein expression levels when quantified by automated means with traditional laboratory tests (such as Fluorescence in situ hybridisation (FISH) or Enzyme-linked immunosorbent assay (ELISA)) or with patient outcome (Conway et al., 2008).

1.3.5 Digital slide scanning

In order to run image analysis programs, high quality images are required. Digital slide scanning (DSS) technology facilitates the creation of an electronic representation of traditional glass slide which can be viewed at different magnifications on a computer. The process is similar to that of examining a glass slide using a traditional microscope and allows pathologists to navigate to any field of view at similar magnifications using images of sufficient resolution to render a correct diagnosis (Costello et al., 2003). A number of alternative methods of acquiring digitized images of slides are commercially available. Usually these are based on one of the following principles:

- taking a series of static images of a glass slide using a conventional light microscope and subsequent collating them together
- remote operation of a microscope capable of dynamically changing its field of view in order to capture a user-defined image
- high resolution scanning of the whole glass slide.

Virtual Slides provide users with similar functionality to that of a microscope, but with numerous additional benefits, including concurrent access for

multiple users, tracking of review movements and image annotation. An enormous benefit of DSS is the ability to share histopathological sections without physical transfer of slides. This process can now be accomplished using either file sharing technology or over a network or internet-based 'virtual' DSS viewing platform (Glatz-Krieger et al., 2006). Such technology has a number of clear benefits, both as a clinical tool and an educational device (Bloodgood and Ogilvie, 2006) and has been termed 'telepathology'.

One of the drawbacks of DSS is the necessity for adequate storage space for the associated large files. Depending on the platform used, a digitized slide that incorporates annotation or algorithm output data can require in excess of 350 Megabytes of storage (personal experience). The memory requirements for storing a full-face DSS are dependent on the area of tissue being scanned, the optical resolution (magnification) at which the scan is performed and the image file format/compression algorithm used for its storage. Data transfer times for such large files can be considerable. In cases where large numbers of slides are being studied, additional file storage computer drives may be necessary. An additional consideration is that some annotation algorithms require the creation of a separate file, necessitating additional storage space and requiring additional transfer time. It is also prudent to consider redundancy/back requirements for image data.

A further disadvantage of digital image analysis is the cost and expertise required for the introduction and maintenance of both software and hardware. However, costs continue to show a gradual trend downward. Appropriate training for investigators using this technology and proper technical support are additional essential requirements for completion of research projects.

1.3.5.1 Aperio Scanscope

Most companies marketing image analysis software also provide accompanying scanning hardware. The Aperio ScanScope CS Slide Scanner (Aperio Technologies, California, USA) was used to acquire whole-slide digitized images for the work described in this thesis. Aperio employs linear array devices (LAD) for image creation in which small number of contiguous overlapping image stripes are acquired. The LAD continuously moves the microscope slide during

image acquisition facilitating rapid slide digitalization and seamless images (Aperio-Technologies, 2008).

In order to highlight regions of interest, image processing software is generally incorporated into digital slide viewing technology. This permits the annotation of sections of tumour, stroma or other regions of interest contained on the slide, which can be subsequently modified as necessary. This software is incorporated as a package in the Aperio ScanScope platform. The Aperio system also incorporates a number of different image analysis packages that can be used (or modified) to identify nuclear, membranous or cytoplasmic cellular staining patterns. The results of algorithmic analyses are displayed as overlays on slide images.

1.3.5.2 Application of Aperio system in clinical studies

A number of studies reporting the use of Aperio's ScanScope® image digitization and different image analysis techniques have been published. Anraku et al used the Aperio ScanScope CS Slide Scanner system to scan human pleural mesothelioma tissue array sections in order to determine the clinical significance of different TIL populations (Anraku et al., 2008). ImageScope (Aperio Technologies) was used to highlight positive cells. However, the number of cells was manually counted by two investigators in this study. It is unclear whether these authors compared lymphocytes detected by the image analysis program to a manually selected method.

Aperio-based image analysis has also been employed to quantitatively determine the expression in breast cancer IHC specimens of survivin, an anti-apoptotic protein that is upregulated in several tumour types (Brennan et al., 2008). Whole-slide scanning with the Aperio ScanScope CS Slide Scanner was used to capture high resolution images which were then analysed using a novel automated algorithm. By incorporating the automated technique, the authors showed that high cytoplasmic-to-nuclear survivin ratio was independently prognostic. Of note, this new prognostic subgroup was not evident following manual analysis.

Rexhepaj et al used the Aperio ScanScope XT slide scanner to capture images of breast cancer tissue (Rexhepaj et al., 2008). Image analysis algorithms

were developed using MatLab 7 (MathWorks, Apple Hill Drive, MA, USA). A fully automated nuclear algorithm was developed to discriminate tumour from normal tissue and to quantify oestrogen receptor (ER) and progesterone receptor (PR) protein expression. Using image analysis, these investigators found, and in a separate patient cohort validated, a cutoff threshold (7%) for ER staining of tumour cells that predicted a better response to tamoxifen treatment.

1.3.6 Selection of regions of interest for immune cell evaluation

A key issue that is frequently overlooked in evaluation of the results of studies assessing the prognostic relevance of lymphoreticular infiltrate is the method used to determine where cell counts are made. However, the critical importance of this methodological consideration is rarely emphasized. Indeed, several methods have been employed and there is no consistent agreement as to which of these is preferable or most representative.

Most commonly, investigators have chosen areas of highest immune density, as subjectively assessed (Hussein and Hassan, 2006, Ruffini et al., 2009). However, these operator-defined 'hot-spots' may not be representative of the immune response to the tumour in general. Recent work by a group of French investigators has highlighted the prognostic significance of distinct intratumoral tertiary lymphoid structures, referred to as tumour-induced bronchus-associated lymphoid tissue (TiBALT) in lung cancer IHC specimens (Dieu-Nosjean et al., 2008). Similar findings have been reported in other tumour types (Coppola and Mule, 2008). It is unclear whether the functional activities of lymphocytes residing in ectopic lymphoid structures mirror those of non-clustered TIL populations. Thus, previously published work that focused on regions of potent inflammatory response within tumour sections may in fact have been inadvertently examining a distinct lymphocyte subset.

Other published work has referred to the selection of regions that are termed 'representative' in order to perform inflammatory infiltrate cell counts (Welsh et al., 2005, Kim et al., 2008). This is, however, by definition, an ambiguous term. It implies not only that high power fields (HPFs) exist in a given IHC section that are

indeed representative (as subjectively assessed) of the pattern of infiltration within the tumour as a whole, but also that the user is capable of making such a selection.

A more straightforward and objective evaluative approach is the selection of random HPFs to perform cell counts (Zeni et al., 2007, Johnson et al., 2000). Such a method would completely eliminate subjective regional selection biases based on either abundant lymphocyte infiltration or complete tumour evaluation.

1.3.7 GENetic Imagery Exploitation

High resolution images created from DSS generate vast volumes of multispectral data. Automated extraction of specific features of interest, such as regions of tumour or stroma from histopathological slides, would accelerate the analytical process, since it would facilitate inclusion only of regions of tissue that are suitable for evaluation, while excluding regions of histologic noise that would compromise the accuracy of cell counts.

The GENetic Imagery Exploitation (GENIE™) algorithm is an adaptive automatic feature extraction (AFE) software tool that was originally developed at the Los Alamos National Laboratory for the analysis of satellite images of the surface of the earth. More recently, this technology has been adapted for use in different biomedical imagery applications. GENIE comprises an artificial intelligence platform that applies statistical machine learning theory and evolutionary computation theory to perform customized and reproducible image analysis using automatically 'learned' spatio-spectral characteristics (Perkins et al., 2000).

GENIE has recently been adapted as a histopathology pattern recognition tool. Modifications to its AFE software have been incorporated to enable preprocessing capable of identifying and categorizing specific histologic tissue types, thus allowing subsequent analysis of target regions by standard image analysis modes.

By considering the spatial context of each computer pixel of a digitized multispectral image, GENIE performs refined morphological filtering in an

evolutionary fashion to distinguish between similar cytopathologic features in a marker of interest, for example immune cells infiltrating tumour islet and stroma of lung cancer sections. This is accomplished through a series of mathematical algorithms termed 'chromosomes' (Harvey et al., 2002). Each chromosome represents a sequence of 'genes', which are basic image analysis morphological and spectral operations that are further optimised by GENIE to meet the desired output. The genes on which the mathematical model is constructed are analogous to those in animal biology. GENIE then assigns an individual fitness score related to how well each chromosome classifies a set of training images. At the end of each successive generation, the chromosomes participate in processes of crossover and mutation, with the probability of selection being based on their fitness. Throughout the evolution process, only appropriate algorithms with appropriate data input will survive. Having completed this process, the most closely matching chromosome, based on both spectral and spatial domain information, is then automatically selected by GENIE, to serve as the final solution.

The study of GENIE in the field biotechnology has increased manifold in recent years and there is an emerging interest in its application in biomolecular research. Use of GENIE to study cancer pathobiology is, however, in its infancy and relatively few investigators have published in the field. Nonetheless, a number of encouraging results using this technology have emerged.

Angeletti et al used GENIE to assess whether detection of malignant urothelial cells in urine samples using a fully automated system was possible (Angeletti et al., 2005). The devised detection algorithm was based on quantitative assessment of both colour (spectral properties) and shape (spatial properties) of cells. In a training set, GENIE was able to differentiate malignant from benign cells and estimate the relative proportions in controlled mixtures. When tested on routine diagnostic cytology specimens, GENIE-based analysis had sensitivity and specificity rates of 85% and 95%, respectively. Furthermore, after training on cases reported as 'atypical' but with definitive confirmation or exclusion of malignancy on clinical follow-up, GENIE was superior to the pathologist with respect to predicting the follow-up result in a patient cohort.

GENIE was also applied by Stromberg et al to examine differential expression of ki67, DLG5 and syntaxin-7 in human melanocytes and malignant melanoma (Stromberg et al., 2009). In this paper, tumour regions of melanoma tissue cores were identified and distinguished from stromal components by GENIE after input of a novel algorithm.

1.3.8 Summary

Computerised image analysis techniques are becoming commonplace in both the research and diagnostic laboratory. There is now an extensive published literature describing the validation of different methods of automated IHC evaluation. It is likely that image analysis technology will show continued refinements in the future. Performing repetitive cell counts in multiple areas of multiple cases is greatly facilitated by automation and computer analysis would remove the inherent subjectivity that may contribute to the inconsistent results of studies evaluating inflammatory cell infiltrates. A move away from the semiquantitative manual scoring models that are currently the norm should in theory lead to less variability in results while increasing throughput. Currently, a variable amount of manual input remains necessary, in particular choosing areas in which to perform cell counts. Increasingly sophisticated software is likely to require less and less operator input which will further improve speed, accuracy and reproducibility of image analysis approaches. In order for automated analytical systems to have wider applicability, however, applications must be easy to use, reproducible, robust and standardized.

1.4 OBJECTIVES

The current TNM staging system for NSCLC remains far from accurate in forecasting survival of individual patients, as 50% of patients treated by surgical resection for early-stage disease will develop recurrent disease. Although significant revisions of the TNM system have been proposed, the prediction of survival at the individual level is not expected to improve significantly. Recent publications suggest that biomarker profiles constructed from patient series with long and accurate follow-up outperform standard pathologic TNM staging in estimating risk of disease recurrence. The overall aim of this work was therefore to examine the prognostic significance of the immune response in lung cancer using different methodologies.

In order to examine the clinicopathological factors associated with outcome in patients who undergo surgical resection for NSCLC, a database incorporating data from patients treated over a 10 year period was constructed and prospectively updated. Patient outcomes after surgery were compared to international published data. The prognostic relevance of differential tumour/stroma lymphocyte infiltration was assessed in a cohort of patients treated at St. James's Hospital from 2001-2005 that were followed prospectively. The quantification of lymphocyte infiltration was made using a novel image analysis system. Algorithms to reliably and accurately quantify cell counts in the different tumour compartments were designed and compared to the standard method of manual counting.

In addition to direct cytotoxic effects, several chemotherapeutic agents used in NSCLC have demonstrated immunomodulatory properties in experimental models. To investigate whether neoadjuvant chemotherapy influences cytokine gene expression in circulating lymphocytes and if these changes correlated with response to therapy, the quantitative real-time polymerase chain reaction (Q-PCR) method was employed to measure circulating monocyte cytokine mRNA levels in patients treated with combination cetuximab/cisplatin/gemcitabine prior to surgical resection.

CHAPTER 2

METHODS AND MATERIALS

2.1 REAGENTS

All laboratory chemicals and reagents were stored according to manufacturers' instructions. They were of analytical grade and were purchased from BDH Laboratories (Poole, Dorset, UK) and Sigma Chemical Company (MO, USA) unless otherwise stated. Solutions were autoclaved following preparation. Gilson pipettes were used for transfer of liquid volumes up to 1 mL (Gilson S.A., France). For volumes greater than 1 mL, an electronic pipette aid (Drummond, PA, USA) and disposable plastic pipettes (Starstedt Ltd., Wexford, Ireland) were used.

2.2 IMMUNOHISTOCHEMICAL STAINING

2.2.1 Study population

This study has received full ethical approval from the St. James's Hospital & Federated Dublin Voluntary Hospitals Joint Research Ethics Committee. NSCLC tissue was obtained from, routinely formalin-fixed, paraffin-embedded archival material (St. James's Hospital Pathology Department, St. James's Hospital, Dublin 8) taken from surgical lung resection specimens between January 2001 and January 2005. The total study population was 197 and consisted of a sequential series of patients with pathologically confirmed stage I-IIIA NSCLC who had underwent surgery with curative intent.

Full clinicopathological information was collected for all patients to include: demographics, smoking status, treatment approach, combined clinical and surgical staging results, histological subtype, tumour grade and outcome data including peri-operative mortality and long term survival. Data was derived from review of case notes and histopathology reports.

Patients who underwent sublobar resection (generally because of medical co-morbidities) or in whom intraoperative mediastinal nodal dissection was not performed were omitted from the dataset. Individuals that were upstaged postoperatively to stage IIIB or stage IV or in whom final histopathological

examination demonstrated patterns other than NSCLC exclusively were similarly excluded.

All surgical candidates were evaluated preoperatively with history and physical examination, blood testing, electrocardiography, flexible bronchoscopy, pulmonary function testing and CT of thorax and upper abdomen. A proportion of cases had preoperative PET scanning performed. This latter technique, based on evaluation of intensity of uptake of ^{18}F-fluorodeoxyglucose, was incorporated as a routine staging procedure at our centre from July 2004 onwards. Where imaging studies (CT or PET) identified possible regional lymph node metastasis, transbronchial nodal sampling and/or cervical mediastinoscopy was performed. Additional investigations including isotope bone scintigraphy and magnetic resonance imaging (MRI) were performed as necessary to exclude the presence of metastatic disease. Where possible, lung conserving bronchoplastic operative techniques (e.g. sleeve resection) were employed.

All cases were individually discussed both before and after surgery at a multidisciplinary lung cancer conference in order to correlate clinical, endoscopic, radiological and histopathological data and thus establish a final pathological stage according to the UICC staging classification (Mountain, 1997). Determination of patient status was by means of chart review or by telephone.

2.2.2 Antibodies

2.2.2.1 CD3

The anti-CD3 antibody was used for detection of the CD3 (cluster of differentiation protein 3) antigen, a protein complex composed of four distinct chains that forms part of the TCR complex common to all mature T-lymphocytes (Mason et al., 1989).

2.2.2.2 CD8

Anti-CD8 antibody was used for detection of cytotoxic $CD8^+$ T-lymphocyte cells (Mason et al., 1992). CD8 is a transmembrane glycoprotein that serves as a co-receptor for the TCR and is specific for the class I MHC.

2.2.2.3 Foxp3

Anti-Foxp3 (forkhead box P3) antibody was used for detection of regulatory T cells (Tregs) (Hori et al., 2003). Foxp3 belongs to the forkhead/winged-helix family of transcriptional regulators and are believed to play a critical role in mediating immune tolerance.

2.2.3 Microtomy

Formalin-fixed, paraffin embedded tissue specimens were taken and placed on an icepack for 30 min. Each specimen was removed and several sequential 5 μm thick sections were cut using a Leica RM 2135 microtome (Laboratory Instruments and Supplies, Dublin, Ireland). The sections were floated out on a waterbath at 45°C and were then mounted onto poly-L-lysine-coated glass slides (BDH Laboratory Supplies, Poole, England). The sections were incubated overnight in a tissue-drying oven (Binder, Tuttlingen, Germany) at 50°C. Dried sections were stored in slide boxes at 4°C until used.

2.2.4 Haematoxylin and eosin staining

To examine tissue morphology, a slide section from each of the lung cancer specimens was stained with haematoxylin and eosin (H&E). Slides to be stained were loaded into a glass slide rack. Sections were dewaxed and rehydrated in the fumehood through the sequential steps listed in Table 2.1.

Slides were then placed on a staining tray and the tissue was incubated with Harris Haematoxylin (BDH Laboratories) using a pasteur pipette for 10 min. Slides were then rinsed with tap water for 1 min. Sections were next covered with acid alcohol (1% hydrochloric acid in 70% alcohol) using a pasteur pipette and incubated for 10 secs to differentiate tissue. Sections were washed in running water for 5 min. Using a pasteur pipette eosin (BDH Laboratories) was applied to the tissue and incubated for 5 min. For some sections a further application was necessary to prevent the tissue from drying out. The slides were then transferred to a glass slide rack and the eosin removed by rinsing thoroughly in tap water for 3 min. Section were subsequently dehydrated and cleared through the steps listed in Table 2.2.

Reagent	Time
Xylene 1	5 min
Xylene 2	5 min
Xylene 3	5 min
100% Methanol	15 sec
100% Methanol	3 min
90% Methanol	3 min
80% Methanol	3 min
70% Methanol	3 min
50% Methanol	3 min
30% Methanol	3 min
Tap Water	3 min

Table 2.1 Sequence for slide-mounted tissue dewaxing and deparaffinization

Reagent	Time
100% Methanol	15 secs
100% Methanol	15 secs
Xylene 4	5 min
Xylene 5	5 min
Xylene 6	5 min

Table 2.2 Sequence for slide dehydration and clearing

Slides were mounted by placing a drop of hydrophobic mounting medium (distyrene/tricresyl phosphate/xylene: DPX) (BDH Laboratories) on a coverslip (VWR International, West Chester, PA, USA) and lowering the slide onto the coverslip. After mounting, slides were left to dry in the fumehood for at least 30 min.

The H&E stained slides were examined using a light microscope (Leica Microsystems GmbH, Weltzar, Germany) with a trained pathologist to ensure that the section contained viable tumour and that the sections were taken from the advancing tumour edge.

2.2.5 Deparaffinization, rehydration and antigen epitope retrieval

A slide rack was loaded with 10 untreated slides and placed into a black plastic staining dish containing 200 mL of 1X Trilogy antigen retrieval solution (Cell Marque, Hot Springs, AZ, USA). A second staining dish was also filled with 200 mL of 1X Trilogy. Both dishes were covered and placed in a pressure cooker, filled with 900 mL of tap water and treated in the pressure cooker for 15 min. The slides from the first staining dish were transferred into the second dish using a forceps to lower the rack into the hot 1x Trilogy solution.

Slides were agitated on the Gyro rocker (Stuart Scientific, Staffordshire, England) at 30 RPM and let sit for 5 min. The solution from the first dish was discarded but rinse solution from the second dish was kept in order to be re-used for the next antigen retrieval run. The slides were rinsed in 1x phosphate buffered saline (PBS) (13.8mM NaCl, 2.7mM KCl, pH 7.4) ensuring total immersion by wash solution.

2.2.6 Immunohistochemical staining

Following deparaffinization and rehydration of the sections, immunohistochemical staining was carried out. The slides were removed from the PBS solution and incubated in a covered slide container for 30 min at room in 200 mL of peroxidase block (3% hydrogen peroxide solution containing 20 mL 30% hydrogen peroxidase/ 180 mL methanol) to block endogenous peroxidase activity. Sections were then directly rinsed 3 times for 5 min in PBS solution.

The Vectastain ABC immunoperoxidase kit (Vector Laboratories, Burlingame, CA, USA) was used for immunodetection according to the manufacturer's instructions. This system uses a biotinylated antibody and a pre-formed avidin: biotinylated enzyme complex, referred to as the ABC technique, to detect tissue antigens. This technique was first described by Hsu et al and has been extensively validated (Hsu et al., 1981). Mixing bottles included in the Vectastain ABC kit were used to prepare working solutions of reagents as follows:

Blocking normal serum	3 drops normal blocking serum + 10 mL buffer in mixing bottle
Biotinylated antibody	3 drops normal blocking serum + 1 buffer in mixing bottle, followed by 1 drop of biotinylated antibody stock
Vectastain ABC Reagent	2 drops Reagent A + 5 mL of buffer in ABC reagent large bottle. Then 2 drops of Reagent B to the same mixing bottle

Table 2.3 Vectastain ABC solution preparation

The ABC Reagent was allowed to stand for 30 min. Sections were incubated with the diluted blocking serum for 30 min after which any excess serum was blotted from the sections. Slides were then incubated with 200 µL of diluted primary antibody for one hour (Table 2.4). Sections were transferred to a slide dish and washed 3 times for 5 min in PBS. Sections were then incubated in diluted biotinylated secondary antibody for 30 min at room temperature. Sections were next transferred to a slide dish, washed again 3 times for 5 min in PBS and incubated for 30 min in avidin-biotin complex reagent. Following this, the sections were washed 3 times for 5 min PBS.

Antibody	Clone	Supplier	Primary Antibody Conditions	Antigen Retrieval Required?
CD3	Polyclonal	Novacastra	1:30 1 hour incubation	Yes
CD8	4B11	Novacastra	1:30 1 hour incubation	Yes
Foxp3	236A/E7	eBiosciences	1:100 1 hour incubation	Yes

Table 2.4 Antibodies and conditions used for immunohistochemical staining.

2.2.7 Staining and counterstaining

One tablet of 3,3'-diaminobenzidine tetrahydrochloride (DAB) was dissolved in 15 mL PBS in a 50 mL plastic tube covered in tinfoil and kept on ice. 12 µL of 30% hydrogen peroxide added. Excess buffer was tapped off and sections were transferred to a staining tray and incubated in DAB solution using a clear plastic pasteur pipette. The tray was covered in tinfoil. After approximately 5 min the DAB was rinsed from the sections using tap water. DAB results in a brown reaction product. Using a pasteur pipette one drop of haematoxylin was added to each section and rinsed off immediately under tap water over a sink

2.2.8 Slide cleaning and mounting

Slides were transferred from the staining tray into a slide holder and placed in a gentle stream of tap water for 5 min. Slides were then transferred to a glass slide holder and dehydrated by dipping 10 times each in 70% methanol, followed by 90% methanol, followed by 100% methanol. Slides were transferred in 100% methanol to the xylene in a fumehood, placed in xylene container 4 for 5 min, then

xylene container 5 for 5 min and left overnight in xylene 6 overnight. The sections were mounted using 1 drop of DPX and coverslipped.

Stained sections were stored in plastic slide boxes in the dark at room temperature until slide scanning was performed.

2.2.9 Controls

Formalin fixed, paraffin embedded skin sections (5 μm thick) from human appendix were used as a positive control for each of the antibodies. Appropriate isotype negative controls, with omission of the primary antibodies were similarly used in all assays.

2.3 LYMPHOCYTE ANALYSIS

2.3.1 Scanning of slides

After antibody staining had been performed, the Aperio ScanScope CS Slide Scanner (Aperio Technologies, California, USA) system was used to acquire whole-slide digitized images with a 20x objective. In this technique, the IHC stained glass slide is placed on a microscope with a motorised stage and an automatic focusing facility that incorporates a specialised scanning device.

Digitized images were stored as ScanScope Virtual Slide (.SVS) files, an image format unique to the Aperio system. The freely available Windows-based software program ImageScope was downloaded and installed to enable viewing of images (http://www.aperio.com/download-imagescope-viewer.asp). Using this program, images can be viewed on any computer with a virtual microscopy interface. The user can press keys or employ a standard USB operated mouse to change magnification from an overall low power view up to the resolution at which the slide was scanned. All subsequent annotations to the DSS were made using a Sony Vaio VGN-FZ21Z laptop computer (Tokyo, Japan).

2.3.2 Development of cell detection algorithm

The standard method of performing a cell count analysis is by means of a manual count of stained cells, the numbers of which are then recorded and expressed per unit area. We developed a novel, quantitative, automated image analysis cell count algorithm program that identifies individual positive immunohistochemically-stained cells on the basis of cell and nuclear size and shape, nuclear to cytoplasmic ration and staining colour intensity. This enables the detection of positive stained cells using a general pattern recognition tool. The technology also facilitates the reproducible categorization and consistent detection of immunohistochemically-stained cells across all regions of tissue (both tumour and stroma) and avoids potential human detection and selection error when such counts are performed manually. Image analysis of digitized images thus provides practical quantification of immune cells by replacing subjective with objective evaluation and therefore enables greater precision of consistent cell counting.

The lymphocyte detection algorithm was developed with the help of Elton Rexhepaj, PhD student, UCD School of Biomolecular and Biomedical Science, University College Dublin. This algorithm employs a set of detection thresholds based on morphometric and colour characteristics to define 'positive' cells based on a user-defined training set and distinguish these cells from adjacent tissue types. In order to perform an accurate count of the number of lymphocytes in a selected section, the image analysis program was required to satisfy a number of requirements. Essentially, this involves the creation of a set of rules that replicate those employed in performing a manual count. In order to accomplish this, the computer must:

1. Identify lymphocytes
2. Ignore objects that are not lymphocytes
3. Recognise separate lymphocytes when they are touching and not count them as a single cell
4. Not count lymphocytes that cross over user-annotated margins of a region or tumour or stroma
5. Express the count per unit area

To ensure validity of the image analysis cell count algorithm program, a manual lymphocyte count from the first 20 sections was initially performed. Five manually selected random HPF (400x magnification) were in turn selected from each digitally scanned slide and the total number of subjectively determined positively stained lymphocytes recorded. Initial training in identification of positively stained lymphocytes was performed under the supervision of a trained pathologist.

The image analysis program was then run and an automated cell count performed. The two sets of data generated from the different methods of cell counting were then directly compared to ensure validity of the automated count method. Once the results obtained showed a significant correlation, the remainder of the slides were examined. This procedure was repeated for each of the markers.

2.3.3 Selection of regions for evaluation

Different methods have been employed to select regions within target tissue from which to perform cell counts. These approaches have generally selected a number of HPFs that show abundant lymphocyte infiltration or are deemed 'representative' of the immunohistochemically stained section. In order to eliminate potential bias in selection of regions in which to perform cell counts, a novel method for random HPF generation was devised. The ImageScope image analysis software (Aperio Technologies) was adapted for this purpose.

A medium power field (10x magnification) of a region of tumour tissue that contained adequate viable tumour and stroma was identified (**Figure** 2.1). Over this region a grid containing 10 x 10 boxes was superimposed. From this grid, a series of 25 randomly-selected regions were sequentially generated by the program. The first five of these HPFs were evaluated (Figure 2.2). This figure was selected as it has been validated by different investigators in previous studies of inflammatory infiltrate quantification (Welsh et al., 2005, Kondratiev et al., 2004, Kim et al., 2008).

If any of the initial random HPFs was characterized by extensive tumour necrosis or displayed prominent lymphoid aggregates an alternative HPF from the initial set of 25 was randomly generated for analysis. Similarly, if a HPF showed a lack of tumour tissue suitable for evaluation (e.g. at the extreme edge of tumour margin or interface with normal lung architecture), an alternative region was selected. Examples of regions excluded from analysis are shown in Figure 2.3. If there were fewer than five HPFs from the initial randomly generated set of 25 suitable for examination, a further random 25 HPFs were created and the evaluative process repeated.

All tumour and stromal regions from each of the five HPFs were then separately annotated at 400x magnification (Figure 2.4) using the Volito2 pen tablet (Wacom, Saitama, Japan). Tumour and stroma layers were distinguished in markup images by separate colours (green for tumour regions, yellow for stroma regions). Each HPF contained variable amounts of tumour and stromal regions.

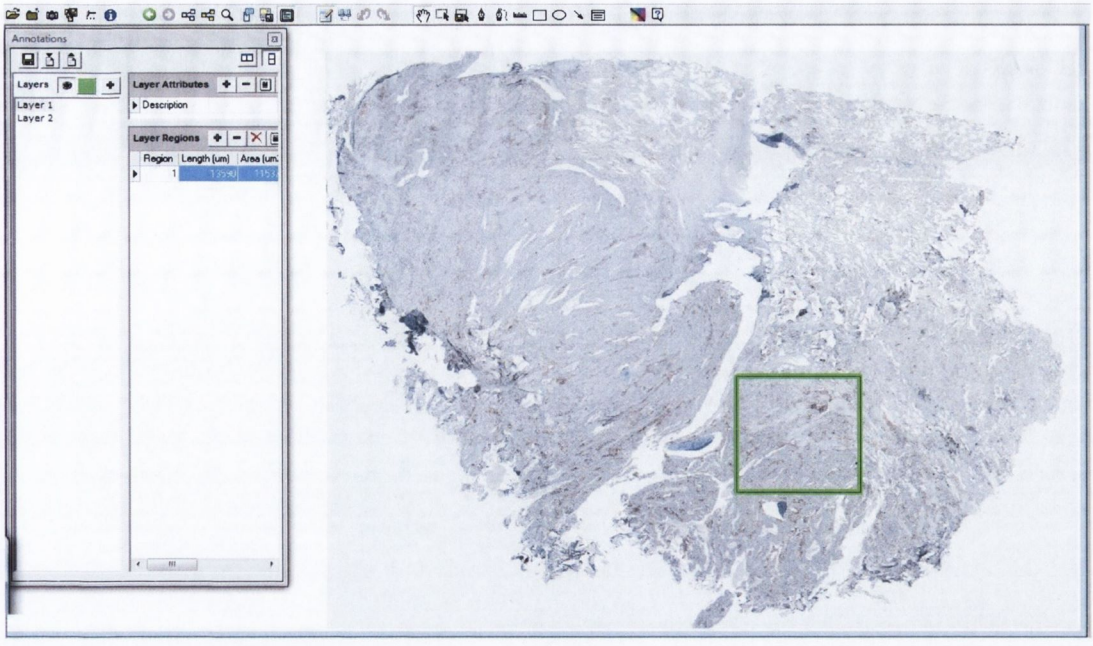

Figure 2.1.1 Medium power (10x) view of immunohistochemically stained NSCLC section with superimposed grid over region demonstrating viable tumour and stroma

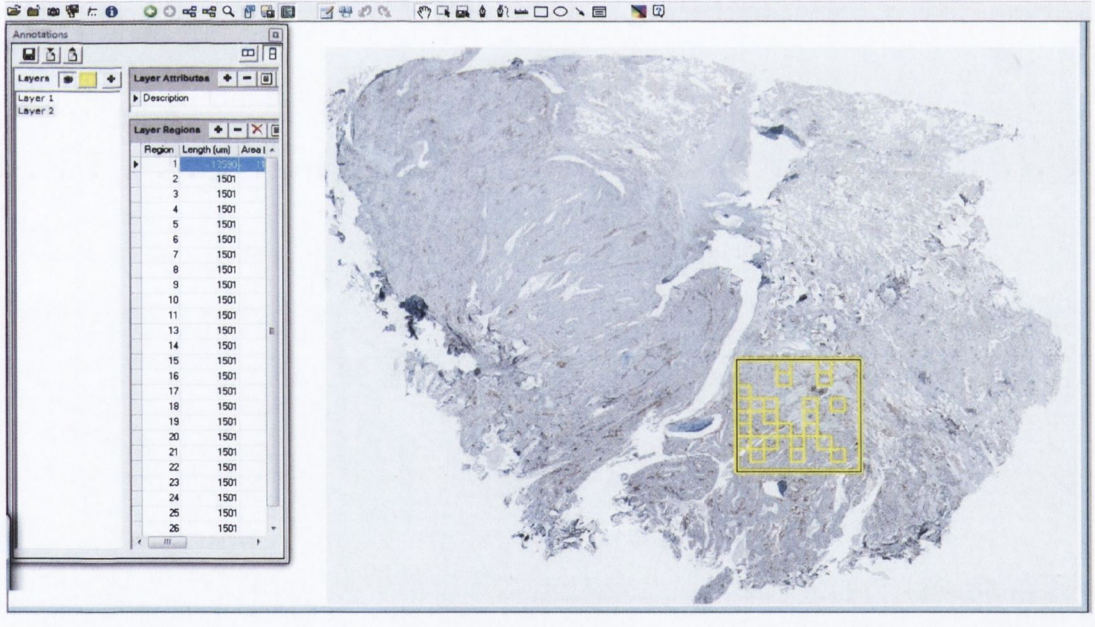

Figure 2.1.2 Medium power view (10x) of immunohistochemically stained NSCLC section with superimposed grid after generation of 25 random HPF boxes

Figure 2.1 Random generation of HPFs

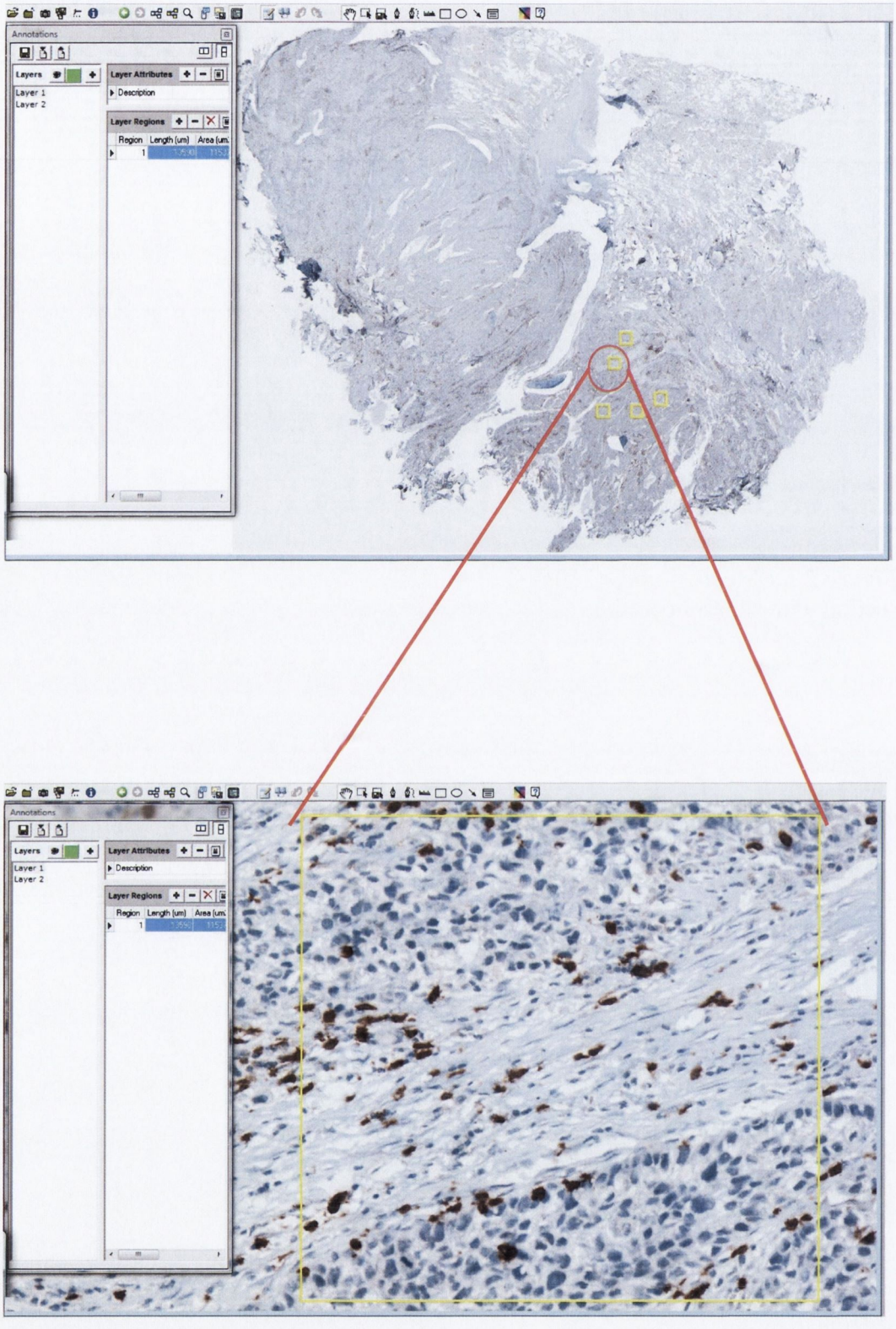

Figure 2.2 Suitable HPFs for analysis Random HPF demonstrating regions of tumour and stroma shown at 10x (upper image) and 400x (bottom image)

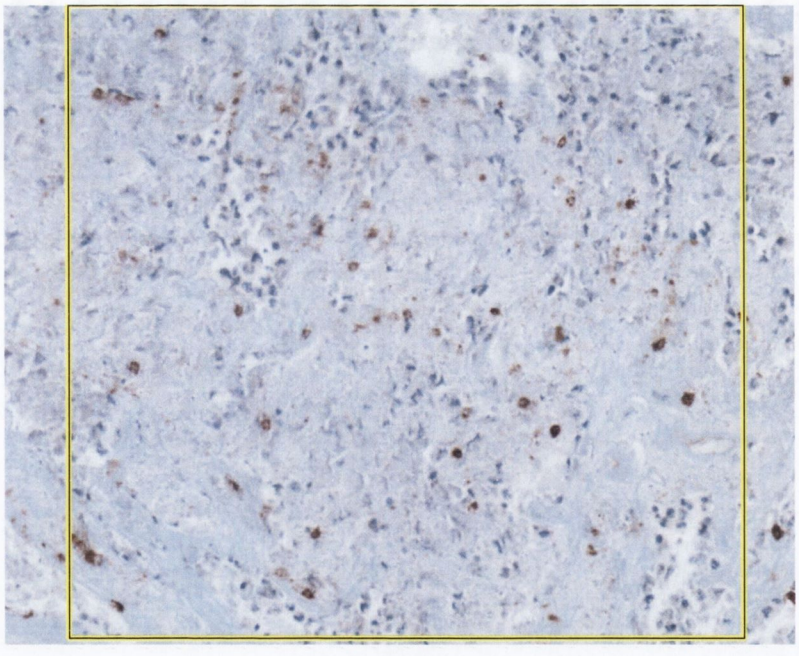

Figure 2.3.1 Randomly selected HPF demonstrating necrotic tumour

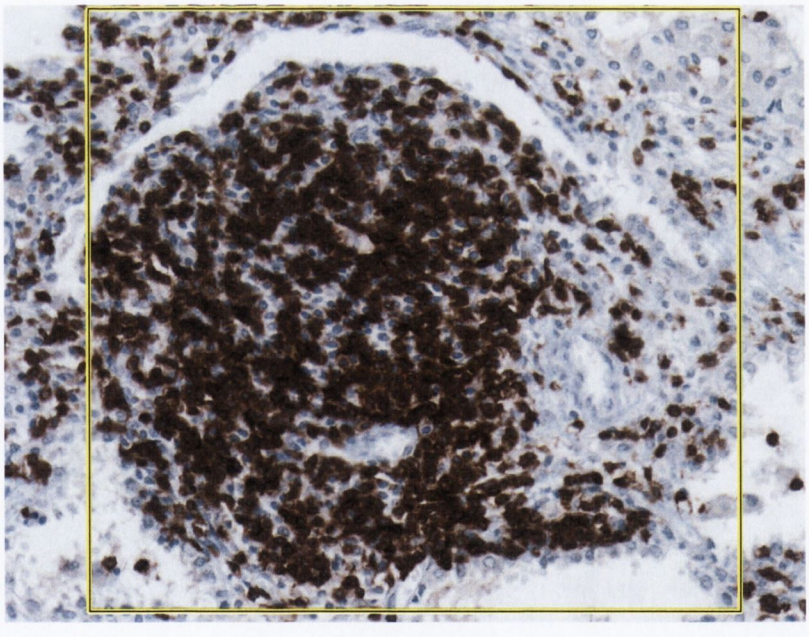

Figure 2.3.2 Randomly selected HPF demonstrating lymphoid aggregate

Figure 2.3 Regions excluded from lymphocyte count evaluation

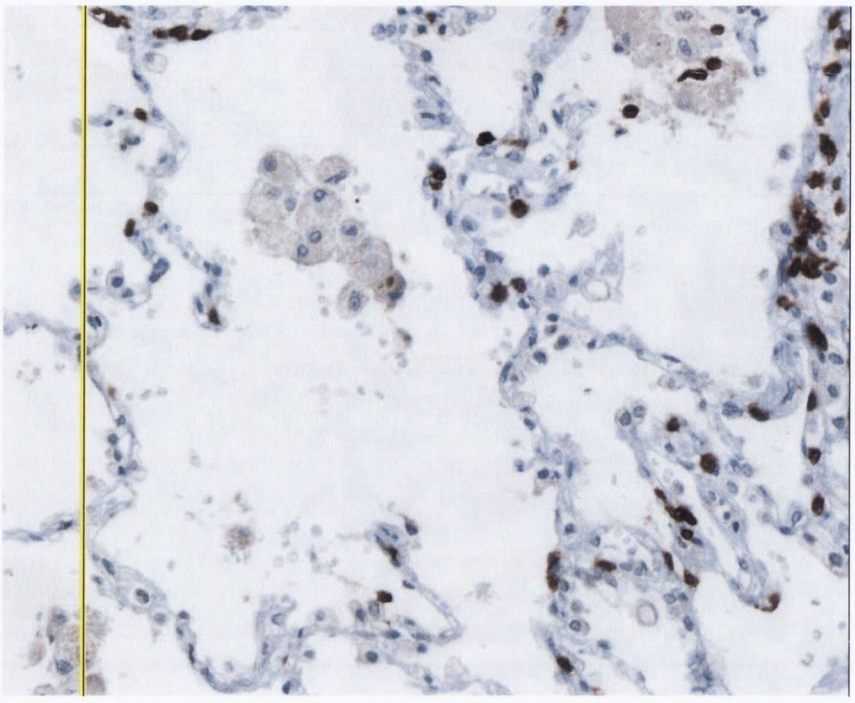

Figure 2.3.3 Randomly selected HPF demonstrating lack of tissue suitable for evaluation

Figure 2.3 (*contd.)* Regions excluded from lymphocyte count evaluation

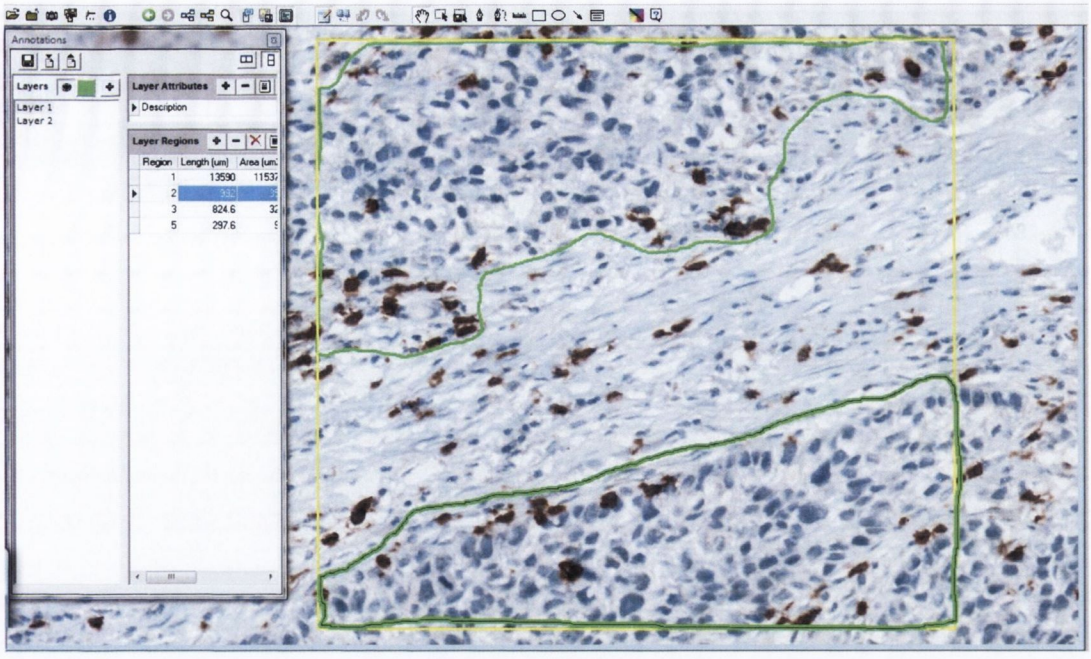

Figure 2.4.1 Markup image of tumour annotation layer (green) within a randomly selected HPF (yellow)

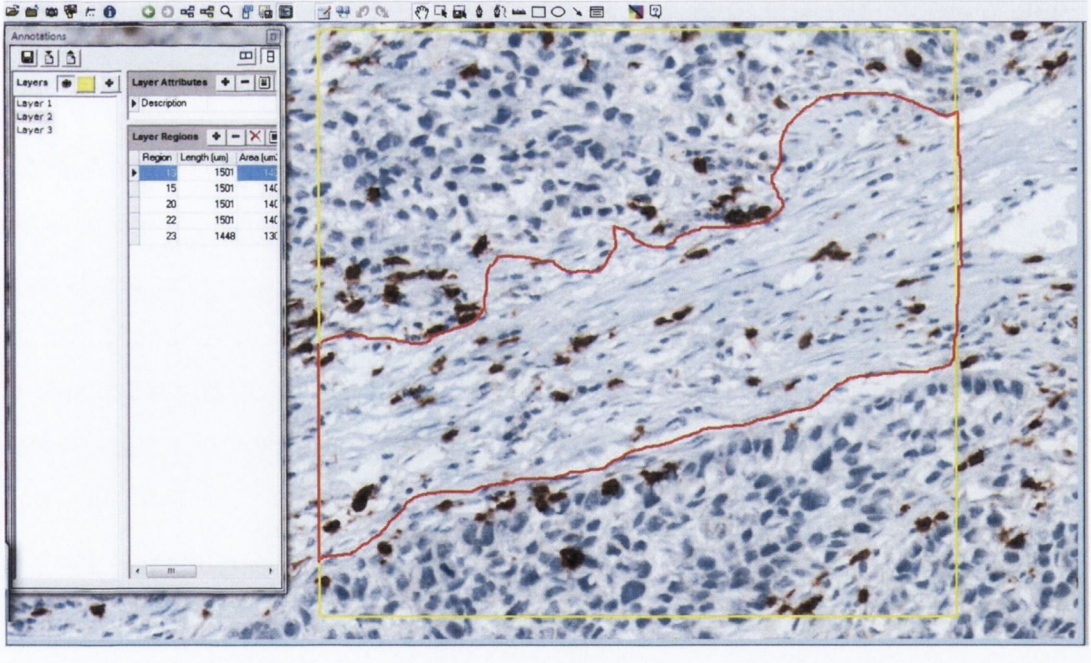

Figure 2.4.2 Markup image of stroma annotation layer (red) within a randomly selected HPF (yellow)

Figure 2.4 Annotation of tumour and stroma layers

2.3.4 Selection of regions of interest with GENIE

A GENIE project and training set was created in Aperio Technologies' Spectrum Plus image analysis software. The program was designed by Elton Rexhepaj and trained to identify different types of tissue in NSCLC slides. For the training set, three region of interest (ROI) classes using the manually defined annotations were used; tumour islets (annotated in yellow), surrounding stroma (annotated in green) and background (white space) as shown in Figure 2.4. An IHC-stained NSCLC section was used to train GENIE to identify the three ROI classes defined in the training set. Once a suitable GENIE classifier tool was created, the training solution was applied to analyse the remainder of the IHC-stained sections.

To identify regions of tissue in which automated lymphocyte count was performed, the GENIE algorithm was selected in ImageScope. The non-tumour non-stromal regions (necrotic tissue and lymphoid aggregates) and background (regions with no tissue) were then deselected to eliminate these classes from the analysis. This allowed a more accurate representation of true tumour- and stroma-infiltrating lymphocyte counts.

2.3.5 Analysis of tumour and stroma lymphocyte counts

Within each of the randomly selected HPFs, all regions of tumour were identified as layer 1 using the ImageScope software and annotated; stromal regions were labelled as layer 2. For each slide, the total number of computer pixels within the annotated tumour regions of each of the five HPFs was summed. The area corresponding to annotated tumour regions was then automatically calculated and expressed in mm^2. The same process was repeated with respect to stromal regions. The total number of HPF images annotated was 196 x 3 x 5 = 2940; this figure represents the numbers of patient samples (196) for each of the antibodies tested (3) that were examined in randomly selected HPFs (5).

The absolute number of positive-stained cells in each layer was determined using the image analysis program. For each section, the number of cells per unit area of tumour and stroma, respectively, was calculated for each of the cell markers and expressed per unit area (μm^2). If the tumour or stroma lymphocyte count was 0 (i.e. the region of analysis contained no lymphocytes) this was changed to 1 in order

to generate a usable ratio. Analysis of lymphocyte count was performed without knowledge of the clinical outcome. **Figure 2.5** illustrates the sequence of steps that was followed for each slide.

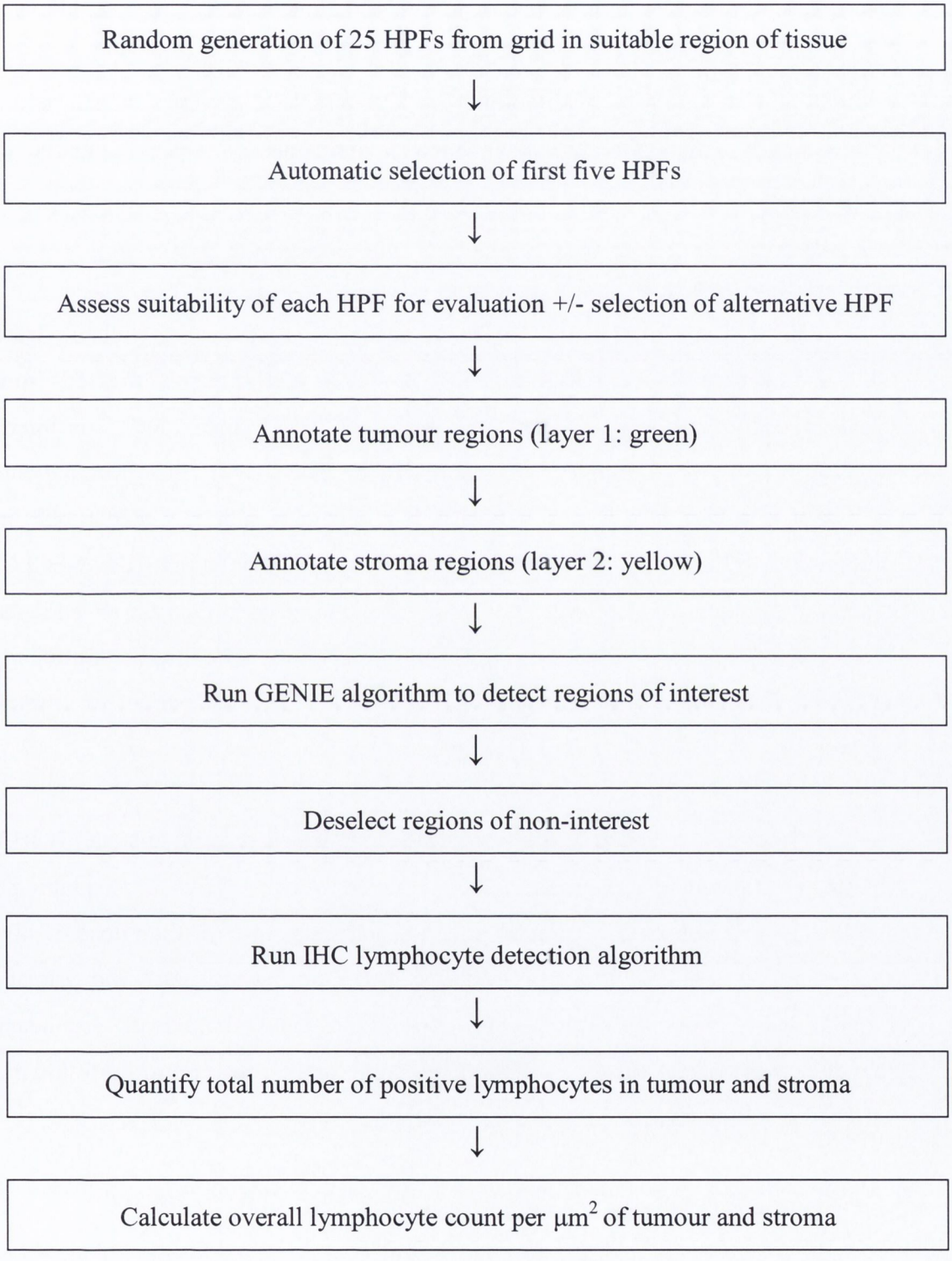

Figure 2.5 Sequence of steps in lymphocyte counting

2.4 QUANTITATIVE POLYMERASE CHAIN REACTION

2.4.1 Aim

A retrospective study to examine changes in a panel of cytokines in circulating lymphocytes from a cohort of NSCLC patients treated with neoadjuvant combination chemotherapy was designed. Specifically, changes in the expression of pro- and anti-inflammmatory cytokine profiles in blood samples taken before initiation of treatment and pre cycle 3 (C3) were quantified and compared to treatment outcomes.

2.4.2 Patient samples

Patient samples stored at the Thoracic Oncology Research Biobank were used for the quantitative real-time PCR (Q-PCR). Cell pellets retrieved from separated buffy coat samples that were prepared from whole blood specimens collected in EDTA-containing tubes were used. Samples were derived from patients that were enrolled in a Phase II pilot study of neoadjuvant cetuximab in combination with cisplatin and gemcitabine in resectable IB-IIIA NSCLC. Sequential samples were collected before, during and after therapy on all patients. Ethics committee approval was received from the institutional ethics committee for the study. Informed written consent was obtained from each patient or from a relative.

All patients included had histologically confirmed resectable stage IB-IIIA NSCLC and adequate end-organ function. Three weekly cycles of cisplatin 80 mg/m^2 D1, gemcitabine 1250 mg/m^2 D1, D8 and cetuximab loading dose of 400 mg/m^2 on first infusion, thereafter weekly 250 mg/m^2 were used. The primary endpoint of the study was response rate (radiological and pathological). Secondary endpoints were safety and tolerability of the combination, resection rate following therapy, overall survival and relapse free survival.

2.4.3 Evaluation of response to treatment

Patients were evaluated after three cycles of chemotherapy with CT imaging. Determination of treatment responses following neoadjuvant chemotherapy were made according to modified WHO response criteria (Miller et al., 1981).

Comparison of baseline and interval CT imaging was made by an expert radiologist to assess for change in tumour volume. Responses were classified as follows:

- Complete response (CR): complete disappearance of all assessable disease
- Partial Response (PR): a decrease of 50% or more in the sum of the products of the greatest perpendicular dimensions (PD) of all assessable lesions, with no increase in the size of any lesions or appearance of new lesions.
- Stable Disease (SD): neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD
- Progressive Disease (PD): an overall increase of at least 25% in the sum of the products of the greatest PD of all assessable lesions.

2.4.4 Chemotherapy

Rationale for assessment of changes in cytokine expression in response to neoadjuvant treatment was as follows:

1. Although the nonimmunogenic mechanisms by which chemotherapy causes cancer cell killing are well established, there is emerging evidence for a pleiotropic effect of several classes of agent on the immune response. Cisplatin, a standard treatment in NSCLC, is capable of enhancing activities of monocytes *in vitro* and *in vivo* (Sodhi et al., 1990, Muenchen and Aggarwal, 1998) and increases peripheral blood mononuclear cell (PBMC) production of IFN-γ, TNF-α, IL-1β and IL-12 *in vitro*. Enhancement of T-cell mediated anti-tumour immune activity has also been demonstrated with gemcitabine, another common chemotherapeutic agent widely used to treat NSCLC patients (Suzuki et al., 2007).

2. The massive tumour cell apoptosis that follows successful chemotherapy increases the amount of cross-presented antigen, may expose *neo*-antigens and provides a variety of immunostimulatory signals. Exposure of dead tumour cells or their apoptotic bodies that are phagocytosed by dendritic cells results in induction of a T_H1 response, in part promoted by the expression of calreticulin, a protein expressed on the plasma membrane of

tumour cells. Platinum derivatives effectively transolocate calreticulin from the cytoplasm to the plasma membrane of dying cells thereby and thereby may enhance this process of antigen presentation (Menard et al., 2008). Furthermore, chemotherapy may abrogate cancer-related immunosuppressive effects that inhibit effective CMI responses by reducing levels of factors such as VEGF and COX-2 (Zou, 2005).

3. Cetuximab has also been shown to mediate antibody dependent cellular cytotoxicity (ADCC), a mechanism of cell-mediated immunity whereby an effector cell actively lyses a target cell bound by specific antibodies. This response involves activation of NK cells which release a host of cytokines, including IFN-γ, and cytotoxic granules after binding to target cells.

2.4.5 Cytokine standards

The DNA standards for IL-4, IL-10, β-actin, IFN-γ, Foxp3, and TGF-β consisted of a cloned PCR product that encompassed the quantified amplicon. These were prepared by PCR from a complimentary (c)-DNA population containing the target messenger ribonucleic acid (mRNA). These standards were a kind gift from Patrick Stordeur, Hopital Erasme, Brussels, Belgium. Primer and probe sequences and reaction conditions are given in Appendix 1.

Stock solutions of standards, containing 10^9 (IL-4, β-actin, IFN-γ, and TGF-β) or 10^{10} (IL-10 and Foxp3) copy numbers per µl, were aliquoted and stored at -20°C. The number of copies was calculated with the use of the molecular weight of each gene amplicon. A dilution series from 10^9 to 10^2 copy numbers per µl was prepared in each case and stored at 4°C. The standards were then diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing double-stranded herring DNA (Sigma) at 10 µg/ mL.

2.4.6 Isolation of RNA from stored lymphocytes

RNA was isolated from cryopreserved white blood cell pellets in batches containing patients' pre-treatment and pre C3 samples using a Qiagen RNeasy kit

(Qiagen Inc. CA, USA). 600 µL of RNA Lysis Buffer was pipetted into a 1.5 mL collection tube that contained the tissue samples, stored in RNAlater at -80°C. To this, 600 µL of 70% ethanol was mixed by pipetting. Up to 700 µL (capacity of the RNeasy mini column) of the sample was then applied to an RNeasy mini column and placed in a 2 mL collection tube. The tube was centrifuged for 15 s at 10,000 RPM and the flow-through discarded. The remainder of the cell/lysis buffer/alcohol mix was then added to the RNeasy mini column. The spin cycle was repeated and the flow-through discarded.

A DNase digestion step was then carried out using an RNase-Free DNase Set (Qiagen Inc., CA, USA). 350 µL wash buffer was added to the RNeasy mini column. The tube was centrifuged for 15 s at 10,000 RPM, and the flow-through discarded. 10 µL DNase was added to 70 µL of RDD buffer for each sample that required DNase digestion. The solution was mixed gently by inversion. 80 µL of the DNase incubation mix was pipetted directly onto the RNeasy silica-gel membrane and incubated at RT for 15 min. A further 350 µL of RWI wash buffer was then pipetted onto the RNeasy column and centrifuged for 15 s at 10,000 RPM. Flow-through was discarded.

The RNeasy column was transferred into a fresh 2 mL collection tube and 500 µL of RPE buffer was pipetted onto the column. The column was centrifuged for 15 s at 10,000 RPM to wash, and the flow-through discarded. A further 500 µL of buffer RPE was added to the column and the tube centrifuged for 2 min at 10,000 RPM. The RNeasy column was transferred into a new 1.5 mL collection tube and centrifuged for 1 min at 10,000 RPM. The columns were then transferred to new collection tubes and total RNA was eluted by pipetting 35 µL of RNase-free water directly onto the silica membrane. The tube was centrifuged for 1 min at 10,000 RPM to elute the RNA. Samples were stored at -80°C until cDNA synthesis.

2.4.7 RNA quantification

RNA concentrations were determined spectrophotometrically, using a Nanodrop 1000 spectrophotometer (version 3.1.0, Nanodrop technologies, DE, USA). 1 µL of RNase-free water was used to blank the instrument after which the analyser was cleaned with tissue. 1 µL of isolated RNA was then loaded onto the

Nanodrop. RNA was measured in ng/μL. 260:280 and 260:230 purity ratios were also recorded. Good quality RNA will have an optical density (OD) 260/280 ratio of 1.8 to 2.0 and an OD 260/230 ratio of 1.8 or greater.

2.4.8 Reverse-transcription reaction

Preparation of cDNA from isolated RNA took place in a laminar flow cabinet separate from that used for experiments involving DNA and Q-PCR. One set of pipettes, reagents and autoclaved disposable pipette tips were used exclusively for this step to prevent contamination of the samples. Reagents and volumes were added to 0.5 mL tubes in the following order to make a final volume of 26 μL:

Reagent	1x volume (μL)
RNA 100ng	*x*
Random Primers	1
dNTP (10mM)	1
RNase-free H_20	24-*x*

Table 2.5 Components of reverse transcription reaction mix

The volume of RNA (*x*) to be added was based on the concentration of diluted RNA derived from spectrophotometry. The dNTPs used were stock prepared as a 1:1:1:1 ratio of dATP, dCTP, dGTP, dTTP (Promega, WI, USA). The reaction mix was vortexed and pulse centrifuged and then incubated at 65°C for 5 min in a thermal cycler. The samples were then immediately chilled on ice for 1 min to denature any RNA secondary structure present. Samples were then pulse centrifuged once more.

The following reagents were added to the master-mix for each reverse-transcription reaction: (1) 4 μl 5X first strand buffer (Invitrogen Corporation, CA, USA); (2) 1 μL 0.1 M DTT (dithiothreitol) (Invitrogen); (3) 1 μL RNasin (RNase inhibitor: 40 U/ μL) (Promega); (4) 1 μL Superscript II (200 U/ μL) (Invitrogen);

and (5) 7 μL H_20. The mixture was vortexed and incubated first at 50°C for 3 hrs and then at 70°C for 15 min. Samples were stored at -20°C.

2.4.9 Quantitative real-time polymerase chain reaction

Real-time polymerase chain reaction is a technique used to amplify and simultaneously quantify numbers of copies of a targeted DNA sequence of interest (**Figure 2.6**). This process occurs in real time, as re-quantification of DNA is made after each round of amplification. The *Taq*man assay is based on detection of a specific PCR product. Amplification proceeds using sequence-specific primers and Taq DNA polymerase. Detection results from hybridisation of the target sequence to a doubly labelled fluorogenic probe. In an intact *Taq*Man probe, the fluorescent dye and the quencher are in close proximity and engage in fluorescence resonance energy transfer (FRET), resulting in a non-fluorescent probe. During amplification, the hybridized probe is cleaved by the inherent 5' to 3' eoxnuclease activity of the Taq DNA polymerase to release reporter dye. This only occurs when the *Taq*Man probe hybridises to the target DNA that is being amplified. As a result, the fluorophore is cleaved from the probe and diffuses away from the quencher allowing a fluorescence signal to be generated. For each unit amplification, 1 U of fluorescence is released. The increase in signal intensity is proportional to the accumulation PCR product.

The ABI Prism 7500 contains a built-in thermal cycler with 96-well positions, and is able to detect fluorescence between 500 nm and 660nm. Fluorescence is induced during the Q-PCR by distributing laser light to all 96 samples contained in thin-walled reaction tubes via a multiplexed array of optical fibres.

The ABI Sequence Detection System (Applied Biosystems) establishes the levels of background fluorescence for each particular run. An algorithm is used to define a fluorescence background threshold. The algorithm then searches the data from each sample for a point that exceeds the baseline. The cycle at which this occurs is defined as the Crossing Threshold (C_T). It is dependent on the starting template copy number and the efficency of the PCR amplification. The fewer cycles

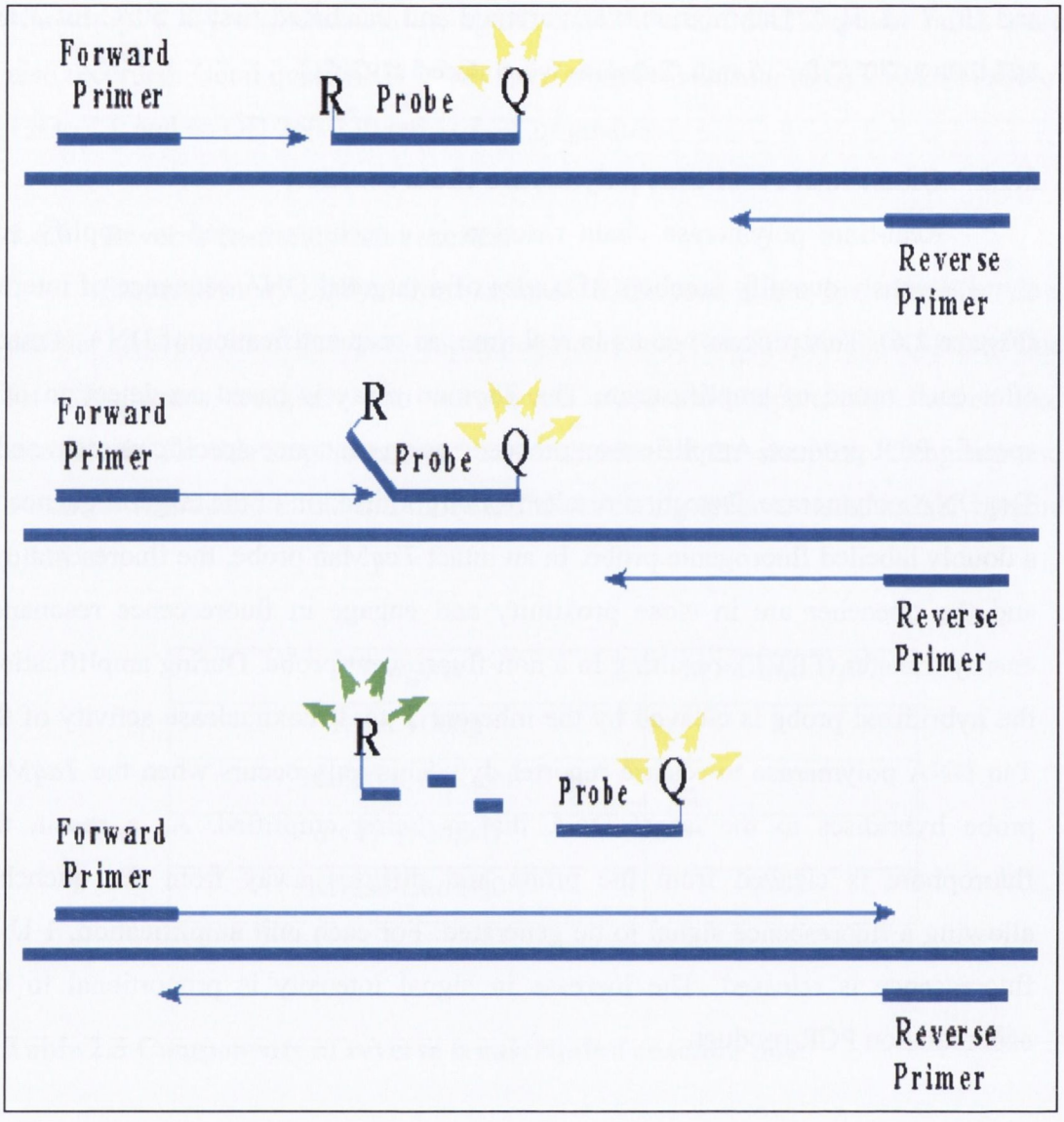

Figure 2.6 Principles of Q-PCR. PCR proceeds using sequence specific primers while a sequence specific probe binds downstream of the primer. The 5'-deoxynuclease activity of the Taq DNA polymerase cleaves the probe and the reporter dye is separated from the quencher resulting in fluorescence. The Taq DNA polymerase then finishes amplifying the area of interest.

it takes to reach a detectable level of fluorescence, the greater the initial copy number.

In order to minimize the inherent error in mRNA transcript quantification due to varying quantities of cDNA loaded into the PCR reaction wells, all RNA values were normalized against the housekeeping reference gene β-actin. β-actin is an ideal standard that encodes for a ubiquitous cytoskeletal protein is expressed at relatively constant levels and served as an internal reference in all reactions. All *Taq*man probes are labelled at the 5' reporter end with the 6-FAM chromophore and at the 3' quencher end with the TAMRA chromophore.

Absolute quantification of the individual amplicons was precisely determined by interpolation from an absolute standard curve constructed using serial dilutions of known concentrations of DNA. Linear regression analysis of all standard curves demonstrated a coefficient of determination (R^2) of 0.99 or higher.

Primers and probes used in the study were synthesized at Applied Biosystems (Foster City, CA: IL-4, IL-10, β-actin, IFN-γ and Foxp3) and Operon (Cologne, Germany: TGF-β) and designed to span exon–intron junctions to prevent amplification of genomic DNA and also to result in amplicons of fewer than 150 base pairs to enhance efficiency of PCR amplification.. Primer and probe stocks were stored at -20°C. The stock for all primers was initially diluted to 100 pmol/ μL. To generate working solutions of the primers for PCR reactions, volumes of water were added to 20 μL of primer stock to yield 1 μL of diluted primer solution as listed in **Table 2.6.**

Primer Concentration (nM)	**Volume of H_20 (μL)**	**Primer quantity per 25 μL reaction (pmol)**
300	246.7	7.5
600	113.4	15
900	68.8	22.5

Table 2.6 Dilutions of primers

The photosensitive probe stock (10 pmol/ µl) was wrapped in tinfoil to prevent degradation. To generate working solutions of the probes for PCR reactions, dilutions were made as listed in **Table 2.7.**

Probe Concentration (nM)	Volume of Probe stock (µL)	Volume of H_20 (µL)	Amount of Probe per 25 µL reaction (pmol)
100	4	12	2.5
150	6	9.6	3.75
200	8	8	5

Table 2.7 Dilution of probes

2.4.10 PCR conditions

The Q-PCR reaction plate is prepared in a cleaned extractor hood separate from DNA experiments. To prevent the accidental introduction of RNases, a number of guidelines were observed when working with RNA:

- Disposable gloves were always worn.
- RNaseAway (Promega) was routinely used to thoroughly wipe down all surfaces.
- A separate set of pipettes, reagents and disposable pipette tips were used exclusively for the Q-PCR step in order to prevent possibility of cross-contamination.

Each thermocycling reaction mixture (25 µL final volume per well) contained the reagents listed in **Table 2.8**:

Reaction Component	Volume (µL) per sample
2X PCR master mix	12.5
Forward Primer	1.0
Reverse Primer	1.0
*Taq*Man probe	1.25
cDNA sample	2
Water	7.25

Table 2.8 PCR reaction mixture components

The 2X *Taq*Man Universal Master Mix (Applied Biosystems) contained AmpliTaq Gold DNA polymerase; AmpErase® -uracil N-glycosylase (UNG) and dNTPs with dUTP. Primers, probes, cDNA and DNA standards were thawed on ice whilst preparing the reaction mixture. All reactions were performed in duplicate and included a dilution series of the DNA standard of the gene being quantified (in concentrations of 10^7, 10^5 and 10^3 copies per µL), as well as no template controls (NTC) containing water in place of the patient sample. The 96-well optical reaction plate (Applied Biosystems) was centrifuged briefly and covered with an optical adhesive cover prior to analysis. The PCR reactions were carried out in an ABI Prism GeneAmp 7500 Sequence Detection System (Applied Biosystems).

The concentrations of the dilution series are designed to encompass the expected range of copy numbers of the genes being examined. Lack of product in the NTC wells confirms an absence of contaminating DNA in the reaction mix.

After an initial denaturation step at 95°C for 10 min, temperature cycling was initiated. Each cycle consisted of 95°C for 15 sec and 60°C for 1 min, the fluorescence being read at the end of this second step. In total, 40 cycles were performed.

2.4.11 Optimization of primers and probes

To ensure an accurate multiplex assay, it is important that the amplification of one species does not dominate over the other. This is avoided by minimizing the primer concentrations utilized to amplify the more abundant species, thereby inhibiting the amplification soon after the C_T has been reached. Suggested primer concentrations for use (personal correspondence: Patrick Stordeur) were confirmed by means of primer optimization curves for all forward and reverse primers to establish the optimal concentrations for use in *Taq*man reactions.

It was necessary to avoid probe sequence limiting concentrations for best reproducibility in *Taq*man reactions. The probe optimization curve was set up similarly, using the optimised primer concentrations. The probe concentrations tested were 100 nM, 150 nM and 200 nM.

2.5 STATISTICAL ANALYSIS

Patient survival time was calculated from the date of surgery to latest follow up date. Survival rate was estimated using the Kaplan-Meier method and compared by the log-rank test. Perioperative mortality was calculated on the basis of patients who died within 30 days of date of surgery. However, all patients were included in the survival analysis.

Between-group comparisons for parametric continuous data was analysed using a paired t-test for matched pairs and a t-test for independent groups. Parametric data were subsequently expressed as the mean with the standard deviation (SD) in parenthesis. Categorical data were analysed using a chi-squared test.

Spearman's ρ correlation, R^2 change and the linear regression model was used to model and estimate the relationship between automated and manual analysis. The median value was selected to define subgroups with respect to immunohistochemical markers. Statistical analysis was performed with Statistical Package for the Social Sciences version 14.0 (SPSS Inc, Chicago, IL). Cox regression analysis, Kaplan–Meier analysis and the log-rank test were used to illustrate the significance of various clinical characteristics.

The influence on overall and disease-free survival of all covariates found to be significant in the univariate analysis was assessed by means of the multivariate Cox proportional-hazards model to identify independent prognostic factors. All *P* values were two-sided, with a value of <0.05 considered statistically significant. Statistical significance of the association of the composite-predicted tree regression analysis groups with outcome was assessed by the Wald test.

CHAPTER 3

RESULTS

3.1 SURGICAL DATABASE

3.1.1 Patient demographics

Data from all cases of NSCLC that underwent surgical resection at our institution between January 1998 and March 2008 were collected and analyzed retrospectively. A total of 648 patients underwent surgical resection with curative intent during the study period. Of these, 566 patients had completely-resected stage I to IIIA NSCLC that was pathologically confirmed. There were 354 males and 212 females (ratio of 1.67:1). The median age at time of surgery of the entire cohort was 65.8 yrs (range 27 to 86) with no significant difference between men and women. A history of tobacco smoke exposure was noted for the majority of patients (current smokers: 38%; former smokers: 55%). Only 7% of patients were confirmed never smokers.

Eighty-two patients were excluded from further analysis due to the following reasons: upstaged to stage IIIB or IV disease (n=49); non-anatomical resections, including wedge resections (n=27); associated small-cell lung cancer on histopathology specimens (n=3); no cancer in resection specimen (n=3).

3.1.2 Surgical approach

Single lobectomy was performed in 75.4% of patients, while in 6.3% of patients, double lobectomy was required. Pneumonectomy was undertaken in the remaining 18.3% (right side: 50 patients; left side: 54 patients). The numbers of patients treated by pneumonectomy decreased from 21.9% (61/278) during the period 1998-2003 compared to 14.9% (43/288) during the period 2004-2008. This fall in the pneumonectomy rate was associated with a corresponding increase in the numbers undergoing lung-conserving surgery. Overall, sleeve resection procedures accounted for 7% of surgeries performed, showing an increase in the later years of the study. There were no anastomotic complications in the sleeve resection subgroup.

3.1.3 Histopathological characteristics

Results showed that squamous cell carcinoma represented the commonest histopathological subtype (51.9%), followed by adenocarcinoma (40.9%), mixed cellularity carcinomas (4.2%) and the large cell neuroendocrine variant (3.0%). In women, the adenocarcinoma cell type was most frequently observed, accounting for 56.8% of cases. By contrast, squamous cell carcinoma was the commonest pattern in males, representing 64.9% of the total.

Tumours were defined as well-differentiated (Grade 1: 6.5%), moderately differentiated (grade 2: 59.5%) or poorly differentiated (grade 2: 34.0%).

Residual disease, defined as the presence of microscopic tumour detectable at the cut resection margin and indicative of incomplete resection, was pathologically confirmed in 11.8% of patients. This included patients with residual tumour that variously involved bronchial, pleural, chest wall, vascular, mediastinal fat and hilar margins.

3.1.4 Staging

Pathologic stage was confirmed as follows: stage IA (T1N0), 19.2%; stage IB (T2N0), 34.8%; stage IIA (T1N1), 4.4%; stage IIB (T2N1, T3N0), 20.8%; stage IIIA (T3N1, T1-T3N2), 20.8%. The median size of the primary tumour (T stage) was 4 cm. The largest primary lesion excised during the study period measured 16 cm in maximum diameter.

3.1.5 Survival factors by univariate analysis

An overall perioperative death rate (defined as deaths within 30 days of surgery) of 7.7% was observed for the study population. However, perioperative mortality rates fell from 9.4% during the period 1998-2003 (n=278) to 6.3% during the period 2004-2008 (n=288). The overall 5-year survival rate for all the patients was 38% (**Figure 3.1**) with an overall median survival of 37.3 months for all stages.

The data series was examined for prognostic factors. These included: age; sex; smoking status; surgical approach; side of surgery; histopathological subtype; tumour grade; T stage; tumour size; N stage; overall stage; and presence of residual

disease. The mean and median survival times for different groups defined by these characteristics are presented in **Table 3.1**. Results for pairwise comparisons of each combination of groups for each variable, and overall comparisons are also presented. For each variable, a Kaplan Meier survival curve was generated (**Figure 3.1**). For all series, survival time estimations were limited to the largest survival time for censored data. Factors affecting survival by univariate analysis are listed in **Table 3.2**.

A number of patient factors were associated with improved postoperative survival. A significantly longer median survival was observed in females compared to their male counterparts (61.9 months vs. 28.1 months, $p < 0.001$).

Patient age strongly influenced outcome, with an inverse correlation shown between survival and advancing age. Median survival times were similar (46-49 months) among patients in the under 50, the 50-59 and the 60-69 age ranges. A shorter survival was observed for those over 70 years, although this was not statistically significant. Patients aged 80 and over had a particularly poor median survival (9.1 months, $p<0.001$ vs. all other groups).

Interestingly, there were similar survivals for patients that were either current or former smokers at time of surgery (43 vs. 45 months, $p= 0.55$). However, never-smokers showed a particularly poor median survival (13.7 months) that was statistically significantly worse compared to those who were current ($p=0.003$) or former ($p<0.001$) smokers.

A trend toward improved survival rates was observed in patients with non-squamous histopathological subtypes though this did not reach statistical significance (34 months vs. 45 months, $p=0.179$). Tumour grade (well, moderately or poorly differentiated) had no influence on survival in univariate analysis.

Type of operation was also a predictor of outcome, such that patients undergoing single or bilobectomy had a median survival more than double that of those requiring pneumonectomy (44.6 months vs. 19.2 months, $P=0.14$). However, there was no significant difference in survival when patients were stratified by side

of surgical resection. Patients with completely resected tumours had a significantly better outcome compared to those with positive surgical margins (43.5 months vs. 21.0 months, p=0.003).

Regarding tumour characteristics, higher T and N descriptors and overall pathological stage conferred an increased risk of death, as anticipated. Increasing size of the primary tumour was also a significant predictor of decreased long-term survival, irrespective of overall stage. Primary tumour size also conferred a worse outcome among the subset of patients with node negative (N0) disease. The prognostic relevance of separating patients with N0 disease according to tumour size as defined by the proposed IASLC update to the TNM staging system was also assessed. Overall, increasing tumour size was associated with a shorter survival (P<0.001). Interestingly, tumours larger than 7 cm were associated with longer median survival compared to tumours 5-7 cm, though numbers in these groups were small.

3.1.6 Survival factors by multivariate analysis

To identify independent prognostic factors, all variables with a P value <0.2 by univariate analysis were included in a multivariate Cox proportional hazards tmodel. Female sex, history of smoking, lower T, N and overall stage, and involvement of resection margins emerged as factors that conferred an improved survival by multivariate analysis (**Table 3.3**).

Mean(a)				Median			
		95% Confidence Interval				95% Confidence Interval	
Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
57.743	2.721	52.410	63.075	37.367	4.395	28.752	45.982

Table 3.1.1 Mean and median overall patient survival rates

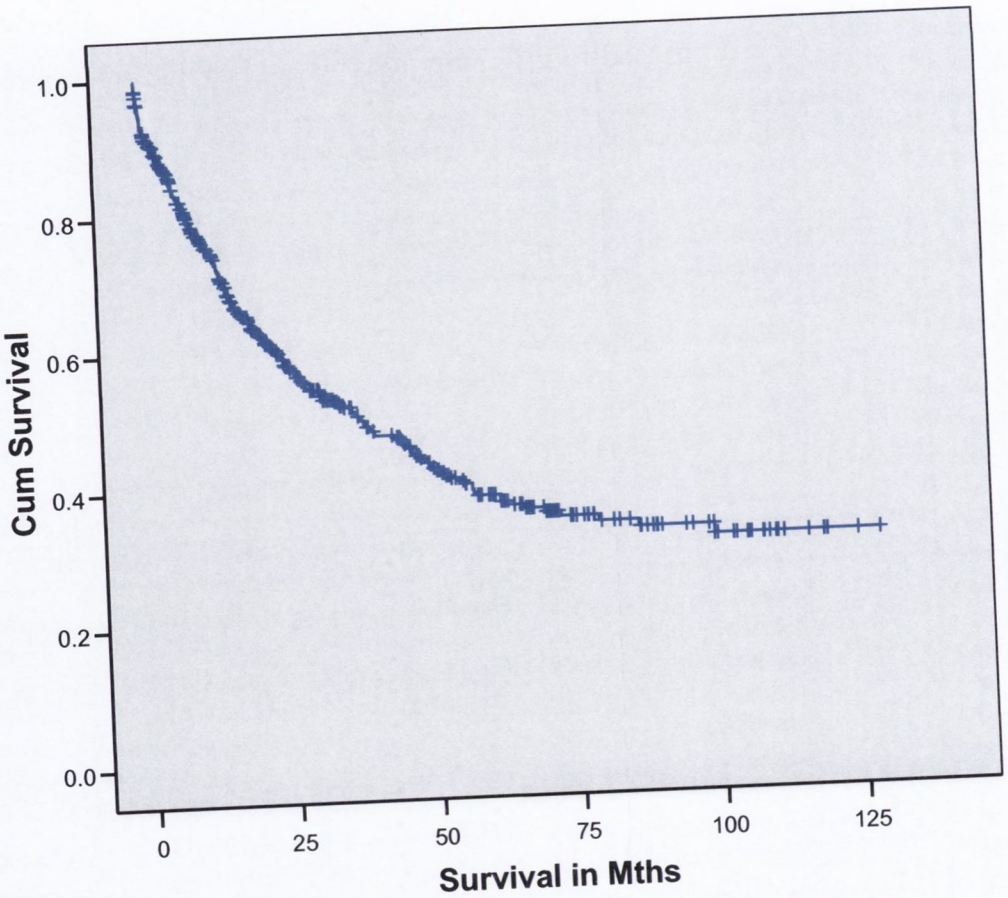

Figure 3.1.1 Kaplan Meier survival curve for overall group

Means and Medians for Survival Time

Sex	Mean(a)				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Male	50.229	3.195	43.967	56.491	28.100	4.414	19.449	36.751
Female	68.016	4.367	59.457	76.576	61.900	14.149	34.168	89.632
Overall	57.743	2.721	52.410	63.075	37.367	4.395	28.752	45.982

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	17.781	1	<.0001

Table 3.1.2 Means and medians and Log Rank test for survival time with respect to sex

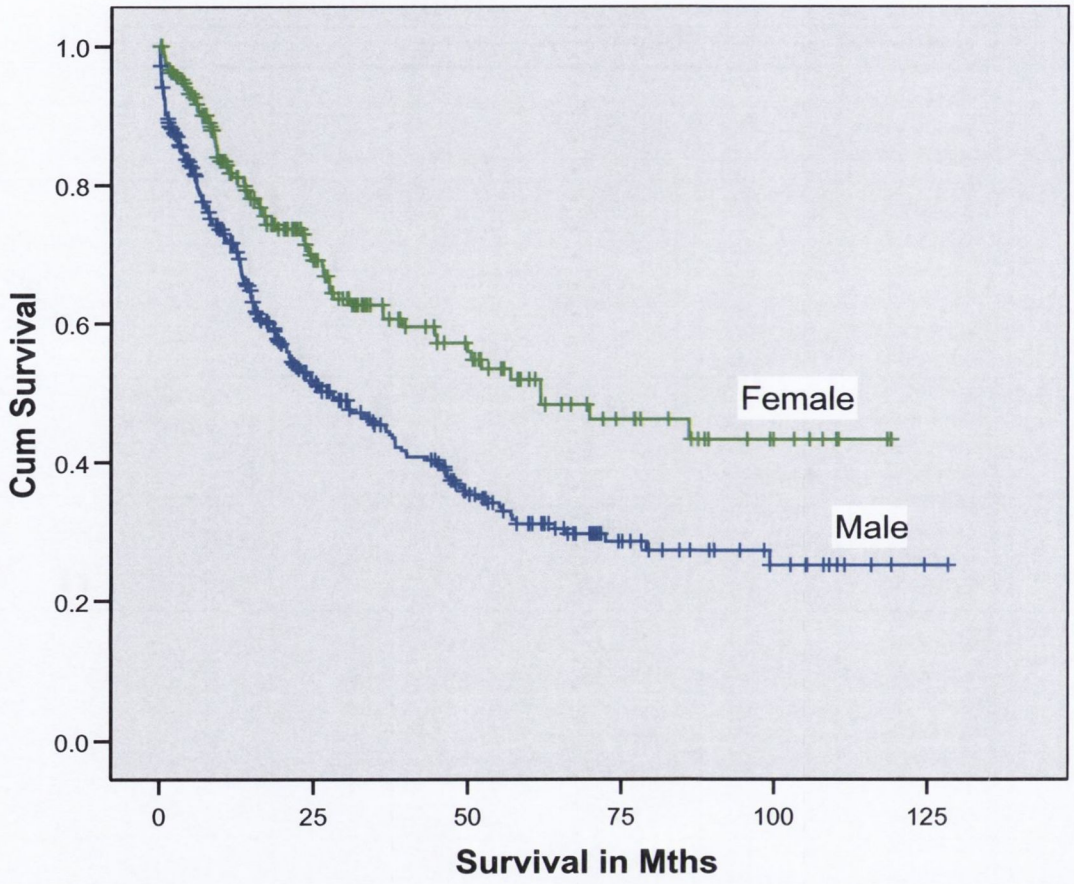

Figure 3.1.2 Kaplan Meier survival curves for patients according to sex

Means and Medians for Survival Time

AGE	Mean(a)				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
<50	68.484	9.980	48.922	88.045	49.967	.	.	.
50-59	65.151	5.743	53.894	76.408	46.367	12.736	21.403	71.330
60-69	56.992	4.032	49.090	64.895	47.067	7.606	32.159	61.975
70-79	50.577	4.743	41.282	59.873	28.100	5.770	16.790	39.410
≥80	17.911	5.969	6.212	29.610	9.100	5.410	<0.001	19.704
Overall	57.845	2.724	52.506	63.183	37.367	4.392	28.758	45.976

Pairwise Comparisons

	AGE	<50		50-59		60-69		70-79		≥80	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	**<50**			.330	.566	.961	.327	2.807	.094	18.373	<0.001
	50-59	.330	.566			.630	.427	3.069	.080	21.519	<0.001
	60-69	.961	.327	.630	.427			1.255	.263	18.055	<0.001
	70-79	2.807	.094	3.069	.080	1.255	.263			15.677	<0.001
	≥80	18.373	<0.001	21.519	<0.001	18.055	<0.001	15.677	<0.001		

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	26.664	4	<0.001

Table 3.1.3 Means and medians and Log Rank test for survival time with respect to age

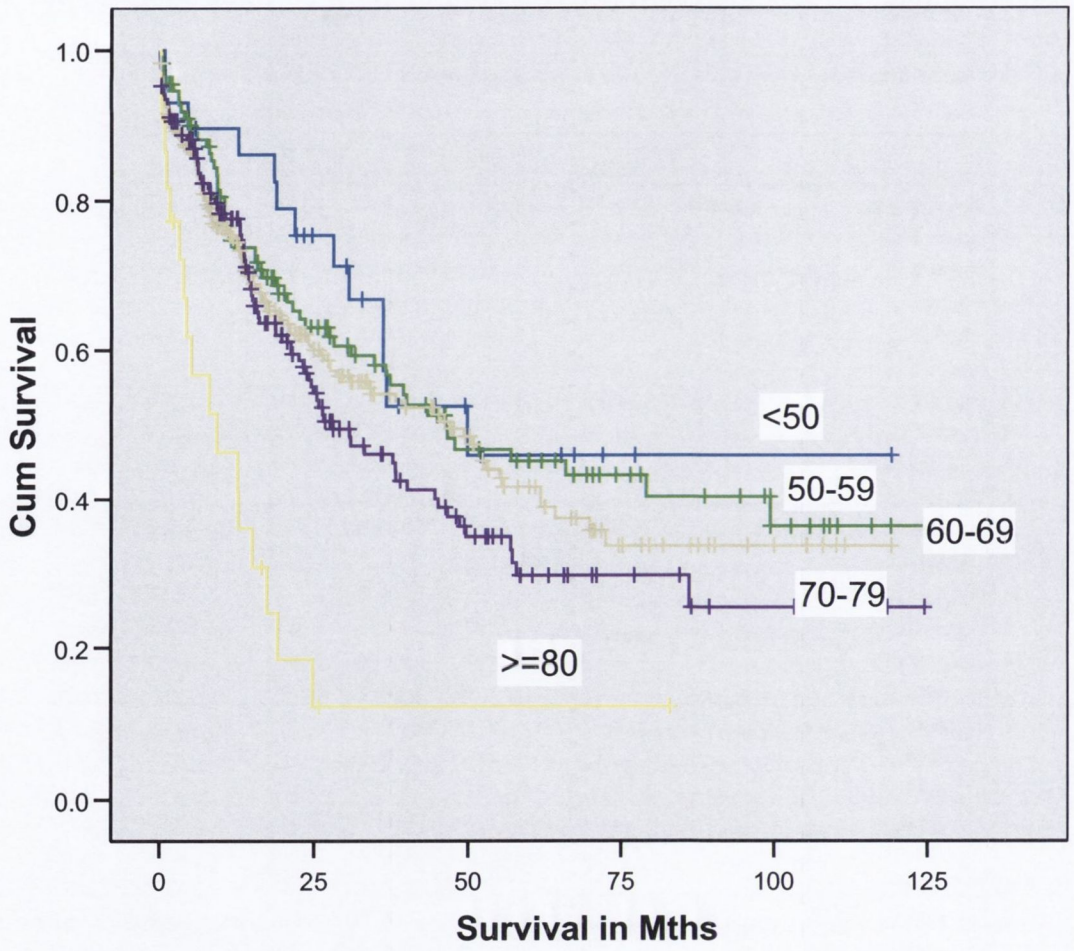

Figure 3.1.3 Kaplan Meier survival curves for patients according to age

Means and Medians for Survival Time

Smoking Status	Mean(a)				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Current	57.696	4.432	49.008	66.383	43.500	5.581	32.562	54.438
Ex-smoker	60.986	3.417	54.289	67.684	45.167	6.035	33.339	56.994
Never	32.080	6.448	19.441	44.719	13.767	5.334	3.313	24.221
Overall	59.352	2.770	53.923	64.780	38.300	4.210	30.048	46.552

Pairwise Comparisons

	Smoking Status	Current		Ex-smoker		Never	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	**Current**			.343	.558	8.897	.003
	Ex-smoker	.343	.558			12.292	<0.001
	Never	8.897	.003	12.292	<0.001		

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	12.355	2	.002

Table 3.1.4 Means and medians and Log Rank test for survival time with respect smoking status

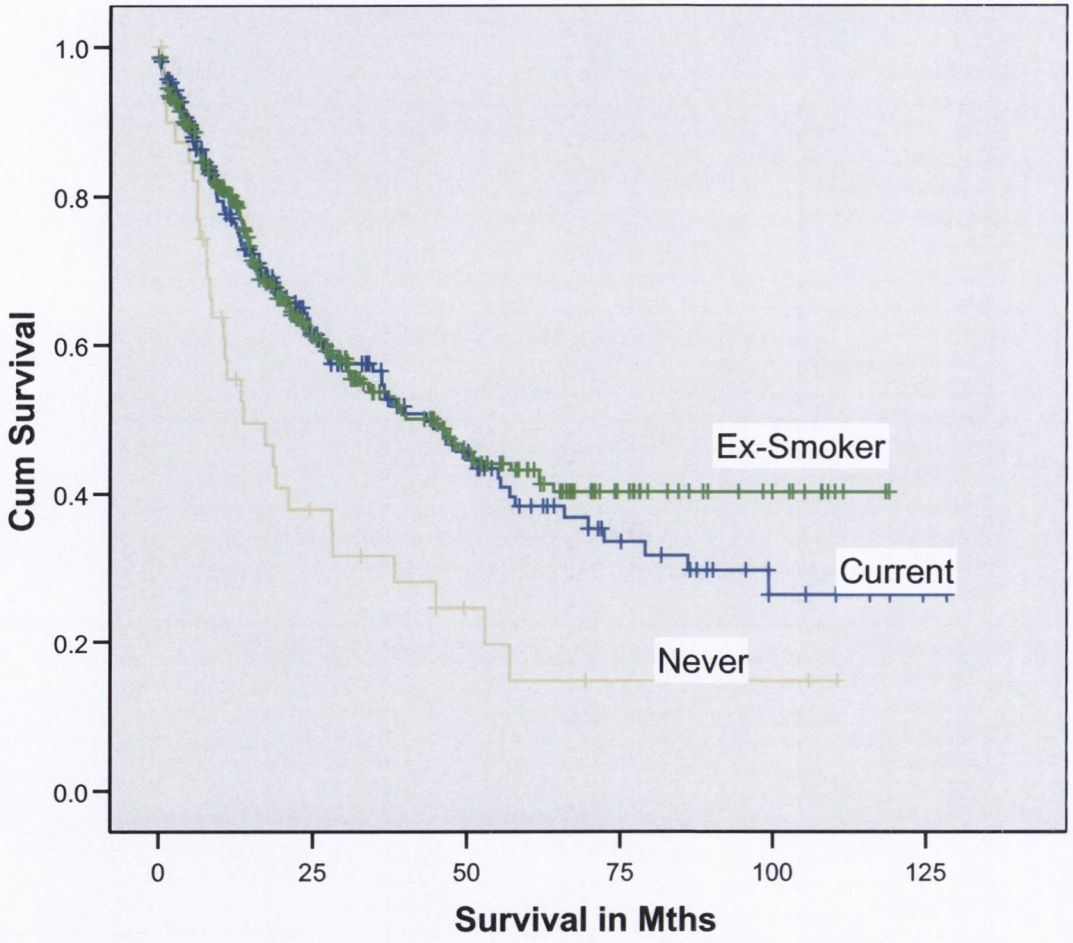

Figure 3.1.4 Kaplan Meier survival curves for patients according to smoking status

Means and Medians for Survival Time

Histopathology	Mean(a)				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Squamous	54.895	3.676	47.690	62.100	34.067	4.123	25.987	42.147
Non-squamous	57.578	3.657	50.411	64.746	45.033	8.292	28.782	61.285
Overall	57.743	2.721	52.410	63.075	37.367	4.395	28.752	45.982

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	1.803	1	.179

Table 3.1.5 Means and medians and Log Rank test for survival time with respect to histopathological subtype

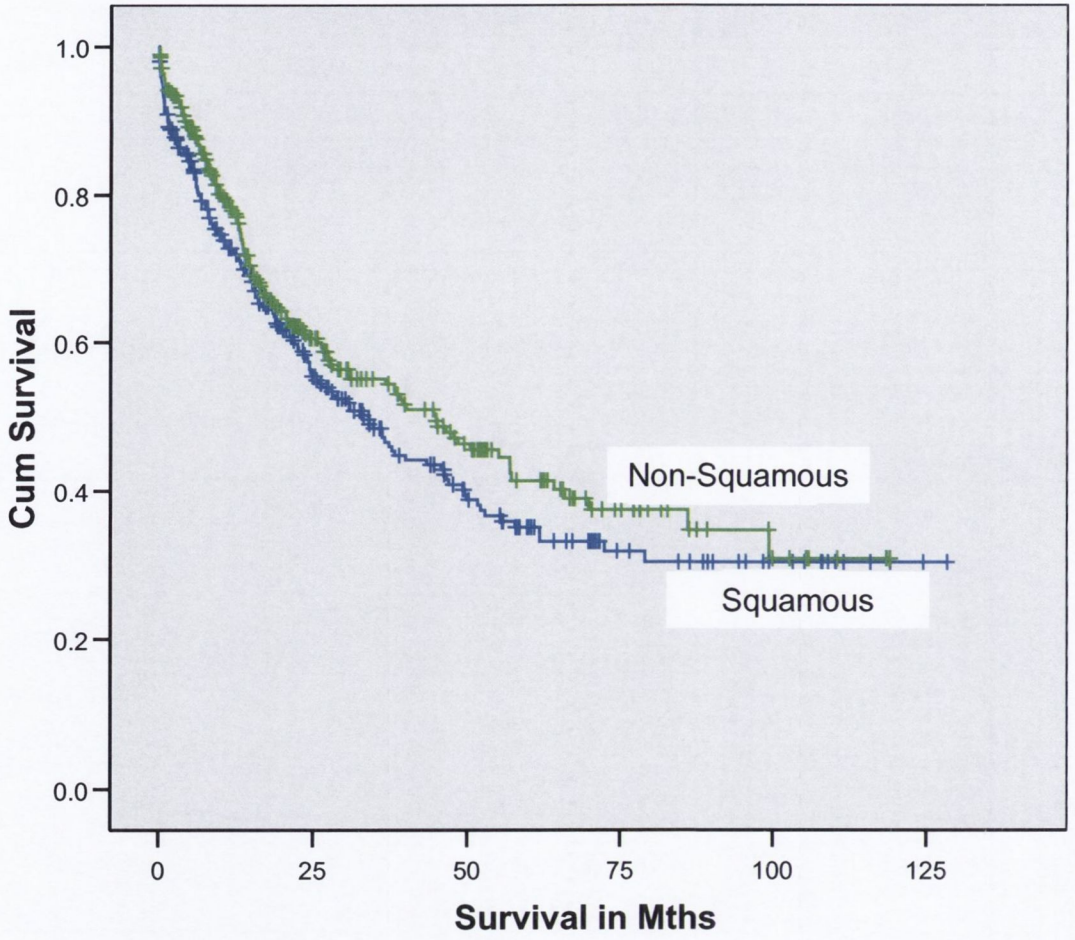

Figure 3.1.5 Kaplan Meier survival curves for patients according to histopathological subtype

Means and Medians for Survival Time

Tumour Grade	Mean(a)				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Well differentiated	59.265	9.082	41.465	77.065	39.267	10.999	17.709	60.825
Moderately differentiated	60.331	3.600	53.276	67.387	40.067	5.767	28.764	51.370
Poorly differentiated	47.231	3.761	39.860	54.603	24.533	5.970	12.832	36.234
Overall	57.743	2.721	52.410	63.075	37.367	4.395	28.752	45.982

Pairwise Comparisons

	Tumour Grade	Well differentiated		Moderately differentiated		Poorly differentiated	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	**Well differentiated**			.001	.975	1.133	.287
	Moderately differentiated	.001	.975			4.444	.035
	Poorly differentiated	1.133	.287	4.444	.035		

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	4.727	2	.094

Table 3.1.6 Means and medians and Log Rank test for survival time with respect tumour grade

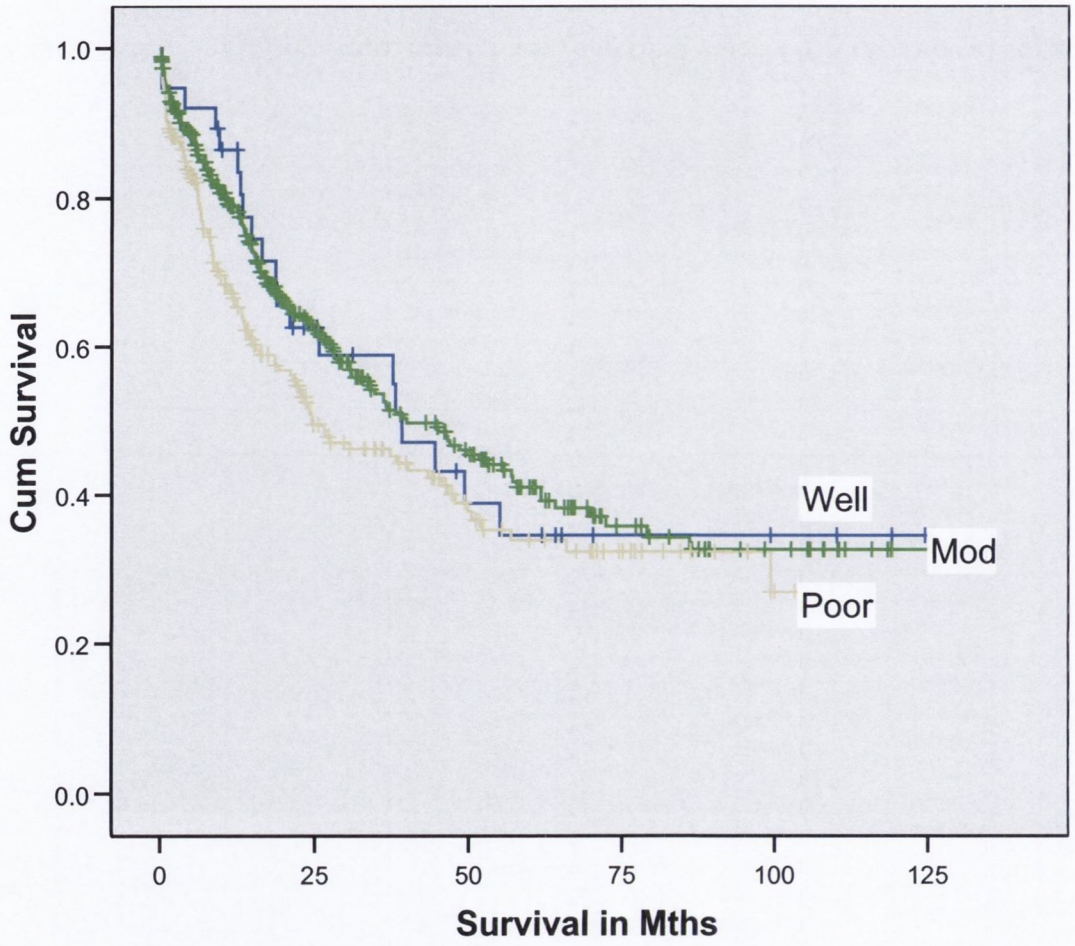

Figure 3.1.6 Kaplan Meier survival curves for patients according to tumour grade

Means and Medians for Survival Time

Operation Type	Mean(a)				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Single/bilobectomy ('other')	58.158	2.924	52.428	63.889	44.633	4.123	36.553	52.714
Pneumonectomy	49.353	5.934	37.722	60.984	19.200	4.957	9.485	28.915
Overall	57.743	2.721	52.410	63.075	37.367	4.395	28.752	45.982

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	6.033	1	.014

Table 3.1.7 Means and medians and Log Rank test for survival time with respect to type of surgery

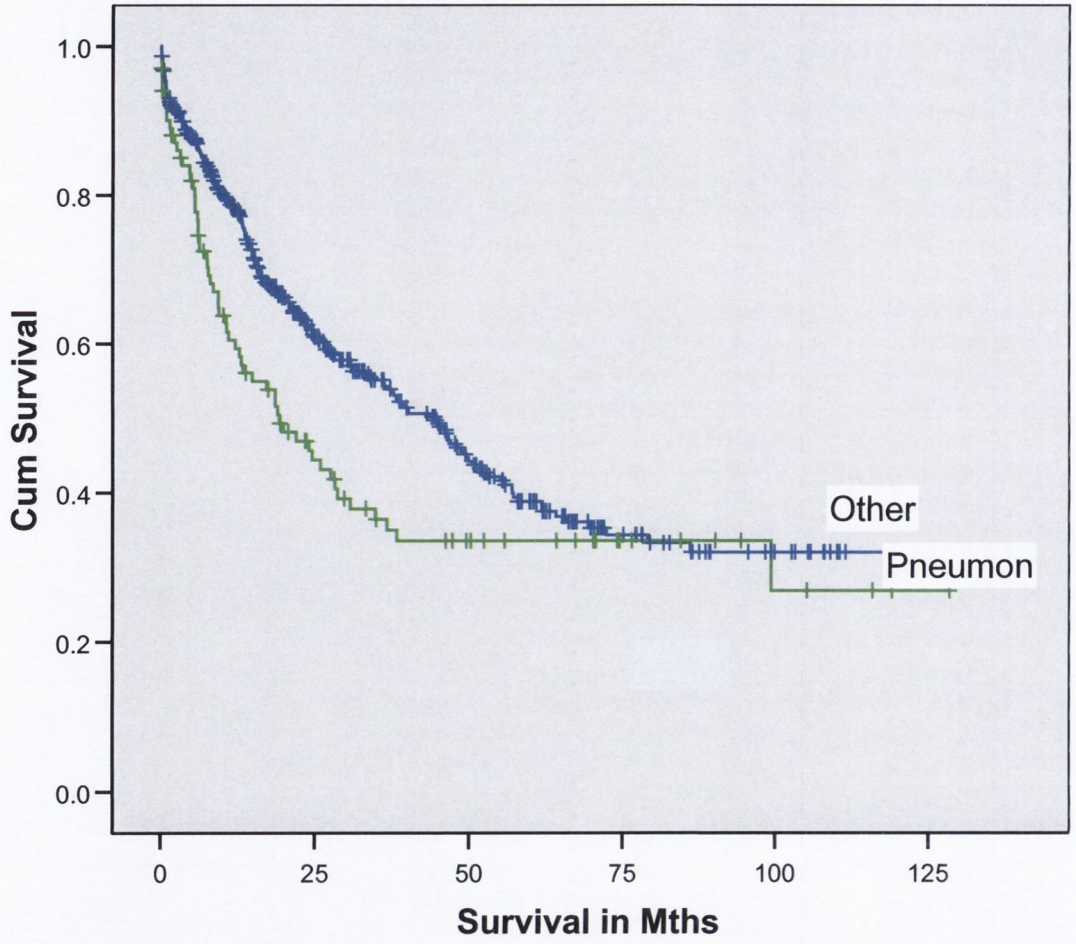

Figure 3.1.7 Kaplan Meier survival curves for patients according to type of surgery

Means and Medians

Side of surgery	Mean(a)				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Left	58.964	3.803	51.511	66.418	36.267	7.641	21.290	51.243
Right	54.865	3.734	47.548	62.183	39.533	5.614	28.530	50.537
Overall	57.726	2.721	52.393	63.058	37.367	4.396	28.750	45.984

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	.129	1	.720

Table 3.1.8 Means and medians and Log Rank test for survival time with respect to side of surgery

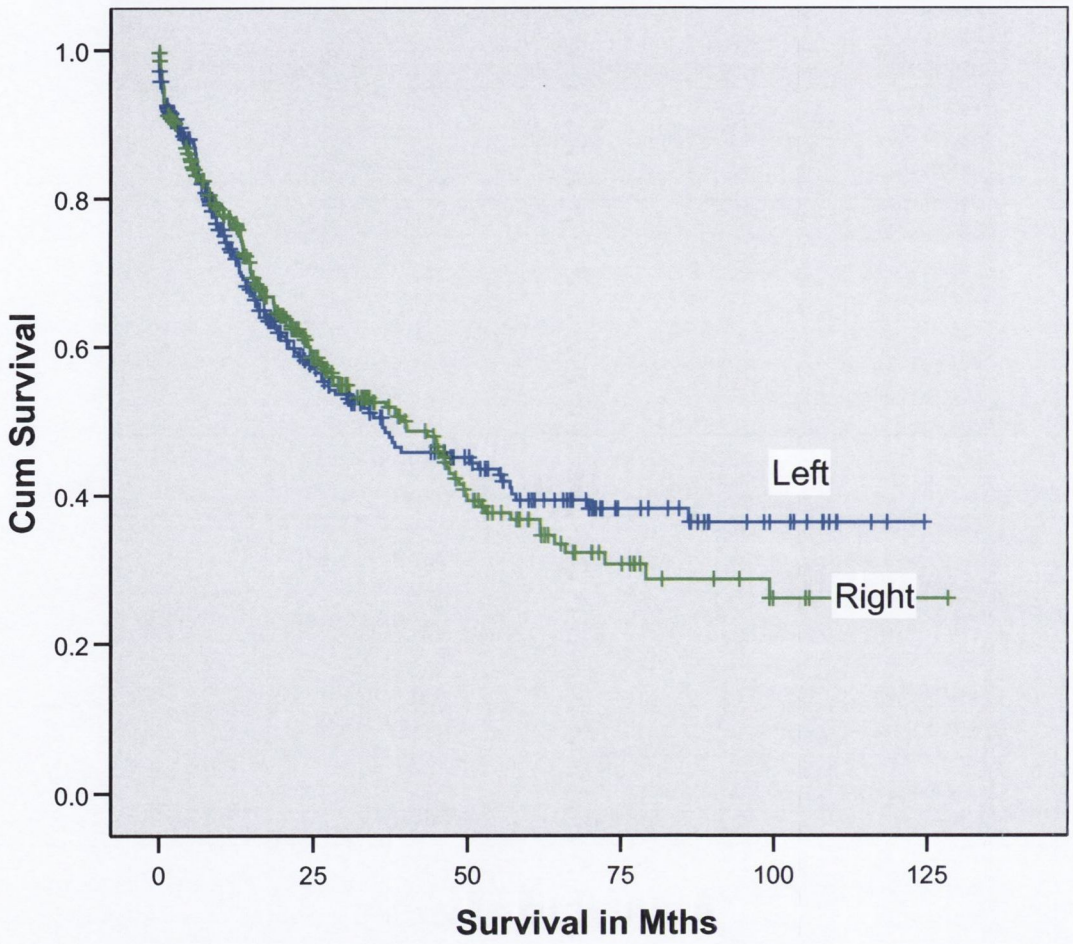

Figure 3.1.8 Kaplan Meier survival curves for patients according to side of surgery

Means and Medians for Survival Time

Residual Microscopic Disease	Mean(a)				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Absent	59.820	2.921	54.094	65.545	43.500	3.941	35.775	51.225
Present	40.044	6.278	27.739	52.349	21.033	4.302	12.601	29.465
Overall	57.743	2.721	52.410	63.075	37.367	4.395	28.752	45.982

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	8.543	1	.003

Table 3.1.9 Means and medians and Log Rank test for survival time with respect to surgical margins

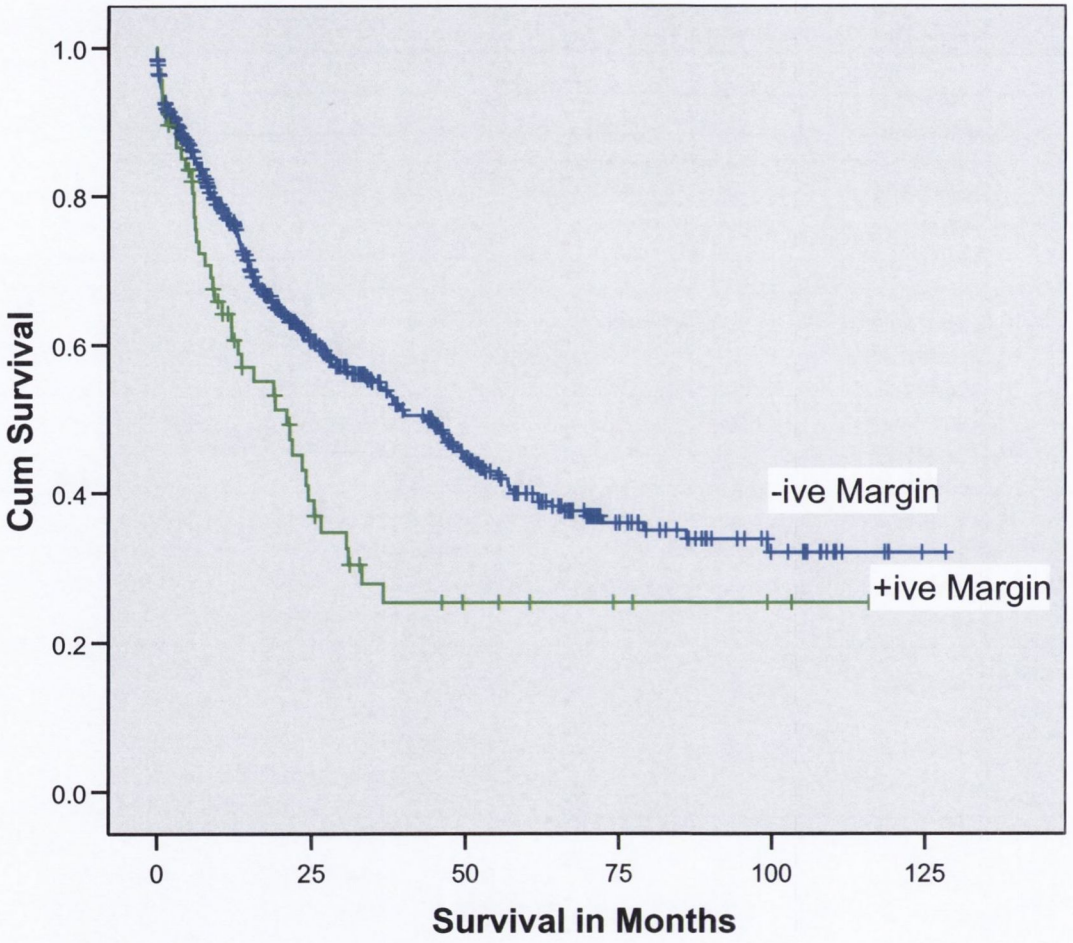

Figure 3.1.9 Kaplan Meier survival curves for patients according to surgical margins

Means and Medians for Survival Time

T stage	Mean(a)				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
1	71.603	5.923	59.993	83.213	72.467	13.797	45.424	99.509
2	55.646	3.260	49.257	62.036	36.267	5.213	26.049	46.485
3	35.407	5.623	24.387	46.427	19.200	2.679	13.949	24.451
Overall	57.743	2.721	52.410	63.075	37.367	4.395	28.752	45.982

Pairwise Comparisons

	T stage	1		2		3	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	**1**			8.101	.004	22.114	<0.001
	2	8.101	.004			7.896	.005
	3	22.114	<0.001	7.896	.005		

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	21.226	2	<0.001

Table 3.1.10 Means and medians and Log Rank test for survival time with respect to T stage

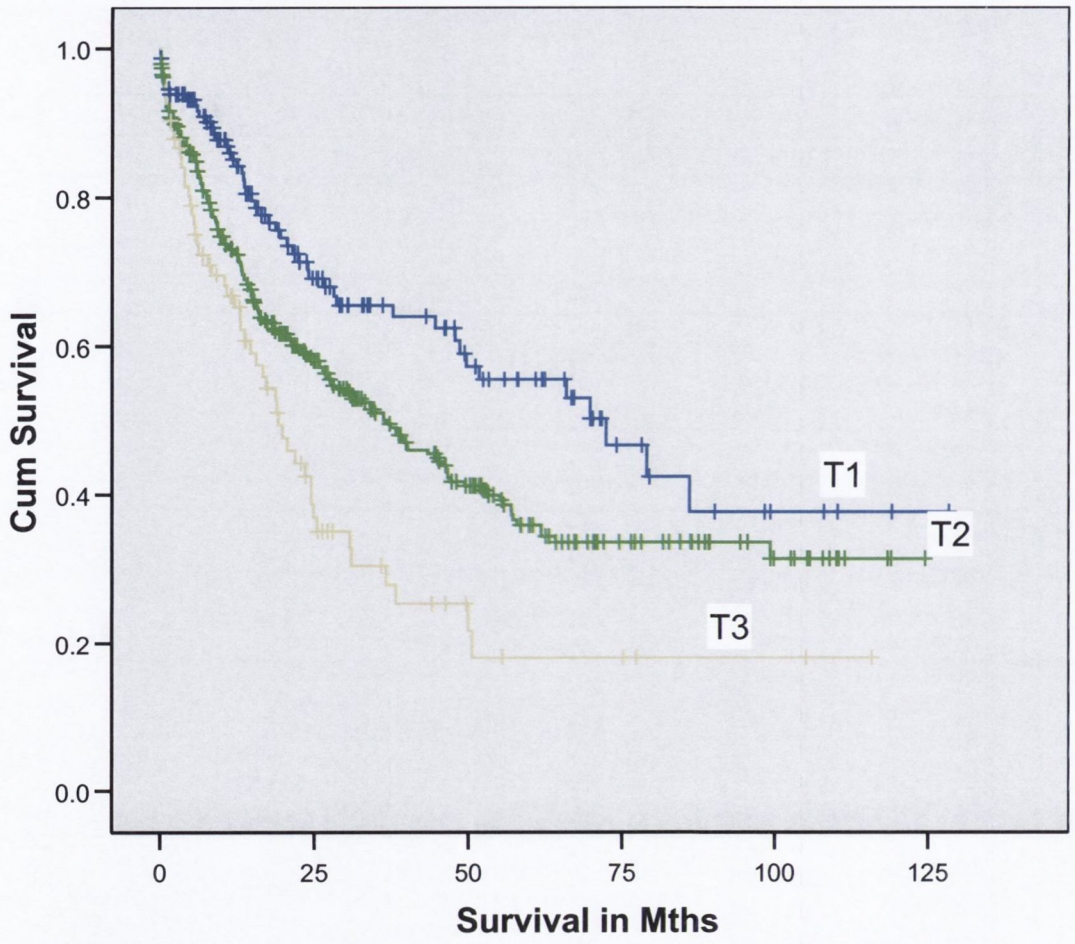

Figure 3.1.10 Kaplan Meier survival curves for patients according to T stage

Means and Medians for Survival Time

N stage	Mean(a)				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
0	62.107	3.521	55.206	69.009	44.633	3.945	36.901	52.366
1	54.063	5.097	44.073	64.054	30.700	8.801	13.449	47.951
2	40.056	5.613	29.054	51.059	19.200	5.281	8.850	29.550
Overall	57.743	2.721	52.410	63.075	37.367	4.395	28.752	45.982

Pairwise Comparisons

	N stage	0		1		2	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	**0**			.783	.376	9.061	.003
	1	.783	.376			3.187	.074
	2	9.061	.003	3.187	.074		

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	8.950	2	.011

Table 3.1.11 Means and medians and Log Rank test for survival time with respect to N stage

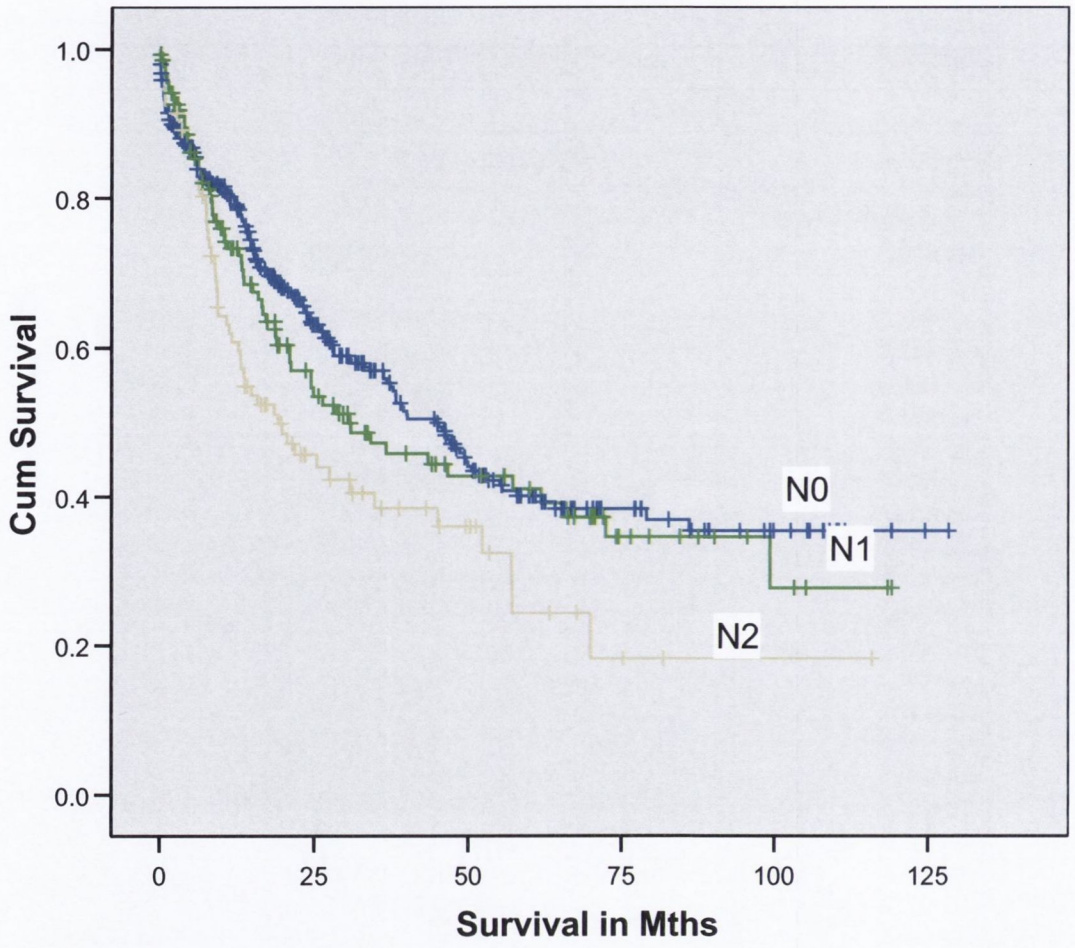

Figure 3.1.11 Kaplan Meier survival curves for patients according to N stage

Means and Medians for Survival Time

Overall stage	Mean(a)				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
IA	72.024	6.786	58.723	85.326	79.133	18.371	43.125	115.141
IB	65.130	11.920	41.767	88.493	72.467	36.344	1.233	143.700
IIA	39.136	5.255	28.836	49.437	19.200	4.576	10.231	28.169
IIB	61.800	4.230	53.509	70.092	45.833	6.548	32.999	58.668
IIIA	42.228	4.409	33.587	50.870	27.533	4.666	18.388	36.679
Overall	57.743	2.721	52.410	63.075	37.367	4.395	28.752	45.982

Pairwise Comparisons

	Overall Stage	IA		IB		IIA		IIB		IIIA	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	**IA**			.090	.764	16.337	<0.001	1.890	.169	10.378	.001
	IB	.090	.764			3.972	.046	.301	.583	2.387	.122
	IIA	16.337	<0.001	3.972	.046			11.159	.001	.699	.403
	IIB	1.890	.169	.301	.583	11.159	.001			5.233	.022
	IIIA	10.378	.001	2.387	.122	.699	.403	5.233	.022		

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	23.158	4	<0.001

Table 3.1.12 Means and medians and Log Rank test for survival time with respect to overall pathologic stage

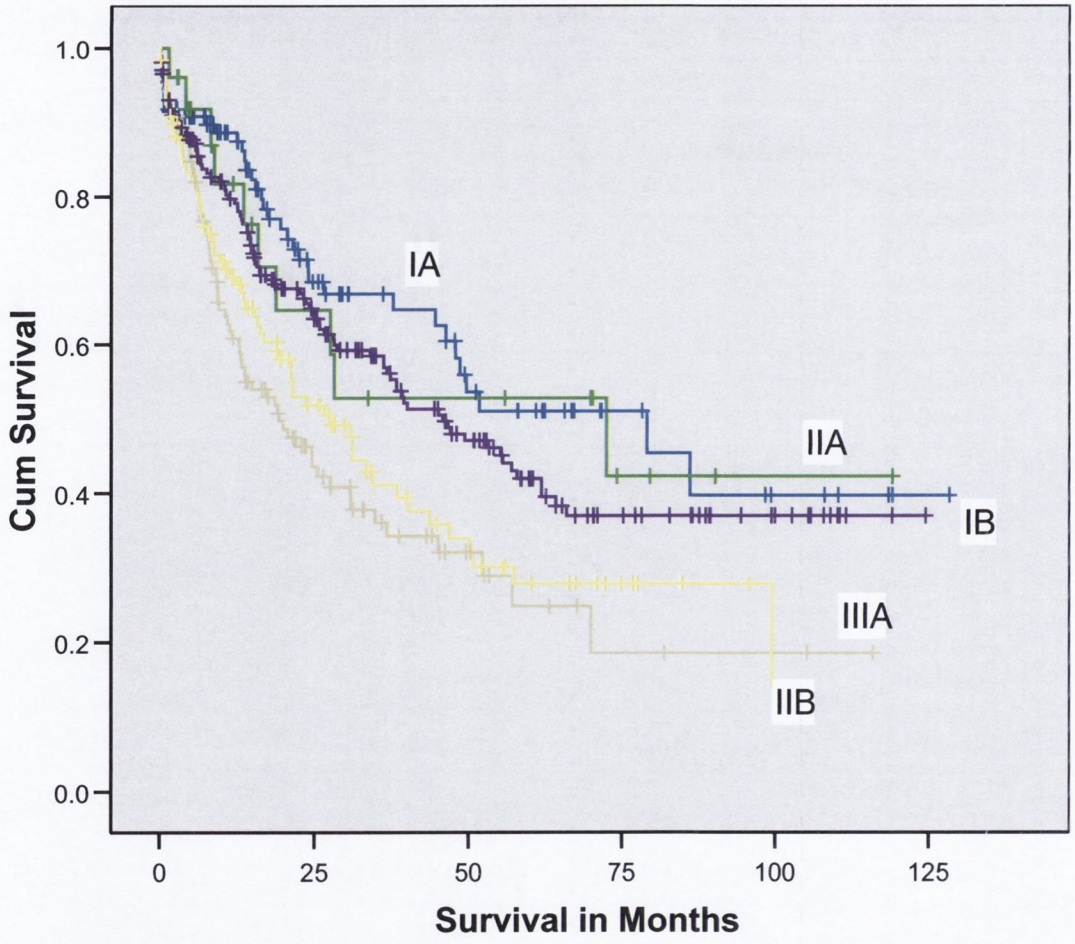

Figure 3.1.12 Kaplan Meier survival curves for patients according to overall pathologic stage

Means and Medians for Survival Time

Tumour size	Mean(a)				Median			
			95% Confidence Interval				95% Confidence Interval	
	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
≤3 cm	74.662	4.863	65.131	84.193	72.467	14.098	44.835	100.098
> 3-5 cm	54.039	4.025	46.149	61.928	34.800	5.647	23.732	45.868
> 5 cm	37.760	4.278	29.375	46.145	16.567	2.189	12.275	20.858
Overall	57.853	2.729	52.504	63.202	37.367	4.375	28.791	45.942

Pairwise Comparisons

	Tumour size	<3 cm		≥3-5 cm		≥5 cm	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	**<3 cm**			11.924	.001	37.983	<0.001
	≥3-5 cm	11.924	.001			9.705	.002
	≥5 cm	37.983	<0.001	9.705	.002		

Overall Comparisons

	Chi-Square	Df	Sig.
Log Rank (Mantel-Cox)	37.678	2	<0.001

Table 3.1.13 Means and medians and Log Rank test for survival time with respect to tumour size

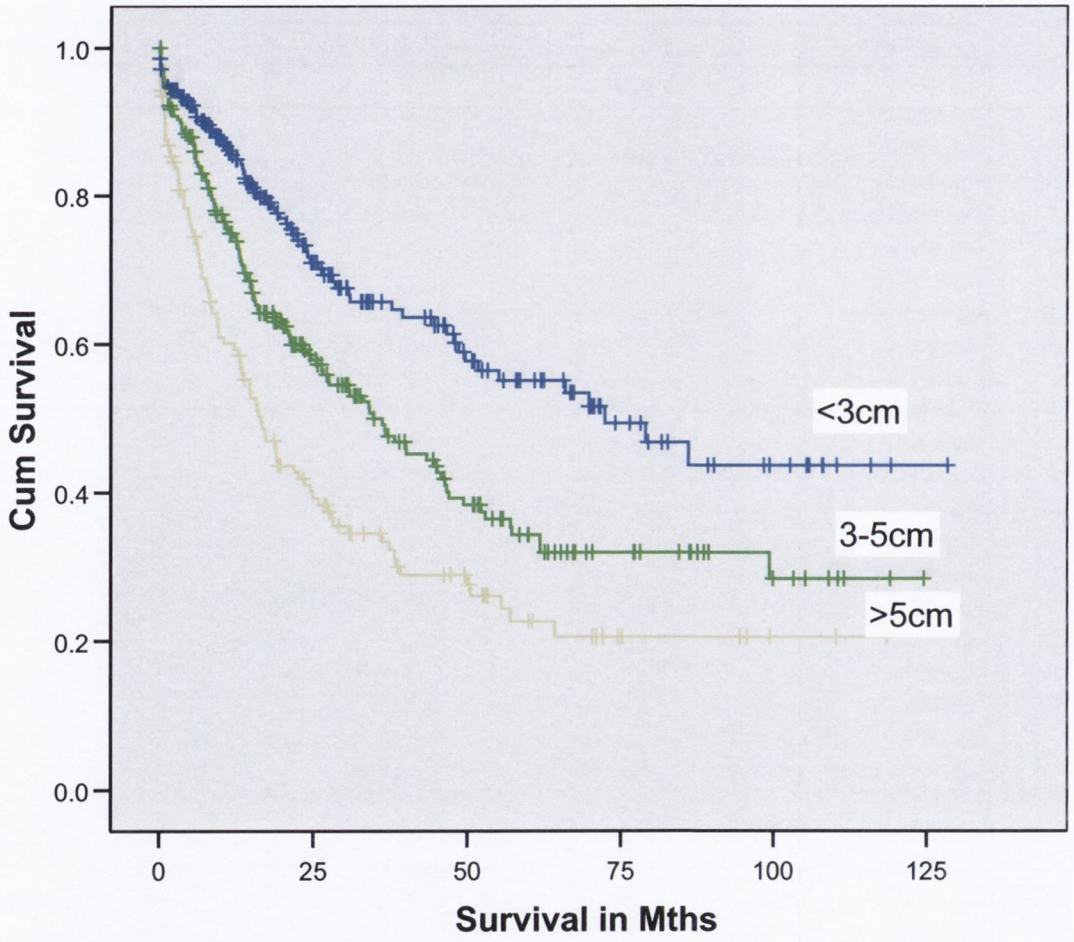

Figure 3.1.13 Kaplan Meier survival curves for patients according to tumour size

Means and Medians for Survival Time

Tumour Size (cm)	Mean(a)				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
≤2	65.519	8.652	48.562	82.477	86.133	25.862	35.443	136.823
>2 to ≤3	79.316	7.614	64.393	94.239	79.133	.	.	.
>3 to ≤5	60.310	5.267	49.987	70.633	40.067	5.511	29.265	50.869
>5 to ≤7	29.429	5.441	18.765	40.092	18.800	7.134	4.817	32.783
>7	54.448	10.337	34.189	74.708	38.300	.	.	.
Overall	62.298	3.537	55.365	69.230	44.633	3.929	36.933	52.333

Pairwise Comparisons

	Tumour Size (cm)	≤2		>2 to ≤3		>3 to ≤5		>5 to ≤7		>7	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	**≤2**			1.272	.259	1.241	.265	12.717	<0.001	.127	.721
	>2 to ≤3	1.272	.259			5.881	.015	25.657	<0.001	1.414	.234
	>3 to ≤5	1.241	.265	5.881	.015			11.462	.001	.136	.712
	>5 to ≤7	12.717	<0.001	25.657	<0.001	11.462	.001			4.623	.032
	>7	.127	.721	1.414	.234	.136	.712	4.623	.032		

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	28.392	4	<0.001

Table 3.1.14 Means and medians and Log Rank test for survival time with respect to tumour size (updated TNM classification)

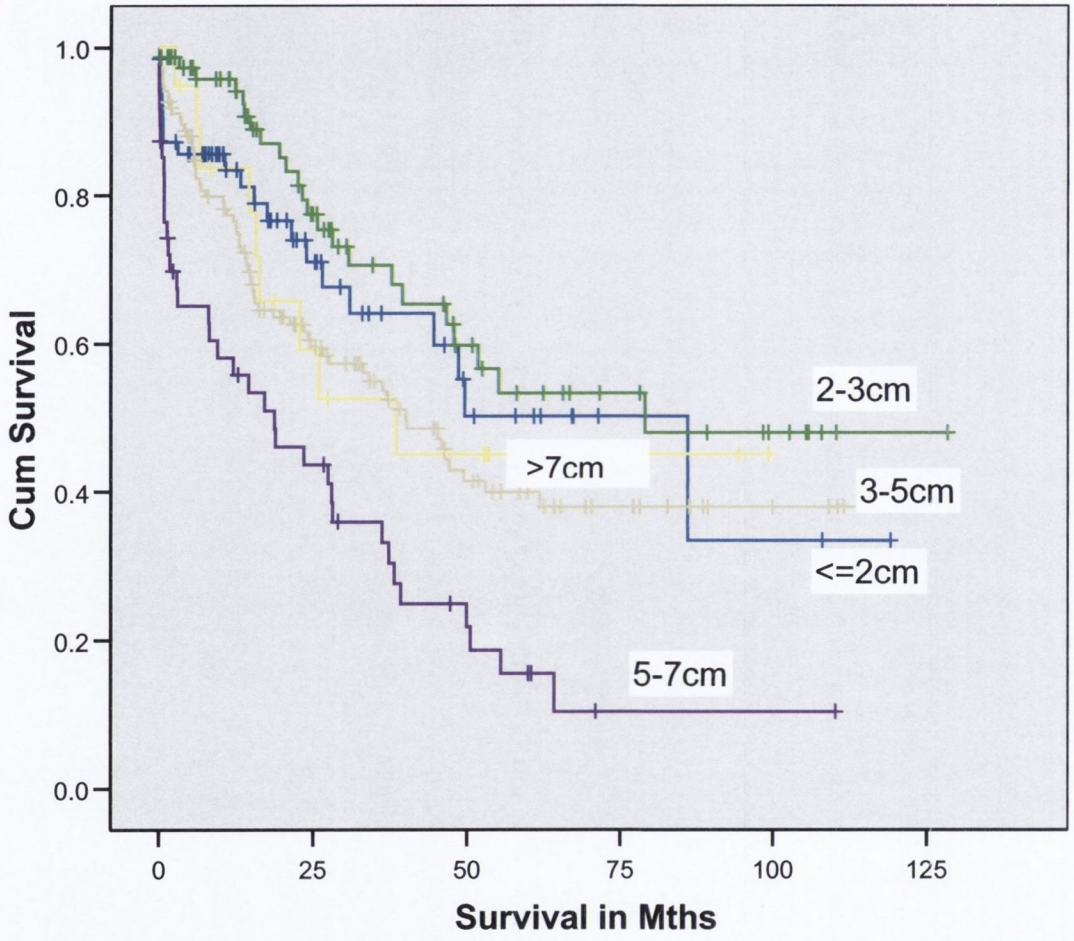

Figure 3.1.14 Kaplan Meier survival curves for patients according to tumour size (updated TNM classification)

Category	N	Description	Hazard Ratio	95% CI	*p*-value
Sex					**<0.001**
	354	Male	1.756	1.347-2.289	
	212	Female	1		
Age (years)					**0.001**
	29	<50	0.238	0.115-0.491	
	115	50-59	0.28	0.161-0.485	
	228	60-69	0.322	0.192-0.539	
	171	70-79	0.383	0.226-0.643	
	22	>80	1		
Smoking status					**0.006**
	206	Current smoker	0.536	0.354-0.813	
	301	Ex-smoker	0.495	0.331-0.740	
	40	Never smoker	1		
Operation type					**0.018**
	464	Other	0.702	0.528-0.932	
	102	Pneumonectomy	1		
Operation side					**0.72**
	281	Left	0.958	0.756-1.213	
	284	Right	1		
Resection margins					
	499	Uninvolved	0.615	0.443-0.855	**0.006**
	67	Involved	1		
Histology pattern					**0.179**
	294	Squamous	1.17	0.928-1.491	
	272	Non-Squamous	1		
Tumour grade					**0.102**
	37	Well differentiated	0.763	0.472-1.232	
	336	Moderately differentiated	0.764	0.595-0.980	
	193	Poorly differentiated	1		
Tumour size (cm)					**<0.001**
	210	<3	0.386	0.282-0.527	
	218	3-5	0.647	0.492-0.852	
	137	>5	1		

Table 3.2 Factors influencing survival by univariate analysis

Category	N	Description	Hazard Ratio	95% CI	*p*-value
T stage					**<0.001**
	147	T1	0.465	0.274-0.599	
	337	T2	0.636	0.465-0.871	
	82	T3	1		
N stage					
	338	N0	0.629	0.462-0.854	**0.017**
	136	N1	0.715	0.500-1.024	
	92	N2	1		
***p* Stage**					**<0.001**
	108	IA	0.526	0.350-0.788	
	197	IB	0.689	0.501-0.946	
	25	IIA	0.582	0.298-1.134	
	118	IIB	1		
	118	IIIA	1.172	0.835-1.645	

Table 3.2 (contd.) Factors influencing survival by univariate analysis

Category	N	Description	Hazard Ratio	95% C.I.	*p*-value
Sex					**<0.001**
	354	Male	1.844	1.385-2.449	
	212	Female	1		
Age (years)					**0.002**
	29	<50	0.202	0.088-0.467	
	115	50-59	0.348	0.184-0.659	
	228	60-69	0.349	0.189-0.643	
	171	70-79	0.417	0.226-0.771	
	22	>80	1		
Smoking status					**0.006**
	206	Current smoker	0.563	0.358-0.885	
	301	Ex-smoker	0.49	0.316-0.758	
	40	Never smoker	1		
Operation type					**0.881**
	464	Other	1.027	0.724-1.456	
	102	Pneumonectomy	1		
Resection margin					**0.174**
	499	Uninvolved	0.776	0.538-1.118	
	67	Involved	1		
Histology pattern					**0.593**
	294	Squamous	1.077	0.821-1.411	
	272	Non-squamous	1		
Tumour grade					**0.42**
	37	Well differentiated	0.834	0.497-1.399	
	336	Moderately differentiated	0.837	1.097	
	193	Poorly differentiated	1		
Tumour size (cm)					**<0.001**
	210	<3	0.372	0.245-0.564	
	218	3-5	0.718	0.535-0.962	
	137	>5	1		

Table 3.3. Independent prognostic factors by multivariate analysis

Category	N	Description	Hazard Ratio	95% C.I.	*p*-value
T stage					**0.93**
	147	T1	1.091	0.518-2.297	
	337	T2	0.956	0.642-1.424	
	82	T3	1		
***p* Stage**					**0.001**
	108	IA	0.540	0.290-1.005	
	197	IB	0.391	0.231-0.661	
	25	IIA	1.077	0.489-2.370	
	118	IIB	0.874	0.534-1.432	
	118	IIIA	1		

Table 3.3. (contd.) Independent prognostic factors by multivariate analysis

3.2 LYMPOCYTE SUBTYPE INFILTRATION IN RESECTED NSCLC

3.2.1 Patient characteristics

To examine the prognostic relevance of lymphocyte infiltration in NSCLC, a subset of the overall NSCLC cohort was selected in which immunohistochemical analysis was performed. Of a total of 255 patients who underwent surgery with curative intent for NSCLC during the period January 2001 to January 2005, 197 patients fulfilled the selection criteria for evaluation on the basis of surgical resection completeness, pathological stage and tumour histopathological type. Archived paraffin embedded tissue from the tumour resection specimens was available for 196 patients. In the case of one individual, no residual tumour was detected in the surgical resection material for evaluation after initial diagnosis by bronchoscopy.

There were 125 males and 72 females (ratio of 1.74:1) in the cohort. The mean age of the patients at time of surgery was 65.5 years (standard deviation, 8.8; range 41-86). Follow-up data was available for a minimum of 36 months in all cases. There was no significant difference between the median ages of male and female patients (66.1y *vs.* 64.3y). Thirty-day mortality rate was 5%. After exclusion of perioperative deaths (n=10), there were 186 cases available for analysis. As of December 2008, 54.3% of the study population had died.

Fifty-eight additional patients who underwent surgery with curative intent for NSCLC during the period 2001-2005 were excluded from the analysis due to the following reasons: intraoperative upstaging (n=22); post-operative upstaging (n=21); non-anatomical/ wedge resections (n=9); no lymph node dissection performed (n=1); associated small-cell lung cancer on histopathology specimens (n=2); and non-lung cancer cases (n=3). Exclusion of patients with non-anatomical wedge resections was deemed necessary as survival might be negatively influenced by the conservative surgical approach adopted. In the case of the patient without lymph node dissection, staging was deemed incomplete and this individual was therefore omitted from the study.

Clinicopathological data are presented in **Table 3.4**. Factors associated with survival by univariate analysis were used to identify independent prognostic factors by multivariate analysis.

3.2.2 Surgical approach

Single lobectomy was performed in 74.1% of patients, of which a sleeve resection technique was undertaken in 16 (8.1%) cases. In 8.6% of patients, double lobectomy was required. Pneumonectomy was necessary in a further 17.3% (right sided: 12 patients; left sided: 22 patients).

3.2.3 Histopathological subtypes

Squamous cell carcinoma was the commonest histopathological subtype (47.7%), followed by adenocarcinoma (43.6%), mixed cellularity carcinomas (6.1%) and the large cell neuroendocrine variant (2.5%). For the purposes of evaluation, patients were grouped as 'squamous', 'adenocarcinoma' and 'other'. There was a greater proportion of adenocarcinoma among women than men (55.6% *v* 36.8%) whereas this trend was reversed for squamous cell carcinoma (37.5% *v* 53.6%). Residual microscopic disease (involving the resection margins of the hilar soft tissue, bronchus, resected major vascular structures or chest wall) was present in 12.3% of patients.

3.2.4 Staging

Pathologic stage was confirmed as follows: stage IA (T1N0), 15.6%; stage IB (T2N0), 39.7%; stage IIA (T1N1), 4.3%; stage IIB (T2N1, T3N0), 19.4%; stage IIIA (T3N1, T1-T3N2), 21.0%. For the purposes of evaluation, patients were grouped by stage according the following: stage I (stage IA and IB); stage II (stage IIA and IIB); and stage IIIA. The median tumour size was 4 cm.

3.2.5 Survival

Patient status was recorded on an ongoing basis. Survival data was censured in December 2008, by which time 53.8% (106/197) of the total cohort had died. Overall median survival of patients included in the immunohistochemical was 37.5 months.

Variable	N	HR	CI	P-value
Sex		0.88	0.57-1.36	0.58
Male	115			
Female	71			
Age		1.31	1.21-1.58	0.04
Mean	65.8			
Range	41-86			
Smoking status		0.94	0.63-1.40	0.79
Current smokers	55			
Former smokers	120			
Never smokers	11			
Type of operation		0.98	0.39-2.41	0.96
Single lobectomy	142			
Bilobectomy	10			
Pneumonectomy	34			
Tumour size		2.34	1.53-3.57	<0.001
≤5 cm	124			
>5 cm	62			
Pathological stage		1.34	1.16-1.55	0.0001
IA & IB	103			
IIA & IIB	44			
IIIA	39			
Tumour grade		1.08	0.76-1.55	0.64
1&2	123			
3	62			
Unknown[a]	1			
Histological subtype		1.12	0.9-1.41	0.29
Squamous	85			
Adenocarcinoma	84			
Other	17			
Residual Microscopic Disease		0.43	0.25-0.74	0.003
Absent	163			
Present	23			
Total number of patients	186			

N denotes number, HR hazard ratio and CI confidence interval
[a] In one patient histological grade was not established

Table 3.4 Clinicopathological characteristics of immunohistochemistry group

3.2.6 Tissue sample quality

Staining for each of the markers of interest was distinct and readily distinguishable from surrounding tumour and stroma (**Figure 3.2**). Patterns of lymphocyte infiltration showed considerable heterogeneity across the study population (**Figure 3.3**). However, for each marker, lymphoid distribution was relatively homogenous within individual tissue sections after exclusion of regions characterised by necrosis and lymphoid aggregates. Appropriate isotype controls with omission of primary antibody were negative in each immunohistochemistry run.

The distribution of $CD3^+$, $CD8^+$ and $Foxp3^+$ lymphocytes was positively skewed and did not show a normal distribution. The numbers of each cell type (medians, range) per unit area of tumour and stroma are detailed in **Table 3.5**.

3.2.7 Validation of automated image analysis algorithm

Visual inspection revealed that the image analysis markup images of positively stained lymphocytes had a distinct appearance and were easily distinguishable from surround tumour and stromal element. An example of a computer-generated mark-up is shown in **Figure 3.4.**

In order to test the validity of image analysis algorithm, a manual count from five random HPFs from the first twenty patients in the dataset was made and recorded counts compared to those generated after the image analysis program was run. There was a strong correlation between counts recorded by the different methods (Spearman's Rho= 0.849; $P < 0.001$; **Figure 3.5**). The automated cell count algorithm was thus deemed a valid method with which to determine lymphocyte counts.

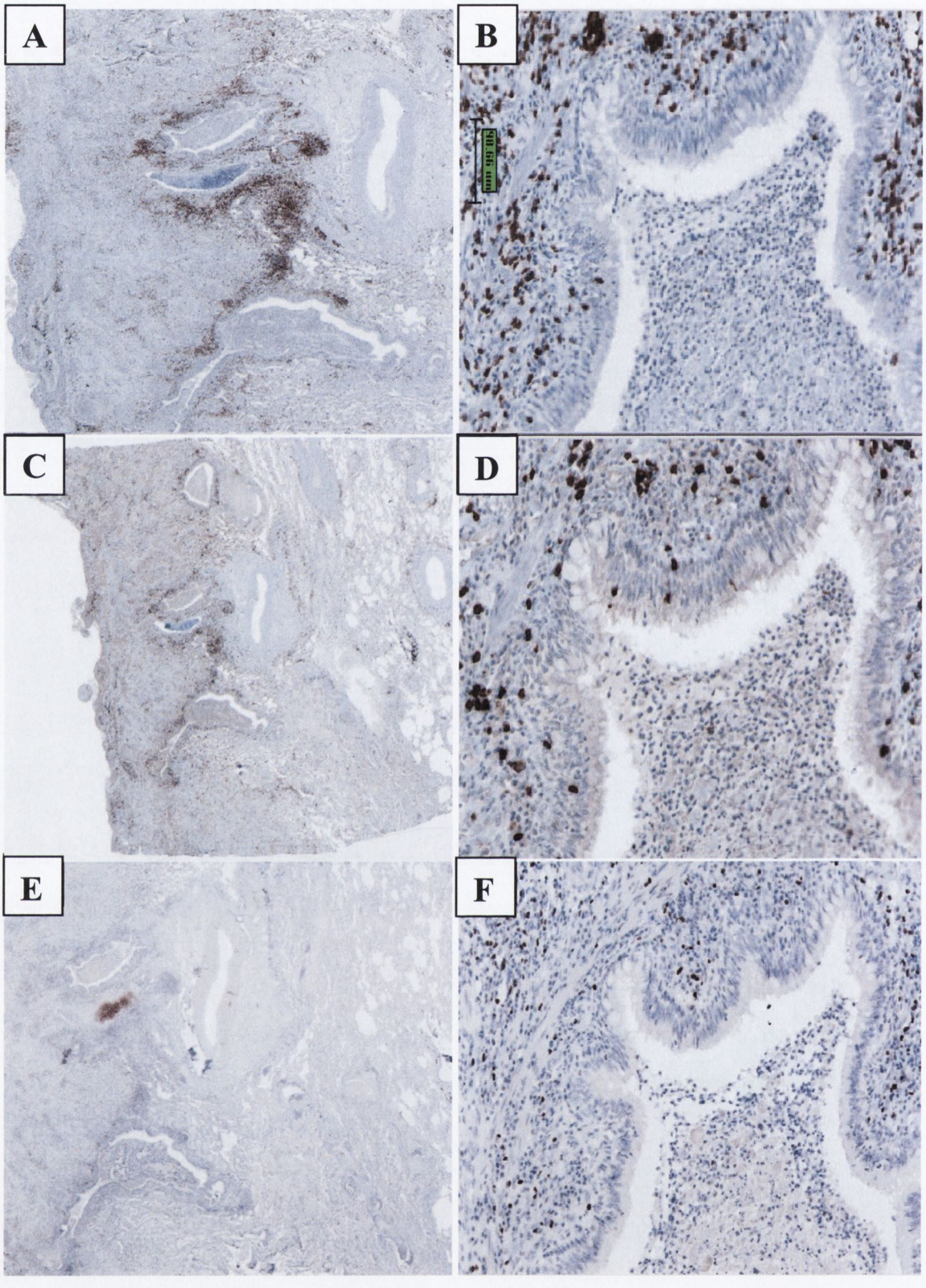

Figure 3.2 Immunohistochemical staining for CD3, CD8 and Foxp3. Representative photomicrographs of sequential sections demonstrating antibody staining for CD3, CD8 and Foxp3 are shown at 20x magnification in images A, C and E. Corresponding sections at 200x magnification are shown in images B, D and F.

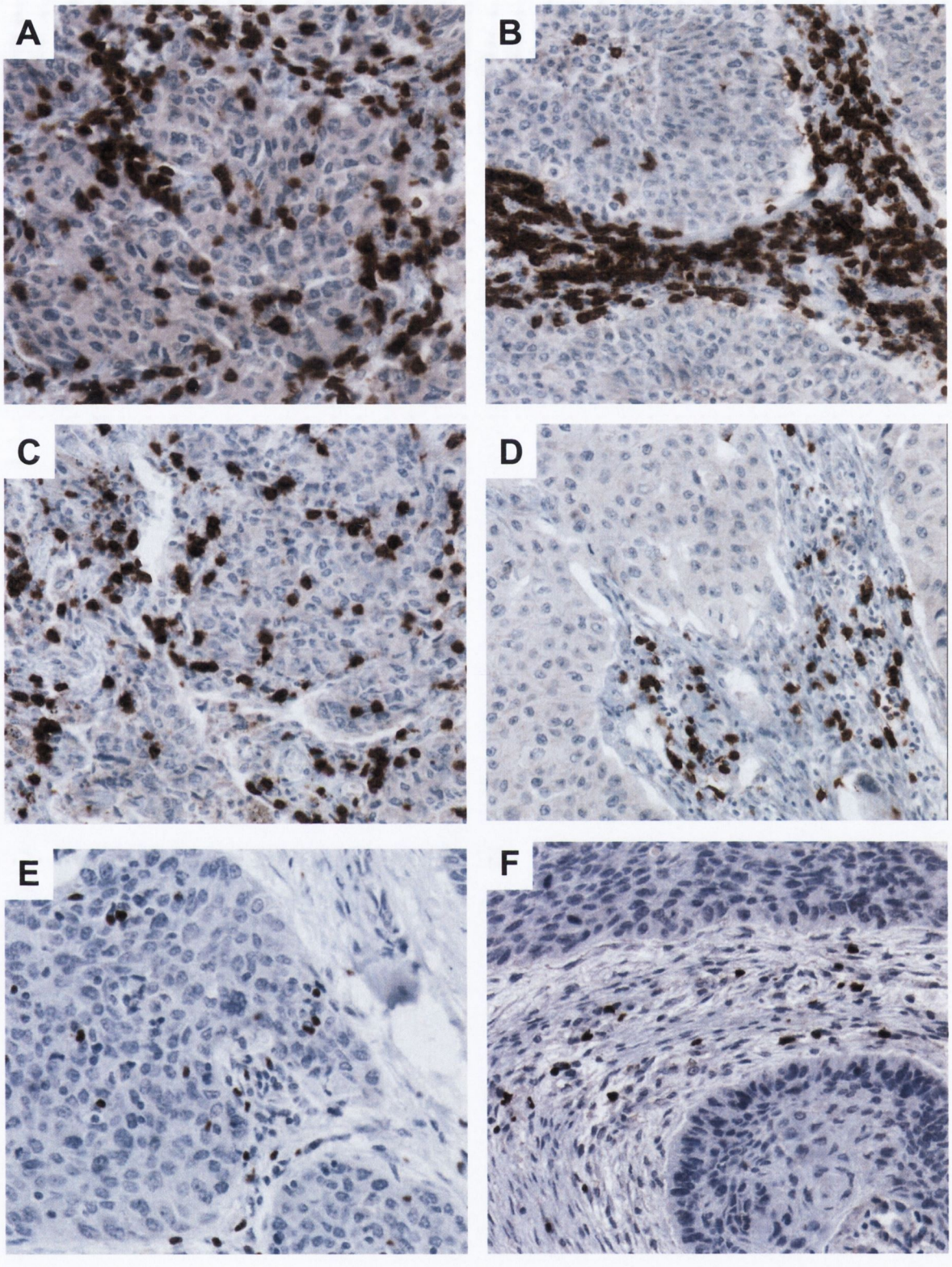

Figure 3.3 Lymphocyte infiltration patterns Immunohistochemistry demonstrating predominant tumour islet infiltration by $CD3^+$ (Panel A), $CD8^+$ (C) and $Foxp3^+$ (E) lymphocytes and predominant stroma predominant infiltration of $CD3^+$ (B), $CD8^+$ (D) and $Foxp3^+$ (F) lymphocytes

Table 3.5 Lymphocyte subset distribution counts

	CD3		CD8		Foxp3	
	Tumour Islet (per mm^2)	Stroma (per mm^2)	Tumour Islet (per mm^2)	Stroma (per mm^2)	Tumour Islet (per mm^2)	Stroma (per mm^2)
Median	518	2734	317	714	14	52
Range	0-4188	0-5625	0-4014	0-4693	0-2099	0-1339

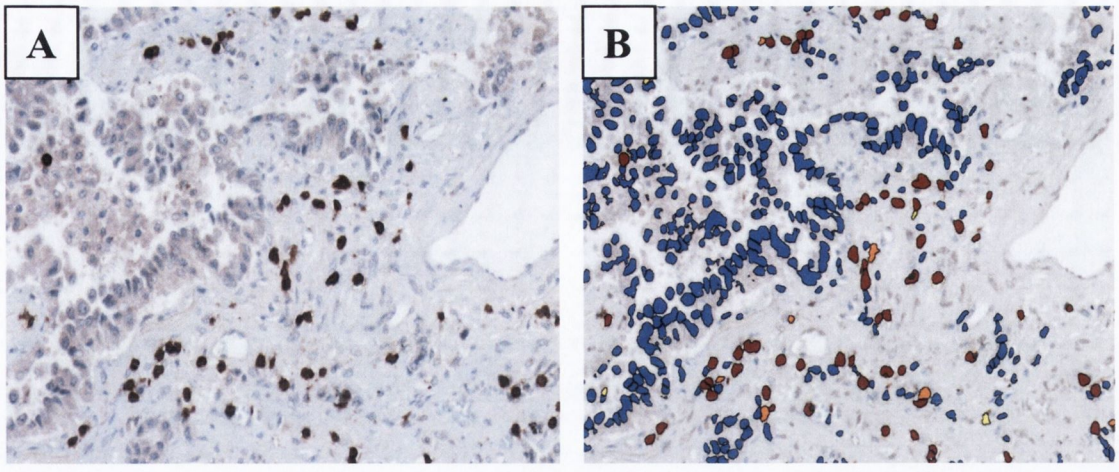

Figure 3.4 Mark up images of NSCLC sections HPF of original IHC section (A). Lymphocyte identification (in red) distinguished from surround tumour cells (in blue) by image analysis (B). The orange and yellow regions represent regions of staining below the imputed threshold for lymphocyte detection

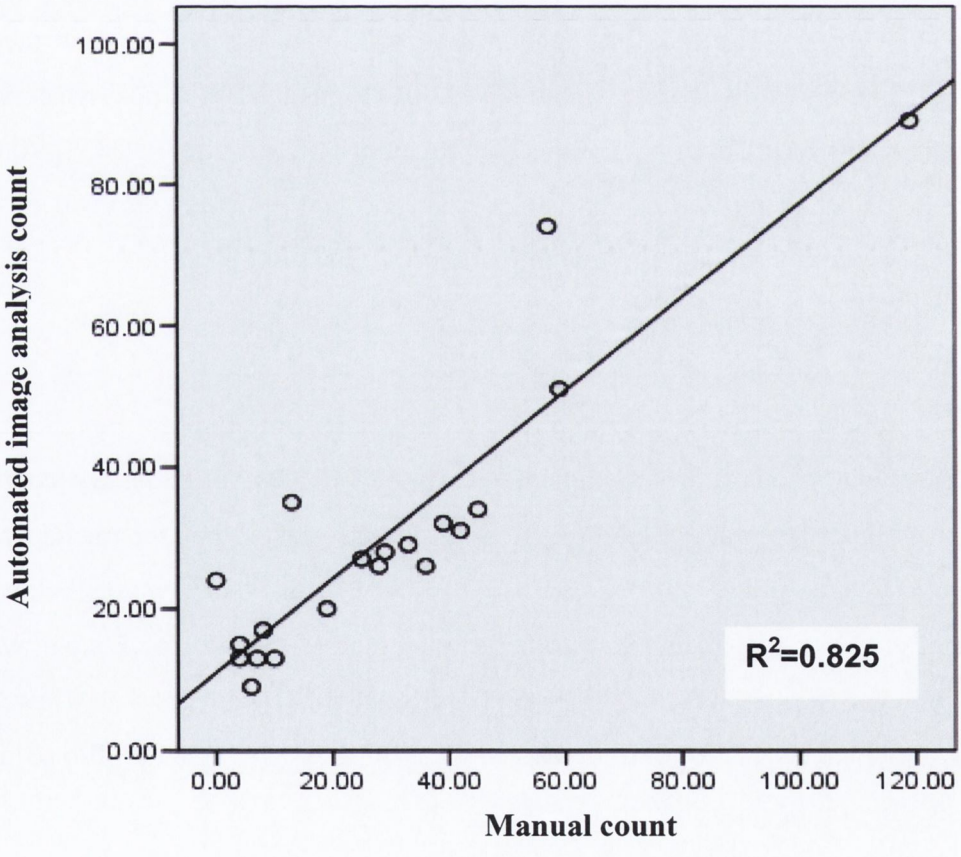

Figure 3.5 Correlation of manual and automated cell counts. Scatter plot demonstrating correlation between automated scores and manual annotation of the same HPFs

3.2.8 Prognostic relevance of lymphocyte subtype infiltration

3.2.8.1 $CD3^+$ lymphocytes

A trend for improved survival was observed for patients with tumour islet $CD3^+$ T cell infiltration counts greater than the median value compared to those with counts below the median (p=0.09; **Figure 3.6**). By contrast a significantly worse survival was seen in the group of patients with $CD3^+$ T cell peritumoral stromal infiltration counts greater than the median (p=0.02; **Figure 3.7**). A positive association between survival and the combination tumour islet/stromal (TI/S) ratio for $CD3^+$ also emerged (p=0.013; **Figure 3.8**). Patients with a high TI/S $CD3^+$ T cell ratio had a median survival of 45.8 months compared with 36.8 months for patients with a low TI/S ratio.

3.2.8.2 $CD8^+$ lymphocytes

A positive association between survival and increased $CD8^+$ T cell tumour islet infiltration counts was observed (p<0.001; **Figure 3.9**). By contrast, patients with $CD8^+$ T cell peritumoral stromal infiltration counts greater than the median value had a significantly worse outcome (p<0.001; **Figure 3.10**). A positive survival association was also observed for the combination TI/S ratio for $CD8^+$ (p<0.001; **Figure 3.11**). Patients with a high TI/S $CD8^+$ ratio had a median survival of 48.4 months, compared with 26.8 months for patients with a low TI/S ratio.

3.2.8.3 $Foxp3^+$ lymphocytes

A striking inverse association between increased intratumoral $Foxp3^+$ T cell infiltration and survival was demonstrated (p<0.001; **Figure 3.12**). By contrast, patients with peritumoral stroma $Foxp3^+$ T cell counts greater than the median value has a significantly better survival compared to those with $Foxp3^+$ counts below the median (p<0.001; **Figure 3.13**). Furthermore, a high TI/S ratio for $Foxp3^+$ was a strongly negative prognostic factor (p<0.001; **Figure 3.14**). Patients with a low TI/S $Foxp3^+$ ratio survived 51.9 months whereas than those with a high TI/S $Foxp3^+$ ratio survived only 20.9 months.

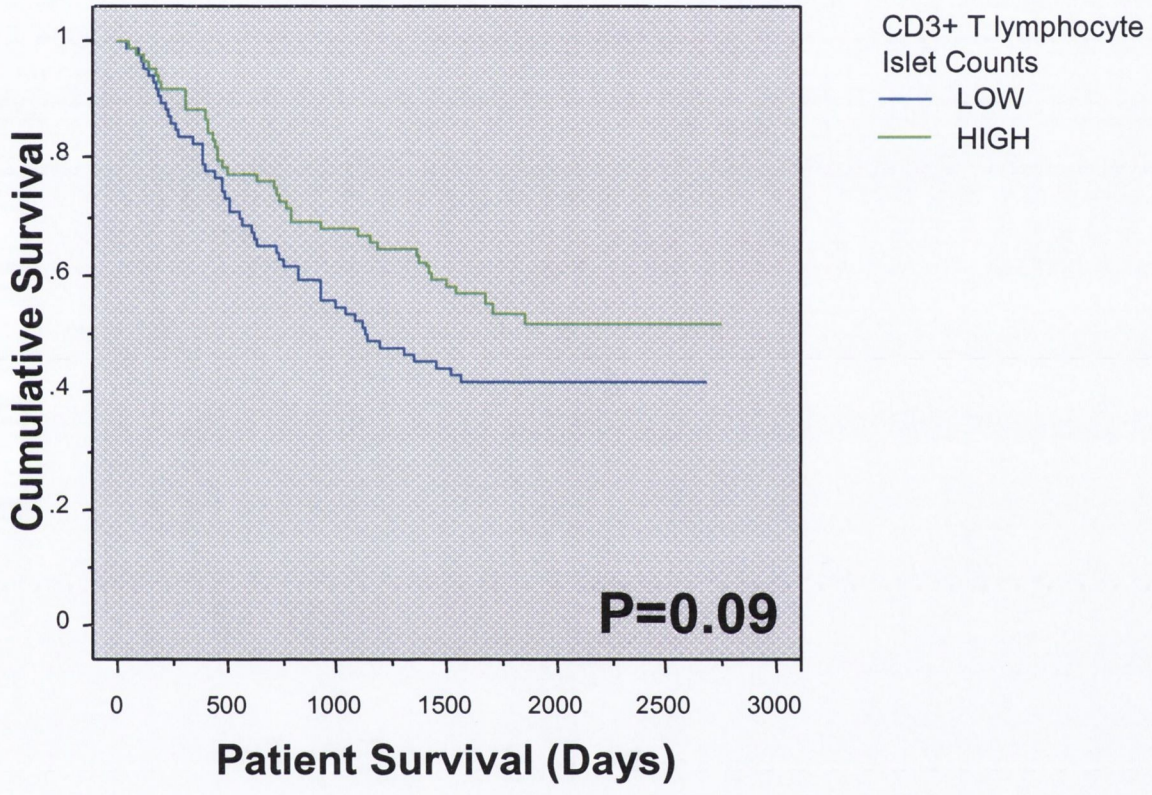

Figure 3.6 Survival according to $CD3^+$ islet infiltration. Kaplan Meier survival curves for patients with $CD3^+$ T-cell tumour islet infiltration ratios above (green) and below (blue) the median value.

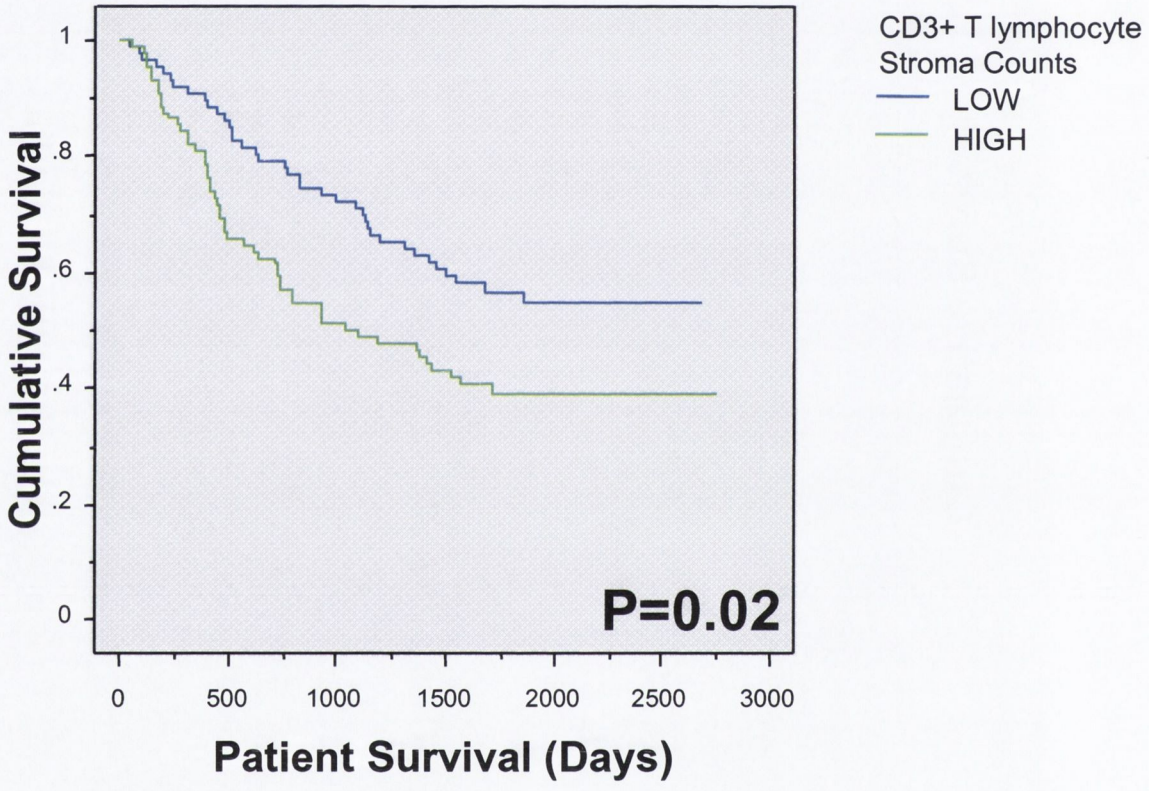

Figure 3.7 Survival according to $CD3^+$ stroma infiltration. Kaplan Meier survival curves for patients with $CD3^+$ T-cell peritumoral stromal infiltration ratios above (green) and below (blue) the median value.

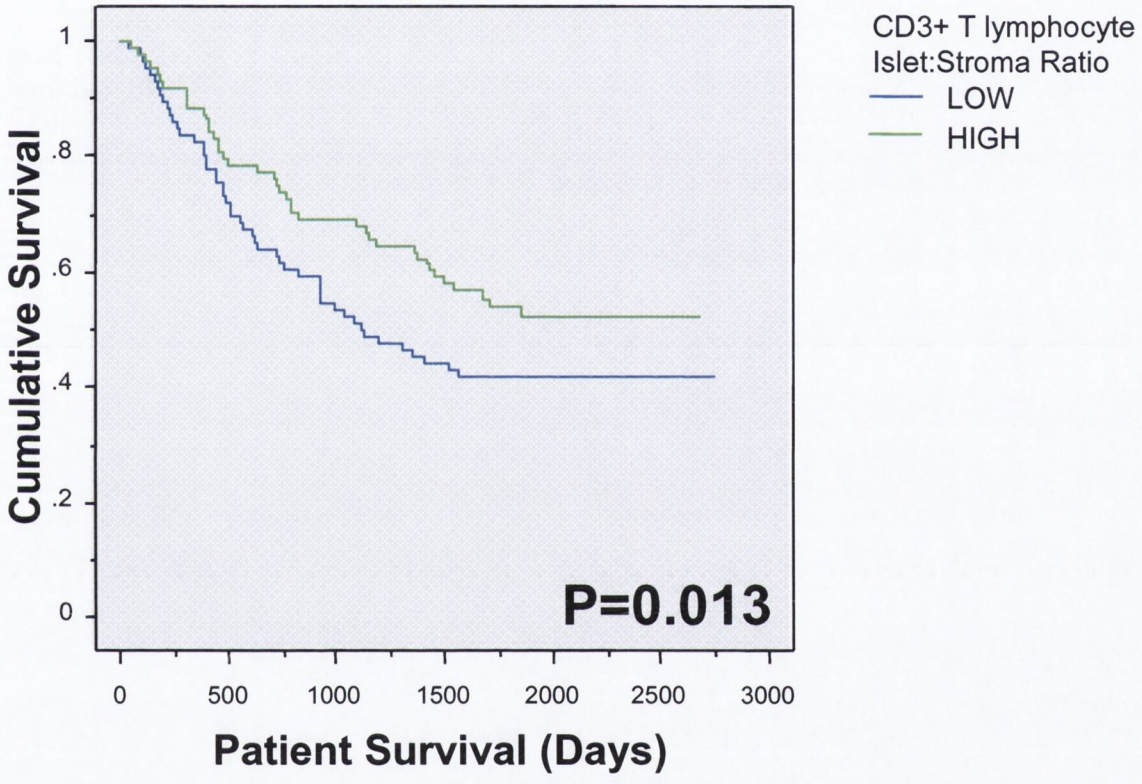

Figure 3.8 Survival according to $CD3^+$ islet/stroma infiltration. Kaplan Meier survival curves for patients with $CD3^+$ T-cell tumour islet/stroma infiltration ratios above (green) and below (blue) the median value.

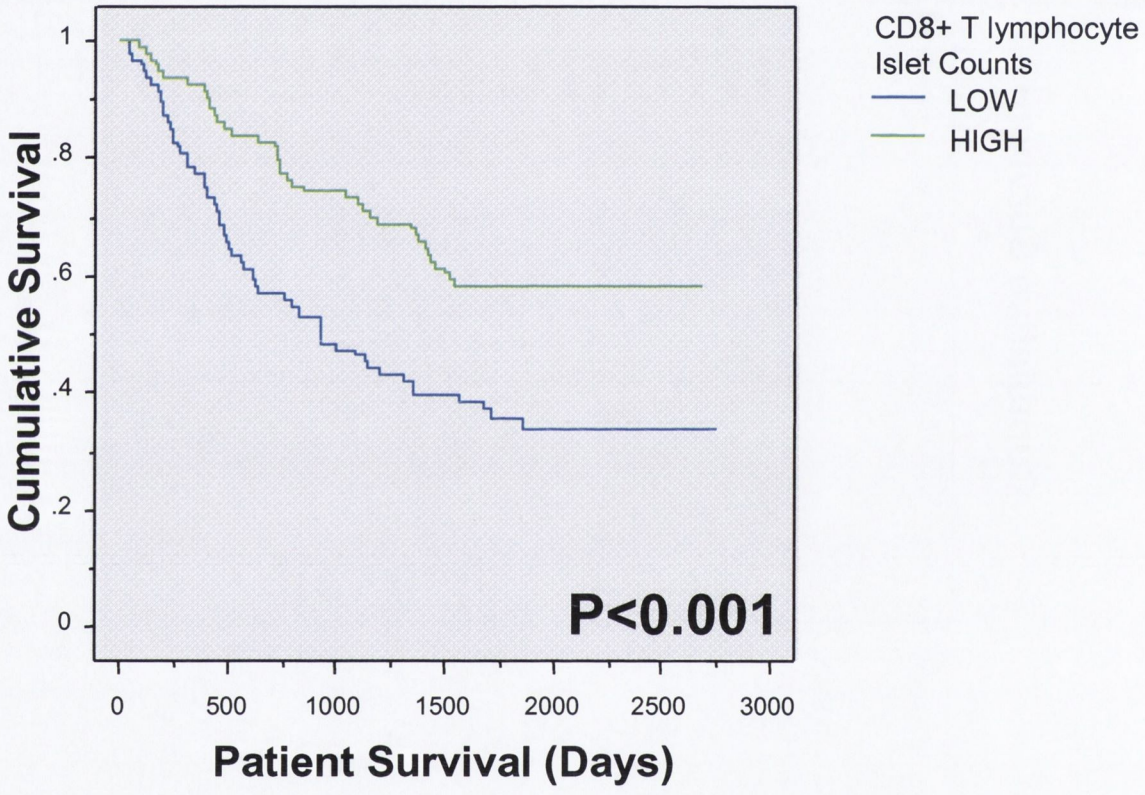

Figure 3.9 Survival according to $CD8^+$ islet infiltration. Kaplan Meier survival curves for patients with $CD8^+$ T-cell tumour islet infiltration ratios above (green) and below (blue) the median value.

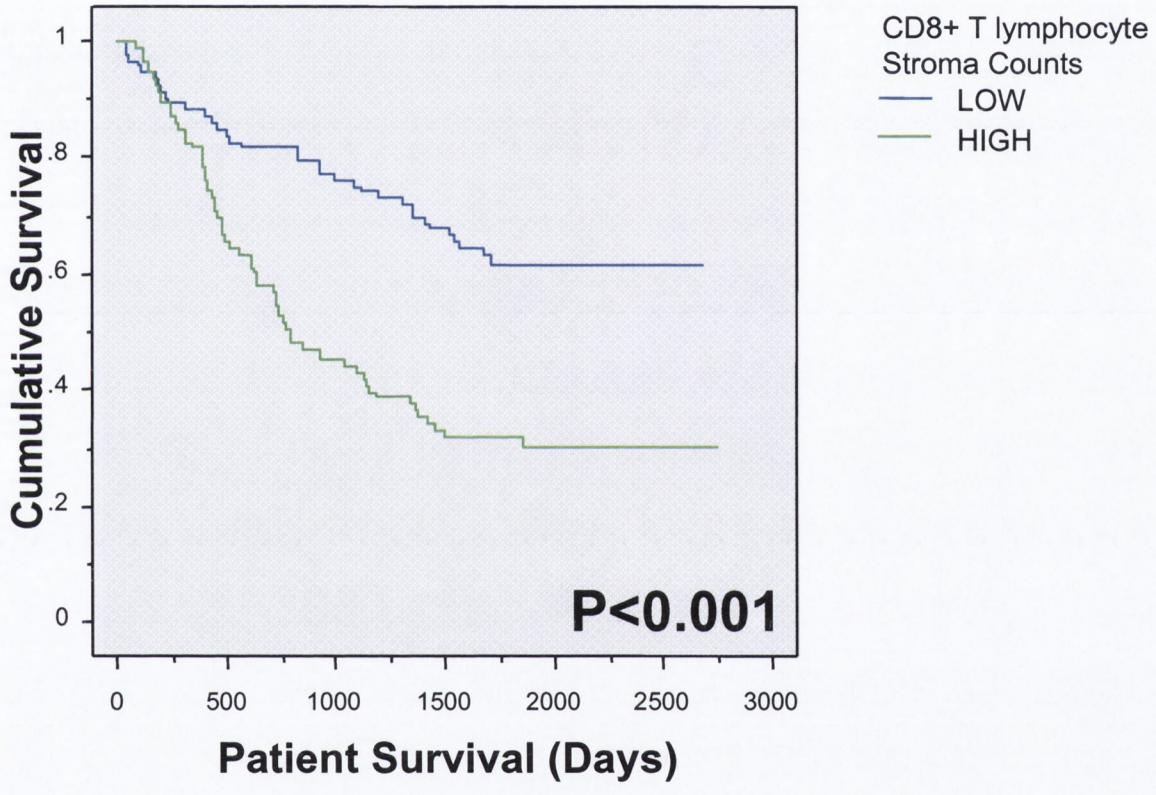

Figure 3.10 Survival according to $CD8^+$ stroma infiltration. Kaplan Meier survival curves for patients with $CD8^+$ T-cell peritumoral stromal infiltration ratios above (green) and below (blue) the median value.

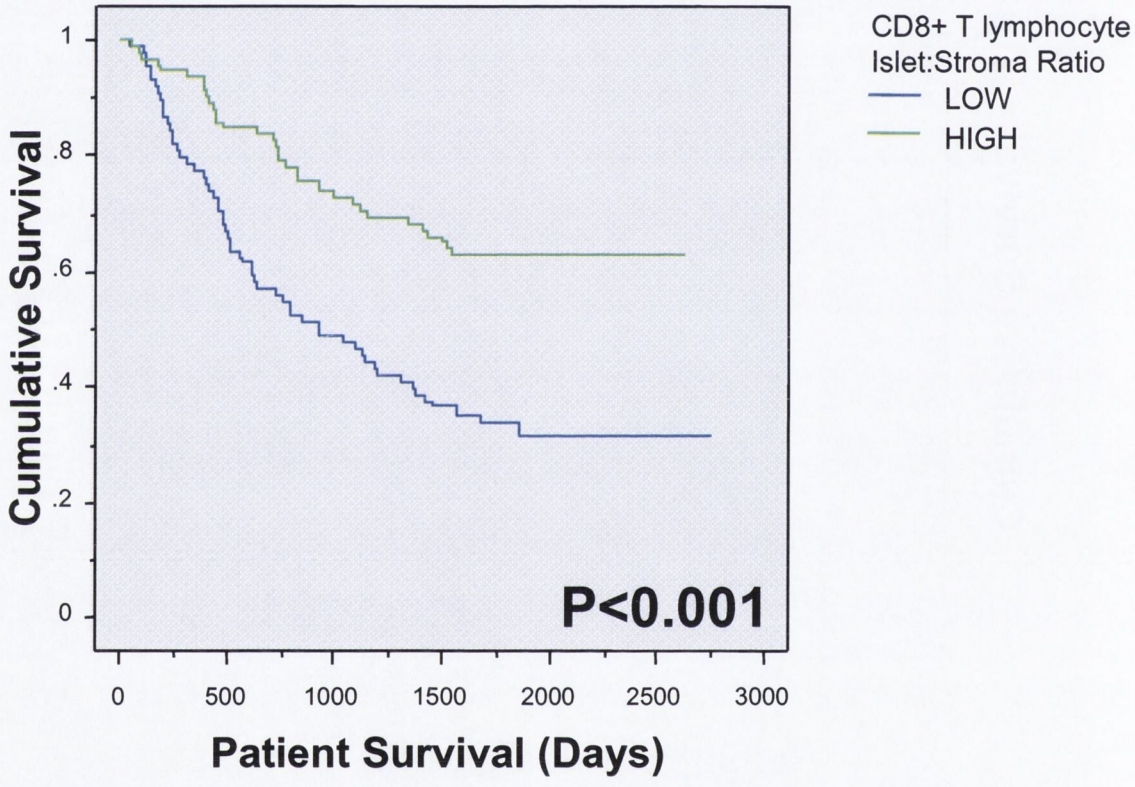

Figure 3.11 Survival according to CD8$^+$ islet/stroma infiltration. Kaplan Meier survival curves for patients with CD8$^+$ T-cell tumour islet/stroma infiltration ratios above (green) and below (blue) the median value.

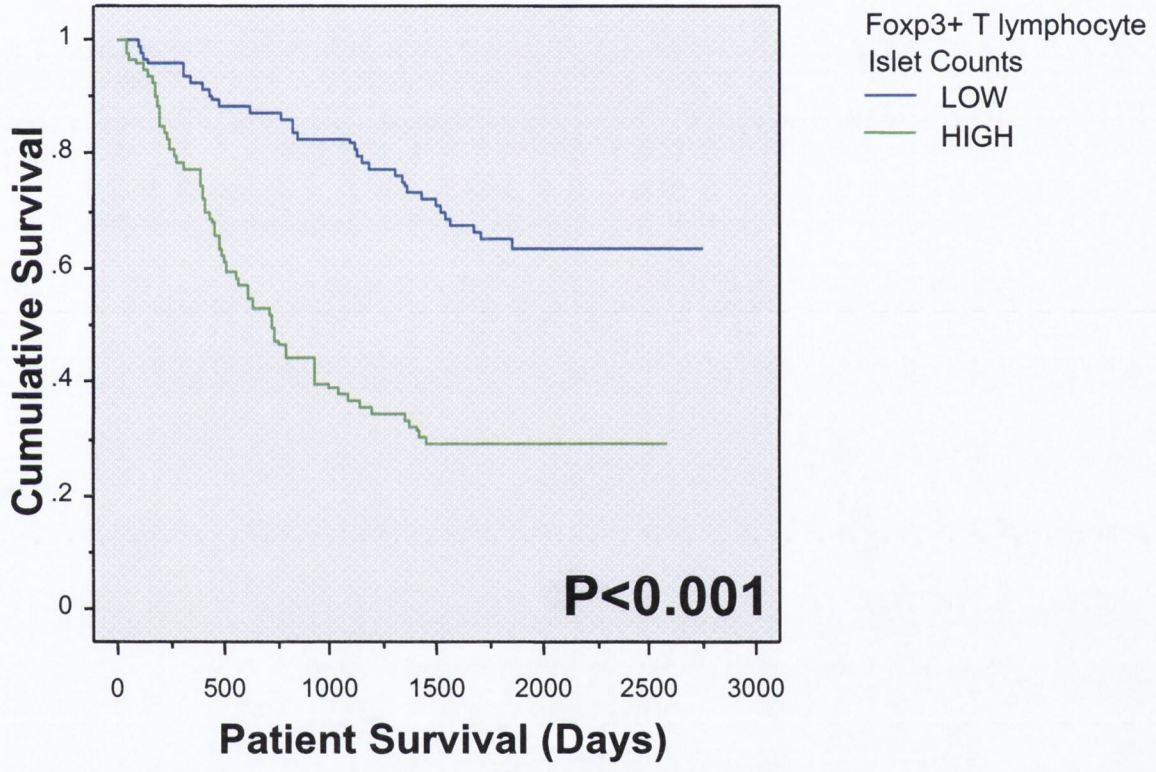

Figure 3.12 Survival according to Foxp3$^+$ islet infiltration. Kaplan Meier survival curves for patients with Foxp3$^+$ T-cell tumour islet infiltration ratios above (green) and below (blue) the median value.

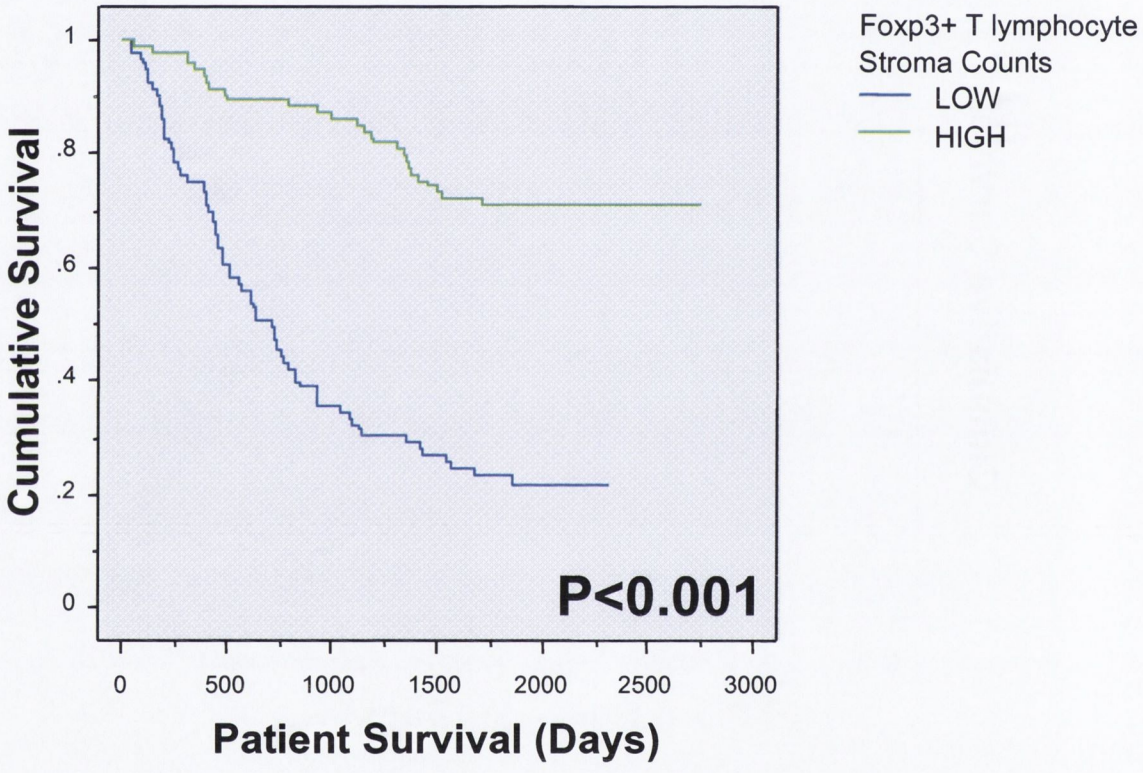

Figure 3.13 Survival according to Foxp3$^+$ stroma infiltration. Kaplan Meier survival curves for patients with Foxp3$^+$ T-cell peritumoral stroma infiltration ratios above (green) and below (blue) the median value.

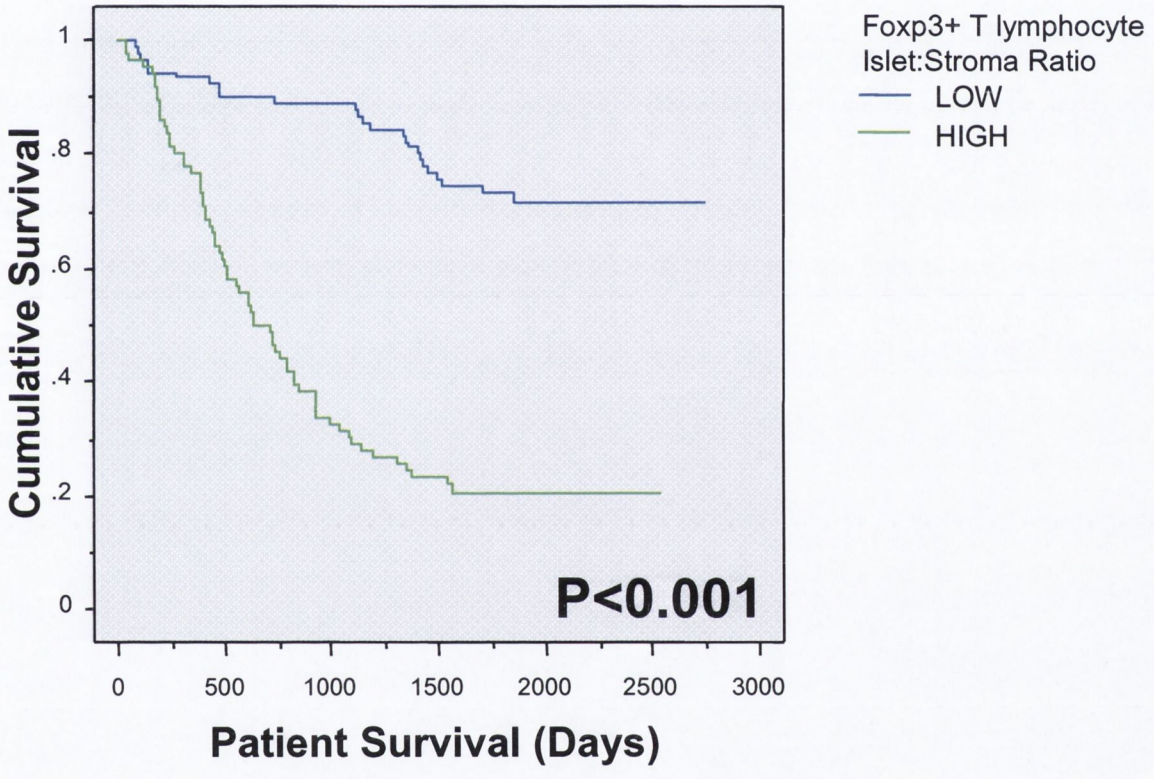

Figure 3.14 Survival according to Foxp3$^+$ islet/stroma infiltration. Kaplan Meier survival curves for patients with Foxp3$^+$ T-cell tumour islet/stroma infiltration ratios above (green) and below (blue) the median value.

3.2.9 Combined $CD8^+/Foxp3^+$ infiltration

The prognostic significance of different patterns of $CD8^+/Foxp3^+$ T-lymphocyte infiltration was also analysed. Patients were classified as 'CD8 HIGH' or 'CD8 LOW' depending on whether the $CD8^+$ TI/S infiltration ratio was above or below the median value. Infiltrative patterns of Tregs were similarly classified as 'Foxp3 HIGH' and 'Foxp3 LOW'. Survival patterns for patients based on the four possible combinations of $CD8^+$ and $Foxp3^+$ infiltration ratios were then examined (**Fig. 3.15**).

Results showed that a CD8 HIGH / Foxp3 LOW pattern was associated with the highest survival rate, with a 91% 3-year survival. By contrast, those with a CD8 LOW/Foxp3 HIGH pattern demonstrated only a 22% 3-year survival. Furthermore, the CD8 LOW/Foxp3 LOW group had a more favourable outcome than the CD8 HIGH/Foxp3 HIGH group (3-year survival 85.2% versus 42.4%, $P<0.001$), suggesting that the distribution of Treg cells exerts a greater influence on outcome than cytotoxic T-cells.

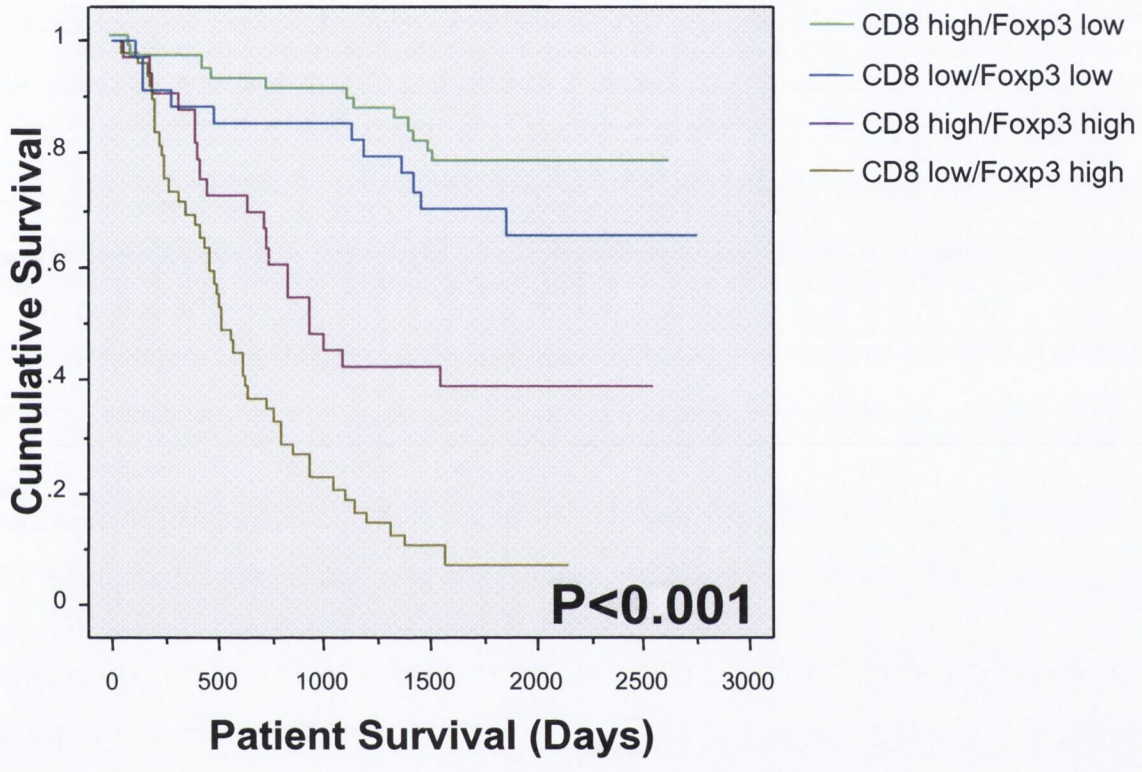

Figure 3.15 Survival according to combined CD8$^+$/Foxp3$^+$ infiltration. Kaplan Meier survival curves for patients with combined CD8$^+$ tumour islet/stroma ratio above the median (high) and Foxp3$^+$ tumour islet/stroma below the median (low) values (green), compared to CD8$^+$ low/Foxp3$^+$ low (blue), CD8$^+$ high/Foxp3$^+$ high (purple) and CD8$^+$ low/Foxp3$^+$ high (gold).

3.2.10 Multivariate analysis

The factors that were significant by univariate analysis were further examined after adjustment for potential confounding factors using multiple linear regression models. The proportional hazards assumption was employed to assess whether the ratio of regional distribution of the lymphocyte subsets were independent predictors of survival. Clinicopathological variables included as covariates in the analysis were age, stage, grade, tumour size, histological type, and presence of residual microscopic disease. These parameters were chosen because of the relative prognostic significance by univariate Cox proportional hazards model (**Table 3.6**).

A high intratumoral accumulation of either $CD3^+$ or $CD8^+$ lymphocytes and a low intratumoral accumulation of $Foxp3^+$ Tregs were independent prognostic factors for improved overall. The presence of combined high intratumoral $CD8^+$ lymphocytes and low intratumoral $Foxp3^+$ Tregs was also an independent positive prognostic factor (**Table 3.6**). The results from univariate Cox Regression analysis indicate that patients with low $CD8^+$ and high $Foxp3^+$ TIL consistently demonstrated the lowest overall survival when compared to the rest of the cohort (HR=5.7, P <0.00001). When we included the different clinicopathological variables above as a covariate in multivariate analyses, HRs and CIs for the prognostic significance of all TIL subtypes and the signatures CD8 LOW/Foxp3 HIGH and CD8 HIGH/Foxp3 LOW did not change significantly.

3.2.11 Tree regression analysis

The observation that TIL subset infiltration ratios identifies patients with poor outcomes is an important finding. However, its application in the clinical setting is limited. A decision model based on absolute counts of $Foxp3^+$and $CD8^+$ lymphocytes that facilitated identification of patients with good versus poor prognosis might be a more useful approach from a prognostication or therapeutic perspective. Decision trees, such as C4.5 are classifiers that predict class labels for data items (Quinlan, 1993). Decision trees are constructed by analyzing a set of training examples for which the class labels are known. They are then applied to

	Median Survival (months)	HR	95% CI	P value
$CD3^+$ TI/S Ratio		0.62	[0.39-0.98]	0.042
Above median	45.86			
Below median	36.83			
$CD8^+$ TI/S Ratio		0.40	[0.25-0.63]	<0.001
Above median	48.76			
Below median	29.33			
$FOXP3^+$ TI/S Ratio		4.73	[2.86-7.85]	<0.001
Above median	21.23			
Below median	52.5			
$CD8^+$ /$FOXP3^+$ groupings		0.63	[0.50-0.79]	<0.001
CD8 HIGH / FOXP3 LOW	52.96			
CD8 LOW / FOXP3 LOW	52.45			
CD8 HIGH / FOXP3 HIGH	31.03			
CD8 LOW / FOXP3 HIGH	17.16			
Regression tree groups: tumour		0.42	[0.33-0.53]	<0.001
CD8 HIGH / FOXP3 LOW	52.76			
CD8 LOW / FOXP3 LOW	44.31			
CD8 HIGH / FOXP3 HIGH	24.19			
CD8 LOW / FOXP3 HIGH	15.91			
Regression tree groups: stroma		5.3	[3.35-8.91]	<0.001
CD8 HIGH / FOXP3 LOW	15.46			
CD8 LOW / FOXP3 LOW	27.05			
CD8 HIGH / FOXP3 HIGH	46.30			
CD8 LOW / FOXP3 HIGH	58.40			

Age, stage, grade, tumour size, histological type, and presence of residual disease were adopted as covariates

TI/S indicates tumour islet/stroma; RFC, random forest clustering; HR, hazard ratio; CI, confidence interval

HIGH indicates a tumour islet/stroma ratio above the median value, LOW indicates a tumour islet/stroma ratio above the median value

Table 3.6 Prognostic significance of different lymphocyte infiltration patterns

classify previously unseen examples. A decision tree regression model was selected that used intratumoral $CD8^+$ and $Foxp3^+$ TILs. Ten-fold cross validation suggested that the model was not overfit to the data (P = .019).

Tree models were created using the RPART routines wherein regression models are derived that can be visualized as binary trees. The trees were pruned to a minimal complexity (fewest terminal nodes but with minimal loss of prognostic ability) by a cross-validation procedure in which models were built on a series of patient groups picked from the total population using a series of increasingly pruned trees (Therneau and Atkinson, 1997). The results over all the groups defined by the decision trees are summed, and the least complex, least error-prone model chosen.

Ten-fold cross validation was performed as an unbiased test for potential overfitting of the tree models. In this test, patients were assigned to ten overlapping groups, each comprising 90% of the total population. Ten prognostic models based on continuous automated were then derived. These ten models were used to predict outcome for the 10% of patients omitted from each model-building episode such that, ultimately, all patients were assigned to two outcome groups (alive and dead). The statistical significance of the association of the composite-predicted groups with outcome was assessed by the Wald test, and if nonsignificant (i.e. P>.05, two sided) it was deemed likely that the derived models were overfit. The least overfitted outcome prediction model was selected (Fig 4.A) and the tree was pruned to one with four terminal nodes corresponding to four distinct patient groups.

Infiltrative patterns of intratumoral $CD8^+$ and $Foxp3^+$ T-lymphocytes were visually different and each group was labelled according to the respective lymphocyte microlocalization pattern (**Fig. 3.16**). Kaplan-Meier survival analysis indicated that patients with a $CD8^+$LOW/$Foxp3^+$ HIGH signature had a significantly worse survival compared to the group of patient with a $CD8^+$HIGH/$Foxp3^+$LOW signature (P<0.001). Cox regression survival analysis showed that the derived tree regression model was as significant (HR=2.14; CI 1.73 to 2.63; P<0.001; **Fig. 3.17**) as the manually derived tumour/stroma infiltration ratio model and independent of clinicopathological parameters (**Table 3.6**).

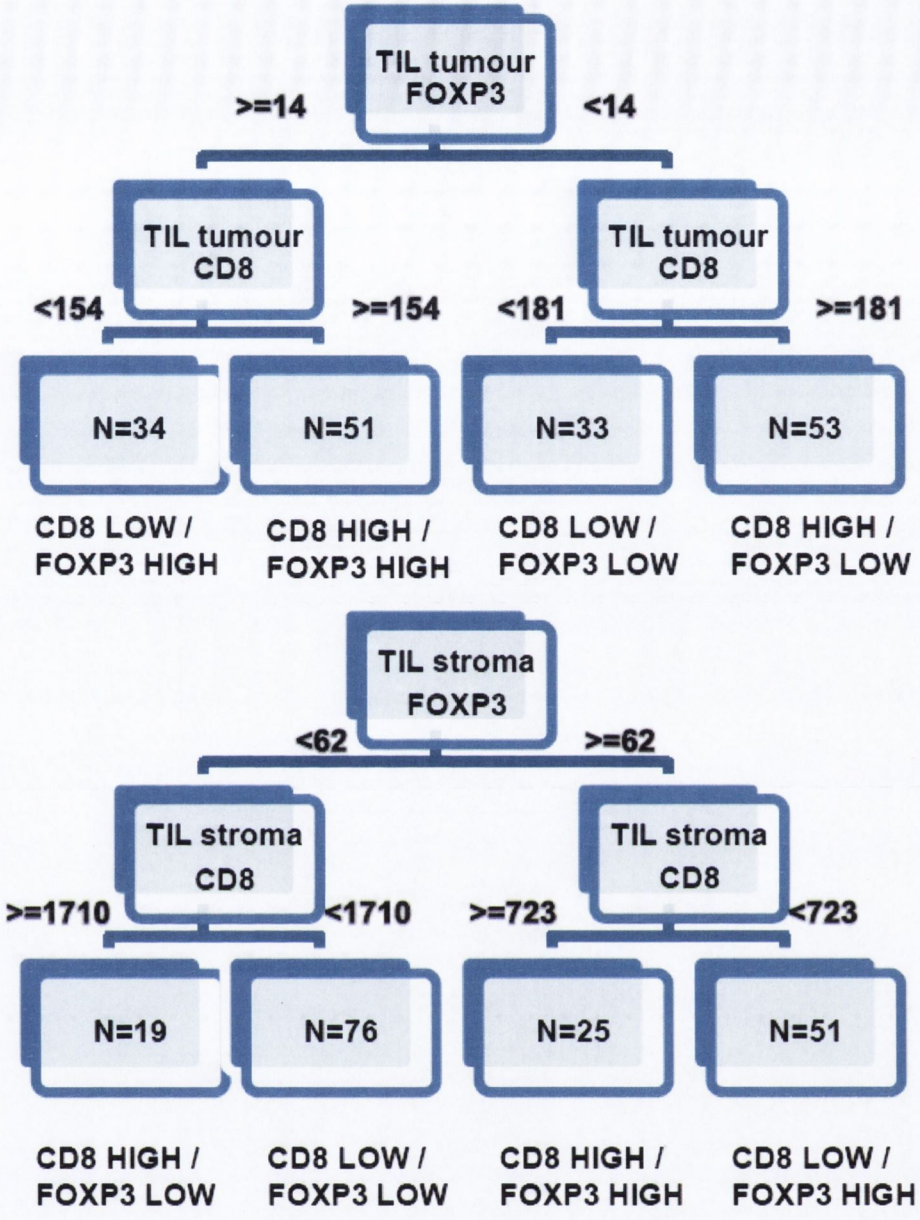

Figure 3.16 Tree regression model of Foxp3 and CD8 infiltration patterns. The final tree model with the four terminal nodes identified by the regression tree analysis according to CD8 and Foxp3 intratumoral (upper tree) and intrastromal (lower tree) infiltration pattern is shown. Numbers represent the lymphocyte count for each marker per unit area of region of analysis.

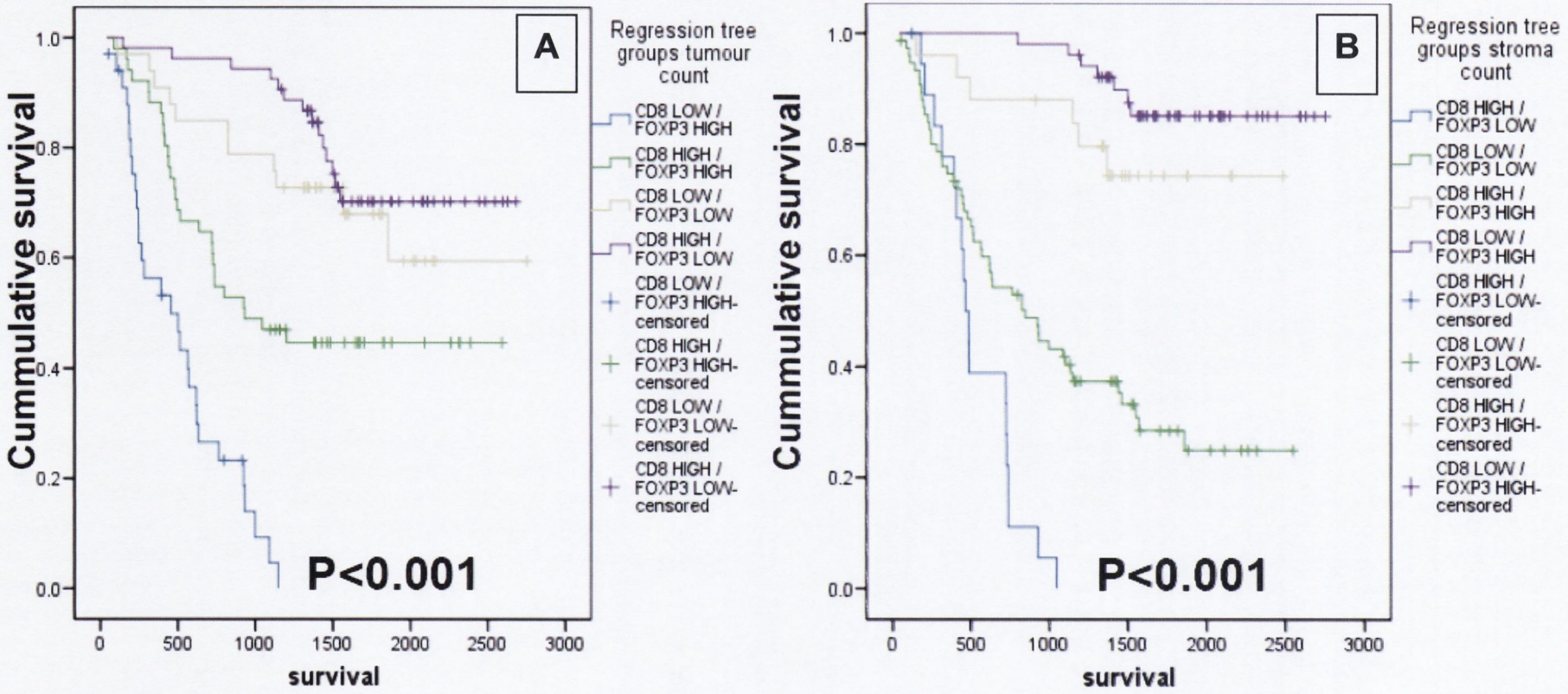

Figure 3.17 Survival according to regression tree CD8/Foxp3 infiltration. Kaplan Meier survival curves for patients with regression tree-defined combination $CD8^+/Foxp3^+$ T-lymphocyte infiltration counts within tumour islets (A) and stroma (B)

3.3 CYTOKINE EXPRESSION PROFILES IN RESPONSE TO NEOADJUVANT CHEMOTHERAPY

3.3.1 Patient characteristics

Samples from twenty one patients enrolled in the neoadjuvant chemotherapy pilot study were used in the PMBC cytokine study. Patient clinicopathological characteristics are presented in **Table 3.7.** All patients were chemotherapy naïve prior to treatment.

In our patient cohort, baseline clinical staging was as follows: stage IB=13; stage IIB=1; stage IIIA=7. After 3 cycles of induction treatment, there was one CR (5%), fourteen cases with PR (67%), 5 cases with SD (23%) and one case of PD (5%). One patient whose resection specimen identified concomitant small cell lung cancer was excluded from the analysis. Of the twenty remaining patients, 13 were men and 7 were women. Mean age at time of starting chemotherapy was 61 yrs +/- 10. Histopatholgoical subtypes were identified as adenocarcinoma (n=9); squamous cell carcinoma (n=9); pleiomorphic carcinoma (n=1); and adenosquamous (n=1).

3.3.2 Cytokines

A range of cytokines were selected to assess different aspects of the immune response in the patient population. The cytokine IL-4 induces differentiation of naive T_H cells to T_H2 cells whereas IFN-γ has potent activating function on T_H1 antitumour immune responses. IL-10 exerts pleiotropic effects but is considered an immunosuppressive cytokine that downregulates T_H1 cytokines. TGF-β and Foxp3 both exert regulatory function on T-cell responses. Levels of mRNA from PBMCs in the patients' blood were determined pre-treatment and pre-cycle 3 (C3) of induction chemotherapy.

In one patient, the quality of RNA extracted from the baselin sample was of insufficient quality to generate cDNA and could not be used. Therefore only pre C3 treatment cytokine levels were available from this patient for evaluation.

No.	Sex/ Age	Histological Pattern	PreCx CT stage	RECIST Response[a]	Pathological stage	Resection type
1	F/70	AC	IB	PR	IIIA	R lower lobectomy
2	M/76	Sq	IB	PR	NA	None [b]
3	M/66	Sq	IIIA	PR	NA	None [c]
4	M/69	Adsq	IIIA	PR	IIIB	L upper lobectomy
5	F/29	Ad	IIIA	SD	IIIA	R upper lobectomy
6	M/62	Ad	IB	PD	NA	None [c]
7	M/74	SCLC	IIIA	PR	IB	R lower lobectomy
8	M/51	Sq	IIIA	PR	IIIA	L lower lobectomy
9	M/56	Ad	IIB	SD	IIIA	None [c]
10	F/72	Ad	IB	SD	IIIA	L upper lobectomy
11	M/57	Pleio	IB	SD	IIIA	L pneumonectomy
12	F/65	Ad	IB	PR	NA	None [d]
13	M/71	Sq	IB	CR	CR	R middle & lower lobectomy
14	M/66	Sq	IB	PR	IIB	L pneumonectomy
15	M/58	Ad	IB	PR	IA	L lower lobectomy
16	M/76	Sq	IIIA	PR	IIIB	L lower lobectomy
17	M/53	Sq	IB	PR	IIIB	L pneumonectomy
18	F/57	Ad	IB	PR	NA	None [e]
19	F/64	Sq	IB	PR	IIB	L upper lobectomy
20	F/58	Ad	IB	SD	NA	None [f]
21	M/62	Sq	IIIA	PR	IB	L lower lobectomy

Key: PreCx=Pre induction chemotherapy; CT=computer tomography; No.=Patient number; M=Male; F=Female; L=Left; R=Right; U=Upper; L= Lower; Md=Middle; NA=Not applicable; CR=Complete response; PR=Partial response; SD=Stable disease; PD=Progressive disease; AC=adenocarcinoma; Sq=Squamous cell carcinoma; Adsq=Adenosquamous; SCLC=small cell lung cancer; Pleio=Pleiomorphic carcinoma

[a] Based on CT assessment; [b] Medically unfit; [c] N2 disease identified at post-induction treatment mediastinoscopy; [d] Open and close; [e] Refused surgery; [f] Metastatic disease identified preoperatively after restaging CT

Table 3.7 Clinicopathological characteristics of neoadjuvant chemotherapy population

3.3.3 PCR reaction amplification efficiency

Measurement of gene expression was performed using use of the ABI Prism 7700 Sequence Detection System to allow real-time PCR monitoring of flourescent emission from the cleavage of sequence-specifice probes by the 5'–nuclease activity of *Taq* polymerase. At any give C_T the amount of PCR product is proportional to the number of initial template copies.

Serial 10-fold dilutions of the different DNA standards of known copy number (CN) were made to create standard curves. The kinetics of PCR amplification of these standards were tested to confirm accuracy of dilutions. Standard curves of each of the cytokines used are illustrated in **Figure 3.18**. Having established dilution accuracy, the serial diluted standards were used in the analysis of patient samples. **Figure 3.19** shows an example of a cytokine standards PCR amplification plot. An example of the layout of a Q-PCR plate is shown in **Figure 3.20**.

PCR amplification efficiency is the rate at which a PCR amplicon is generated and is conventionally expressed as a percentage value. If a particular PCR amplicon doubles in quantity during the geometric phase of its PCR amplification then the PCR assay has 100% efficiency.

The slope of a standard curve is commonly used to estimate the PCR amplification efficiency of a real-time PCR reaction. A real-time PCR standard curve is graphically represented as a semi-log regression line plot of C_T value vs. log of input nucleic acid. A standard curve slope of exactly -3.32 indicates a PCR reaction with 100% efficiency. Slopes that are more negative than -3.32 (e.g -3.9) indicate reactions that are less than 100% efficient. Slopes that are more positive than -3.32 (e.g. -2.5) may indicate sample quality or pipetting problems. A calculation for estimating the efficiency (E) of a real-time PCR assay is:

$$E = (10^{-1/\text{slope}} - 1) \times 100$$

As shown in **Figure 3.18 and Table 3.8** the standard curves (plotted as copies of DNA standards versus C_T) were found to have excellent PCR amplication

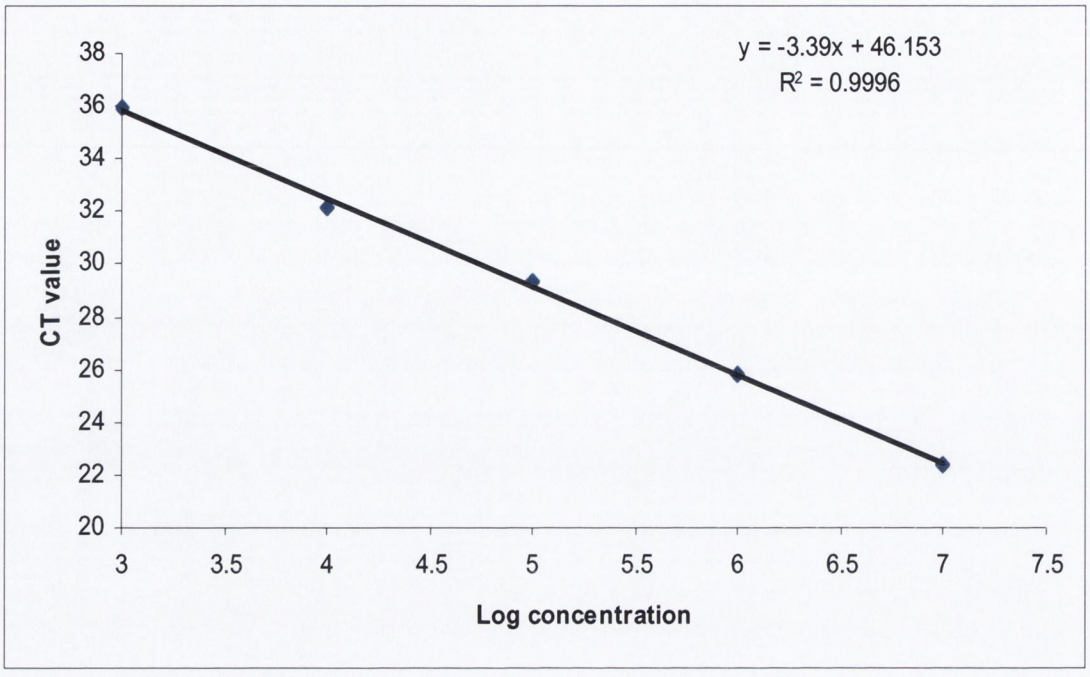

Figure 3.18.1 Standard curve of serial dilutions of IFN-γ. When the known concentrations (expressed in logarithmic form, X axis) of target gene are plotted against the corresponding cycle threshold (C_T, Y axis) obtained by Q-PCR, the result is a line representing the linear correlation between the parameters. The gene copy numbers of patient samples can be calculated from the linear regression of that standard curve, with the y-intercept giving the sensitivity and the slope giving the amplification efficiency.

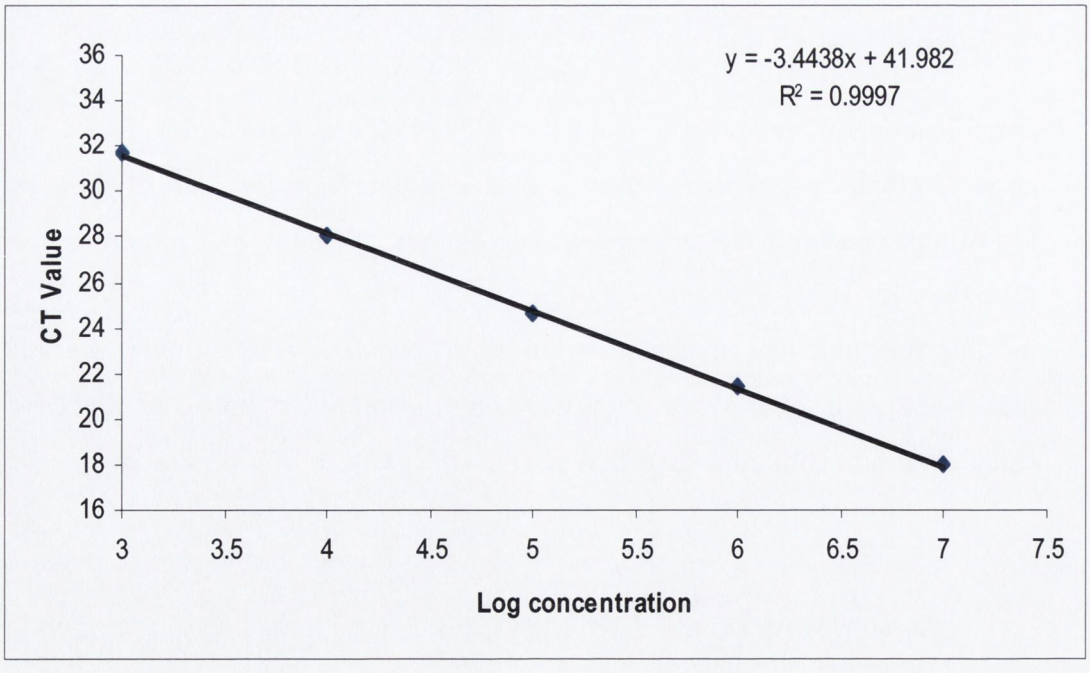

Figure 3.18.2 Standard curve of serial dilutions of IL-10.

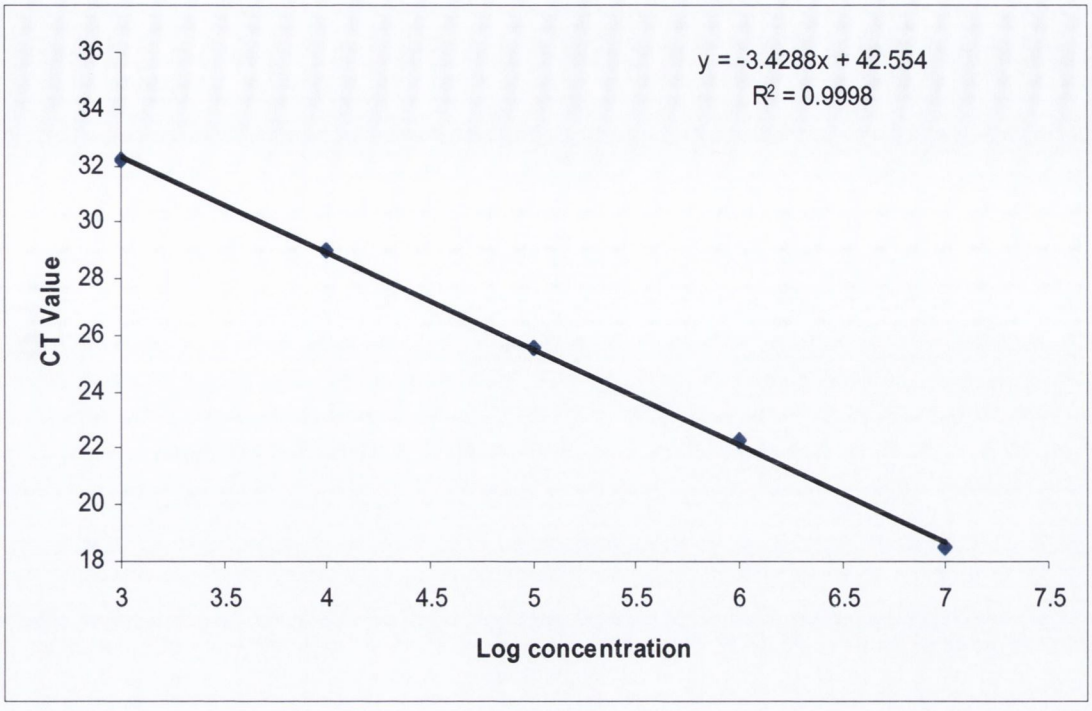

Figure 3.18.3 Standard curve of serial dilutions of TGF-β

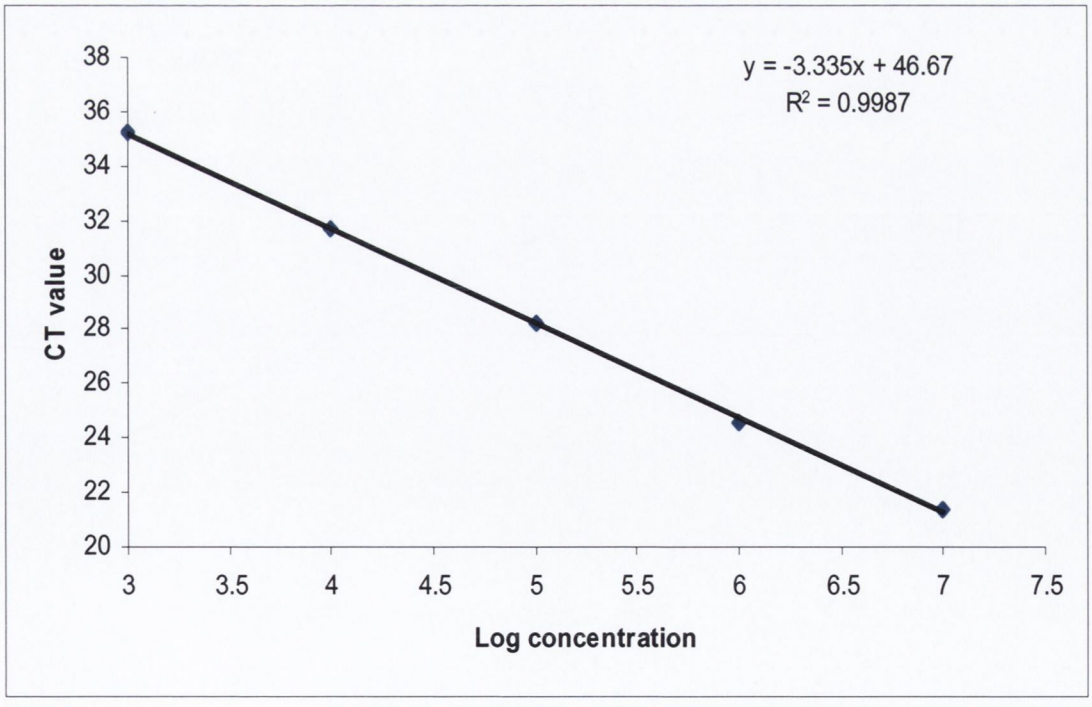

Figure 3.18.4 Standard curve of serial dilutions of β-actin

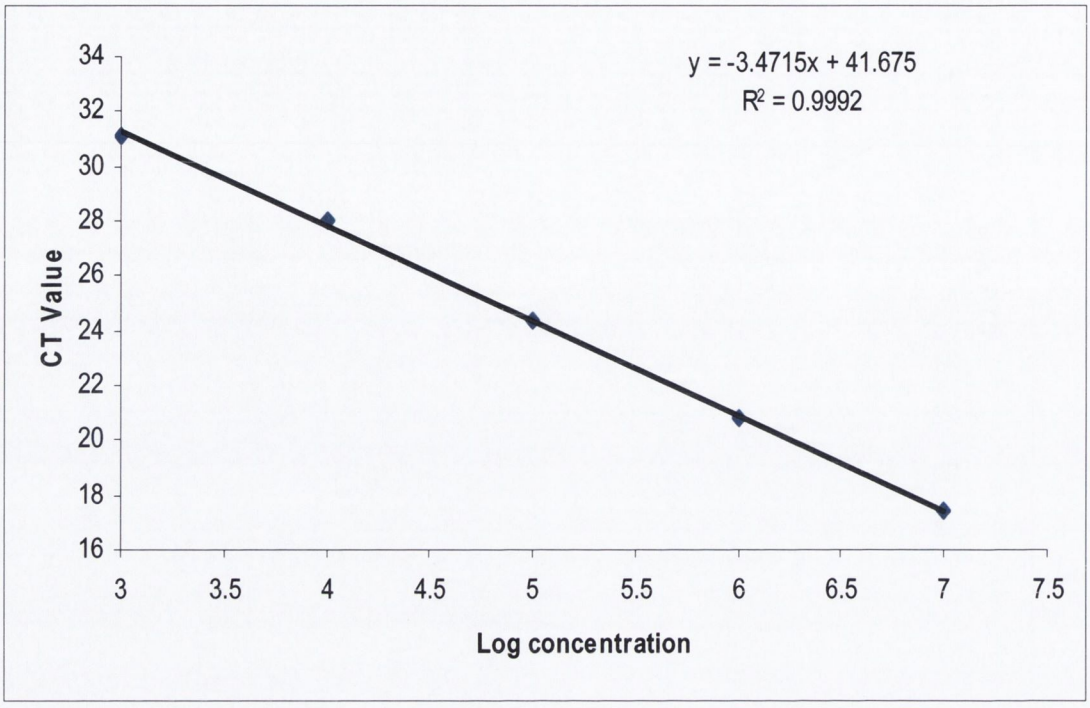

Figure 3.18.5 Standard curve of serial dilutions of IL-4

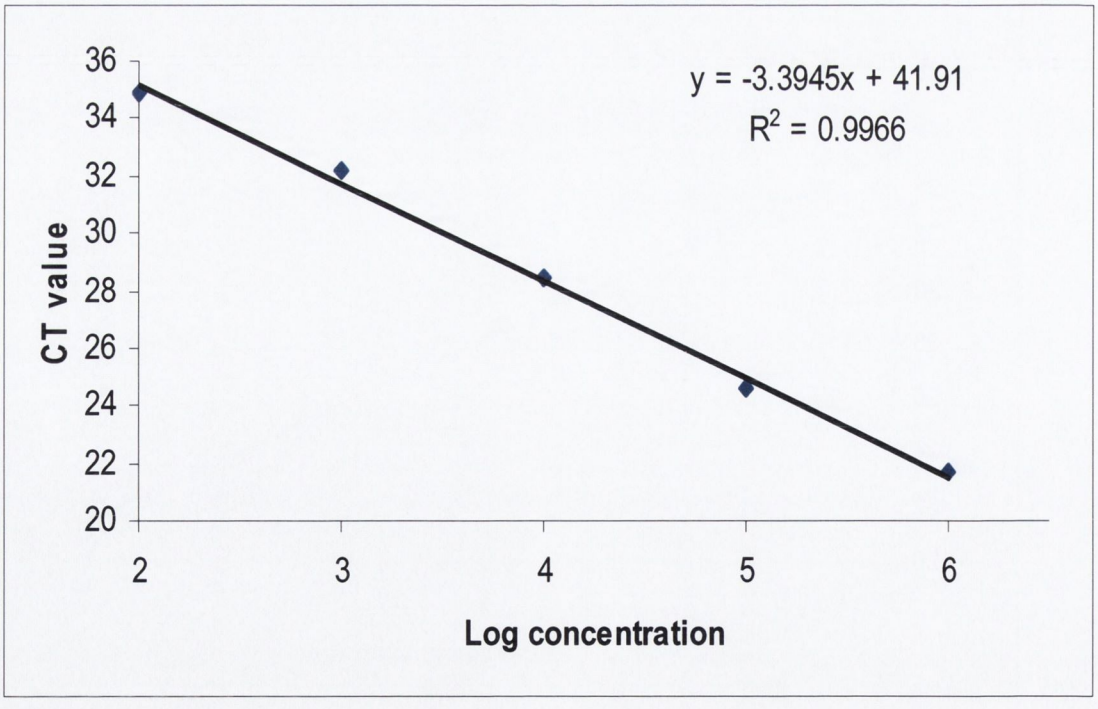

Figure 3.18.6 Standard curve of serial dilutions of Foxp3

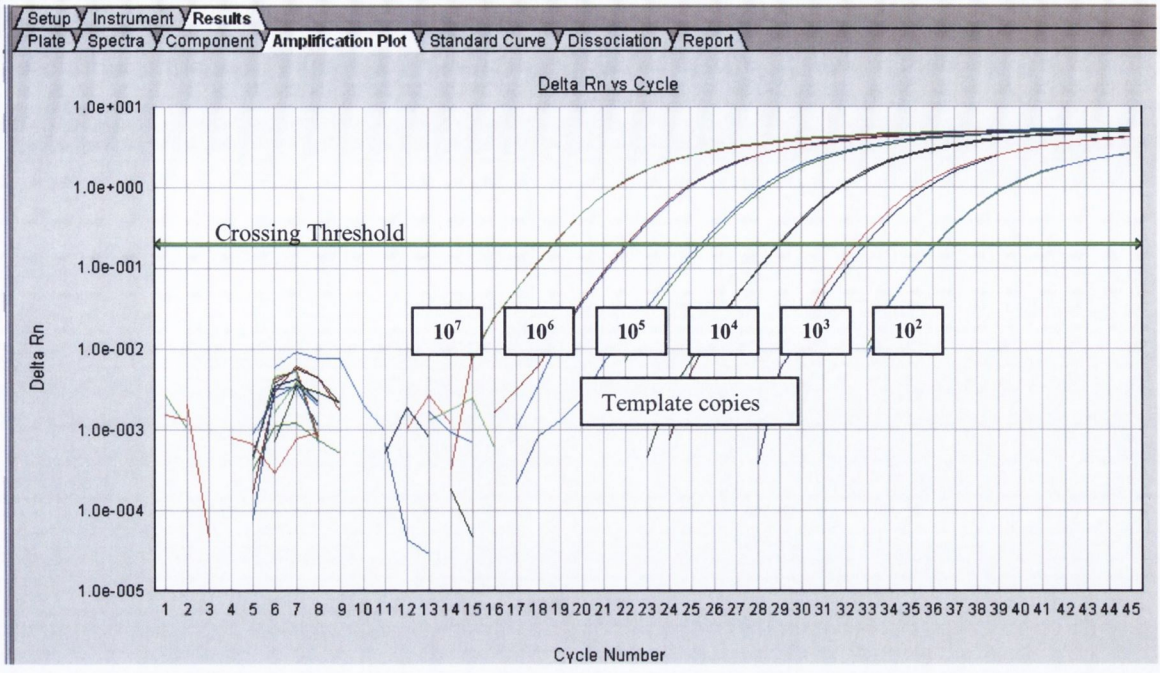

Figure 3.19 Q-PCR cytokine standards amplification plot. Boxes indicate the template copies (TGF-β in this example) at the start of the reaction

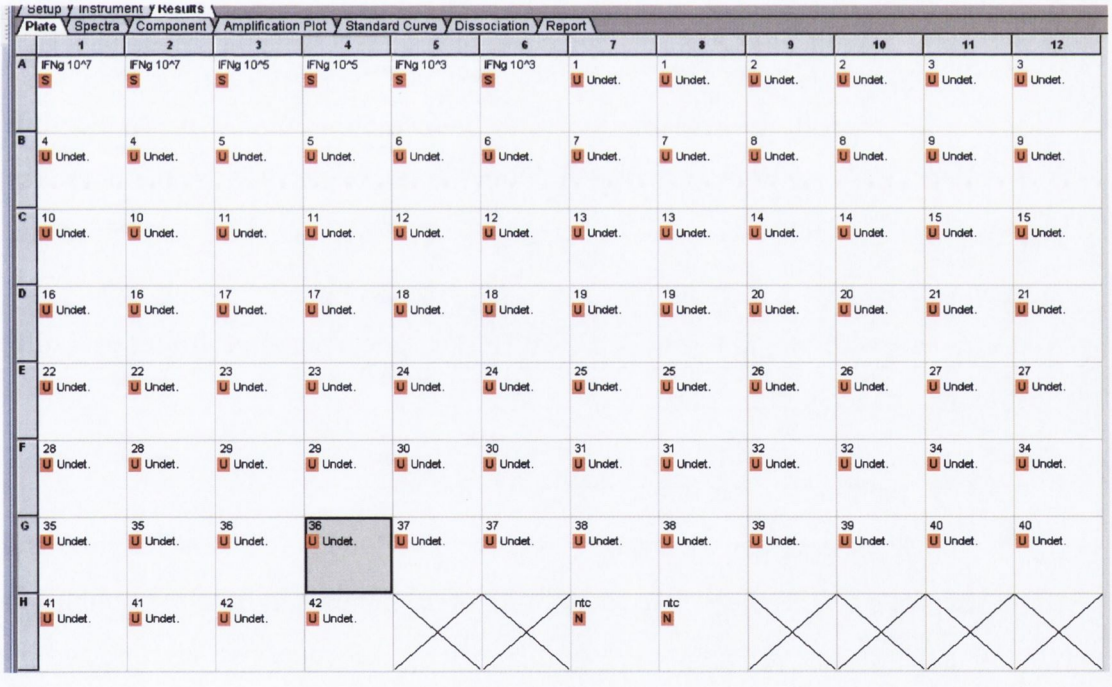

Figure 3.20 Example Q-PCR plate layout. Wells A1 through A6 contain known concentration of standards of the gene of interest (A1/A2=10^7 copies; A3/A4=10^5 copies; A5/A6=10^3 copies). Wells A7 through H4 contain patient cDNA samples and primers and probe for the gene being quantified. Wells H7/8 contain contains primers, probe and master-mix (no cDNA), and serve as the PCR no-template control (NTC), to estimate the level of DNA contamination in the system

efficacy (95% - 99%) as determined by the slope of the standard curve. All standard curves showed correlation coefficients of greater than 0.99 indicating a precise log-linear relationship. The mean efficiency of the standard curves for all the target cDNA was 96.4% +/- 1.9%.

Cytokine	Efficiency (%)	R^2
INF-α	97.2	0.99
IL-10	95.2	0.99
TGF-β	95.8	0.99
Foxp3	96.7	0.99
IL-4	94.1	0.99
B-actin	99.5	0.99

Table 3.8 Cytokine standard curve amplification efficiency

3.3.4 Normalization of target gene CN for housekeeping gene.

To correct for the variability in the amount of cDNA in the different samples, data were normalised by dividing the target gene CN by the housekeeping gene (β-actin) CN. Results for target gene were therefore expressed in CN normalised against β-actin. For each patient sample, the target gene CN was calculated according to a standard curve of that gene, prepared from serial dilutions of known concentration of purified DNA.

3.3.5 Cytokine expression

All patients had detectable levels of INF-γ and TGF-β at baseline and pre C3. Levels of IL-10 were detectable in 17 of 20 patients in baseline samples and in 16 patients in pre C3 samples. Copy number of these genes at baseline and pre-C3 are shown in **Table 3.9.** Analysis of circulating PMBC expression of IL-4 and Foxp3 revealed C_T values of greater than 40 indicating that gene copy numbers were below reliable detection levels of the assay, indicating extremely low expression of these cytokines. As a result meaningful quantification of changes in these amplicons was not possible.

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	Baseline copy no.		Pre C3 copy no.	
Cytokine	median	(range)	median	(range)
INF-γ	15455	(176 – 1582982)	9577	(50 – 676016)
TGF-β	6648	(680 – 15072)	7925	(3427 – 31837)
IL-10	619	(20- 54142)	1001	(24 – 19877)

Table 3.9 Changes in cytokine expression with chemotherapy

Overall, there was a trend toward reduced INF-γ with corresponding increased TGF-β and IL-10 expression in the group overall (**Figure 3.21**). However, these changes were not statistically significant. No correlation between baseline and pre C3 levels between patients who showed a clinical response to induction chemotherapy according to WHO response criteria (complete and partial responses) and those who did not (those with stable and progressive disease) was observed.

Assessment of changes in gene expression profiles according to histopathological subgroup, baseline stage and sex did not reveal any statistically significance.

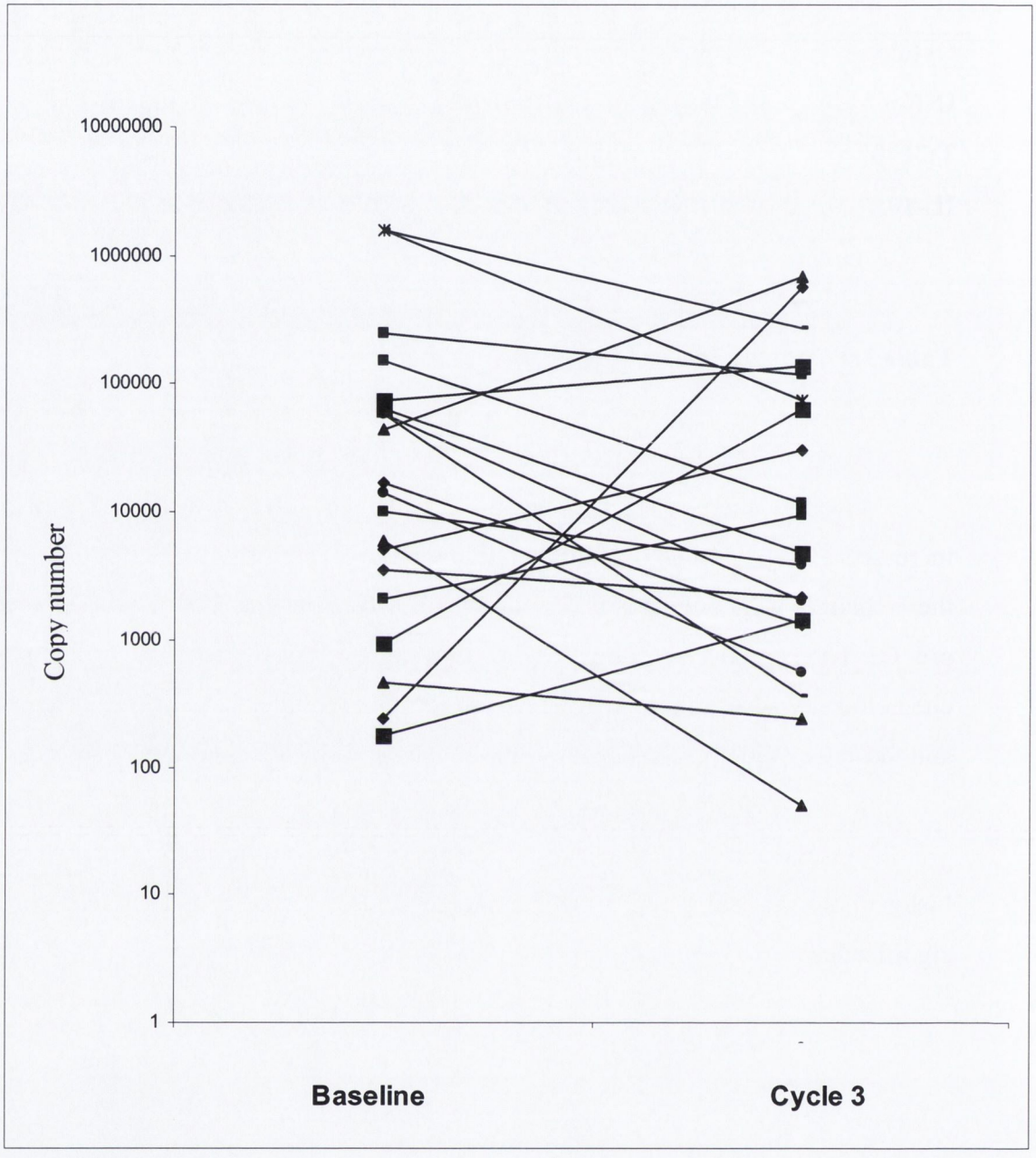

Figure 3.21.1 Change in IFN-γ absolute CN with chemotherapy

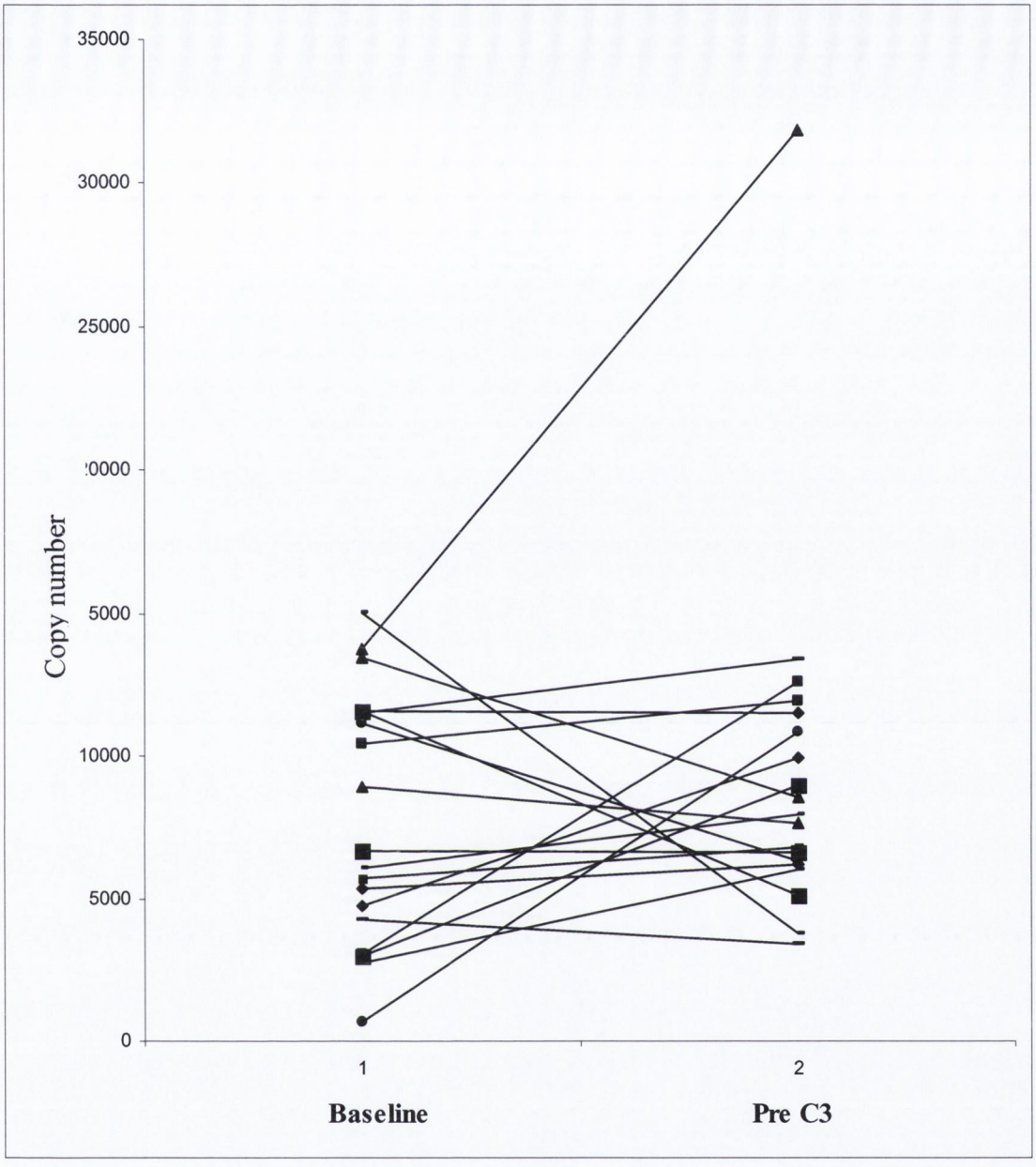

Figure 3.21.2 Change in TGF-β absolute CN with chemotherapy

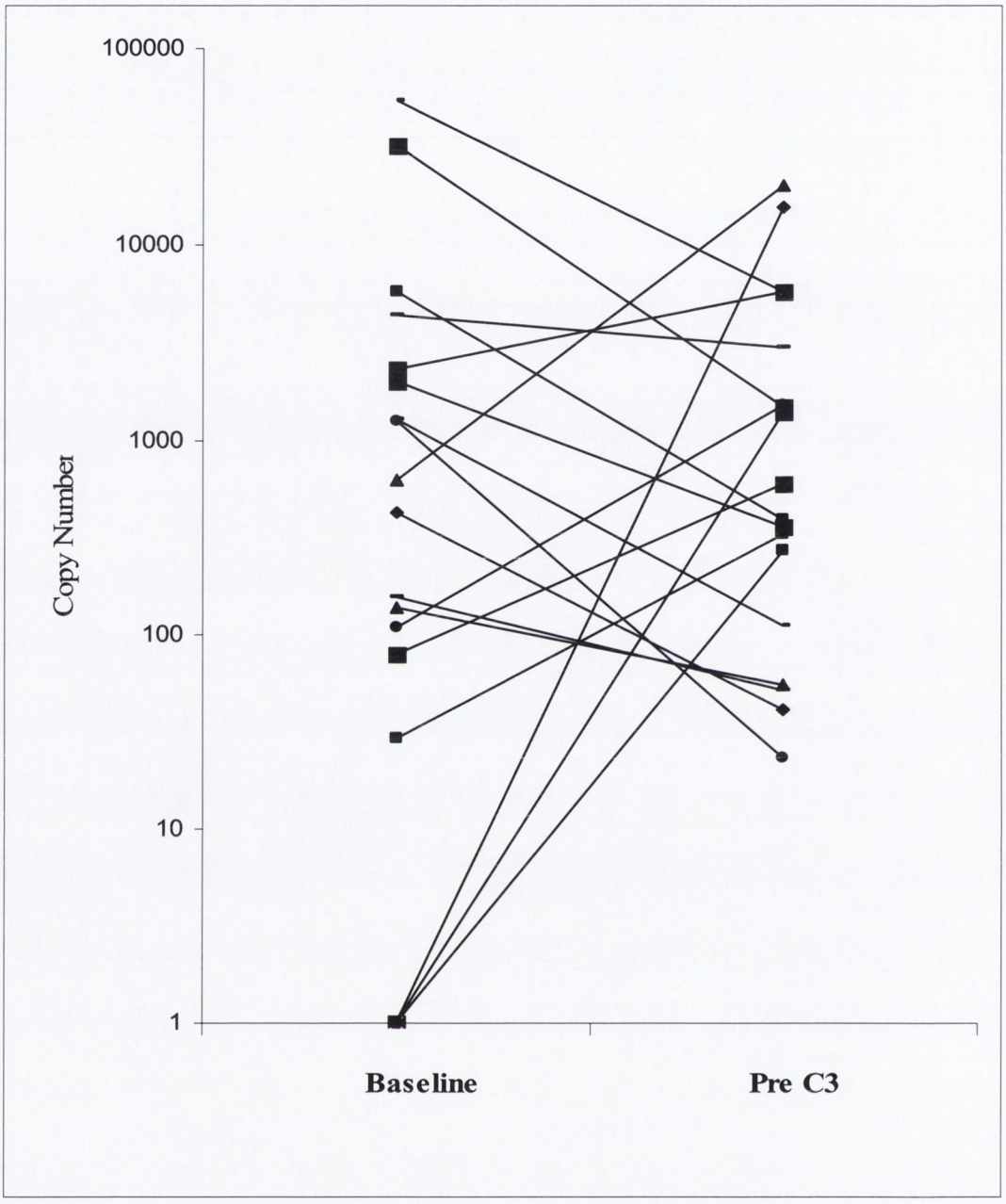

Figure 3.21.3 Change in IL-10 absolute CN with chemotherapy

CHAPTER 4

DISCUSSION

4.1 PROGNOSTIC FACTORS FOR RESECTED NSCLC

There are limited data reporting the long-term outcomes of NSCLC patients treated by surgery in the Republic of Ireland. The clinical features and outcomes of a consecutive series that underwent curative intent resection over a ten-year period at the largest tertiary referral centre in Ireland have been presented here. The results show similarities to those of other international thoracic oncology centres. The overall 5-year survival for the entire cohort was 38%, a figure that compares favourably with survival rates published elsewhere. For example, Martin-Ucar *et al* reported a survival rate of 32% at 5 years (Martin-Ucar et al., 2004) in their operative population at a large teaching hospital in the United Kingdom.

Our figures are also in keeping with survival rates of patients randomized to the observation arm of the large International Adjuvant Lung Cancer Trial Collaborative Group (IALT) cisplatin-based adjuvant chemotherapy trial (Arriagada et al., 2004), a population selected as being of sufficient performance status to tolerate chemotherapy following surgical resection. This study contained a greater proportion of patients with stage III disease than in our population, but had a lower median age (59 versus 65.8 yrs). Moreover, all patients were included in our survival analysis, including early postoperative deaths. In this regard, it would be expected that survival rates in the control arms clinical trials such as the IALT study and others would be higher since perioperative deaths that occur prior to randomization are not considered in outcome analysis.

It is important to note that the majority of patients in the series presented here underwent surgical resection without adjuvant treatments. Specifically, post-operative chemotherapy was not routinely administered to our study population until mid-2005, toward the end of our study period. This is because the majority of surgeries were performed before the positive results of the IALT and other adjuvant chemotherapy trials were confirmed and this intervention became standard of care (Arriagada et al., 2004, Winton et al., 2005). Further studies have now shown that postoperative chemotherapy confers a statistically significant gain in survival in those with stages II and IIIA disease, though probably not earlier stages. In this

regard, it is likely that the overall survival rates reported for the cohort of patients operated at St. James's Hospital would have been further increased had they received adjuvant chemotherapy and an overall relative reduction in mortality of 11% would be expected with a cisplatin-based doublet regimen (Sedrakyan et al., 2004). Since 2005, adjuvant platinum-based doublet cytotoxic chemotherapy is offered to all patients at our institution with stage II and IIIA disease.

In Ireland, as elsewhere, most patients with lung cancer are symptomatic at time of presentation accounting for the fact that the disease is generally diagnosed at an advanced stage. Similarly, the majority of very early stage cancers (stage IA) are detected incidentally. The patients reported in this study presented, in the overwhelming majority of cases, with symptoms related to their cancer. It has been proposed that early detection of lung cancer by population screening might result in reduced mortality rates. Whereas impressive survival rates in screen-detected early stage lung cancers have been reported by some authors (Henschke et al., 2006, Sato et al., 2005), there is no evidence to date that screening reduces the risk of advanced disease or has a favourable effect mortality rates in at-risk populations (Bach et al., 2007). This fact is of critical importance, since reduction in population mortality is the ultimate gauge of the success or otherwise of a national screening program. Given that this measure has yet to be conclusively demonstrated, in Ireland, as elsewhere, there is no national lung cancer screening strategy. Results from a number of randomized controlled trials that are underway in the United States and Europe should help clarify whether interventions such as annual chest radiography or low-dose thoracic CT can significantly impact on disease burden.

The observed overall perioperative mortality rate in our series was similar to that reported in large surgical series in the United States, and reflects the significant caseload at St. James's (Bach et al., 2001). This latter fact is important, since patients who undergo resection for lung cancer at centres that perform large numbers of procedures are likely to survive longer than patients who have such surgery at centres with a low volume of lung cancer surgeries (Bach et al., 2001). The improvement in perioperative mortality rate over the study period is likely explained by several factors, including improved preoperative patient selection,

refinements in perioperative interventions (in particular intensive care unit management) and increased operator experience. This experience also allows a relatively high number of complex surgical procedures to be successfully undertaken at centres such as our own.

Lung conserving resections in those with borderline pulmonary reserve and operations involving radical chest wall resection and/or major vascular reconstruction constitute a significant proportion of the operative caseload at our institution. In particular, the proportion of cases treated by sleeve lobectomy has increased to approximately 10% of the annual NSCLC workload. This bronchoplastic technique is an acceptable alternative to pneumonectomy for tumours involving a main bronchus. Long-term survival rates for this sleeve resections are at least as good as for traditional surgical approaches (Suen et al., 1999). In concert with the increasing sleeve resection rate, we also observed a significant decrease in the numbers undergoing pneumonectomy in the latter part of study. This is of particular importance given the higher perioperative risk associated with pneumonectomy compared to lobectomy (Melloul et al., 2008).

A number of independently prognostic survival factors were identified in the study population. Results showed that women fared better than men across all stages, showing a striking difference in median survival times (61.9 versus 28.1 mo, $P<0.001$). Similar findings have been previously reported and have led to the suggestion that lung cancer affecting females may be a different disease to that which occurs in males. Posited hypotheses to explain the apparent abrogated lethality of lung cancer in women include genetic factors; hormonal influences; altered susceptibility to carcinogens; or differences in tumour biology (Zang and Wynder, 1996, Kure et al., 1996, Ryberg et al., 1994, Wisnivesky and Halm, 2007).

The increased survival for women has been observed in both those with early stage NSCLC treated by surgery and among those with advanced disease (Fu et al., 2005). Although the preponderance of cases of NSCLC still occurs in males, as was the case in our study cohort, epidemiological data from several countries (including Ireland) confirm that the incidence rate of NSCLC among men has begun

to fall. However, there has been a dramatic increase in the incidence of the disease among women over the last two decades as a result of increased cigarette smoking prevalence (Devesa et al., 2005). This trend was similarly reflected in the demographics of those treated by surgery in our series, with 37.4% of the cohort represented by women, a proportion similar to that observed elsewhere (Strand et al., 2006).

The finding of improved overall survival for current and former smokers compared to never-smokers in this series was unexpected and to the best of our knowledge has not been previously reported in the Irish population. Previous investigators have highlighted improved survival for non- versus current smokers with NSCLC (Bryant and Cerfolio, 2007), while others found no differences in outcome (Subramanian et al., 2007). Similar to other populations of non-smokers who develop lung cancer, those who had no prior history of cigarette smoking were more likely to be women (60%) and have adenocarcinoma (62.5%) but were not older (median age 66.8 yrs). These data support the contention that NSCLC affecting never smokers may in fact represent a different entity from that develops in smokers, which may account for the different outcomes in the patients studied here (Sun et al., 2007, Subramanian and Govindan, 2008).

Consistent with findings of others, patients in our series with larger tumours had less favourable outcomes, whether analysed in the context of overall stage or within stage I disease alone. Larger tumours conferred an increased risk of death independent of pathological stage. Increased tumour size has been previously been reported as a negative prognostic feature within patients of the same stage groups. Ou and colleagues analysed outcomes of patients with stage I NSCLC and also identified tumour size of 4 cm or greater as an independent predictor of worse outcome in early stage disease (Ou et al., 2007). Furthermore, enrolment criteria for patients entered into randomized trials of adjuvant combination chemotherapy has begun to reflect the importance of tumour size (Wakelee et al., 2006).

Currently, stage I tumours are divided, somewhat arbitrarily, into lesions of greater or smaller than 3 cm in maximum dimension. Although others have

suggested that tumour size criteria should be altered to reflect the prognostic relevance of tumours greater than 4 cm in diameter (Turbin et al., 2008), we observed a marked survival difference using the current 3 cm size cut-off. The proposals by the IASCLC committee for updating of the T component of the TNM staging system for NSCLC reflect the differences in survival according to primary tumour size. In this regard, we also showed that tumours above 5 cm in size conferred a worse prognosis whereas patients with tumours smaller than 2 cm showed comparatively improved survival. Interestingly, tumours greater than 7 cm in maximum diameter were associated with better survival rates than those in the 5-7 cm range, though the proportion of patients in our cohort with very large (i.e. >7 cm) tumours was very small. Nevertheless, our data supports the proposal to set additional size limits in the new TNM classification system to reflect these associated differences in outcome.

There was a significantly correlation between risk of death and age of patients at time of surgery, with similar findings reported elsewhere (Chang et al., 2007). It is likely that much of this excess mortality is the result of comorbidities, which are common among older populations. Even though nearly 35% of our study population were aged 70 years or older, there is nonetheless broad consensus that age alone should not be used as an exclusionary criteria for operability (Cerfolio and Bryant, 2006). As expected, patients with more advanced disease had a worse outcome with higher overall stage associated with higher risk of death. However, nearly 20% patients in the St. James's series with stage IIIA disease survived at least three years after complete resection, underscoring the fact that surgery should still be considered in this population if technically possible. Indeed, it is crucially important that such 'borderline' cases are assessed by experienced lung cancer clinicians, since a significant number of patients that may initially be deemed unsuitable for surgery (either on the basis of extent of disease or co-morbid conditions) may in fact be candidates for resection.

In recent decades, a shift in the distribution of lung cancer morphologies has been observed with several studies reporting an increase in the rate of lung adenocarcinoma (Thun et al., 1997). Although the most frequently observed

histological pattern identified in our series was squamous carcinoma, cases of adenocarcinoma represented over 40% of the total. Moreover, rates of adenocarcinoma in women were significantly greater than in their male counterparts. Indeed, a gradual increase in the proportion of adenocarcinoma diagnosed in women over the last 20 years has been previously reported (Wahbah et al., 2007). This increase has been attributed, at least in part, to increased exposure to tobacco-specific ntirosamines characteristic of so-called 'low-tar' cigarettes (Stellman et al., 1997). However, it is likely the increased incidence is multifactorial, with other environmental factors as well as genetic and hormonal influences contributing to this finding (Osann, 1998). Other groups have identified adenocarcinoma as a risk factor for reduced survival (Strand et al., 2006). A trend toward improved survival for non-squamous subtypes was also demonstrated in this series, though this was not significant.

All patients with lung cancer should have access to high quality diagnostic, therapeutic and support facilities under the direction of a multidisciplinary team. Ideally, operable candidates should be treated by a dedicated lung cancer surgeon in specialist cancer units offering the only real prospect of extended survival. Early diagnosis together with timely and accurate staging can identify increased numbers of surgical candidates and thereby to improved long term survival for patients with this disease.

4.2 PROGNOSTIC RELEVANCE OF LYMPHOCYTE INFILTRATION IN RESECTED NSCLC

There is increasing evidence that the interplay between host immune components and tumour cells is a critical determinant of outcome in a variety of human cancers. Although an abundant T-lymphocyte intratumoral infiltration is a frequent observation, there is conflicting data with regard to its prognostic importance. Using a novel objective method to detect and quantify different lymphoid populations, we performed a detailed evaluation of the tumour microenvironment in NSCLC specimens. Focusing in particular on immune cell microlocalization, our results showed that regional distribution of T-lymphocyte subsets is a strong predictor of clinical outcome in a large population of surgically resected NSCLC patients, irrespective of tumour stage.

These results confirm that enumeration of distinct populations of lymphocytes within different compartments of NSCLC tumours shows prognostic relevance and provides strong though indirect evidence of immune-mediated cancer control mechanisms. The observation that higher numbers of $CD8^+$ T lymphocytes within tumour islets, coupled with a reduced infiltration of $Foxp3^+$ Tregs in these regions confers a distinct survival advantage has not been previously reported for surgically-resected NSCLC. This can also be interpreted as showing that a lower tumour islet Treg infiltration effectively reduces the risk of local and systemic spread of disease after curative-intent surgery. Furthermore, a strong reciprocal relationship between effector and regulatory lymphocyte subsets with respect to tumour islet as opposed to peritumoral stromal infiltration was evident in our study population. However, the underlying molecular signalling pathways that account for the development of these different patterns are poorly understood.

Both positive and negative effects on tumour invasion, growth, metastasis and clinical outcome have been attached to the presence of TILs in solid tumours. Although accumulation of TILs has been linked to improved prognosis in several studies, there is also convincing data indicating that TILs may also facilitate tumour escape from immune surveillance mechanisms. As a result, the predominant prognostic implication of TILs remains the subject of debate. Failure to fully

characterise and identify specific T-cell phenotypes and their precise location within tumours may have contributed to the seeming incongruity with respect to the clinical relevance of TILs in earlier studies. Interestingly, previous experimental work also yielded conflicting data with regard to the impact of tumour-associated macrophages (TAM) in lung cancer. However, when determination is made of the exact microanatomic infiltrative pattern, a more clear relationship to prognosis emerges (Welsh et al., 2005). Specifically, an increase in macrophages populating tumour islets confers an improved outcome, whereas stromal macrophages are associated with reduced survival.

Numerous investigators have demonstrated that intratumoral infiltration by lymphocytes expressing the cytotoxic phenotype correlates with outcome. Indeed, a marked survival advantage has been shown for increased intratumoral $CD8^+$ T-cell accumulation of in patients with colorectal, ovarian, endometrial and cervical cancers, among others (Galon et al., 2006, Zhang et al., 2003, Kondratiev et al., 2004, Piersma et al., 2007). An effective antitumour TIL activity has been proposed as the biological explanation for these findings, which likely accounts for the significant survival gain in patients in our series with a high intratumoral $CD8^+$ counts.

The results presented here show some similarities to those of other investigators evaluating immunomechanisms of TILs in lung cancer. Johnson et al showed that quantification of different lymphocyte subset in surgically resected lung cancer specimens also provided prognostic information (Johnson et al., 2000). Using image analysis to evaluate immunohistochemically stained lymphocyte populations in 95 cases these authors showed that high intratumoral accumulation of $CD3^+$ cells, but not $CD8^+$ cells was associated with longer post-operative survival. However this analysis was performed using semi-quantitative assessment of intratumoral T-cell populations, grouped as 'absent', 'low' (<10 positive cells per HPF) or 'high' ($\geq$10 positive cells). Furthermore, the relevance of intrastromal infiltration patterns was not assessed.

Trojan et al found that assessment of $CD8^+$ lymphocyte infiltrative patterns did not provide additional prognostic information leading these authors to conclude that these cells were unable to mount effective anti-tumour responses (Trojan et al., 2004). However, only 31 patients were included in this study and effector cell populations were also assessed in a semi-quantitative fashion.

Kawai et al examined tumour biopsy specimens from 199 patients with stage IV NSCLC and found that a predominant $CD8^+$ T-cell infiltration within tumour islets was associated with improved survival (Kawai et al., 2008). By contrast, Al-Shibli et al found that high number of stromal $CD8^+$ lymphocytes was an independent favourable prognostic marker and that an increase in epithelial $CD8^+$ lymphocytes correlated with improved survival, though only in univariate analysis (Al-Shibli et al., 2008). Immunohistochemical staining of tumour microarrays, rather than full tissue sections were used by these investigators. In addition, the lymphocyte infiltration scoring system used to quantify tumour and stroma immune cell counts was based on approximate percentages of lymphocytes as a proportion of total nucleated cells per compartment.

In contrast to these most of these studies, however, the absolute numbers of lymphocytes in our patient population were automatically counted in each region and expressed per unit area to yield a precise value for each patient. Moreover, using the Genie image analysis method, we were able to exclude non-tumour and non-stromal regions within tissue sections from the evaluation, thereby generating a more precise lymphocyte count per unit area of ROI.

In order to identify Treg cells, we used an antibody directed against Foxp3, a member of the forkhead family of transcription factors. Intracellular Foxp3 has been shown to be critical to the maturation and immunosuppressive functionality that is characteristic of Tregs. A key feature of these cells is their ability to inhibit other immune populations, including $C8^+$ T-cells, $CD4^+CD25^-$ T-cells NK cells, dendritic cells and B-lymphocytes, by direct cellular contact (Beyer and Schultze, 2006). This function is critically important in countering the activity of autoreactive cells to prevent autoimmune disease. However, active recruitment of Tregs to the tumour

microenvironment facilitates evasion of cancer cells from immune detection and destruction.

The finding that high levels of tumour islet $Foxp3^+$ Treg cells predict a strikingly worse outcome in NSCLC is consistent with results from studies examining their role in several other cancer types. Woo et al were the first to demonstrate an increased percentage of $CD4^+CD25^+$ Tregs in NSCLC and ovarian cancer (Woo et al., 2001). Subsequent studies have shown that increased intratumoral Treg infiltration is a poor prognostic marker in several cancer types (Perrone et al., 2008) (Gao et al., 2007). With respect to lung cancer, other investigators have shown that stage I NSCLC patients with a high proportion of intratumoral Treg cells relative to total TIL counts are at increased risk of disease relapse (Petersen et al., 2006). However, assessments were made using a semi-quantitative scoring system to grade TIL counts and employed a stylised combination risk index of different TIL populations rather than absolute numbers alone. Interestingly, assessment of intratumoral $Foxp3^+$ Treg cell in isolation did not provide additional prognostic information.

The data presented here also suggests an interrelationship between effector and suppressor cell ratios and prognosis exists. Patients with the combination of a high tumour islet/stroma $CD8^+$ ratio and low tumour islet/stroma $Foxp3^+$ ratio showed significantly longer median survivals compared to individuals with the opposite pattern. Interestingly, patients with both a low $Foxp3^+$ and low $CD8^+$ tumour/stroma ratio fared better than those with corresponding high conjoint immune infiltration ratios. These results suggest that while cytotoxic $CD8^+$ T-cell microlocalization is an important determinant of postoperative survival, it is presence or absence of Tregs that exerts the greatest influence on outcome.

The relationship between Treg tumour infiltration and outcome is clearly complex. There are now numerous reports that a worse survival is observed in different tumour types that are characterised by a high density of intratumoral Tregs. However, as already discussed, there is also mounting evidence that this pattern may also be associated with longer disease-free and overall survival in several

malignancies, including colorectal cancer, lymphoma and head and neck cancer. Because it is generally presumed that the deleterious effect of Tregs is mediated by their inhibitory activity on antitumour effector cells, it is not surprising that various studies have reported that the ratio of $CD8^+$ T lymphocytes to Tregs had more impact on patient survival that the absolute numbers of either cell type when examined in isolation. Focusing only on Tregs without knowledge of the balance between Tregs and effector cells may have lead to some bias in interpretation of results from previous studies. The approach used in this work was therefore to analyse both effector and regulatory populations and compare outcomes when patients were grouped according to different patterns of tumour islet and stroma lymphocytic infiltration. However, a further study to establish precise monocyte co-localisation patterns, perhaps using a double-stain immunohistochemical technique, may provide additional interesting information.

Our results are similar to those obtained by Gao et al, who showed that hepatocellular carcinoma (HCC) tumour islets that simultaneously contained both low Tregs counts and high $CD8^+$ cytotoxic T-cells was associated with improved disease-free and overall survival (Gao et al., 2007). Thus, while the absolute numbers of different lymphocyte populations infiltrating NSCLC is important, the balance of tumour-promoting (Tregs) and tumour-limiting ($CD8^+$ cytotoxic cells) mechanisms may be of even greater relevance. Additional data from experimental cancer models appear to support this hypothesis (Bui et al., 2006).

There are several possible biological explanations that may account for the apparent opposing effects of $CD8^+$ and $Foxp3^+$ TILs in lung cancer. A simple hypothesis is that $CD8^+$ cells within tumour islets are functionally activated and control tumour growth through elaboration of cytokines that target and kill cancer cells (Finn, 2008) or recruitment of other cytotoxic immune cells such as macrophages (Welsh et al., 2005, Ohri et al., 2009) whereas Tregs exhibit an immunosuppressive phenotype that facilitates cancer cell survival and proliferation (Sharma et al., 2005). Thus, a relative increase in the intratumoral accumulation of Tregs over effector populations might be sufficient to overcome cytotoxic T-cell function.

Experimental evidence indicates that Tregs inhibit effective $CD8^+$ T-cell cytotoxic activity via the TGF-β signalling pathway, since expression of a dominant-negative TGF-β receptor by tumour-specific $CD8^+$ T lymphocytes renders them resistant to suppression and is associated with unimpaired cytotoxicity and successful tumour rejection in animal models (Chen et al., 2005a). In this manner, an excess of Tregs within tumour islets may blunt host effector mechanisms, resulting in cancer progression. Similarly, immunotherapeutic strategies that result in depletion of Tregs without concomitant modulation of effector T-cell activity are insufficiently capable of restoring effective anti-tumour immunity (Quezada et al., 2008). By contrast, an increased cytotoxic T-cell infiltration in conjunction with complete abolishment of Tregs is characteristic in patients with a complete pathological response to neoadjuvant chemotherapy in breast cancer (Ladoire et al., 2008).

Tregs are capable of suppressing secretion of different cytokines, as well as interfere with the proliferation and possibly survival of other lymphocyte populations during the host immune response. However, this particular mechanism may not be typical of Treg populations that abundantly populate the peritumoral stroma in human NSCLC, since those patients displaying this inflammatory pattern have a significantly better outcome. Therefore, additional examination of the relationship between stromal $Foxp3^+$ T cell density and the expression of both suppressor signalling molecules, such as TGF-β, as well as characteristic effector cytotoxic molecules such as granzyme and perforin, would be particularly useful and would extend the current findings.

The regions chosen and the methods employed in order to quantify TILs has varied greatly in different studies, which may account for some of the discrepancies between results from previous studies. We deliberately chose randomly generated HPFs from which to make determinations of lymphocytes within regions of tumour and stroma, as this avoids the potential for selection bias. Other investigators have tended to select so-called 'representative' regions which generally involve counting regions characterised by abundant TIL infiltration. Clearly, this has the potential to yield significant variations in lymphocyte numbers, depending on the pattern of TIL

in the area from which analysis of cell counts are made. Furthermore, the automated image analysis approach we employed ensured a consistent recognition of different immunohistochemically stained lymphocyte populations and avoided any potential inter-observer variations in cell counts.

Lymphocyte counts were made only from regions that contained the advancing tumour edge. This is important, since deeper parts of the tumour often display necrotic regions characterised by large numbers of host immune cells recruited in response to dying and dead cancer cells. Counts performed in such areas of the tumour may not representative of specific effective host anti-tumour response and was therefore avoided.

In order to fully characterise the distinct biological mechanisms that are responsible for this phenomenon, additional functional studies are required so that the interactions between host immune cells, cancer cells and surrounding peritumoral stromal elements may be characterised. Furthermore, an improved understanding of the interactions between different immune populations in these separate microregions, in particular between effector and regulatory lymphocyte subsets, is of critical importance. If the mechanism and consequences of these observations can be determined, then there is the potential to develop effective immunotherapies for NSCLC, be they vaccine, adoptive or cytokine based strategies.

Further detailed studies in both the tumour islets and stroma, in addition to the peripheral blood may help clarify the processes that account for the different patterns observed in this study. Assessment of expression of cytokine/chemokine proteins and their associated receptors may be informative, since chemokines are prime candidates for the recruitment of immune cells; for example, the CXCR3 ligands are known to recruit cytotoxic $CD8^+$ cells into various tumours (Hensbergen et al., 2005). Indeed, accumulated evidence has now firmly established that many if not most of the members of the CC and CXC chemokine families are involved in mediating critical features of cancer biology, such as cell growth, angiogenesis, and metastasis, in addition to regulating tumour immunity. Recently published work in a

relatively small study population revealed an association between five-year survival and NSCLC tumour islet CXCR2, CXCR3 and CCR1 density as well as stromal CXCR3 density (Ohri et al., 2010). Assessment for these chemokine receptors in the context of differential $Foxp3^+$ and $CD8^+$ T lymphocyte infiltration patterns, as was observed in the current study, therefore appears warranted.

A detailed study of the proteome of NSCLC cells from patients with tumour islets that contain high numbers of $CD8^+$ lymphocytes versus those with high numbers of $Foxp3^+$ Tregs may be another useful approach, given the marked differences in survival observed in these different groups. Such investigation would help facilitate the delineation of the interaction of tumour and immune cells in order to detect differences in terms of protein expression between those patients with the different infiltration patterns that have been the focus of the current work. Furthermore, such an approach could be adopted to determine whether chemokine expression varies between tumours of good versus poor prognosis. Additional immunohistochemical study of T_H1 cytokines (e.g. IFN-γ, IL-2, TNF-α) and T_H2 cytokines (e.g. IL-4, IL-13) focusing in particular on the tumour islet and stromal compartments of NSCLC would also inform us about the relative contributions of T_H1 to T_H2 immunity and outcome.

Although the analysis of cytokine mRNA expression in circulating monocytes among patients who responded to neoadjuvant chemotherapy versus those that did not yielded no additional prognostic information in a separate population that was examined as part of this study, a similar analysis in blood from patients with the different $CD8^+$/$Foxp3^+$ T lymphocyte infiltration patterns identified in the immuno-histochemical study described might be worthwhile. It would also be interesting to establish whether levels of Foxp3 expression in peripheral lymphocytes (which showed considerable heterogeneity in patients receiving cytotoxic agents prior to surgical resection) correlates with intratumoral or intrastromal Treg distribution. Clearly, this would be an interesting area for additional study, particularly in relation to possible changes in blood and tissue Tregs caused by surgery, radiation therapy and/or cytotoxic chemotherapy and whether this modulates the antitumour immune response. The contrasting

prognostic associations observed in this study for Tregs localized within tumour and adjacent stroma underscore the critical importance for simultaneous investigation of both compartments, perhaps also in conjunction with the level of circulating Tregs. If a correlation exists between circulating Foxp3 expression levels and intratumoral Treg infiltration patterns, it may ultimately be possible to identify different prognostic subgroups by blood testing prior to any proposed therapeutic intervention and tailor treatment strategies accordingly.

The phenotype of different immune populations is clearly modified by their microenvironment, and there is evidence that this applies also within tumours. In gastric carcinoma for example, the degree of $CD8^+$ T cell infiltration directly correlates with macrophage infiltration suggesting that macrophages play an important part in the activation of T cells and the subsequent tumour cell destruction (Ohno et al., 2002). Furthermore, increased infiltration of macrophages and mast cells within tumour islets confers a marked survival advantage in NSCLC, irrespective of stage (Welsh et al., 2005). It would be interesting therefore to examine in our population whether those tumours characterised by high intratumoral $CD8^+$ T cells or high intrastromal Tregs counts (patterns which confer a favourable prognosis) also exhibit similar skewed infiltration patterns by macrophages and/or mast cells. Indeed, a recent microarray study that also focused on the prognostic relevance of regional inflammatory infiltrate patterns revealed that dense $CD56^+$ NK cell and $CD1a^+$ dendritic cell infiltration in the tumour stroma positively influences prognosis in NSCLC (Al-Shibli et al., 2009). A similar immunohistological assessment for the presence of these cell populations in our large study series of surgically resected NSCLC patients in order to establish whether such a pattern also correlates with Treg infiltration patterns may help further clarify the complex inter-cellular interactions that exist in this disease.

Why a higher degree of intrastromal Foxp3 expression conferred a survival gain in our series in not known. It would be important in future studies to determine not only the mechanisms that drive increased Treg cells recruitment and/or expansion within the stroma of NSCLC in certain patients (or indeed reduced Treg tumour islet accumulation), but also to establish the cellular interactions at play

once this population have localized there, and thus determine how this pattern might be advantageous. Experimental evidence from previous studies that have previously exposed a favourable role of Tregs in tumour control in other malignant disorders may be informative in designing future experimental studies to help explain the underlying potential mechanisms at work in lung cancer. For example, haematologic malignancies and some solid tumours (in which the presence of Tregs correlates with good clinical outcome) are abundantly infiltrated by innate inflammatory populations that elaborate a range of growth factors and pro-angiogenic molecules that favour tumour progression. It is possible that Tregs inhabiting the peritumoral stroma attenuate these pro-tumoral responses preferentially, and additional study to assess for variations in tumour islet versus stromal distribution of such markers may help clarify why Treg accumulation in tumour stroma might be advantageous, as was the case in the current patient series.

It is also important to stress that whereas Foxp3 expression has been widely employed in experimental studies of human cancer in order to define Tregs, this marker is not confined exclusively to certain lymphocyte populations. Indeed, it has been shown that activated $CD4^+CD25^+$ effector T cells may transiently express Foxp3 with or without acquisition of suppressive functions (Roncador et al., 2005). Furthermore, subsets of $CD8^+$ T cells may also co-express the Foxp3 marker. Accordingly, while Foxp3 is currently considered the best marker available for immunohistochemical staining to characterize Tregs, the simple use of this marker on its own may potentially overestimate this Treg population. Depending on the study and proportion of $Foxp3^+$-activated $CD4^+$ T cells or $CD8^+$ T cells in tissue specimens, the simple enumeration of $Foxp3^+$ cells without functional analysis does not necessarily correspond to the same levels of Tregs in the resected NSCLC samples.

Regardless of why the density of tumour-infiltrating $CD8^+$ and $Foxp3^+$ lymphocytes appears to have different prognostic value for different cancer types, the fact remains that it is a strong and independent marker in the surgically resected NSCLC population evaluated in the current study. Confirmation of these findings by independent validation cohort studies could eventually lead to the routine

examination of $CD8^+/Foxp3^+$ lymphocyte regional density in order to further refine prognostic stratification in NSCLC.

In conclusion, our data suggest that assessment of T-lymphocyte microlocalization may help identify individuals at risk of early disease relapse despite apparent complete surgical resection. Future immunotherapeutic strategies capable of downregulating intratumoral Treg responses while enhancing effector $CD8^+$ activity may be worthy of pursuit.

4.3 IMPACT OF NEOADJUVANT CHEMOTHERAPY ON CYTOKINE EXPRESSION IN NSCLC

There is now a wealth of accumulated evidence highlighting the key role played by cytokines in the development and progression of lung cancer. In recent years, techniques to quantify cytokine expression have attracted considerable interest as a means to help identify different prognostic subgroups of NSCLC patients and predict responses to established and novel treatment approaches. However, this is not a simple task because interactions between inflammatory cells, cytokines, antibodies, and other biologically significant immune products and the host are not fully understood. Despite this, molecular targeted therapy is beginning to be integrated into routine management of subgroups of NSCLC patients and there remains a need to accurately and reliably assess the *in vivo* immune effects of these treatments.

The development of prognostic biomarkers has helped identify cancer patients that might benefit from adjuvant or neoadjuvant chemotherapy. Unfortunately, the predictive efficiencies of the various biomarkers employed for this purpose show considerable variation and few have been rigorously tested in validation studies. Furthermore, patients displaying similar clinicopathological features often exhibit dissimilar responses. This is likely due to the heterogeneity of tumour genetic and epigenetic characteristics and the variability of host response to the tumour. These factors likely account for the disparity between the numbers of published reports of predictive biomarkers in retrospective series and the numbers of these biomarkers that are applied in day-to-day clinical practises.

As the malignant process evolves, there is a corresponding switch in the cytokine profile from a T_H1 pattern, characterised by elaboration of IL-2, IL-12 and IFN-γ to a T_H 2 pattern, with associated increased levels of IL-4, IL-5, IL-6, IL-10 and IL-13) (O'Byrne and Dalgleish, 2001). In addition to direct cytotoxic effects, it is firmly established that conventional chemotherapeutic agents used in the treatment of NSCLC may also promote specific immune-mediated cytotoxic mechanisms through several different pathways (Suzuki et al., 2007, Bae et al.,

2007, Menard et al., 2008). Many of the novel targeted therapies also act on molecular and cellular immunologic pathways. However, these observations are largely based on extrapolations from in vitro studies or animal models, with a relatively small contribution from studies in humans. As a result, influences of different neoadjuvant approaches on the immune repertoire of patients with cancer remain poorly defined.

Detection and measurement of changes in host anti-tumour immunity is now an important adjunct in clinical studies of investigational treatments and may allow correlation with clinical responses and help identify different prognostic/predictive patient subgroups. We hypothesised that NSCLC patients demonstrating a favourable response to neoadjuvant chemotherapy might show an upregulation of T_H1 cytokines and a corresponding reduction in T_H2 cytokine production. The goal of this study therefore was to assess the impact of neoadjuvant combination chemotherapy on the inflammatory cytokine profile of patients with resectable NSCLC, based on the premise that cell-mediated immune function is integral to an effective host antitumour response. We sought to determine whether the clinical response of patients receiving preoperative cetuximab in combination with cisplatin and gemcitabine correlated with cytokine gene expression as assessed by Q-PCR. Signature cytokines for $CD4^+$ T_H1 (IFN-γ) and T_H2 (Foxp3, TGF-β and IL-10) responses were selected for this purpose. Levels of mRNA from PBMCs in the patients' peripheral blood were determined pre-treatment and pre-cycle 3 (C3) of induction chemotherapy. For each amplicon, absolute quantification was precisely determined by interpolation from a standard curve included in each experiment constructed using serial dilutions of known concentrations of DNA.

Taken together, analysis of PBMC cytokine RNA levels in our highly-selected population did not reveal a significant correlation with therapeutic responses to the treatment regimen used in this pilot study. Though quantifiable levels of several different PBMC cytokines were detected in most patients, these results indicate that circulating T-cells in patients that demonstrated therapeutic responses were not consistently polarized to a T_H1 phenotype.

Effective CMI responses are characterised by the production of IFN-γ by both circulating and intratumoral T_H1 cells (Finn, 2008). In this study, use of the PCR technique to analyse IFN-γ levels revealed that expression levels in PBMCs is highly variable between different patients. Previous investigators have shown that IFN-γ PCR quantification represents a fairly reliable parameter for monitoring T-cell specific immune function (Hartel et al., 2001). Although no statistically significant correlation between IFN-γ expression and response to neoadjuvant chemotherapy was shown in the overall group, treatment resulted in a definite change in levels of all patients. Furthermore, patterns of change of IFN-γ after two cycles of treatment showed considerable heterogeneity. Previous investigators have used other methods to quantify IFN-γ levels in NSCLC patients but results have been inconsistent. Using 3-colour flow cytometry, Ito et al measured IFN-γ and IL-4 in PBMCs to show that T_H1/T_H2 ratios were significantly depressed in peripheral blood of NSCLC patients that developed tumour recurrences after surgical resection, suggesting that a predominant CMI response is beneficial (Ito et al., 1999). However, subsequent results from these investigators revealed that a high T_H1/T_H2 ratio in patients with stage II or III NSCLC in peripheral blood was in fact a negative prognostic factor (Ito et al., 2005).

It is conceivable that activation of tumour-specific lymphocytes in patients that demonstrate a response to chemotherapy may occur in the absence of consistent changes in circulating PMBC cytokine mRNA profiles. In NSCLC, the proportion of lymphocytes with intracellular IFN-γ expression has also been shown to be significantly higher in TILs compared to peripheral lymphocytes (Ito et al., 2001). Thus, a possible explanation for the inconsistent changes in IFN-γ in response to neoadjuvant treatment is that biological processes governing upregulation of genes in TILs differs from those of PBMCs and that local proinflammatory effects are not consistently projected in the host. Systemic immunity to tumour as measured in the peripheral blood in patients with cancer is difficult to demonstrate and tumour-specific responses are particularly elusive. In order to more fully characterise the levels of functional IFN-γ, it may be necessary to examine protein levels since changes at the mRNA level that are small or short-lived may nonetheless have relevant immunomodulatory effects.

Use of Q-PCR to monitor IFN-γ gene expression has been previously shown to be a sensitive method to evaluate precursor cytotoxic T-cell reactivity in the blood of cancer patients (Kammula et al., 1999). However, a low frequency of cytotoxic T-cell numbers in peripheral blood has been observed by others. As a result the testing of CTL reactivity is often preceded by in vitro antigen-driven expansion of CTL precursors using tumour peptides (Rivoltini et al., 1995). As less than 2% of total body mononuclear cells are represented by PBMCs and it has been suggested that cells derived from disease sites (tumour and associated lymph nodes) might better reflect extent of disease- or treatment-induced alterations in cellular characteristics (Whiteside, 2008). Kammula et al used PCR to examine gene expression in sequential tumour biopsy specimens and blood from patients immunized with peptides derived from melanoma proteins and found treatment-related increases in intratumoral IFN-γ but not in peripheral blood (Kammula et al., 1999). Discordance between IFN-γ expression in tumours and peripheral blood has also been observed in studies of ovarian (Santin et al., 2001a), cervical (Santin et al., 2001b), breast (Wong et al., 1998) and renal carcinomas (Kowalczyk et al., 1997).

Abundant expression of the immunosuppressive cytokine TGF-β promotes maturation of T_H2 $CD4^+$ cells (O'Byrne et al., 2000) and also directly inhibits cytotoxic $CD8^+$ T-lymphocyte-mediated cancer killing mechanisms (Chen et al., 2005a). Furthermore, host cytotoxic activity is reduced in a TGF-β-dependent manner via cross-talk with local Tregs that express Foxp3 (Wahl et al., 2006). Expression of TGF-β was observed in all patients in this study both pretreatment and prior to the third chemotherapy cycle. Surprisingly, there was no consistent relationship between changes in TGF-β expression and treatment response. Levels of TGF-β increased in 11 patients but fell in 9 patients.

Considerable inter-patients variation in the differences of expression in response to treatment was also observed. The reasons why some patients showed dramatic changes in levels of PBMC expression of TGF-β between baseline and C3 whereas in others treatment had apparently only minimal effect are unclear though

may in part be explained by the heterogeneity of the population studied. However, it is possible that assessment of TGF-β levels at different timepoints to those chosen in this study may reveal more pronounced changes in expression profiles in the remaining individuals. In this regard serial measurements at additional timepoints may be worthwhile.

Interestingly, other investigators have found a similar lack of correlation between circulating TGF-β and response to chemotherapy in NSCLC patients. Ciszak et al also showed that cisplatin-based combination chemotherapy did not produce significant differences in serum levels of TGF-β when measured by ELISA in patients with advanced disease (Ciszak et al., 2009). Although no consistent change in circulating TGF-β expression was found in the study by these investigators or in our patient population, this does not exclude the possibility that the local tumour microenvironment cytokine profile may be profoundly influenced by neoadjuvant treatments, particularly in the subgroup who demonstrate favourable responses.

In our study no prognostic significance with respect to treatment and IL-10 levels was observed. Most, though not all patients, had detectable IL-10 at baseline and pre C3 in circulating PBMCs. Our results differ from those of De Vita et al who found that blood IL-10 levels may have predictive value in NSCLC (De Vita et al., 2000). Sixty patients treated with combination cisplatin and etoposide and advanced NSCLC were assessed for response to therapy and compared to healthy controls in this retrospective study. The authors showed that there was a significant difference in basal IL-10 levels between responders and non-responders. There was also a marked increase in serum IL-10 levels in nonresponders whereas values in responders significantly decreased. However this study evaluated patients with inoperable disease and used the ELISA immunoassay to quantify serum cytokine profiles. Thus, the results of De Vita et al may not be applicable in a population with operable disease.

The lack of consistent treatment-related changes in IL-10 expression in our population is perhaps not surprising since there is considerable debate as to precise

role played by IL-10 with respect to tumour development and progression. Indeed, there is experimental evidence to support both pro- and antitumour activity and classification of cytokines as pro- versus anti-inflammatory might not apply to the pleiotropic effects of this cytokine. For example, in murine models of ovarian carcinoma, expression of IL-10 results in inhibition of tumour growth and metastasis (Kohno et al., 2003). The antitumour activity of IL-10 is mostly attributed to NK-cell activation, possibly via a synergistic effect on $CD8^+$ T-cells (Mocellin et al., 2003). Downregulation of pro-angiogenic activity via the suppression of macrophage-derived VEGF through MMPs has also been proposed as a possible mechanism by which IL-10 inhibits cancer cell growth (Huang et al., 1999). However, IL-10 may also exert a key role in tumour development via its potent immunosuppressive effects that blunt effective host antitumour immunity. Specifically, suppression of NK and NKT cells by IL-10, a process involving cross-talk with TGF-β, results in impaired activation of IFN-γ producing T_H1 CD4+ cells and fosters tumour immune privilege (Seo et al., 2002). A negative correlation between circulating IL-10 levels and outcome has been observed in patients with a variety of tumour types (Moore et al., 2001).

Recently published data from Enewold et al also suggests that measurement of IL-10 levels does not provide consistent prognostic information. These investigators measured cytokine levels in 353 NSCLC patients from the US using an ultrasensitive electrochemiluminescence immunoassay and showed that IL-10 was associated with survival in African American patients and not Caucasians (Enewold et al., 2009). Evaluation of the impact of chemotherapy on expression of IL-10 in other tumour types has also yielded inconclusive results. De Vita et al showed that there was no significant difference between pre and post treatment serum levels of IL-10 in patients with advanced gastrointestinal malignancy that showed a response to chemotherapy (De Vita et al., 1999). Tong et al showed that whereas administration of paclitaxel in breast cancer patients significantly reduced serum IL-10 levels, this treatment had no effect on IFN-γ, IL-2 or IL-12 levels (Tong et al., 2000).

There are limited data on biomarker patterns that predict response to neoadjuvant chemotherapy in lung cancer. Interestingly, studies on the association of the EGFR gene copy number with clinical benefit from cetuximab have also yielded inconsistent results (Takano et al., 2009). To date, the sole positive predictive factor for response to cetuximab-based therapies in NSCLC is an increase in EGFR gene copy number (≥ 4) detected by fluorescence in situ hybridization (FISH) analysis (Hirsch et al., 2008). However, this study evaluated only patients with advanced-stage disease who were unsuitable for surgery leaving the generalizability of the findings in question.

An additional potential explanation for the lack of consistent signal in cytokine profile changes may be a possible tolerogenic effect related to an inadequate bioavailability of drug in bulky tumours. In cases where treatment results in a modest cell death rate, chronic exposure of low antigen dose may result in generation of tolerogenic signals to the immune system, thereby blunting effective cytokine responses through T-cell anergy (Formenti and Demaria, 2008). In this respect, a larger study to assess for treatment-related differences in T_H1 and T_H2 cytokines in patients with large versus small tumours may be more informative.

The suitability of Q-PCR as a method of quantification of in vivo cytokine gene expression in different disorders is becoming increasingly recognised as an appropriate and acceptable assay (Stordeur, 2007). Quantifying gene expression by traditional methods presents several problems. Firstly, detection of mRNA on a Northern blot or PCR products on a gel or Southern blot is time-consuming and does not allow precise quantification. Also, over the 20-40 cycles of a typical PCR, the amount of product reaches a plateau determined more by the amount of primers in the reaction mix than by the input template/sample. One of the major advantages of Q-PCR is its high sensitivity to detect minor changes in cytokine mRNA patterns. However, since reproducibility is influenced by parameters such as distribution statistics, Q-PCR data are theoretically less reproducible when working with very low copy numbers due to the stochastic sampling effects (Peccoud and Jacob, 1996). As expression of Foxp3 and IL-4 was found to be extremely low in the

patient population studied here, it was not possible to confidently assess for a change in levels of these cytokines after treatment.

Since it was hypothesised that treatment response would coincide with a reduction in immunosuppressive responses, the finding of undetectable levels of IL-4 and Foxp3 in our patient population was somewhat surprising and the biological mechanisms to account for this finding require additional investigation. A simple explanation is that circulating PBMC mRNA levels do not reflect the biologic effects mediated by IL-4 or Foxp3. Interestingly, cervical cancer patients also appear to have reduced levels of IL-4 in circulating T-lymphocytes compared to IL-4 levels expressed in TILs (Santin et al., 2001b). Others have shown that quantitative expression of IFN-γ is up to several log-fold higher than other commonly studied cytokines, a finding consistent with our results (Kammula et al., 1999).

Several other groups have similarly attempted to identify prognostic and predictive NSCLC patient subgroups by examining mRNA expression profiles assayed by PCR (Beer et al., 2002, Bhattacharjee et al., 2001, Larsen et al., 2007). However, results have shown considerable heterogeneity, likely due to the diversity of tumour and host cellular components. Methodological considerations are also likely to play a role in the lack of overlap in conclusions drawn from these various studies (Boutros et al., 2009).

The choice of assay used to gain insight into the immunomechanisms associated with pretreatment of surgical NSCLC patients may also influence the results. Profiling of mRNA expression may provide an incomplete picture of cytokine profiles in vivo. Levels of mRNA do not always correlate with protein levels and provide no information on protein-protein interaction or any post-translational modifications that may be critical for regulating protein activity (Yildiz et al., 2007). In this regard, a lack of change of circulating PMBC cytokine mRNA levels does not exclude differences in activities of released pro- and anti-inflammatory mediators either systemically or in the tumour itself. By contrast, immunohistochemistry is a reliable method to assess individual protein expression

and has been shown by others to be useful as a prognostic and predictive too (Zheng et al., 2007, Olaussen et al., 2006). However, obtaining serial tumour biopsy specimens from NSCLC patients to assess for potential treatment-associated changes in intratumoral cytokine expression is a much more invasive approach than serial blood testing.

Tumour molecular heterogeneity is a major reason that patients with NSCLC with a similar clinical stage and tumour histology are often characterised by dramatically different clinical outcomes and responses to treatment. The population examined in this retrospective study was relatively small and as such, sample size may have contributed to the lack of correlation between cytokine profile and treatment responses. The size of study population was, however, limited by the numbers successfully recruited to the pilot study in our hospital. Patient samples were selected on the basis of availability of suitable specimens rather than on response or other clinical characteristics. An additional consideration is that clinicopathological characteristics of the patients evaluated also showed considerable heterogeneity, limiting useful subgroup analysis.

A further confounder that may have potentially influenced the analyses is the possible contribution to PBMC mRNA cytokine expression made by cigarette smoking. Inhalation of smoke particles and chemical irritants contained in tobacco smoke can induce a host response that can change cytokine concentrations (Kuschner et al., 1996). Since most of the patients in this study were current (8) or former (11) smokers, this may have influenced results. Controlling for the potential effects of smoking on cytokine expression in NSCLC populations is appropriate in future studies of this nature.

The development of techniques to accurately monitor the immunologic effects of investigational treatment strategies has increased our understanding of the complex host-tumour interactions in NSCLC patients. Although quantification of total PBMC cytokine levels did not show a significant correlation with tumour responses after induction chemotherapy in the patients assessed in this retrospective analysis, future studies to assess for changes in cytokine patterns in a larger patient

cohort may be worthy of consideration. Whether the lack of consistent cytokine response is particular to the treatment combination used in our population will also require clarification. In this regard, assessment of immunologic responses to alternative cytotoxic regimens measured at additional timepoints by Q-PCR and by quantification of protein expression may be of prognostic relevance. Additional investigation to clarify why some patients show marked changes in different pro- and anti-inflammatory mediators examined in this study whereas others do not is also warranted.

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Appendix 1

PCR oligonucleotides sequences

mRNA targets	Primer Sequences[a]	Final Concentration (nM)
β-Actin:	Fwd:5'-AACCCCAAGGCCAACCGCGAGA-3' Rev:5'-GGTGATGACCTGGCCGTCAGGC-3' Probe:6-FAM-TGACCCAGATCATGTTTGAGACCTTCA-TAMRA-P	F: 300 R:300 Probe: 100
TGF-β	Fwd: 5'-GACTACTACGCCAAGGAGGTCA-3' Rev: 5'-TGCTGTGTGTACTCTGCTTGAAC -3' Probe: 6-FAM-CGCGTGCTAATGGTGGAAACCC-TAMRA	F: 900 R:600 Probe: 200
Foxp3	Fwd: 5'-GAGTTCCTCCACAACATGGACT-3' Rev: 5'-ATGGTTTCTGAAGAAGGCAAAC -3' Probe:6-FAM- CAACATGCGACCCCCTTTCACC -TAMRA-P	F: 600 R: 600 Probe: 150
IL-4	Fwd: 5'-ACTTTGAACAGCCTCACAGAG -3' Rev: 5'-TTGGAGGCAGCAAAGATGTC -3' Probe: 6-FAM -CTGTGCACCGAGTTGACCGTA – TAMRA-P	F: 900 R: 900 Probe: 200
IL-10	Fwd: 5'-CATCGATTTCTTCCCTGTGAA -3' Rev: 5'-TCTTGGAGCTTATTAAAGGCATTC -3' Probe: 6-FAM-ACAAGAGCAAGGCCGTGGAGCA-TAMRA-P	F: 900 R: 900 Probe: 100
IFN-γ	Fwd: 5'-CTAATTATTCGGTAACTGACTTGA -3' Rev: 5'-ACAGTTCAGCCATCACTTGGA -3' Probe:6-FAM-TCCAACGCAAAGCAATACATGAAC-TAMRA-P	F: 600 R: 900 Probe: 100

Primer sequences and concentrations of oligonucleotides used for reverse-transcription Q-PCR

[a] Fwd and Rev indicate forward and reverse primers;

[b] Final concentration of forward (F) and reverse (R) primers and probe (P)

Appendix 2

Publications and presentations

Abstracts and presentations related to this thesis

O'Callaghan DS, Rexhepaj E, Gately K, Delaney D, O'Connell F, Kay E, Gallagher W, O'Byrne KJ. Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis in resected non–small cell lung cancer. *Eur Resp J* 2009. 34(3):746s. *Selected for Oral Presentation, European Respiratory Society Congress, Berlin 2009.*

O'Callaghan DS, Rexhepaj E, Gately K, Gallagher W, Delaney D, Kay E, O'Byrne KJ. Effect of pattern of lymphocyte infiltration in NSCLC on outcome. *J Clin Oncol* 2009; 27:15s, (suppl; abstr 11079).

Al-Alao B, O'Callaghan DS, Gately K, Nicholson S, O'Connell F, McGovern E, O'Byrne KJ, Young VK. Audit of Surgically Resected Non Small Cell Lung Cancer, Survivals and Prongostic Factors. *J Thor Oncol* 2009. 2(9): 59s.

O'Callaghan DS. Immune response as a prognostic factor in NSCLC. *Invited speaker, British Thoracic Oncology Group Winter Meeting 2009, Dublin.*

O'Callaghan DS, Rexhepaj E, Gately K, Delaney D, O'Connell F, Gallagher WM, Kay E, McGovern E, Young VK, KJ O'Byrne. Pattern of CD8+ lymphocyte infiltration in non-small cell lung cancer strongly influences outcome. *Ir J Med Sci* 2008. 177(13): 440s. *Selected for Oral Presentation, Irish Thoracic Society Annual Meeting, Belfast, 2009.*

O'Callaghan DS, Coate L, Rowley S, Gately K, Devlin M, Nicholson S, O'Connell F, O'Byrne KJ, McGovern E, Young VK. Surgical resection for non-small cell lung cancer: clinical features and outcomes for stage I-IIIA disease. *Ir J Med Sci* 2007. 176(10): 408s.

Coate L, O'Callaghan D, Barr M, Barrett C, O'Byrne KJ, Gately K. A retrospective clinicopathological study of the epidermal growth factor receptor profile in patients with resectable non-small cell lung cancer. *J Thor Oncol* 2007. 2(8): 69s.

Publications related to this thesis

O'Callaghan DS, O'Donnell D, O'Connell F, O'Byrne KJ. The role of inflammation in the pathogenesis of non-small cell lung cancer. *J Thor Oncol 2010 (accepted for publication).*

Book chapter

O'Callaghan DS, O'Connell F. Lung Cancer. Heggenhougen K (ed.). International Encyclopedia of Public Health. Amsterdam: Elsevier, 2008. ISBN: 0-12-227225-0.

Manuscripts in preparation

O'Callaghan DS, Rexhepaj E, Gately K, Delaney D, O'Connell F, Kay E, McGovern E, Young VK, Gallagher WM, O'Byrne KJ. Pattern of adaptive immune response determines outcome in non-small cell lung cancer. *In preparation.*

O'Callaghan DS, Al-Alao B, Gately K, Coate L, Rowley S, Nicholson S, O'Connell F, McGovern E, Young VK, O'Byrne KJ Surgical resection for non-small cell lung cancer: clinical features and outcomes for a consecutive series at an Irish tertiary referral centre. *In preparation.*