ANGIOGENESIS, CYCLOOXYGENASE-2 AND MATRIX METALLOPROTEINASES IN MALIGNANT MESOTHELIOMA

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and Aoife.

DECLARATION

This thesis was composed by myself. The work of which it is record was planned and performed entirely by myself except for the specific instances quoted in the Acknowledgements or text, and was carried out between 1998 and 2002. All sources of information have been acknowledged and referenced.

The tissue samples collected and used for this work were handled as "surplus tissue" under the terms of the report *Human tissue: ethical and legal issues* (Nuffield Council on Bioethics, London, 1995) which were the guidelines followed at the commencement of this project. This was with agreement of the chairman of the Local Ethical and Research Committee. Latterly, written patient consent was gained for prospective tissue collection on the hospital operation consent forms.

The computerised database was registered according to the Data Protection Act and hardcopy data anonymised to protect patient confidentiality.

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John G Edwards

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Angiogenesis, Cyclooxygenase-2 and Matrix Metalloproteinases in Malignant Mesothelioma

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Malignant Mesothelioma (MM) is a fatal tumour, related to prior asbestos exposure, of increasing incidence. Current treatment modalities may provide symptom palliation but survival benefits remain unclear.

Angiogenesis is essential for tumour growth of greater than 1-2mm in diameter and is stimulated by hypoxia, which is reflected by tumour necrosis (TN). Angiogenesis can be assessed by the intratumoural microvessel density (MVD). Cyclooxygenase(COX)-2 plays a central role in the upregulation of angiogenic growth factors, such as vascular endothelial growth factor and matrix metalloproteinases (MMPs). Epidermal Growth Factor Receptor (EGFR) is overexpressed in many solid tumours and participates both in COX-2 and MMP upregulation. These factors may be prognostic in solid tumours.

A database of MM cases in Leicester from 1987 to 2001 was created. Clinical and pathological prognostic factors were derived. Angiogenesis, TN and EGFR were assessed in 171 MM cases by immunohistochemistry and/or microscopy. COX-2 and MMP expression were analysed prospectively by semi-quantitative Western blotting and gelatin zymography, respectively, in up to 47 snap-frozen samples.

TN, COX-2 and MMPs were identified for the first time in MM. MVD, COX-2, and EGFR correlated with TN but not with each other. The prognostic significance of MVD and EGFR were confirmed in the largest series of MM studied. TN, COX-2 and MMP-2 were novel prognostic factors. MVD, TN, EGFR, COX-2 and MMP-2 each contributed both to the CALGB and EORTC prognostic scoring systems in multivariate analyses.

In addition to establishing new laboratory methods and the prognostic importance of these factors, this work has identified novel targeted therapies for MM. These include anti-angiogenic therapies, such as thalidomide, and COX-2, EGFR and MMP inhibition, all of which are now either under current or future investigation in clinical trials in Leicester and other international centres.

ACKNOWLEDGEMENTS

ABBREVIATIONS

^{3}HT   tritiated-thymidine

ALP   alkaline phosphatase

ABC   Streptavidin-biotin complex

APS   ammonium persulphate

BCRU   Breast Cancer Research Unit

bFGF   basic fibroblast growth factor

BSA   bovine serum albumin

CA   carbonic anhydrase

CALGB   Cancer and Leukemia Group B

cAMP   cyclic adenosine monophosphate

CD   cluster of differentiation antigen

CEA   carcino-embryonic antigen

CI   confidence interval

cm   centimetre

CMHT   Centre for Mechanisms of Human Toxicity, University of Leicester

CMI   cell-mediated immunity

COX   cyclooxygenase

CSF   colony-stimulating factor

CT   computed tomography

CXR   chest X-ray

DAB   diaminobenzidine tetrachloride

dl   decilitre

DNA   deoxyribonucleic acid

d-PAS	diastase-periodic acid-Schiff
DTPA	diethylenetriamine penta-acetic acid
DTT	Dithiothreitol
ECL	enhanced chemiluminescence
ECM	extracellular matrix
ECOG	Eastern Co-operative Oncology Group
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EIA	enzyme immunoassay
EORTC	European Organisation for Research and Treatment of Cancer
EPP	extrapleural pneumonectomy
FDG	fluorodeoxyglucose
FFPE	formalin-fixed, paraffin-embedded
FITC	fluoroscein isothiocyanate
FN	fibronectin
g	gram
GFR	growth factor receptor
GGH	Glenfield General Hospital, Leicester
GP	general practitioner
Gy	Grays
H&E	haematoxylin and eosin
H2O	water
HA	hyaluronic acid
HAB	hyaluronidase-alcian blue
Hb	Haemoglobin

HGF/SF	hepatocyte growth factor/scatter factor
HI	humoural immunity
HIF	hypoxia inducible factor
HMC	human mesothelial cells
HR	hazard ratio
HSV-tk	Herpes simplex virus-thymidine kinase
ICRF	Imperial Cancer Research Fund
IFN	interferon
Ig	immunoglobulin
IGF	insulin-like growth factor
IGF-IR	insulin-like growth factor-I receptor
IL	interleukin
IMIG	International Mesothelioma Interest Group
IMRT	intensity-modulated radiotherapy
IMS	industrial methylated spirit
IP	inflamed pleura
IRS	insulin receptor substrate
kDa	kiloDaltons
l	litre
LAK	lymphokine activated killer
LDH	lactate dehydrogenase
LPF	low power field
M	Molar
mA	milliamps
µg	microgram

μl	microlitre
mg	milligram
MHC	major histocompatability complex
ml	millilitre
MM	malignant mesothelioma
mm	millimetre
mM	millimolar
MMP	matrix metalloproteinase
MMPI	matrix metalloproteinase inhibitor
MoAb	monoclonal antibody
MRI	magnetic resonance imaging
MT-MMP	membrane-type matrix metalloproteinase
MVD	microvessel density
NFKB	nuclear-factor-KB
NK	natural killer
NGS	normal goat serum
NRS	normal rabbit serum
NSCLC	non-small cell lung cancer
O+G	Department of Obstetrics and Gynaecology, University of Leicester
°C	degrees centigrade
OD 595	optical density at, e.g., 595nm
P/D	radical pleurectomy and decortication
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline

PD-ECGF	platelet-derived endothelial cell growth factor
PDGF	platelet-derived growth factor
PDT	photodynamic therapy
PET	positron emission tomography
PF	platelet factor
PG	prostaglandin
PMSF	phenylmethylsulphonyl fluoride
PolyAb	polyclonal antibody
PRL	prolactin
PS	performance status
Rb	retinoblastoma
RNA	ribonucleic acid
RT	room temperature
SD	standard deviation
SDS	sodium dodecyl sulphate
SV40	Simian virus 40
Tag	large-T antigen
tag	small-t antigen
TBS	tris-buffered saline
TBST	tris-buffered saline with Tween 20
TCR	T-cell receptor
TEMED	N,N,N',N'-tetramethylenediamine
TGF	transforming growth factor
Th	T-helper
TIMP	tissue inhibitor of metalloproteinase

TN	tumour necrosis
TNF	tumour necrosis factor
TNM	tumour-nodes-metastasis
tPA	tissue plasminogen inhibitor
TSP	thrombospondin
UICC	International Union Against Cancer
UK	United Kingdom
UP	uniflamed pleura
uPA	urokinase plasminogen activator
uPAR	urokinase plasminogen activator receptor
USA	United States of America
V	volts
VATS	video assisted thoracoscopic surgery
VEGF	vascular endothelial growth factor
WBC	white blood cell count
wt	wild type

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Chapter One

Introduction

1.1. Background

Malignant Mesothelioma (MM) is an aggressive tumour with a poor prognosis. MM most commonly involves the pleura and is usually associated with asbestos exposure. MM responds poorly to conventional modes of therapy, including surgery, chemotherapy and radiotherapy. A number of small-scale trials of these conventional treatments have been published, but no real impact on survival has been made. Given the poor prognosis in MM and other solid tumours, research has focused on the development of new treatment modalities, such as immunotherapy, gene therapy, photodynamic therapy and anti-angiogenesis therapy. Developments in our understanding of the molecular biology of MM are expected to continue to lead to novel treatment strategies.

1.1.1. Epidemiology

Until recently, MM was considered to be a rare tumour. In 1960, an association was noted in South Africa between occupational or environmental exposure to asbestos, particularly the crocidolite asbestos fibre, and MM (Wagner 1993). This observation subsequently was confirmed in several other studies. The usual duration of exposure to asbestos may vary from a few months to many years. During that time there is usually a period of intense exposure to asbestos fibres. A history of asbestos exposure is given in about 80% of cases of MM (Aisner 1995). More recently, exposure to Simian Virus 40 (SV40) has also been postulated as an aetiological factor. Contaminated polio and adenovirus vaccines exposed a portion of the population to SV40 between 1955 and 1963 (Carbone et al. 1997a). The role of SV40 in the pathogenesis of MM will be considered further later.

Evidence of a genetic susceptibility to MM has been reported (Roushdy-Hammady

et al. 2001) amongst Turkish villagers exposed to the carcinogenic zeolite fibre, erionite, which is found in the stone used for house construction there. The incidence of MM in the villages of Karain and Tuzkoy is approximately 50%. MM was associated with "houses of death", which did not have a greater amount of erionite but whose residents were closely related. Genetic pedigree analysis has suggested that, in this setting, susceptibility to MM is transmitted genetically, possibly in an autosomal dominant manner reported (Roushdy-Hammady *et al.* 2001).

The UK Health and Safety Executive have kept a register of deaths where mesothelioma was mentioned on the death certificate since 1968. Annual deaths have risen from 154 in 1968 to 1527 in 1998. There is a long latent period between the first exposure to asbestos and onset of symptoms: this is usually greater than twenty years and frequently more than forty years. Taking into account the likely exposure of the population to asbestos, judged historically by the importation and industrial use of asbestos, the incidence of MM is expected to rise well into the next century. It has been estimated that there may be a peak of over 3000 deaths per year at about 2020 (Peto *et al.* 1999). For the cohort of men born in the UK in the 1940's, MM may account for approximately 1% of all deaths (Peto *et al.* 1995).

1.1.2. Clinical Features

The most common primary site is the pleura, although MM can also occur in the peritoneum (Corson 1997). MM is characterised by extensive primary growth and invasion into intrathoracic tissues and organs. Eventually the pleura may be replaced with a thick "rind" of tumour, which constricts lung expansion. Symptoms of dyspnoea occur in 80%, cough in 60% and chest pain in 40% of patients. There may be other symptoms such as

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weight loss or an abdominal mass. Respiratory examination reveals reduced breath sounds and a dull percussion note over the area of the tumour. A mass may be palpable in the abdomen or in intercostal spaces. Distant metastasis occurs late in the course of the disease, but haematogenous spread is present in about 50% of patients at the time of death. Nodal metastasis is seen in about 40% of patients who undergo radical surgery in the form of extrapleural pneumonectomy. Death is usually due to the local invasion by the primary tumour. Cytological analysis of aspirated pleural fluid may suggest a diagnosis of MM, but this alone is rarely diagnostic. Closed (percutaneous) biopsy may be inadequate due to poor tissue sampling, whereas Video Assisted Thoracoscopic Surgery (VATS) not only produces an adequate tissue sample but usually allows a full intrathoracic assessment of the tumour (Sugarbaker *et al.* 1997).

1.1.3. Histopathologcial features

There are three main cellular types of MM. Epithelioid cell (epithelial, carcinomatous) MM is the most common, accounting for approximately 50% of cases. Cells may be in tubular, papillary or solid forms. Biphasic (mixed cell) MM consists of sarcomatoid and epithelioid cell elements and is the pattern in a third of cases. Sarcomatoid (sarcomatous) MM, in which the cells may display one or more of a variety of connective tissue phenotypes, accounts for the remainder. Most commonly, sarcomatoid MM consists of spindle-shaped cells, although it may be difficult to distinguish the desmoplastic variant of sarcomatoid MM from florid fibrous proliferations and even osseous differentiation can occur. There is a high proliferation rate, suggested by a high mitotic index, staining for nuclear antigens and the pattern of nucleolar organisation (Attanoos and Gibbs 1997).

The difficulty in distinguishing between MM and its differential diagnoses, in particular adenocarcinoma, may be overcome using histochemistry, immunohistochemistry, electron microscopy, or cytogenetic techniques (Corson 1997; Attanoos and Gibbs 1997). Positivity for histochemical stains such as diastase-periodic acid-Schiff (d-PAS) is found in approximately 5% of cases of MM, whereas it is positive in 50-60% of adenocarcinomata. Hyaluronidase-alcian blue (HAB) is very specific for adenocarcinoma. The pattern of keratin staining in epithelial MM is diffuse and perinuclear, whereas it is peripheral and membrane bound in adenocarcinoma. Carcino-embryonic antigen (CEA) is positive in only 10% of MM and it is usually weakly so and focal. It is positive in 93% of lung adenocarcinomata and 84% of metastatic adenocarcinomata from other primary tumours. CEA is often used initially to help exclude MM. Similarly, Leu-M1, B72.3 Sialyl-TN, CD15 and Ber-EP4 are rarely positive in MM but usually so in adenocarcinoma (Dejmek *et al.* 1997). The differential expression of cadherins has recently been described. N-Cadherin appears sensitive and specific for mesothelioma, whereas E-Cadherin is so for adenocarcinoma (Peralta Soler *et al.* 1995). Calretinin positivity has been proposed recently as specific for MM rather than adenocarcinoma (Cury *et al.* 2000). Electron microscopy may be useful in differentiating epithelial MM and adenocarcinomata, or sarcomatoid MM and sarcomas. For example, the length to diameter ratio of microvilli may aid the identification of epithelial MM (Burns *et al.* 1985).

There are a number of chromosomal deletions which may be present in MM (Fletcher 1995). For example, deletion of the short arms of chromosome 1 or 3, or the long arm of chromosome 22, are common. Deletions of the long arm of chromosome 6 or the short arm of chromosome 9 are less common. These deletions are similar to those found in

lung cancers, although there is no evidence regarding mesothelioma specific chromosomal

abnormalities. Chromosomal deletions are not found in reactive mesothelial proliferations.

1.2.   Current Management of Malignant Mesothelioma

1.2.1. Staging

1.2.1.1. Tumour-Nodes-Metastasis staging

Deriving the Tumour-Nodes-Metastasis (TNM) stage of a tumour may provide

prognostic information, as well as defining the extent of the tumour. Accurate TNM

staging is necessary before planning treatment, particularly if surgical resection with

curative intent is contemplated. Plain chest radiography may show pleural thickening, a

pleural effusion or pleural calcification. Computed Tomography (CT) is used to assess the

tumour burden and extent of the disease, but Magnetic Resonance Imaging (MRI) has a

higher sensitivity than CT when assessing tumour extension into the chest wall, interlobar

fissures, diaphragm and mediastinum (Marom *et al.* 2002; Knuuttila *et al.* 1998). However,

the experience in Leicester was that, although MRI with gadolinium-DTPA contrast

enhancement was of value in predicting the resectability of MM by radical surgery, it was

accurate at judging the pathological TNM stage in only 38% of patients (Figure 1.1)

(Edwards *et al.* 2000). Positron Emission Tomography (PET) with fluorodeoxyglucose

(FDG) has been proposed recently as a specific and sensitive imaging modality, with

possible roles in diagnosis, staging, and assessment of disease progression and treatment

response, although it is not widely available (Benard *et al.* 1998). It may be possible to

assess the transpericardial extent of the tumour, as well as cardiac function, by

echocardiography. It remains difficult, however, to gauge an accurate TNM stage by

radiological means.

Figure 1.1: Contrast-Enhanced Magnetic Resonance Imaging (CE-MRI). Sagittal T1-weighted images a) pre- and b) post-gadolinium DTPA contrast demonstrating the benefits of CE-MRI in visualising the plane of the diaphragm. The posterior diaphragm is shown not to be breached by tumour following contrast (arrow).
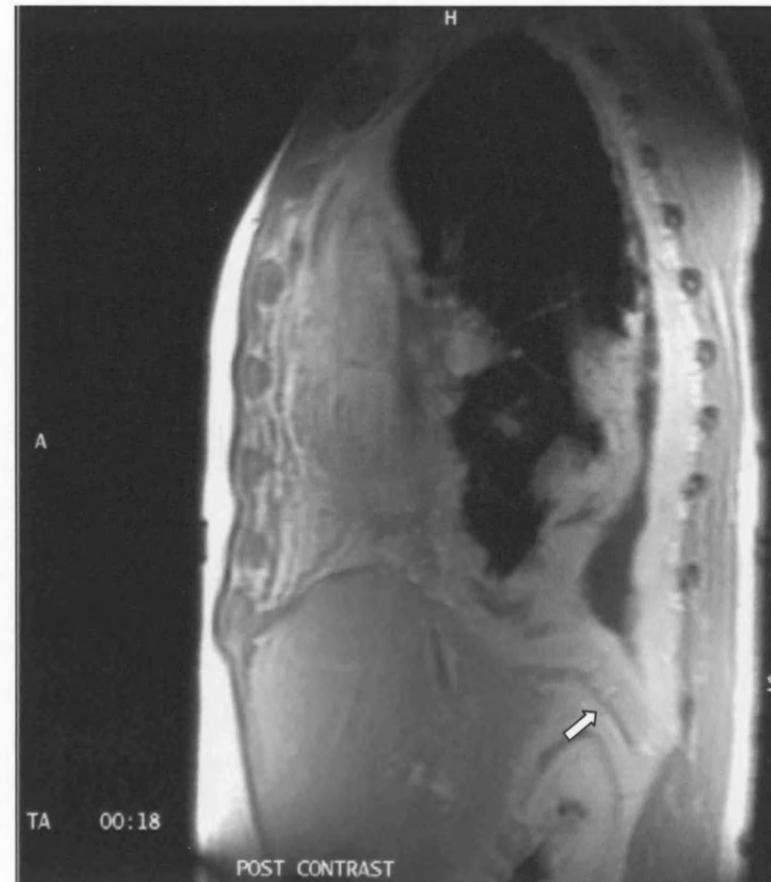
Therefore a combination of surgical staging, by VATS in conjunction with mediastinoscopy, and radiological imaging techniques is probably required before planning radical therapy. VATS may yield an adequate tissue sample for a definitive diagnosis to be made, when closed pleural biopsy has been unhelpful, as well as allow biopsy of intrathoracic lymph node groups. Mediastinoscopy may be useful in assessing pre- and paratracheal lymphadenopathy, but not all mediastinal nodes which drain the pleura are accessible by this technique (Sugarbaker *et al.* 1999). There are no specific published data regarding the use of mediastinoscopy in MM.

Several staging systems have been proposed but often they have been difficult to validate (Sugarbaker *et al.* 1997). The first widely used system was devised in 1976 by Butchart (Butchart *et al.* 1976). This was based on a small, historical series of extrapleural pneumonectomy for MM, dating back to the 1950's. In 1983, Chahinian proposed the first TNM-based system, but it was not possible to correlate pre-operative and pathological staging. The International Union Against Cancer (UICC) published a modified TNM system in 1990 but this had similar limitations. The International Mesothelioma Interest Group (IMIG) proposed revisions to the UICC TNM system in 1994 (Table 1.1) (Rusch 1995). This system is used increasingly in clinical practice, but there is still difficulty in differentiating the local extent of the tumour without resorting to surgical staging. Sugarbaker *et al*, from the Brigham and Women's Hospital in Boston, USA, have validated their Brigham staging system in 183 patients who underwent a multimodality treatment program incorporating extrapleural pneumonectomy (Sugarbaker *et al.* 1999). This surgical system, which is only applicable after extrapleural pneumonectomy, assesses the involvement of resection margins by tumour, as well as nodal status (Table 1.2). The Brigham stage stratified survival successfully in their series, which is the largest of radical

Table 1.1: The International Mesothelioma Interest Group staging system for malignant

mesothelioma (Rusch 1995).

T1	T1a	Tumour limited to the ipsilateral parietal pleura, including mediastinal and diaphragmatic pleura; no involvement of the visceral pleura
	T1b	Tumour involving the ipsilateral pleura, including mediastinal and diaphragmatic pleura; scattered foci of tumour also involving the visceral pleura
T2		Tumour involving each of the ipsilateral pleural surfaces (parietal, mediastinal, diaphragmatic, and visceral pleura) with at least one of the following features: Involvement of diaphragmatic muscle Confluent visceral pleural tumour (including the fissures) or extension of tumour from visceral pleura into the underlying pulmonary parenchyma
T3		Describes locally advanced but potentially resectable tumour Tumour involving all the ipsilateral pleural surfaces (parietal, mediastinal, diaphragmatic, and visceral pleura) with at least one of the following features: Involvement of the endothoracic fascia Extension into mediastinal fat Solitary, completely resectable focus of tumour extending into the soft tissues of the chest wall Non-transmural involvement of the pericardium
T4		Describes locally advanced technically unresectable tumour Tumour involving all the ipsilateral pleural surfaces (parietal, mediastinal, diaphragmatic, and visceral pleura) with at least one of the following features: Diffuse extension or multifocal masses of tumour in the chest wall, with or without rib destruction Direct transdiaphragmatic extension of tumour to the peritoneum Direct extension of tumour to the contralateral pleura, one or more mediastinal organs or the spine Tumour extending through to the internal surface of the pericardium with or without a pericardial effusion, or tumour involving the myocardium
NX		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastases
N1		Metastases in the ipsilateral bronchopulmonary or hilar lymph nodes
N2		Metastases in the subcarinal or ipsilateral mediastinal lymph nodes, including the ipsilateral internal mammary nodes
N3		Metastases in the contralateral mediastinal, contralateral internal mammary, ipsilateral, or contralateral supraclavicular nodes
MX		Presence of distant metastases cannot be assessed
M0		No distant metastasis
M1		Distant metastasis present
Stage Ia		T1a N0 M0
Ib		T1b N0 M0
Stage II		T2 N0 M0
Stage III		Any T3 M0
		Any N1 M0
		Any N2 M0
Stage IV		Any T4
		Any N3
		Any M1

Table 1.2: The Brigham staging system for malignant mesothelioma (adapted from Sugarbaker *et el.* 1999). Stage I represents resectable disease with negative nodes, whereas Stage II indicates resectable disease but positive nodes.

Stage	Definition
I	Disease completely resected within the capsule of the parietal pleura without adenopathy: ipsilateral pleura, lung, pericardium, diaphragm, or chest wall disease limited to previous biopsy sites.
II	All of stage I with positive resection margins and/or intrapleural adenopathy
III	Local extension into the chest wall or mediastinum; into the heart or through the diaphragm or peritoneum; or with extrapleural lymph node involvement
IV	Distant metastatic disease

surgery to date, whereas the IMIG stage failed to do so. An effective TNM staging system, for which extensive surgical staging or resection is not required, is yet to be developed.

1.2.1.2. Prognostic factors

Radical surgery is either not appropriate for or available to the vast majority of patients in the UK and therefore a TNM stage, confirmed with pathological data, is rarely obtained. Observations that simple clinical and pathological variables had a bearing on the outcome of the disease have led to the establishment of a number of them as prognostic factors. These include age, gender, the presence of pleuritic chest pain, weight loss of greater than 5%, performance status, histopathological cell type and the pretreatment white blood cell (WBC) count and platelet count (Steele 2002).

Two prognostic scoring systems have been developed in series of patients undergoing chemotherapy for mesothelioma. The Cancer And Leukemia Group B (CALGB) in the USA proposed a system which places patients into six groups according to performance status, presence of chest pain and weight loss, white blood cell count and haemoglobin (Herndon *et al.* 1998). The European Organisation for Research and Treatment of Cancer (EORTC) system defined high and low risk groups, which are derived from a score incorporating gender, performance status, cell type and white blood cell count (Curran *et al.* 1998).

1.2.2. Surgery

1.2.2.1. Diagnostic and palliative surgery

Surgery may be used to establish the tissue diagnosis and tumour stage, to palliate symptoms, or to attempt a curative resection of the tumour. VATS permits biopsy and

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staging of the tumour, as well as offering symptom palliation through pleurodesis, parietal pleurectomy or visceral decortication (Figure 1.2) (Waller *et al.* 1995). Parietal pleurectomy enables a partial debulking of the tumour mass, a durable, surgical pleurodesis and relief from cough, dyspnoea and pain (Brancatisano *et al.* 1991; Soysal *et al.* 1997; Martin-Ucar *et al.* 2001).

1.2.2.2. Radical Surgery

Extrapleural pneumonectomy (EPP) is the most radical and therefore most cytoreductive surgical option. Only 10% to 30% of patients are of suitable stage to be resectable (Kaiser 1997; Sugarbaker *et al.* 1999; Aziz *et al.* 2002) and patients must also demonstrate sufficient cardiopulmonary reserve to be medically operable. The parietal pleura is dissected from the chest wall and resected *en bloc* with the lung, pericardium and diaphragm. The diaphragm and pericardium are then reconstructed. The operative mortality is about 6%.

Radical pleurectomy and decortication (P/D), preserving the lung parenchyma, is an alternative to EPP, with a lower reported operative mortality of 1.5 to 5% (Rusch 1997). Following a posterolateral thoracotomy, the parietal pleura is stripped from the chest wall, diaphragm and mediastinum; the pleural mass adherent to the lung is decorticated and reconstruction of the diaphragm and pericardium is carried out, as for an EPP.

As well as being the most cytoreductive operation, EPP allows a greater post-operative dose of radiotherapy, since lung toxicity is no longer a problem. However, chemotherapy is better tolerated following P/D, especially if regimens require significant pre-treatment hydration.

Figure 1.2:   Intraoperative photographs of Video Assisted Thoracoscopic Surgery (VATS).

a)   VATS assessment of the pleural cavity. This is a T1b tumour, with scattered, non-confluent, visceral pleural nodules but no lung parenchymal invasion.

b)   It is also possible to decorticate the lung with VATS. Note the hyaline plaques on the parietal pleura indicating the previous asbestos exposure.
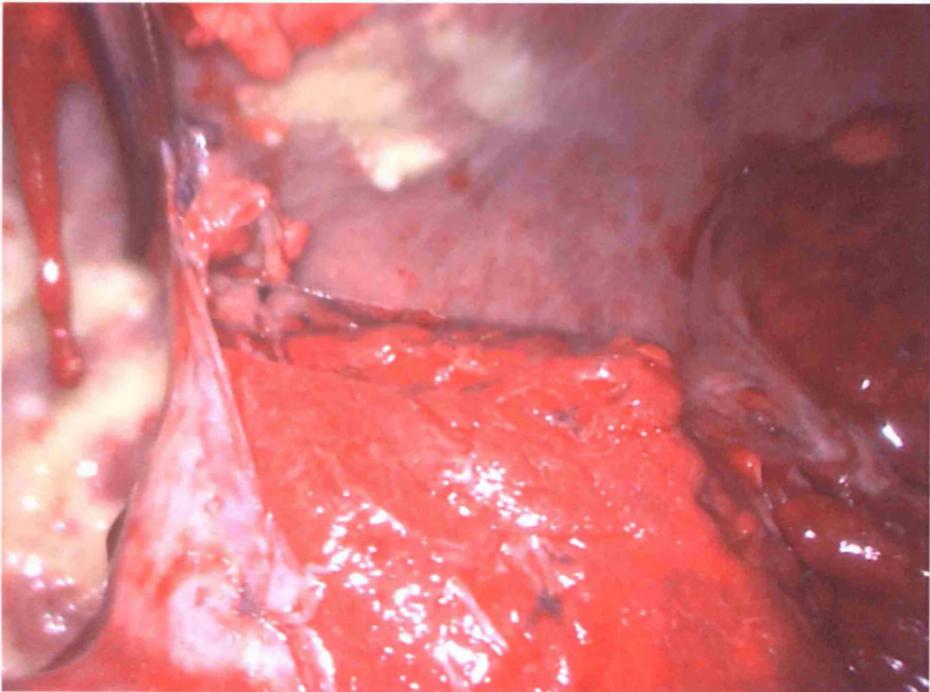
Survival data from series of radical surgery must be treated with caution as these patients are, by definition, carefully selected and are likely to be in the better prognostic groups before surgery. Neither palliative nor radical surgery has been subjected to randomised trials. Although some patients do appear to enjoy prolonged survival after resection, it is not clear whether this is as a reflection of surgery, tumour stage or patient selection. For example, Sugarbaker quoted a five year survival rate of 46% for the 31 patients (of 183) who had epithelioid cell type, clear resection margins and no extrapleural lymph node involvement (Sugarbaker *et al.* 1999).

1.2.3. Chemotherapy

Single agent and combination chemotherapy has been used extensively in an attempt to control MM (Baas 2002; Taub and Antman 1997; Ryan *et al.* 1998), but the results remain disappointing. No regimen, using either single agent or combination therapy, has emerged as a standard or has demonstrated a survival benefit in a randomised trial. However, chemotherapy may have a role in the symptomatic palliation of the disease (Middleton *et al.* 1998). The response rate of single agents, such as cisplatin, doxorubicin and methotrexate, rarely exceeds 20%. The recently introduced vinca alkaloid vinorelbine holds promise with a response rate of 20% reported when used as a single agent in a phase II setting (Steele *et al.* 2000). Combination chemotherapy, which is usually doxorubicin or cisplatin based, tends to gives a response rate of 15 to 40% (Baas 2002; Hunt *et al.* 1996; Samuels *et al.* 1998; Middleton *et al.* 1998).

A number of new cytotoxic agents are under evaluation. For example, gemcitabine as a single agent has reported response rates of 0% to 31% (van Meerbeeck *et al.* 1999). A

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response rate of 47.6% (confidence interval 26.2% to 69.0%) has been reported in 21 patients in a phase II study of gemcitabine and cisplatin (Byrne *et al.* 1999), but these results were not confirmed in a second study of 32 patients with a 15% response rate (van Haarst *et al.* 2000). The investigators from the former study have repeated their phase II trial in a larger, multicentre setting and recently have reported their results. In 52 evaluable patients, a partial response was seen in 17 (33%) and stable disease in 31 (60%) (Nowak *et al.* 2002a).

Recent results for the novel multitargeted antifolates raltitrexed and pemetrexed appear promising (Fizazi *et al.* 2002). In phase I studies of pemetrexed with cisplatin or carboplatin, response rates of 31% and 45% were reported (Calvert *et al.* 2000; Thodtmann *et al.* 1999). The largest randomised phase III trial to date in MM examined pemetrexed and cisplatin versus cisplatin alone in 472 patients, of whom 448 were evaluable. The overall response rate was 41% in the pemetrexed and cisplatin arm, compared to 17% for cisplatin alone. The median survival was 12.1 months vs. 9.3 months (p=0.02). Improvements in folate and vitamin B12 supplementation significantly reduced the toxicity seen with combination therapy, improved response rates and possibly improved survival (Vogelzang *et al.* 2002). Following encouraging response rates of the raltitrexed/oxaliplatin combination (Fizazi *et al.* 2000), the EORTC has initiated a phase III trial of cisplatin with or without raltitrexed with an accrual goal of 240 patients.

Intrapleural chemotherapy may improve the response rate and may be useful to improve local control over residual disease after cytoreductive surgery. Pharmacokinetic data of cisplatin with or without mitomycin has suggested that a 3 to 5 times greater concentration is generated in the pleural space than in plasma and that the "Area Under the

Curve" is 50 times greater (Bogliolo *et al.* 1991). Phase I studies have been reported but no evidence has yet been published demonstrating a significant improvement in symptoms or survival over systemic administration (Ratto *et al.* 1999; Shoji *et al.* 2002; Yellin *et al.* 2001).

A recent study confirmed the feasibility of three cycles of gemcitabine and cisplatin as neoadjuvant chemotherapy, prior to EPP (Kestenholz *et al.* 2002). 17 patients underwent EPP, including 9 who had a partial response and 5 stable disease. Median survival was 22 months, compared to that in their patients previously treated by EPP alone of 12 months, which is identical to the experience in Leicester (Edwards *et al.* 2002). Early reports of operative technical difficulty following chemotherapy (IMIG 1997; Sugarbaker *et al.* 1999) were not confirmed by this study but further data are required in larger series.

None of the above trials have compared chemotherapeutic regimes with active supportive care. There are no data from randomised controlled trials to confirm whether chemotherapy prolongs survival or results in better symptom palliation than active supportive care alone. The trials being conducted by the Medical Research Council and British Thoracic Society aim to answer this question by randomising patients between two chemotherapy arms and active supportive care (Girling *et al.* 2002).

1.2.4. Radiotherapy

The diffuse nature of MM limits the usefulness of radiotherapy to treat the whole tumour. Furthermore, radiotherapy is hindered by intrathoracic dose limiting structures. The dose limit of the lung is about 20 Gy and the heart and spinal cord 45 Gy (Sugarbaker *et al.* 1995). The response rate is about 15%. Radiotherapy alone does not increase

survival but may have a role in symptom palliation, in preventing tumour seeding in needle

and intercostal tube tracts (Boutin *et al.* 1995), or as part of multimodality management

(Rusch 1997; Sugarbaker *et al.* 1991). Novel techniques, such as intensity-modulated

radiotherapy (IMRT) are under investigation in MM in the US but are yet to be reported.

IMRT has the ability to conform the radiation dose distribution closely to anatomical

structures by delivering the dose in small beamlets of varying intensity, which results in

reduction of the field size (Brugmans *et al.* 1999). IMRT is advantageous for large,

irregular targets with critical structures in close proximity (Meeks *et al.* 1998), conditions

which certainly apply to MM.

1.2.5. Multimodality therapy

A promising series of trimodality therapy in 183 carefully selected patients has been

published by Sugarbaker *et al.* (Sugarbaker *et al.* 1999). Combination chemotherapy and

external beam radiotherapy followed maximal surgical debulking by EPP. The operative

mortality was 3.8% and the median survival 19 months (range: 1 to 96 months). Survival

at 2 years was 38 % overall, at 5 years 15%. After multivariate analysis, poor prognostic

factors included biphasic or sarcomatoid cell type, metastatic extrapleural lymph nodes and

positive resection margins. The group of patients with none of these factors had a median

survival of 51 months, with 68% 2 year and 46% 5-year survival rates.

However, current management remains disappointing. Multimodality therapy

generates fair response rates, but still there are few long-term survivors and only a small

proportion of patients is suitable. It is difficult to determine the response to treatment for

two main reasons. Firstly, there are no prospective, randomised, controlled trials in the

literature to substantiate the quoted "prolonged survival" of trimodality therapy. Secondly,

comparisons between the series are difficult because they tend to be of small numbers with different criteria for staging, entry into treatment arms and response assessment. As TNM staging is difficult, trials need to be stratified by validated prognostic criteria, which have not yet been fully elucidated. Some favourable prognostic factors, such as female gender, age and haematological indices, are not consistent between studies. The natural course of MM is short but also variable: there are cases of a few long term survivors who have received little more than minor palliative procedures, such as pleurodesis. The median survival ranges from 7 to 17 months in different series with supportive care only, making an assessment of the different management strategies in these small, unrandomised trials difficult. No single treatment modality has increased the overall survival significantly.

Local recurrence of disease occurs in most cases even after trimodality therapy (Baldini *et al.* 1997). This apparent failure to gain local control has lead to new approaches in the management of MM. These include photodynamic therapy, gene therapy and immunotherapy, which will be discussed later. An understanding of the pathology and molecular biology of malignant mesothelioma has lead to the development of these novel therapeutic strategies.

1.3. Molecular Biology of Malignant Mesothelioma

1.3.1. Angiogenesis

The three components essential for tumour growth and metastasis are tumour cell proliferation, invasion and angiogenesis. Angiogenesis is the formation of new blood vessels from existing vasculature. Normally quiescent endothelial cells proliferate and/or elongate and gain invasive characteristics following basement membrane degradation and remodelling of the extracellular matrix (ECM). Angiogenesis is essential for tumour

growth of greater than 1 to 2mm in diameter (Hanahan and Folkman 1996). Metastasis is a

dynamic process by which a malignant cell leaves its original environment, invades locally

and disseminates. The processes of angiogenesis and metastasis are closely inter-related

and are regulated by autocrine, paracrine and endocrine factors (Cox *et al.* 1999; Price *et al.*

1997; Folkman 1995; Folkman 1996). There is a balance of angiogenic and angiostatic

signals, which may be secreted either by tumour cells themselves or by surrounding stromal

tissue. Regulators of angiogenesis include angiogenic growth factors and their receptors,

Type 1 Growth Factors and their receptors, basement membrane and ECM proteolytic

enzymes and their inhibitors, cell surface receptors and immunomodulatory cytokines (Cox

et al. 1999; Folkman 1995; Liotta *et al.* 1991; O'Reilly *et al.* 1994; Bicknell and Harris

1996; Brooks *et al.* 1994a).

The intensity of the angiogenic process can be measured indirectly by counting

microvessels. Vascular endothelial cells are identified in tumour sections by

immunohistochemistry using antibodies to CD34, CD31 or Factor VIII (Vermeulen *et al.*

1996). Under low power, vascular "hot spots" are identified and microvessels within these

areas are counted at high power, with or without the aid of a grid eyepiece graticule

(Weidner *et al.* 1991). Other techniques which have been evaluated include the use of a

Chalkley eyepiece graticule, subjective eye appraisal and computer-assisted image analysis

(Fox *et al.* 1995; Giatromanolaki *et al.* 1996; Charpin *et al.* 1995). High intratumoural

microvessel density (MVD) has been associated with a poor prognosis in many solid

tumours, including non-small-cell lung (Meert *et al.* 2002; Giatromanolaki *et al.* 1996; Cox

et al. 2000b) and breast cancer (Weidner *et al.* 1991; Horak *et al.* 1992; Fox *et al.* 1995).

Before the start of this study, a single preliminary report of the prognostic value of

MVD in malignant mesothelioma was published. Kumar-Singh *et al.* (Kumar-Singh *et al.*

1997) described a relationship of intratumoural MVD with prognosis in 25 MM patients.

Microvessels were visualised with anti-CD34 and anti-CD31 antibodies and counted

visually (per x200 power field) and with computer-aided image analysis. The MVD was

significantly higher in MM than in non-neoplastic mesothelium and there was a correlation

with survival when the 25 cases were divided into tertiles of MVD.

There is evidence of asbestos induced angiogenesis in an animal model.

Intraperitoneal injection of long crocidolite fibres resulted in formation of nodular lesions

characterised by clustering of fibres, activation of macrophages and mesenchymal

proliferation. After six weeks, a network of neo-vessels surrounded 30% of the lesions.

Shorter crocidolite fibres and chrysotile fibres were less effective at promoting

angiogenesis (Branchaud *et al.* 1989).

1.3.2. Hypoxia and Necrosis

Tumour cell hypoxia has been associated with resistance to radiotherapy and a poor

outcome for many years. Gray *et al.* demonstrated in 1953 that the absence of oxygen at

the time of irradiation reduced tumour cell sensitivity to radiotherapy (Gray *et al.* 1953).

Hypoxia occurs frequently in tumours due to the imbalance of the increased oxygen

demand of the rapidly proliferating cells and a relatively poor oxygen supply by the

neovasculature. Hypoxia induces a number of angiogenic growth factors, such as VEGF,

bFGF, PDGF, PD-ECGF and COX-2 (Griffiths *et al.* 1997; Kuwabara *et al.* 1995; Shweiki

et al. 1992; Harris 2002; Chiarugi *et al.* 1999). Surrogate markers of hypoxia, such as

carbonic anhydrase(CA)-IX and hypoxia inducible factor(HIF)-1α, have been correlated

with increased MVD in solid tumours (Beasley *et al.* 2001; Giatromanolaki *et al.* 2001a; Birner *et al.* 2001).

Coagulative necrosis, which is caused by chronic ischaemic injury, is a common feature of solid tumours and reflects the level of intratumoural hypoxia. Tumour necrosis (TN) has been correlated with poor prognosis in a number of solid tumours (Swinson *et al.* 2002; Muro-Cacho *et al.*, 2000; Llombart-Bosch *et al.* 1986). TN has been studied extensively in breast cancer and correlates with stage, grade and both angiogenesis and macrophages which secrete angiogenic growth factors (Leek *et al.* 1999; Lewis *et al.* 2000). Although MM is recognised clinically to be a fast growing tumour which responds poorly to radiotherapy, TN has not been characterised beyond the recognition that it can help differentiate malignant from benign pleural proliferations (Churg *et al.* 2000).

1.3.3. Growth Factors and Growth Factor Receptors

Vascular Endothelial Growth Factor (VEGF) is a secreted endothelial cell specific mitogen, which has multiple roles in tumour growth. There are five active isoforms, created from a single gene by alternative splicing (Houck et al. 1991; Olofsson et al. 1996). VEGF expression may be dependent on loss of the tumour suppressor gene, wild type p53 (Giatromanolaki et al. 1998; Fontanini et al. 1997). VEGF expression is associated with an increase of vessel permeability, mitosis and migration of endothelial cells and formation of tubular vessel-like structures. VEGF may also be associated with lymph node metastasis (Ohta et al. 1997). Inhibition of VEGF with monoclonal antibodies has been shown to suppress angiogenesis in a murine *in vivo* tumour model (Kim et al. 1993). Blockade of the VEGF-R2 receptor (flk-1/KDR) has also reduced tumour invasion and angiogenesis, reversing a malignant tumour into a benign phenotype

(Skobe et al. 1997). VEGF stimulates urokinase plasminogen activator (uPA) activity in endothelial cells (Olofsson et al. 1998), plasminogen activator inhibitor and interstitial collagenase (matrix metalloproteinase (MMP) -1), which increase vascular permeability (Mandriota et al. 1995).

Although described as "*the*" angiogenic switch molecule (Czubayko *et al.* 1997), the concept that basic Fibroblast Growth Factor (bFGF) could hold sole responsibility for the induction of angiogenesis is unlikely. bFGF may be released by proteolysis of the ECM (Miao *et al.* 1996), rather than displacement from its binding site on heparan sulphate. bFGF stimulates expression of $\alpha_v\beta_3$ integrins (Brooks *et al.* 1994b). Integrins are adhesion molecules expressed on many cells, including endothelial cells, which are involved in cell to cell and cell to matrix adhesion and communication. bFGF also synergizes with Transforming Growth Factor-β (TGF-β) and VEGF in the induction of angiogenesis (Price *et al.* 1997).

Both bFGF and VEGF have been found to be expressed by a wide range of tumours: the factors have been detected in the serum, plasma and urine of patients with malignant disease (Folkman 1995; Banks *et al.* 1998). Overexpression of VEGF and bFGF has been demonstrated in mesothelioma cell lines. Four mesothelioma lines each produced significantly greater concentrations of these angiogenic factors than either normal mesothelial cells or fibroblasts (Strizzi *et al.* 2001). Serum concentrations of VEGF in MM were twice those of patients with breast and colorectal carcinoma (Vermeulen *et al.* 1996). Similarly, pleural fluid VEGF concentrations in MM were twice those of non-malignant controls (Thickett *et al.* 1999). Immunohistochemical evaluation of 52 cases of MM for

bFGF and VEGF expression revealed a correlation between the intensity of bFGF staining

and short survival (Kumar-Singh *et al.* 1999). Tumour cell VEGF expression, but not

pleural fluid or serum concentrations, also correlated with MVD in MM (Konig *et al.* 1999;

Ohta *et al.* 1999a; Strizzi *et al.* 2001).

Transforming Growth Factor-β is one of a family of growth factors, which elicit

different responses from different cell types. TGF-β usually inhibits growth of

haematopoietic cells and enhances mesenchymal growth. In the progression from

transformed to malignant cells, TGF-β may switch from mediating inhibition of growth to

stimulation (Price *et al.* 1997). TGF-β is one of several factors secreted by MM cells *in*

vivo and by normal mesothelial cells or macrophages *in vitro* after exposure to asbestos

fibres (Bielefeldt-Ohmann *et al.* 1996; Kumar-Singh *et al.* 1999).

IGF-I, like TGF-β, is a growth factor which has well-established roles in cell

transformation and tumour growth (Baserga *et al.* 1997). IGF-I is an established

angiogenic factor which stimulates migration and tube-forming activity of endothelial cells

(Nakao-Hayashi *et al.* 1992). IGF-I is considered a major growth factor in the pathogenesis

of MM and the IGF-I Receptor (IGF-IR) has attracted interest as a target of immune

therapy (Pass *et al.* 1996).

Hepatocyte Growth Factor/Scatter Factor (HGF/SF) is a cytokine which stimulates

proliferation and motility of epithelial cells and tumour cells (Rosen and Goldberg 1997).

Its secretion is upregulated by bFGF (Roletto *et al.* 1996) and cleavage by uPA is required

for its activation (Naldini *et al.* 1992). HGF/SF may stimulate all three aspects of

metastasis: growth, invasion and angiogenesis. Its angiogenic action is mediated via a tyrosine kinase receptor, *met*, resulting in endothelial cell proliferation, secretion of proteases and invasion. HGF/SF stimulates production of matrix metalloproteinases (MMPs) in colorectal cancer cell lines (Aparicio *et al.* 1998) and induces synthesis of MMP-1 and MMP-3, but not MMP-9, in keratinocytes (Dunsmore *et al.* 1996). HGF/SF may also be implicated in the activation of the angiogenic integrin $\alpha_v\beta_3$ (Trusolino *et al.* 1998). HGF/SF is over secreted in a range of tumours (Rosen and Goldberg 1997). Mesothelioma samples stain strongly for HGF/SF and *met* with immunohistochemical techniques (Klominek *et al.* 1998; Harvey *et al.* 1996). HGF/SF has been found in the pleural effusions of patients with MM (Eagles *et al.* 1996; Harvey *et al.* 1996) and may be important for both invasion and angiogenesis. HGF/SF stimulates chemotactic and chemokinetic mobility in mesothelioma cell lines (Klominek *et al.* 1998) and has been associated with increased microvessel density in MM tumours (Tolnay *et al.* 1998).

Both Interleukins(IL)-6 and IL-8 and Colony-Stimulating Factors (CSFs) have been identified as paracrine angiogenic growth factors in malignant mesothelioma, as well as in other tumours. IL-6 is produced by MM cell lines (Schmitter *et al.* 1992). Concentrations of IL-6 are markedly elevated in the pleural effusions of mesothelioma patients (Nakano *et al.* 1998). IL-6 may induce angiogenesis indirectly by the upregulation of VEGF expression (Cohen *et al.* 1996). IL-8 levels are raised significantly in pleural effusions due to MM compared to other malignant pleural effusions (9.4 +/-0.9 ng/ml versus 0.7+/-0.3 ng/ml) (Antony *et al.* 1996). IL-8 has also been demonstrated in the supernatant of MM cell lines and localised to MM cells of pleural biopsies (Antony *et al.* 1996). IL-8 has been shown to induce activation of MMP-2 (Luca *et al.* 1997) and its expression may be

upregulated by TNF-α (Yoshida *et al.* 1997). Granulocyte-macrophage CSF and granulocyte CSF are related to angiogenesis in the progression of solid tumours (Bussolino *et al.* 1993).

The Type 1 Growth Factor Receptor (GFR) family includes Epidermal Growth Factor Receptor (EGFR, HER1, ErbB-1), ErbB-2 (HER2, neu), ErbB-3 (HER3) and ErbB-4 (HER4) (Walker 1998). These are cell membrane situated receptors, which have an extracellular ligand binding domain, a transmembrane domain and an internal domain with tyrosine kinase enzyme activity. They are central to cell proliferation, differentiation, migration, adhesion and survival (Yarden 2001). Inappropriate over-expression may contribute to tumour growth and invasion (Walker 1998). EGFR immunoreactivity may reflect gene amplification, upregulated transcription, increased translation and/or reduced degradation of the protein, all of which may be present in tumours (Arteaga 2001; Slichenmyer and Fry 2001; Yarden 2001; Gill *et al.* 1987). Aberrant activation of the EGFR family, as a result of these processes, is well recognised as a predictor of poor prognosis in solid tumours (Nicholson *et al.* 2001).

Studies correlating EGFR expression in tumour samples and MVD are conflicting. EGFR and MVD were positively correlated in breast cancer (de Jong *et al.* 1998) although there are studies in NSCLC, for example, where no correlation (Giatromanolaki *et al.* 1996) or an inverse correlation (Cox 2000) was found. However, there are several facets of *in vitro* and *in vivo* data eluding to mechanistic links with angiogenesis. Inhibition of EGFR with specific tyrosine kinase inhibitors (TKIs) or anti-EGFR antibodies results in reduction of angiogenic growth factor production, inhibition of angiogenesis and antiangiogenic effects on endothelial cells (Ciardiello *et al.* 2001; Bruns *et al.* 2000; Hirata *et al.* 2002;

Bruns *et al.* 2000; Petit *et al.* 1997; Ciardiello and Tortora 2001). EGFR and HER-2 may

exert some of their effects via induction of cyclooxygenase(COX)-2 (Mestre *et al.* 1997;

Coffey *et al.* 1997; Vadlamudi *et al.* 1999). EGFR autophosphorylation results in

activation of the transcription factor nuclear factor-κB (NF-κB) (Faux *et al.* 2001), which

can act as a promoter for COX-2 gene expression (Kosaka *et al.* 1994).

Amplification of the EGFR gene has been demonstrated in MM (Ramael *et al.*

1993), but the relationship of angiogenesis to Type 1 GFRs has not been studied in this

disease. There have been four small studies which have investigated EGFR expression in

MM tumour samples. Dazzi *et al.* found EGFR expression in 23 of 34 (68%) cases

examined, with positivity more common in the epithelioid cell type. In contrast with

studies in many other tumours, Dazzi found that the loss of EGFR staining was associated

with a worse outcome, but that this was not independent of the effect of cell type (Dazzi *et

al.* 1990). Ramael *et al.* (Ramael *et al.* 1991) described both cytoplasmic and nuclear

immunoreactivity in mesothelial cells of all 32 MM specimens. Govindan *et al.* examined

paraffin embedded sections of 24 cases of MM (21 pleural MM) by immunohistochemistry

and detected HER-2 in none but EGFR in 14 (58%). A second study found ErbB-2 (HER-

2) expression in 28 of 29 cases (Thirkettle *et al.* 2000). EGFR expression has also been

noted in MM cell lines (Morocz *et al.* 1994).

A high platelet count is associated with a poor prognosis in solid tumours, including

MM (Ruffie *et al.* 1989). The contribution of platelets towards angiogenesis may be

significant, in that platelets are a source of angiogenic factors, such as VEGF, bFGF, PDGF

and PD-ECGF, and angiostatic factors, such as thrombospondin and platelet factor 4

(Pinedo *et al.* 1998). A positive correlation has been established between serum VEGF and

platelet count in cancer patients (Vermeulen *et al.* 1999) and these have been further

correlated to prognosis in renal cancer (O'Byrne *et al.* 1999). The role of platelet

extravasation through the leaky tumour neovessels in the control of angiogenesis requires

further investigation.

Platelet-derived growth factor (PDGF) is potent mitogen of cells of mesenchymal

origin. MM cells not only overexpress the PDGF A and B subunits but also express the

PDGF-β receptor (Langerak *et al.* 1996; Versnel *et al.* 1991). Thus PDGF forms the basis

for a potential autocrine growth loop in mesothelioma cells. Expression of the PDGF-α

receptor is down-regulated in MM cells. There is recent evidence to suggest that activation

of this receptor modulates an anti-proliferative effect in vitro in conjunction with the

PDGF-A subunit. *In vivo*, however, PDGF-A expression is associated with tumour

formation and growth (Metheny-Barlow *et al.* 2001).

1.3.4. Extracellular Matrix Proteinases

Proteolysis of the ECM and basement membrane, by proteinases such as matrix

metalloproteinases, cathepsins and enzymes of the fibrinolytic system, is a central part of

metastasis. These enzymes not only facilitate tumour cell mobility and extravasation but

also the stromal remodelling around endothelial cells as a central part of angiogenesis.

1.3.4.1. Matrix Metalloproteinases

Matrix Metalloproteinases (MMPs) are a family of zinc dependent enzymes, which

are implicated in the growth of primary and secondary tumours (Chambers and Matrisian

1997). Between them, they are capable of digesting all the components of the ECM and

basement membrane. They have a distinctive PRCGVPD sequence in the proenzyme

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domain with an unpaired cysteine residue which co-binds the zinc atom. Disruption of this sulphur-zinc bond activates the enzyme. MMP family members differ in their structure and substrate specificity and interactions with their inhibitors (Table 1.3). Most MMPs are secreted as latent proenzymes, which undergo extracellular proteolytic activation by cleavage of their N-terminal sequence. For example, MMP-2 activation occurs at the cell surface, with pro-MMP-2 interacting with tissue inhibitor of metalloproteinases-2 (TIMP-2) bound to MT1-MMP by forming a ternary complex. Free MT1-MMP, closely located to the ternary complex, then activates pro-MMP-2 on the cell surface (Corcoran *et al.* 1996; Nagase 1998). MMPs may be secreted by stromal cells near to tumour cells, rather than by tumour cells themselves, with diffusion through the tissue to the site of action (Heppner *et al.* 1996; Pyke *et al.* 1993).

MMPs are regulated in a number of different ways (Jones and Walker 1997; Cox *et al.* 1999). MMPs are inhibited directly by the TIMPs (Chambers and Matrisian 1997; Johnson *et al.* 1994), the balance between activated MMPs and TIMPs determining the net MMP activity. At low levels, TIMP-2 promotes the complex formation with MT1-MMP and pro-MMP-2 as described above. However, at high concentrations, TIMP-2 inhibits MMP-2 activation (Strongin *et al.* 1995). TIMPs inhibit the extravasation of metastatic tumour cells, reduce the growth of both the primary and secondary tumours and also reduce angiogenesis (Johnson *et al.* 1994; Gomez *et al.* 1997). With regard to upregulation of MMPs, angiogenic factors, such as acidic FGF, bFGF, EGF, VEGF, Tumour Necrosis Factor(TNF)-α, TGF-α and -β, have been shown to increase expression of MMPs. Specific growth factors increase the expression of some, but not all MMPs. For example, bFGF upregulates the expression of the gelatinases (MMP-2 and-9) and interstitial collagenase (MMP-1) but not MMP-3 (Mignatti *et al.* 1989). TNFα increases MMP-1, -3 and -9

Table 1.3: Matrix metalloproteinases and their substrate specificity

Subsets	Name	MMP no.	major substrates
CLASSIC MMP FAMILY MEMBERS			
COLLAGENASES	interstitial collagenase	MMP-1	fibrillar collagens
	neutrophil collagenase	MMP-8	fibrillar collagens
	collagenase-3	MMP-13	fibrillar collagens
	?	MMP-18	collagens I, II, III, VII denatured collagen
STROMELYSINS	stromelysin-1	MMP-3	laminin, fibronectin,
	stromelysin-2	MMP-10	proteoglycans, type IV collagen
	matrilysin (PUMP-1)	MMP-7	proteoglycans, type IV collagen, gelatins, elastin, ECM glycoproteins
GELATINASES	gelatinase A	MMP-2	type I & IV collagen, gelatin
	gelatinase B	MMP-9	type IV & V collagen, gelatin
ELASTASES	metalloelastase	MMP-12	elastin
	?	MMP-19	elastin
	enamelysin	MMP-20	amelogenin
NOVEL MMP FAMILY MEMBERS			
RXKR SECRETED TYPE	stromelysin-3	MMP-11	laminin, fibronectin, proteoglycans, alpha 1-antitrypsin
RXKR MEMBRANE TYPE	MT1-MMP	MMP-14	proMMP-2, proMMP-13, fibronectin, collagen I & III
	MT2-MMP	MMP-15	proMMP-2, laminin
	MT3-MMP	MMP-16	proMMP-2
	MT4-MMP	MMP-17	proMMP-2
	MT5-MMP	MMP-21	proMMP-2

expression, but not that of MMP-2 (Hanemaaijer *et al.* 1993; Esteve *et al.* 1998; Qin *et al.* 1998).

Overexpression of MMPs and their relation to disease progression has been described in many solid tumours, including NSCLC (Cox *et al.* 2000a; Passlick *et al.* 2000; Iizasa *et al.* 1999; Anderson *et al.* 1995), breast (Jones *et al.* 1999), gastric (Murray *et al.* 1998), pancreatic (Yamamoto *et al.* 2001), colorectal (Baker *et al.* 2000), ovarian (Wu *et al.* 2002) and head and neck cancers (O-Charoenrat *et al.* 2001). The pattern of overexpression varies greatly between tumour types, however. MMPs have not been characterised in MM tumour samples, although the expression of MMPs in MM cell lines has recently been reported (Harvey *et al.* 2000; Liu *et al.* 2001).

1.3.4.2. MMPs and angiogenesis

Angiogenesis has three principle steps, similar to those of tumour metastasis. Endothelial cells proliferate, there is local basement membrane and ECM proteolysis and then endothelial cell migration. MMPs are expressed by endothelial cells adjacent to tumour cells (Pyke *et al.* 1993) and MMP-2 can bind to endothelial cells via integrin $\alpha_v\beta_3$ (Brooks *et al.* 1996). Murine tumour allograft studies have demonstrated that stromal production of MMP-2 is important in tumour growth, invasion and angiogenesis (Kato *et al.* 1992; Itoh *et al.* 1998). MMP-2 expression correlated with VEGF and MMP-9 with PD-ECGF in breast cancer (Kurizaki *et al.* 1998). MMP-2 and −9 expression have been correlated with increased vessel counts in colorectal cancer (Kim and Kim 1999).

However, MMPs may also be involved in down-regulation of angiogenesis, demonstrated by the observations that MMP-3, -7, -9 and -12 generate angiostatin from

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plasminogen (Patteson and Sang 1997; Lijnen *et al.* 1998). Similarly, endostatin is formed

by the proteolysis of collagen XVIII (O'Reilly *et al.* 1997).

1.3.4.3. Fibrinolytic enzymes

The balance of the fibrinolytic enzymes, uPA and tissue plasminogen activator

(tPA), and their regulator, plasminogen activator inhibitor-1 (PAI-1), is important in

tumour invasion and angiogenesis. Plasmin is an enzyme implicated in the proteolysis of

the major protein components of the ECM (Price *et al.* 1997), enabling invasion of tumour

cells and angiogenesis. tPA binds strongly to fibrin and is involved primarily in

fibrinolysis. However, uPA is associated more with tissue remodelling and cellular

invasion. Migrating endothelial cells express elevated levels of uPA *in vitro*. Anti-uPA

antibodies and blockade of uPAR have been observed to reduce tumour growth and

metastasis *in vivo* (Ossowski *et al.* 1991; Rabbani 1998). uPA is secreted as a pro-enzyme

and activated by plasmin and cathepsins, amongst other enzymes. Induction of uPA

activity may be mediated by TNF-α and TGF-β, which also activate the uPA receptor in

vitro (Shetty *et al.* 1995). uPA is also implicated in the activation of metalloproteinases

such as MMP-2 and MMP-9 (Mignatti and Rifkin 1996; Mazzieri *et al.* 1997). uPA is

produced by a number of tumours and its expression can be linked to tumour size, lymph

node metastasis and angiogenesis (Rabbani 1998; Bolon *et al.* 1997; Rifkin *et al.* 1997).

Lung cancers which do not express PAI activity are associated with a poor prognosis (Gris

et al. 1993). The role of the fibrinolytic system is being investigated in MM. MM often

produces a fibrinous pleural effusion. However, although the levels of the antigens of uPA,

tPA and PAI-1 are increased in the plasma of MM patients, the activity of the enzymes is

not significantly greater than normal (Ozdemir *et al.* 1996). Activity of uPA appears to be

higher in the pleural fluid of these patients, but the importance of this is unclear. This

"balance" of the fibrinolytic system seen with MM is absent in lung carcinoma (Gris *et al.* 1993), which may explain the relatively increased metastatic potential of the latter.

1.3.4.4. Hyaluronic Acid

Hyaluronic Acid (HA) is a glycosaminoglycan of the ECM (Price *et al.* 1997), which plays a role in angiogenesis and may be involved in the pathogenesis of MM. Degradation of HA occurs to form oligosaccharide fragments which are angiogenic: this process is inhibited by native HA (Deed *et al.* 1997). HA oligosaccharides have synergistic angiogenic effects with VEGF *in vitro* (Montesano *et al.* 1996). Mesothelioma tumour cells produce factors, such as Platelet Derived Growth Factor (PDGF), TGF-β and basic fibroblast growth factor (bFGF), which may stimulate HA synthesis (Tzanakakis *et al.* 1997). CD44 is a widely expressed cell surface glycoprotein, which acts as an adhesion molecule in cell-ECM and cell-cell interactions and is a receptor for HA. CD44 is produced by mesothelioma cells and reactive mesothelium (Attanoos *et al.* 1997), but HA receptors are not a feature of normal mesothelial cells (Asplund and Heldin 1994). The interaction between HA and CD44 may be involved in mobility of mesothelioma cells through the ECM. The expression of CD44 is a poor prognostic factor in a number of human cancers including gastric, colorectal and breast (Mulder *et al.* 1997; Streit *et al.* 1996; Sleeman *et al.* 1995), although this has not yet been established in MM. CD44 expression is also upregulated in tumor endothelial cells, compared to those of surrounding vessels, and these cells are a target for anti-CD44 cytotoxic therapy with immunotoxins (Griffoen *et al.* 1997).

1.3.5. Cyclooxygenase-2

Epidemiological evidence linking a reduction in the risk of colorectal cancer to the use of aspirin (Thun *et al.* 1991) led to investigation of the role of the cyclooxygenase enzymes in carcinogenesis. Cyclooxygenase-1 and –2 both catalyse the initial two steps of prostanoid synthesis (Figure 1.3) (Taketo 1998). COX-1 is constitutively expressed in nearly all cell types and plays a central role in homeostatic processes through the synthesis of prostaglandins and prostacyclin. COX-2 is expressed in response to inflammatory stimuli, being up-regulated by cytokines including IL-1β, IL-2, IFN-α, -β and -γ and TNF-α. The particular prostaglandin synthesised following COX-2 activity is cell-specific (Brock *et al.* 1999). Prostaglandin E_2 (PGE$_2$), which is synthesised by prostaglandin E_2 synthase, may stimulate cell growth, alter cell adhesion and inhibit apoptosis (Tsujii and DuBois 1995). Recent evidence suggests that the overexpression of COX-2 plays an important role in the pathogenesis of solid tumours, including breast (Vainio and Morgan 1998) and colorectal carcinomata (Watson 1998). COX-2 has been shown to play a central role in angiogenesis (Fosslien 2001). Expression of a number of angiogenic growth factors, including VEGF, bFGF, TGF-β and PDGF, is upregulated by COX-2 expressing colorectal cancer cells. Synthesis and release of these angiogenic factors is inhibited by aspirin and specific COX-2 inhibitors (Tsujii *et al.* 1998). COX-2 expression correlated with angiogenesis, as assessed by MVD, in NSCLC (Marrogi *et al.* 2000b) and breast (Costa *et al.* 2002), gastric (Uefuji *et al.* 2000) and colorectal cancers (Masunaga *et al.* 2000). Furthermore, hypoxia induces COX-2 transcription in human umbilical vein endothelial cells (HUVECs) (Ji *et al.* 1998). COX-2 inhibition may reduce angiogenesis and stimulate apoptosis (Seed *et al.* 1997; Tsujii and DuBois 1995) and reduce tumour invasion (Reich and Martin 1996; Tsujii *et al.* 1997).

Figure 1.3: The role of Cyclooxygenases(COX) in arachidonic acid metabolism. Both the constitutive COX-1 and inducible COX-2 catalyse the two steps of PGH_2 synthesis from arachidonic acid. The balance of prostanoids synthesised from PGH_2 is dependent on the degree of COX-2 induction, as indicated by the width of the arrows.

(Adapted from Brock *et al.* 1999 and Taketo *et al.* 1998)

Arachidonic Acid

| COX-1 | COX-2 |
| di-oxygenase activity |

Prostaglandin G_2

| COX-1 | COX-2 |
| peroxidase activity |

Prostaglandin H_2

TXA$_2$ PGD$_2$ PGI$_2$ PGF$_{2\alpha}$ PGE$_2$
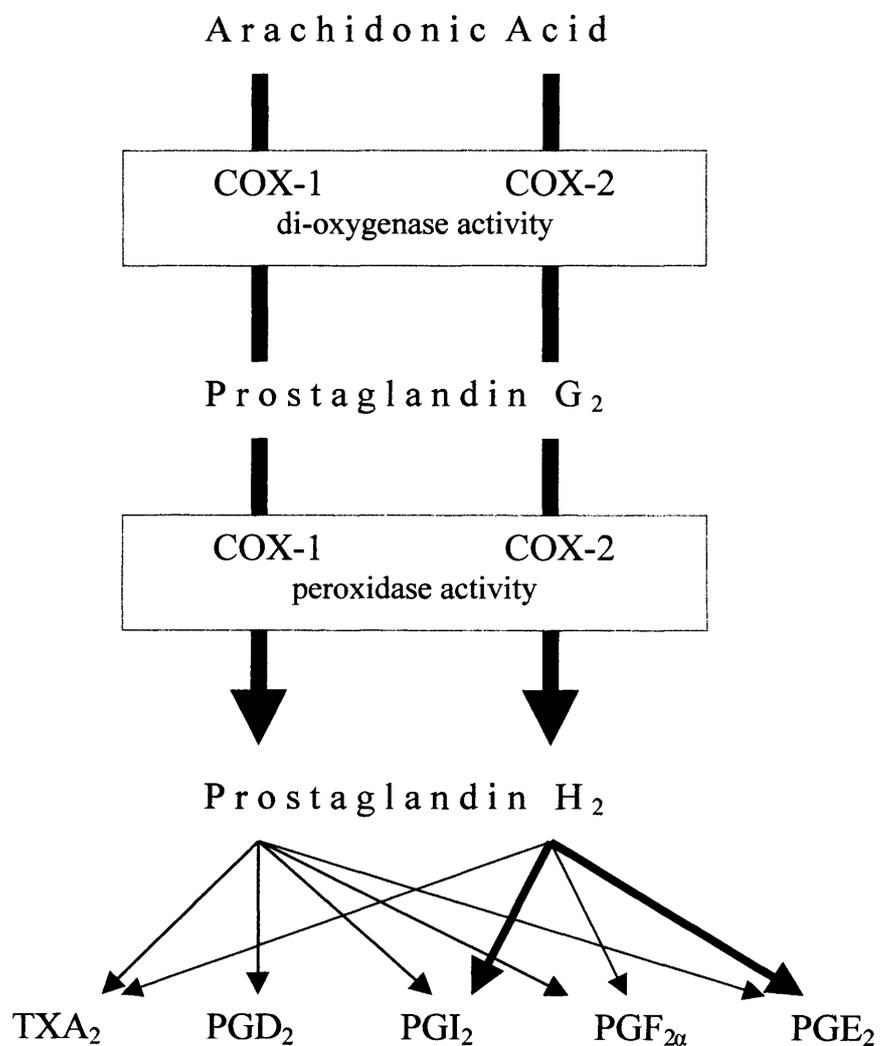
There is emerging evidence of a putative COX-2 autocrine loop, which may exist in conjunction with EGFR. EGFR has been shown to stimulate COX-2 transcription in squamous carcinoma (Mestre *et al.* 1997) and cholangiocarcinoma cells (Yoon *et al.* 2002). Activation of COX-2 induced EGFR transcription, which was abolished by the addition of indomethacin (Kinoshita *et al.* 1999). Furthermore, PGE_2 is capable of transactivation of EGFR (Pai *et al.* 2002)

COX-2 is implicated in the activation of MMP-2. In colorectal cancer, COX-2 expressing cells were associated with activation of MMP-2 whilst sulindac sulfide, a COX-2 inhibitor, blocked the increase in RNA levels of the MMP-2 activator, MT-MMP-1 (Tsujii *et al.* 1997). PGE_2 may also regulate expression of MMP-9 (Zeng *et al.* 1996).

1.3.6. Angiostatic factors

A change of the balance between angiogenic and angiostatic factors is responsible for the induction of angiogenesis. Angiostatin is a plasminogen fragment generated by elastase (MMP-12) activity (O'Reilly *et al.* 1994). Angiostatin inhibits angiogenesis in vitro and induces dormancy of primary and secondary tumours in a mouse model (O'Reilly *et al.* 1996). Angiostatin-like fragments may also be generated by other members of the MMP family, including MMP-7 and MMP-9 (Patteson and Sang 1997), MMP-3 (Lijnen *et al.* 1998) and macrophage metalloelastase (Rivas *et al.* 1998). A similar fragment of Collagen XVIII, termed endostatin, has also been identified. Like angiostatin, endostatin causes suppression of tumour induced angiogenesis (O'Reilly *et al.* 1997). A number of fragments of other proteins have been identified as inhibitors of angiogenesis, although they appear less specific than angiostatin and endostatin. They include fragments of

prolactin (16kDa PRL) (Clapp *et al.* 1993), fibronectin (29kDa FN), PF-4 (Gupta *et al.* 1995) and epidermal growth factor (EGF) (Nelson *et al.* 1995). These parent molecules are often stored in the ECM (Hanahan and Folkman 1996). The ECM may therefore be considered as a reservoir storing molecules which, following proteolysis, have potential angiogenic or angiostatic activities.

Thrombospondins (TSP) are a family of five platelet and matrix proteins found in blood clots and wounds, where they stabilise platelet aggregation. They have the ability to inhibit endothelial cell migration and angiogenesis (Wienstat-Saslow *et al.* 1994). TSP-1 blocked the usual angiogenic response to bFGF or VEGF (Good *et al.* 1990). Expression of TSP-1 is regulated by the well-characterised tumour suppressor gene wild-type p53 (wt p53) (Dameron *et al.* 1996; Volpert *et al.* 1995). Restoration of the normal wt p53 increased TSP-1, which led to blockade of endogenous angiogenic factors and angiogenesis stimulated by added bFGF (Dameron *et al.* 1996). p53 is also linked with other factors involved in the angiogenic balance. For example, mutant p53 may stimulate VEGF expression (Kieser *et al.* 1994). The p53 protein is an inducer of apoptosis. Therefore, p53 provides a link between angiogenesis and apoptosis. TSP-2 is also correlated with reduced angiogenesis and metastasis of colorectal cancer; this effect was probably due a shift in the balance between TSP-2 and VEGF (Tokunaga *et al.* 1999).

Expression of TSP-1, as assessed by the reverse transcriptase-polymerase chain reaction, has been reported in 74 of 78 (95%) cases of MM. Levels were upregulated compared to benign controls and positively correlated with VEGF expression. TSP-1 was inversely related to stage and lymph node metastasis. Increased TSP-1 was associated with

good prognosis in tumours with low VEGF expression, although overall it was not prognostic (Ohta *et al.* 1999b).

1.3.7. SV40, p53 and Apoptosis

1.3.7.1. Simian Virus 40

In recent years, there has been a much interest in the role of Simian Virus 40 (SV40) in the pathogenesis of MM. SV40 is a DNA tumour virus capable of causing mesothelioma in hamsters and of transforming human mesothelial cells *in vitro* (Carbone *et al.* 1998). The SV40 genome can be divided into early and late regions, according to the order of transcription. The early region encodes the large T-antigen (Tag), the small t-antigen (tag) and 17kT, which are responsible for the transforming activity of the virus. In particular, Tag binds to and inactivates the p53 protein (Carbone *et al.* 1997a; Procopio *et al.* 1998). Tag also binds to members of the cell growth suppressive retinoblastoma (Rb) gene family (De Luca *et al.* 1997). Therefore, inactivation of the p53 and Rb suppressor genes by SV40 may occur, predisposing the affected cells to malignant transformation (Stenton 1997; De Luca *et al.* 1997). The activity of SV40 in different cell types depends on how permissive are the cells to viral replication. Monkey cells are permissive allowing SV40 replication and cell death, whereas rodent cells are not, with no SV40 DNA replication but intergration of the viral DNA into the host cell genome. Human mesothelial cells (HMCs) are semi-permissive, but, interestingly, were infected much more readily than other human cell types, such as fibroblasts and epithelial cells (Bocchetta *et al.* 2000). However, rather than being followed by viral replication and cell lysis, SV40 infection resulted in Tag binding to the relatively high levels of p53 in HMCs, which prevented SV40 replication. The reduction of p53 function is shown by the inactivity of p21 in MM tumours: treatment of SV40 positive MM cell lines with SV40 early region antisense

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transcripts resulted in the induction of the cell cycle inhibitor p21 and growth arrest

(Waheed *et al.* 1999). The accumulation of Tag in HMCs caused transformation in culture

1000 to 100,000 times more frequently than in other SV40 infected cell types (Bocchetta *et al.* 2000).

In a murine model, SV40 Tag appeared to interact with Insulin Receptor Substrate-1

(IRS-1) to transform IGF-IR deficient mice (Fei *et al.* 1995). IRS-1 is a signalling protein,

with multiple functions, which is an immediate substrate of IGF-IR (Baserga *et al.* 1997).

MM may resist apoptosis through the action of IGF-I, alone or in synergy with SV40.

Therefore, as well as inhibiting the regulators of apoptosis, SV40 may act as a co-factor

with asbestos in upregulating the IGF-I pathway. This may result in the inhibition of

programmed cell death and induction of malignant transformation of mesothelial cells.

Evidence for the possible importance of SV40 as a cofactor in the pathogenesis of

MM is gained from studies investigating the prevalence of SV40 DNA sequences in MM

tumour samples. The initial report of 48 cases found SV40 sequences in 60% of cases

(Carbone *et al.* 1994). Several studies confirmed the presence of SV40 but some did not

(Pepper *et al.* 1996; Mulatero *et al.* 1999). However, there may be geographic differences

in the prevalence of SV40 sequences in MM. In a Finnish study of 49 cases, no SV40

sequences were found, whereas the United States-sourced samples tested in the study were

positive (Hirvonen *et al.* 1999).

Despite the theoretical contribution of SV40 to the pathogenesis of MM, however, it

has not been possible to link mesothelioma deaths with exposure to polio vaccine

contaminated with SV40 by epidemiological methods, despite follow-up of over 30 years in

two large studies (Strickler *et al.* 1998; Olin and Giesecke 1998).

1.3.7.2. p53

p53 is a tumour suppressor gene which is located on chromosome 17p13.1

(McBride *et al.* 1986). p53 mutations are the most common genetic alterations in human

malignancies (Bray *et al.* 1998). Studies investigating genetic variation in p53 in MM have

been hampered by small numbers. Two of four MM cell lines displayed point mutations

(Cote *et al.* 1991). Point mutations in the p53 gene were revealed in 2 of 17 MM cell lines

and one was p53 null (Metcalf *et al.* 1992). Analysis of the conserved exons of the p53

gene did not reveal any point mutations in 13 specimens of MM examined (Mor *et al.*

1997). A silent p53 mutation was found amongst 11 cases (Mayall *et al.* 1999). It appears

that, although p53 immunostaining is fairly common, mutations are relatively rare in MM.

As well as point mutations and binding to SV40 Tag, p53 may be inactivated by the

formation of complexes with the oncogene product *mdm2*. There are inconsistent reports of

mdm2 overexpression in MM. Segers *et al.* (Segers *et al.* 1995) found that, in 60% of

mesothelioma tumours expressing non-mutated p53, overexpression of *mdm2* was present.

However, a second study did not find *mdm2* gene amplification or protein overexpression

in a study of 18 MM cell lines no association was seen (Ungar *et al.* 1996).

1.3.7.3. Apoptosis

Apoptosis is a genetically encoded, physiological program of cell death. The

imbalance of induction and inhibition of apoptosis plays a central role in tumour growth,

with apoptosis reduced in malignant cells. An inverse relationship between intratumoural

microvascular density and apoptosis index has been demonstrated in a number of tumours

including breast and gastric carcinomata (Wu 1996; Lu and Tanigawa 1997). Inhibition of

angiogenesis limits tumour growth by increasing apoptosis of malignant cells (Wu 1996).

The role of p53, the master controller of apoptosis, has been described above. The

oncogene *bcl-2* is an anti-apoptotic protein. However, expression of *bcl-2* has been related

to an improved prognosis in solid tumours including non-small cell lung carcinoma

(Fontanini *et al.* 1995; Koukourakis *et al.* 1997). An inverse relationship was noted

between vascular grade and *bcl-2* expression. The *bax* protein is pro-apoptotic and related

to *bcl-2*. The ratio between *bax* and *bcl-2* may be important prognostically in NSCLC

(Pezzela *et al.* 1993).

Expression of *bcl-2* is not a common feature in MM. *Bcl-2* immunoreactivity has

been demonstrated in 5 of 62 (Segers *et al.* 1994) and 7 of 35 MM cases (Soini *et al.* 1999).

A third study demonstrated absence of immunostaining for *bcl-2* in MM, in contrast to

benign solitary fibrous tumours of the pleura (Chilosi *et al.* 1997). A study of

mesothelioma cell lines found that *bcl-2* was expressed in only 3 of 14 lines (Narasimhan *et

al.* 1998), in contrast to the universal expression of *bax* (Narasimhan *et al.* 1998; Soini *et

al.* 1999). Although *bcl-2* expression is variable in other solid tumours, it has been shown

to be both a good (Inada *et al.* 1998; Kaklamanis *et al.* 1998) and a poor prognostic factor

(Keshgegian *et al.* 1998; Silvestrini *et al.* 1998). The role of the *bcl-2* family in the

regulation of apoptosis in MM requires further evaluation. The studies in MM have been

too small to evaluate a correlation between *bcl-2* and prognosis, although patients with a

high apoptotic index had a worse prognosis (Soini *et al.* 1999).

1.3.8. Immune responses

1.3.8.1. Cytokine response to asbestos inhalation

Within a few hours after inhalation, asbestos particles form aggregates at alveolar duct bifurcations (Brody *et al.* 1985). Macrophages accumulate around these aggregates and are thought to mediate both the acute and chronic reactions of inhaled asbestos particles (Pinkerton *et al.* 1984; Warheit *et al.* 1984). The precise mechanism for the induction of mesothelial cell proliferation after fibre deposition in the airways is not known but it is widely thought to involve fibre penetration through lung tissue and translocation to the pleura, probably *via* the lymphatic system (Davis and Jones 1988). Asbestos is concentrated in the parietal pleura within "black spots," in association with macrophages (Boutin *et al.* 1996). Although it seems likely that considerable time would be required to accumulate enough fibres at the pleural surface to induce cell injury and mesothelial cell proliferation, evidence indicates that these cells divide soon after one inhalation exposure to chrysotile asbestos (Coin *et al.* 1991) or after a single instillation of crocidolite or amosite asbestos (Adamson *et al.* 1993). These observations have been confirmed by Adamson *et al.* (Adamson 1997). In lavage fluid taken from rats following a one week exposure to crocidolite fibres, uptake of ^{3}HT by mesothelial cells was 10-fold above that of mesothelial cells exposed to lavage fluid from sham treated rats. Where mesothelial cell proliferation was under way, no fibres were seen by light or electron microscopy in the pleura. Because of the early timing of this response, it was postulated that the mesothelial cells were responding to a secretory product produced by macrophages exposed to long fibres.

Alveolar macrophages produce a number of secretory products including cytokines. A large body of evidence has accumulated to implicate these agents in asbestos and other fibre-induced pathogenicity. Macrophages exposed *in vitro* to crocidolite and chrysotile

asbestos release increased amounts of various cytokines including IL-1 and TNF-α. The levels of these substances are also found to be increased following inhalation of crocidolite and chrysotile by rats (Kagan and Brody 1996). TNF-α is a potent mitogen to mesothelial cells and this is a potential candidate for the early proliferation that occurs in mesothelial cells *in vivo* (Faux and Howden 1999) via alterations in cell signalling pathways and gene expression that are critical for the development of tumourigenesis (Mossman *et al.* 1997).

1.3.8.2. Cell mediated and humoural immunity in MM

T-helper (CD4+) lymphocytes (Th) have two patterns of activity. Th1 lymphocytes synthesise IL-2, IFN-γ and TNF-α, which, with the production of the cytokines IL-1α, IL-1β, IFN-α and IL-12 by other cells, characterise cell mediated immunity (CMI). Th2 lymphocytes synthesise IL-4, IL-5, IL-6, IL-10 and IL-13, which are anti-inflammatory and are associated with humoural immunity (HI) (Romagnani 1996). There is evidence that in solid tumours, including MM, the balance in Th1 and Th2 responses is altered such that there is suppression of CMI (O'Byrne *et al.* 2000). Tumour cells may fail to elicit a CMI response by the host in a number of ways. T-cell activation via the T-cell receptor complex (TCR) may be reduced and there is evidence of this in MM (Valle *et al.* 1998). MM tumour cells may underexpress co-stimulatory molecules, such as B7-1 and B7-2, and other adhesion molecules, as well as downregulating major histocompatability complex (MHC) expression (Valle *et al.* 1998). In addition, lymphocyte activated killer (LAK) cell activity is reduced in MM (Manning *et al.* 1989). There is increasing evidence of depression of systemic Th1 responses following asbestos exposure (Kagan *et al.* 1977; Miller *et al.* 1983; Morris *et al.* 1985; Manning *et al.* 1991), and natural killer (NK) activity is downregulated by asbestos fibres, which was restored with administration of IL-2 (Robinson 1989).

Suppression of CMI may therefore predate malignant transformation in the development of

MM.

There are several immunosuppressive factors produced following asbestos

administration in experimental models. *In vitro* binding and phagocytosis of asbestos by

macrophages induces the release of growth factors such IGF-I, TGF-β and PDGF

(Bielefeldt-Ohmann *et al.* 1996). Exposure *in vitro* of normal mesothelial cells to asbestos

results in an inflammatory response associated with synthesis and release of angiogenic

factors, such as IGF-I, TGF-β, IL-6, and TNF-α, which are also produced by malignant

mesothelioma (Fitzpatrick *et al.* 1995; Gerwin 1994). IGF-I and TGF-β depress CMI

(Valle *et al.* 1998) and are angiogenic. TGF-β is expressed at high levels by MM. TGF-β

may be induced by TNF-α and perhaps also through induction of COX-2. TGF-β is a

powerful immunosuppressant of anti-tumour effector cells, such as cytotoxic/helper T-

lymphocytes and macrophages, and is angiogenic.

In addition to its angiogenic effects described previously, COX-2 is associated with

immune responses through the induction of cyclic Adenosine Monophosphate (cAMP)

(Uotila 1996). PGE$_2$ downregulates the Th1 response, inhibiting the production by T-

lymphocytes of the Th1 cytokines IL-2, IFN-γ and TNF-α, whereas it upregulates cytokines

associated with the humoural immune (Th2) response, such as IL-6 (Della Bella *et al.* 1997;

Chung *et al.* 1998; Hilkens *et al.* 1996). Although the immunological roles of COX-2 have

not been investigated directly in MM, there is evidence suggestive of COX-2 activity.

Exposure of mesothelial cells to asbestos induced NF-κB, which is present in the promoter

region of the COX-2 gene (Janssen *et al.* 1997). Asbestos causes release of PGE$_2$ from

alveolar macrophages and inhibition of mediated cytotoxicity (Bissonnette *et al.* 1990;

Leikauf *et al.* 1995). Indomethacin, a COX-2 inhibitor, restored cell mediated immune

responses in an *ex vivo* MM model (Manning *et al.* 1989) and those mediated by alveolar

macrophages (Bissonnette *et al.* 1990). These findings suggest that COX-2 plays a role in

both the carcinogenesis of MM and in established tumours.

IGF-I, an established mitogenic and transforming agent which is secreted in

malignant mesothelioma, is angiogenic and enhances IL-1β induction of COX-2 expression

(Guan *et al.* 1998). Although a lymphoproliferative factor, IGF-I has been shown to

downregulate the pro-inflammatory response in an *in vivo* ischaemic renal cell model (Goes

et al. 1996). IGF-I may inhibit T-cell activation and cytotoxic T-cell activity (Valle *et al.*

1998). Administration of antisense IGF-I transfectants was associated with a curative anti-

tumour response in a murine teratocarcinoma model (Trojan *et al.* 1994). These findings

suggest that angiogenesis and impaired Th1 responses may play a role in the pathogenesis

of MM, with growth factors and cytokines such as IGF-I, TGF-β and IL-6 important in this

regard (Figure 1.4).

Further evidence of links between Th1 responses and angiogenesis are provided by

the role of IL-12, a Th1 cytokine. IL-12 has been shown to be a potent inhibitor of

angiogenesis in a murine model of breast cancer (Dias *et al.* 1998), via downregulation of

VEGF expression. There was also a fall in MMP-9 activity with an increase of TIMP-1

production. In a murine mesothelioma model with similar characteristics to human

mesothelioma, IL-12 has been proposed as an effective anti-tumour agent (Caminschi *et al.*

1998).

1.4. Novel Therapies for MM

1.4.1. Immunotherapy

The therapeutic cytokines under investigation in MM include IL-2, IL-12 and IFN's. These are CMI cytokines which aim both to upregulate cell mediated immunity and to inhibit angiogenesis. TNF-α, IFN-α-2a and IFN-γ have been found to inhibit *in vitro* growth of human mesothelioma cell lines. IFN-γ has been infused into the pleural space of patients (Boutin *et al.* 1994). A 61% response rate was noted in patients with Stage IA disease. IFN-α-2a administered subcutaneously resulted in one complete and two partial responses in 25 patients with previously untreated disease (Christmas *et al.* 1993). The disappointing results obtained with IFN-α2a are similar to those for IFN-γ, where only an 11% response rate was achieved (Monnet *et al.* 2002). However, 55% of patients were responders in one study which investigated intrapleural administration of IL-2 in a phase II setting (Astoul *et al.* 1998), although these results have not been repeatable by other groups (Goey *et al.* 1995). IL-12 has been investigated in a murine model similar in behaviour to human mesothelioma (Caminschi *et al.* 1998).

A combination of immune therapy and chemotherapy might have great efficacy in the management of MM due to theoretical synergistic effects. A National Cancer Institute (USA) phase II trial has been completed using cisplatin, IFN-γ and tamoxifen, after surgical debulking (Pass *et al.* 1995). A 19% partial response rate was observed. Cisplatin and subcutaneous IFN-α2a is also being assessed (Soulie *et al.* 1996). There are concerns regarding the apparent short duration of action once the treatment is stopped.

Figure 1.4: A model of the cytokine responses of mesothelial cells and macrophages to asbestos

Mesothelial Cell

Asbestos

CSFs

PDGF

IGF

IL-6

TGF-β

COX-2

Platelets

Growth and Transformation

Apoptosis

Angiogenesis

Cell Mediated Immunity

Macrophage

Expression

Stimulation

Inhibition
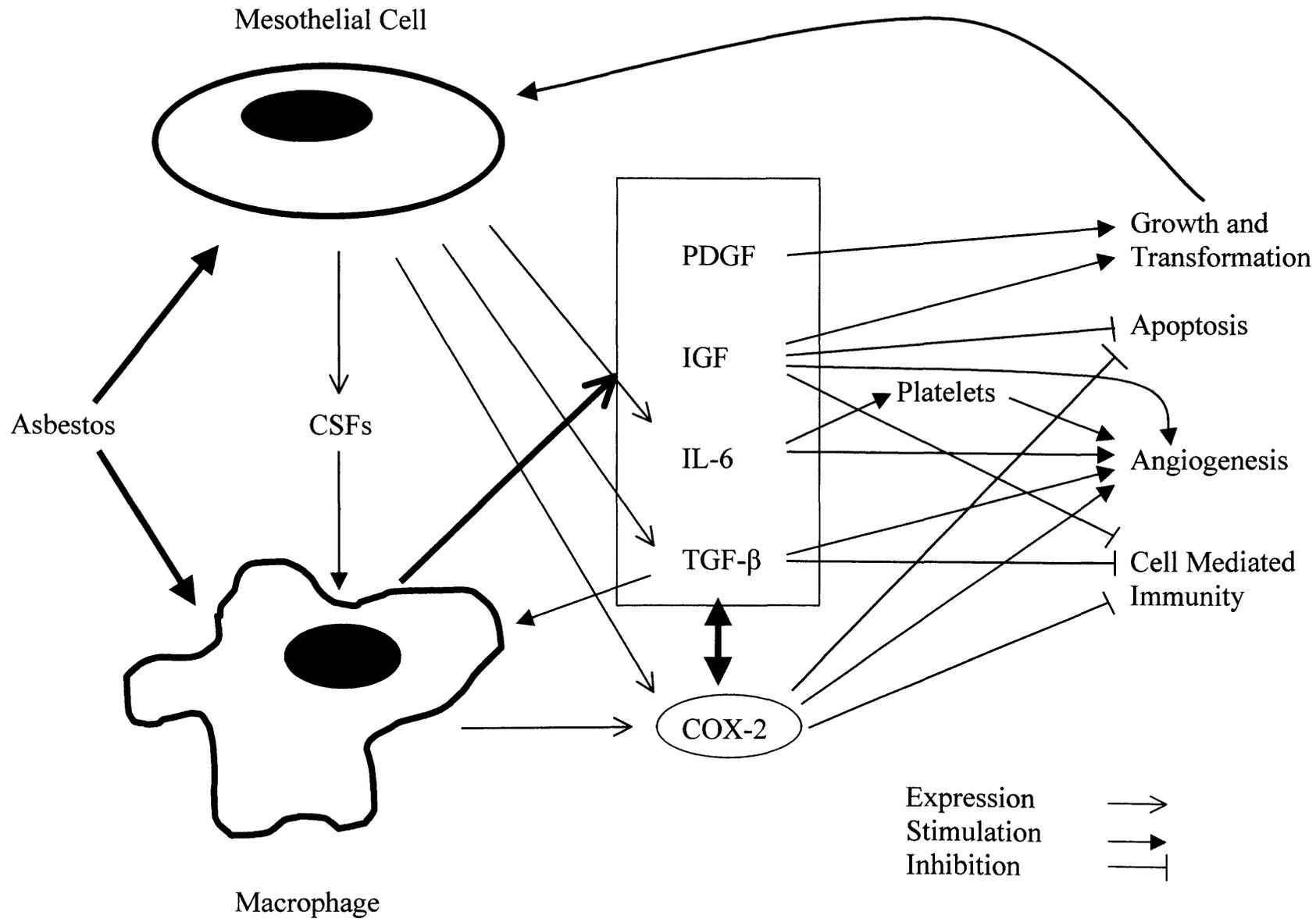
It is possible to speculate that Th1 cytokine therapy ultimately fails because of the stimulation of COX-2 activity. This would result in overexpression of prostaglandins, including PGE_2, which in turn would downregulate the Th1 response, thereby blocking the induction of Th1 responses by therapeutic Th1 cytokines. The combination of IFN-α and Non-Steroidal Anti-Inflammatory Drugs inhibiting COX-1 and COX-2 appear to have additive effects in the setting of chronic viral hepatitis (Andreone *et al.* 1993) and these require further evaluation in the setting of immunotherapy for malignant tumours such as MM.

IGF-I is an important growth factor in MM, as described above. IGF-IR antisense transcripts have been evaluated in a murine model of malignant mesothelioma (Pass *et al.* 1996), in which massive apoptosis was demonstrated, suggesting that IGF-I is a necessary factor for tumour growth. Other factors antagonising IGF-I, such as somatostatin analogues, may have a therapeutic role in MM. Similarly, antisense oligonucleotides specific for TGF-β_2 have been shown to reduce mesothelioma growth *in vivo* in a mouse model (Marzo *et al.* 1997).

Combinations of IL-2, IFN, Somatostatin analogues and COX-2 inhibitors, with or without other cytokines, may emerge as a more efficacious approach to immunotherapy in the chemoprevention and management of MM.

Immunotherapy strategies based upon non-specific immunostimulation were sidelined with the increased understanding of NK and LAK cell activities over the last 20 years. There have been, however, promising results of the administration of

Mycobacterium vaccae (SRL-172) in combination with chemotherapy in MM. A response

rate of 37.5% was noted, with corresponding improvement in NK activity and reduction in

thrombocytosis (Mendes *et al.* 2002). The approach is under further investigation.

1.4.2. Photodynamic therapy

Photodynamic Therapy (PDT) consists of administration of a protoporphyrin dye,

which is absorbed preferentially by the tumour cells. It is then activated by light of a

certain wavelength delivered by a laser. Toxic metabolites are produced which lead to cell

death. It is possible that this is due to the induction of apoptotic pathways. MM is an ideal

candidate for PDT, being a tumour directly related to the pleural cavity. A number of series

have been published (Takita *et al.* 1994; Takita and Dougherty 1995; Baas *et al.* 1997;

Schouwink *et al.* 2001). A prospective randomised controlled trial has been undertaken by

the National Cancer Institute in the United States. Debulking surgery (EPP or P/D) was

followed by immunochemotherapy (see below) and patients were randomised to receive

PDT or not (Pass *et al.* 1997). No significant increase in time to disease recurrence or

survival time following PDT was seen. However, the concept of PDT is worthy of further

research. New photosensitive compounds are being developed which are hoped to have

less systemic absorption yet penetrate the tumour more deeply (Hahn *et al.* 2001). The role

of genetic susceptibility to apoptosis following PDT is being investigated.

1.4.3. Gene therapy

Gene therapy uses the uptake of a gene into the tumour cell genome, using a vector

(such as a virus). Incorporation and subsequent expression of a "suicide gene", which

induces a toxic pathway, may lead to tumour cell death (Pass 1994). MM would appear to

be well suited for gene therapy for a number of reasons. MM is a tumour which spreads by

local invasion and the bulk of the tumour is responsible for its pathophysiological effects. The tumour remains localised with only late metastasis. The relationship to the pleural cavity allows local administration of vectors with theoretical minimisation of systemic side effects. Preliminary results of a phase I trial of Gene Therapy for MM have been reported (Albelda 1997). Transfection with an adenovirus, which carried the Herpes Simplex Virus thymidine kinase (HSV-tk) gene, was carried out by its introduction into the pleural cavity. Incorporation of viral DNA into superficial cells was noted. Thymidine kinase phosphorylates the drug ganciclovir to an extremely toxic triphosphorylated form. A Phase II trial involving patients with minimal disease continues and a trial of gene therapy as an adjuvant to surgical debulking is planned (Nowak *et al.* 2002b). Although transfection rates are good with the adenoviral vector, uptake into the full tumour thickness has not been demonstrated *in vivo*. In addition, production of anti-adenoviral antibodies by the host may reduce the efficacy of subsequent treatments. Administration of the permanently transduced cell line, PA-1-STK, which expresses the HSV-tk gene, may permit gene uptake by neighbouring cells, increasing the transduction efficiency (Schwarzenberger *et al.* 1998). Results of a phase I trial are awaited. Other genes which are being investigated are those which encode IFN-γ, IL-2, B7, antisense TGF-β (Kaiser 1997; Bignon *et al.* 1994), p53 (Giuliano *et al.* 2000; Procopio *et al.* 1998) and Heat-Shock Protein 65 (Lukacs *et al.* 1998).

1.4.4. Anti-angiogenesis therapy

Inhibition of the angiogenic process may provide a novel therapeutic approach to the management of solid tumours, including MM. Research into the mechanisms underlying angiogenesis have indicated a number of potential anti-angiogenic agents and endogenous angiostatic peptides which are currently undergoing investigation in other

tumours (Bicknell and Harris 1996; Twardowski and Gradishar 1997). These include: proteinase inhibitors (TIMPs and synthetic matrix metalloproteinase inhibitors (MMPIs) such as Batimastat (Johnson *et al.* 1994; Macaulay *et al.* 1999) and Marimastat (Steward 1999; Rasmussen and McCann 1997)) and cytokines and their modulators (IFNα-2a, IL-12). Angiostatic factors in development include thalidomide (Calabrese and Fleischer 2000), angiostatin, endostatin (Harris 1998), TNP-470 (Gervaz and Fontolliet 1998), PF-4 (Gupta *et al.* 1995)and TSP-1(Streit *et al.* 2002).

With regard to MM, a number of these approaches are being considered. A phase I trial of batimastat in malignant pleural effusions produced good symptomatic relief from dyspnoea (Macaulay *et al.* 1999). MMPIs may be most efficacious as an adjunct to debulking surgery (Steward 1999) and they require evaluation in MM in this setting. Preliminary laboratory work investigating the effects of the EGFR-TKI ZD1839 (AstraZeneca, UK) has been recently published (Janne *et al.* 2002). A phase II trial is underway under the auspices of the CALGB in 40 patients with MM (Nowak *et al.* 2002b). Three anti-angiogenesis agents are being tested in MM (Table 1.4). SU5416 is a potent selective inhibitor of the tyrosine kinase activity of the VEGF receptor flk-1, which is under investigation in a phase II study at the University of Chicago, USA (Kindler *et al.* 2001). This institution is also examining the clinical effects of the anti-VEGF monoclonal antibody Bevacizumab (Avastin, Genetech, San Francisco, USA) in a double-blind, placebo-controlled randomized phase II trial of 106 patients. There are two on-going phase II studies of thalidomide for MM. Thalidomide is thought to inhibit the induction of angiogenesis mediated by VEGF, TNF-α and bFGF (Calabrese and Fleischer 2000). STI-571 (Gleevec, Novartis, Swizerland), a highly selective PDGF TKI, is under evaluation, targeting the putative autocrine growth stimulation based on PDGF (Nowak *et al.* 2002b).

Table 1.4: Antiangiogenic therapies in clinical trials for malignant mesothelioma

(adapted from Nowak *et al.* 2002)

Agent	Mechanism	Trial centres
ZD1839	EGFR tyrosine kinase inhibitor	CALGB
SU5416	flk-1 tyrosine kinase inhibitor	University of Chicago
Bevacizumab	Anti-VEGF monoclonal antibody	University of Chicago University of Pennsylvania MD Anderson Cancer Center
Thalidomide	Inhibitor of VEGF, bFGF, TNF-α	University of Maryland Netherlands Cancer Institute
STI-571	PDGF tyrosine kinase inhibitor	University of Chicago

1.5.   Conclusions

Malignant mesothelioma is an, as yet, incurable disease of increasing incidence. Improvements in techniques of diagnosis, surgery, chemotherapy and radiotherapy have had no impact on survival. Randomised clinical trials to investigate the relative importance of these modalities in both palliation and survival are proposed, but novel approaches to the management of MM are required urgently. Through the understanding of the molecular biology of solid tumours, promising new treatment modalities may emerge. There is increasing evidence that there is a state of depressed cell mediated immunity and an angiogenic environment in MM. Asbestos induces release of cytokines and growth factors, such as IL-6, IGF-I and TGF-β, from normal mesothelial cells and/or inflammatory cells, which may be central to these processes (Figure 1.4). These observations indicate that anti-angiogenic and/or immunomodulatory agents, including IL-2, IL-12, IFNs, COX-2 inhibitors, somatostatin analogues, MMPIs and thalidomide, alone, in combination or in a multi-modality approach, offer fresh hope in the treatment of MM and require evaluation.

1.6.   Aims of the study

The aims of the study were:

1   To establish a database of MM patients in Leicester, identifying cases both retrospectively and prospectively

2   To evaluate clinical and pathological prognostic factors in this series, using data retrieved from case notes or recorded prospectively

3   To validate the CALGB and EORTC prognostic scoring systems by the derivation of prognostic risk groups and comparison of data from the Leicester and reference series

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4   To evaluate angiogenesis in MM, as assessed by the Chalkley counting method of

        anti-CD34 immunostained tumour sections, as a prognostic factor in univariate and

        multivariate models

5   To characterise the incidence and correlations of tumour necrosis in MM, identified

        in routinely processed, haematoxylin and eosin-stained tumour sections

6   To investigate EGFR expression by immunohistochemistry in MM, evaluating its

        correlations with survival

7   To determine the presence of COX-2 in MM, using immunohistochemistry and a

        semi-quantitative Western blotting technique

8   To determine the presence and activities of MMP-2 and –9 in MM by gelatin

        zymography

9   To assess the correlations between the above factors in this patient series

Chapter Two

Materials and Methods

2.1. Materials

2.1.1. General chemicals and reagents

All the general chemicals and reagents were purchased from Sigma-Aldrich Company Ltd (Poole, UK), unless otherwise stated.

2.1.2. Primary antibodies

CD34: anti-CD34 purified mouse monoclonal antibody (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK). Clone QB-END/10 (isotype IgG_1) recognises human endothelial cells (some cross reactivity with basement membrane collagen) (Martin *et al.* 1997).

CD31: anti-CD31 mouse monoclonal antibody, M 0823, $330\mu g/ml$ (Dako, Ely, UK). Clone JC/70A reacts with a formalin-resistant epitope on CD31 in endothelial cells (Parums *et al.* 1990).

EGFR (epidermal growth factor receptor): anti-EGFR purified mouse monoclonal antibody NCL-EGFR (Novocastra Laboratories Ltd). Clone EGFR.113 (isotype IgG_{2a}) recognises the extracellular region of the EGFR molecule (Fox *et al.* 1994).

COX-2 (cyclooxygenase-2): anti-COX-2 rabbit polyclonal antibody, PG27B, 9mg/ml (Oxford Molecular Research / Biogenesis, Poole, UK), recognises the C-20 terminal amino acids of the human COX-2 molecule (Muller-Decker *et al.* 1998).

COX-2: anti-COX-2 goat polyclonal antibody, SC-1745, 200µg/ml (Santa-Cruz

Biotechnology / Autogen Bioclear, Calne, UK). The epitope maps the carboxyterminus of

human COX-2 (Tsujii and DuBois 1995).

α-Tubulin: anti-α-tubulin mouse monoclonal antibody, Clone DM 1A, T-9026 (Sigma)

(Blose *et al.* 1984).

2.1.3. Secondary antibodies

biotinylated rabbit anti-mouse whole immunoglobulins: E 0354, 1:400 (Dako)

fluorescein isothiocyanate (FITC) labelled rabbit anti-goat immunoglobulins: F2016, 1:50

(Sigma)

peroxidase-conjugated goat anti-rabbit IgG's: A8275, 1:1000 (Sigma)

peroxidase-conjugated goat anti-mouse IgG's: A8924, 1:1000 (Sigma)

peroxidase-conjugated rabbit anti-mouse IgG's: P 0260, 1:1000 (Dako)

peroxidase-conjugated swine anti-rabbit IgG's: P 0217, 1:1000 (Dako)

peroxidase-conjugated donkey anti-goat IgG's: SC-2020, 1:1000 (Santa-Cruz

Biotechnology)

2.1.4. Standards

COX-2 standards: ovine COX-2 protein, NP-04, 100µg/ml (Oxford Molecular Research) or

recombinant human COX-2 protein RP-02 (Oxford Molecular Research)

COX-2 blocking peptide: blocking peptide SC-1745p for the SC-1745 anti-COX-2 antibody (Santa-Cruz Biotechnology).

Gelatinase Zymography standards: human MMP-2 and human MMP-9, CC073, 100µg/ml (Chemicon International, Harrow, UK). Gelatinolytic bands correspond with pro-MMP-9 at 92kDa, pro- and active MMP-2:

Molecular Weight markers: Rainbow™ coloured protein molecular weight markers, RPN 756 (Amersham Life Science, Little Chalfont, UK) and SeeBlue™ pre-stained standards, EP057 (Novex, Frankfurt, Germany).

2.1.5. Kits

Streptavidin-biotin peroxidase complex (ABC) kits were purchased from Dako for immunohistochemistry. The enhanced chemiluminescence (ECL) detection kit was purchased from Amersham Life Science. Bicyclo-PGE$_2$ was measured using an enzyme immunoassay kit (Cayman Chemical /Alexis Corporation, Nottingham, UK).

2.1.6. Buffers

Buffers and stock solutions were made from analytical grade chemicals and reagents and stored, where appropriate, until use.

2.2. Patients

2.2.1. Case Selection and Data Collection

At the commencement of this project in August 1998, the Department of Pathology at Glenfield General Hospital (GGH) provided a list of all pathological specimens with a diagnosis of MM since October 1987, from hence the diagnostic records had been computerised. Case notes were identified and original notes or stored microfilm copies were reviewed. Where data was missing from GGH hospital notes, arrangements were made to review case notes from referring hospitals. Northampton General Hospital, Kettering General Hospital and the Derby Royal Infirmary were visited to inspect case notes. Case notes were also obtained from Market Harborough, Loughborough and Hinckley General Hospitals and from Queen's Hospital, Burton. Relevant demographic, clinical and pathological data, as well as management and survival data were retrieved and entered into a computer database (Table 2.1). The detailed histopathological report was reviewed for each case. The majority of these cases represented patients passing through the Department of Cardiothoracic Surgery at Groby Road Hospital, and latterly GGH, Leicester. Patients were referred for surgical biopsy, management of pleural effusion or empyema, or radical surgery. Case notes of 119 cases were reviewed retrospectively. From August 1998, data was collected prospectively. A total of 200 cases were entered into the database, the date of diagnosis of the last patient to be entered being 25th July 2001.

2.2.2. Data Validation

A validation exercise was carried with the Trent Cancer Registry (see Section 3.4.1), to ensure that:

1   Demographic and survival data within the database were correct,

Table 2.1:   List of the data fields retrieved from case notes for the MM database.

Name
Sex
Date of Birth
Referring Hospital
Hospital Unit Numbers
General Practitioner - name, address, telephone number
Date of first symptoms
Age at diagnosis
Symptoms at diagnosis – pain, dyspnoea, cough, weight loss, ECOG performance status
Asbestos history
Dates first seen by GP and of referral to physician / surgeon / oncologist
Date and results of radiological examinations – CXR, CT, MRI
Clinical International Mesothelioma Interest Group TNM stage
Date of diagnostic biopsy
Date and results of closed pleural biopsy
Date and results of VATS / Open pleural biopsy
Definitive operative details
Details of pleurodesis
Operative morbidity
Postoperative hospital stay
Symptomatic improvement postop (dyspneoa, chest pain) at 6/52, 3/12, 6/12, 12/12
GGH Histopathology reference number
Histopathology report – tumour cell type, results of diagnostic immunohistochemistry
Haematological and Biochemical indices immediately prior to diagnostic biopsy -
        Hb, WBC, Platelet count,
        ALP, LDH
Postoperative chemotherapy – dates, agents, response
Postoperative radiotherapy -   dates, dose, response
Relapse – dates, symptoms, sites
Patient status
Date of death
Cause of death
Miscellaneous comments

2   All cases had been correctly entered into the Trent Cancer Registry and that

there were no data discrepancies between the two databases,

3   Data regarding whether post-mortem examinations had been performed were

also obtained.

2.3. Methods

Immunohistochemistry and light microscopy was performed initially in the Breast

Cancer Research Unit, Glenfield General Hospital and later in the adjoining Department of

Oncology laboratory.   Gelatin zymography and the homogenisation of snap-frozen tissue

samples for this were carried out in the laboratories of the Department of Obstetrics and

Gynaecology, at the Leicester Royal Infirmary.   The snap-frozen tissue sample

homogenisation for COX-2 Western blots and PGE_2 EIAs, and all the experiments in

Chapter 7 were performed in the Centre for Mechanisms of Human Toxicity, Medical

Research Council Toxicology laboratories, at the University of Leicester.

2.3.1. Paraffin-embedded blocks

2.3.1.1. Block selection

From the Department of Pathology slide store at GGH, the Haematoxylin and Eosin

(H&E) stained tumour sections were acquired for each case. All H&E slides were reviewed

with guidance from Prof. Rosemary Walker to choose the most suitable blocks for

performing immunohistochemistry.   Approximately three fields of tumour at x250

magnification were deemed acceptable.   Of the original 200 patients entered into the

database, 29 cases had to be excluded from further study, due to the lack of survival data in

1, the unavailability of tissue block in 6, the available paraffin-embedded block having

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insufficient tumour for analysis in 19 cases and 3 cases with missing survival data and an insufficient block. This left 171 cases for entry into the study, who had accurate survival data and at least one paraffin-embedded tumour block suitable for microtomy and immunohistochemistry. Details of the block selected, on which block diagnostic immunohistochemistry was performed and reasons for rejection were entered into the database.

2.3.1.2. Processing of formalin-fixed paraffin-embedded blocks

Formalin-fixed paraffin-embedded (FFPE) blocks had been made previously by the Department of Pathology, GGH, according to standard protocols. In brief, specimens were fixed in formalin/saline for 24 to 72 hours prior to cutting-up and block selection by the histopathologist. Paraffin wax embedding of the tissue blocks was carried out in a standard automated fashion at 53-56°C. Blocks selected for this study were kept in the dark at room temperature (RT) prior to sectioning.

2.3.1.3. Microtomy

4μm sections were cut with a microtome onto glass slides which had been treated previously with 2% 3-aminopropylethoxysilane (in methanol). Paraffin sections were cut and dried overnight at 37°C followed by incubation at 56°C for 6 hours to assist adhesion of the tissue to the slide. Slides were stored in the dark at 4°C in a cold room until required.

2.3.2. Snap-frozen tissue blocks

2.3.2.1. Collection

Collection of snap-frozen surgical biopsy specimens of benign and malignant pleura was commenced in March 1998. Fresh biopsies were obtained in the operating theatre and

taken to the Department of Pathology, GGH, where they were immediately cut up by an histopathologist. After sufficient blocks had been taken for diagnostic purposes, waste tissue, which would have been discarded, was assessed and suitable blocks taken. These were mounted onto slices of cork with O.C.T. mountant (TISSUE-Tek, RA Lamb, London UK) and snap frozen in liquid nitrogen. Between 3 and 5 blocks, of approximately 60mm^3, of each case were thus frozen and stored in cryotubes in a liquid nitrogen storage dewar, located in the Breast Cancer Research Unit (BCRU) at GGH. In total, 92 cases were collected (Table 2.2).

2.3.2.2. Microtomy

Snap frozen tissue blocks were mounted on a cryostat microtome chuck and cut into 7μm sections, maintaining a chamber temperature of –27°C. Sections were mounted on silane-treated slides and dried overnight at –20°C in a sealed box containing silica gel.

2.3.2.3. Acetone fixation

Sections were warmed at room temperature for 10 minutes. Acetone was pre-cooled for 30 minutes at 4°C in a screw-topped bottle. Cut sections were fixed with acetone for 10 minutes in a Hellendahl jar at 4°C, before removal and air-drying.

2.3.3. Immunohistochemistry

2.3.3.1. Dewaxing of FFPE slides

Slides were warmed to RT and then heated to 60°C for 5 minutes, to ensure firm bonding of tissue sections. Slides were passed rehydrated by passing through graded alcohols (Xylene x 2, 99% IMS x 2, 95% IMS x 2), with 2 mins in each and periodic agitation.

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Table 2.2: The inventory of snap-frozen pleural samples which were taken at the time of surgery and snap-frozen in liquid nitrogen.

	n
Epithelioid MM	29
Mixed / sarcomatoid MM	20
Total MM	*49*
Inflamed pleura (empyema)	16
Non-inflamed pleura	22
Metastatic adenocarcinoma	5
Total	*92*

2.3.3.2. Antigen Retrieval - Pressure Cooking

Formaldehyde fixation of tissues results in the formation of hydroxyl-methylene cross links which may mask antigen sites. It may be necessary to unmask these sites, most commonly using heat-mediated or enzymatic disruption.

Pressure cooking sections briefly in citrate buffer at 130°C is standard heat-mediated method of antigen retrieval. Heating provides the energy to break the hydroxyl bonds formed by the fixative and the protein antigen. Dewaxed slides were rinsed in de-ionised water and immersed into a pressure cooker containing 1500ml of boiling 10mM citric acid buffer. When the pressure indicator rose, the sections were treated for 2 minutes. The slides were removed and washed in running tap water for 10 minutes, then rinsed in de-ionised water for 5 minutes.

2.3.3.3. Antigen Retrieval – trypsin digestion

Use of enzymes, such as trypsin or pronase, prior to immunostaining breaks cross-linking bonds of the fixative with the protein to reveal antigenic sites. Over digestion however, may lead to destruction of the tissue matrix.

Sections were dewaxed and rehydrated before incubation for 20 minutes at 37°C in the trypsin pre-treatment buffer. Sections were rinsed in running and then de-ionised water for 5 minutes each.

2.3.3.4. Immunohistochemical methods

Two different techniques of immunohistochemistry were employed. On formalin-fixed, paraffin-embedded tissue sections, the indirect streptavidin-biotin complex peroxidase technique was used to amplify the primary antibodies directed against either CD34, CD31 or EGFR (Tables 2.3 and 2.4). This technique involves a biotin-labelled secondary antibody which forms a complex with the streptavidin-peroxidase complex. The chromogen 3-diaminobenzidine tetrahydrochloride (DAB) was used for visualisation with haematoxylin counterstaining. For COX-2 immunohistochemistry, immunofluorescence was used, with an FITC-conjugated secondary antibody (Table 2.5). Both techniques had been were optimised previously by other researchers in the laboratory. However, when results were not satisfactory under the suggested conditions, appropriate optimisation of the methods was performed. Negative and positive controls were used in each batch. Initially, positive controls were from other tumour types which had been stained by other investigators in the laboratory: thereafter several "internal positive control" MM tumour samples were included in each batch to ensure reproducibility of the techniques.

2.3.3.5. Light Microscopy

All light microscopy was carried out on a Leitz Wetzlar SM-Lux microscope.

2.3.3.5.1. Assessment of Angiogenesis

It is possible to assess angiogenesis indirectly in solid tumours by counting microvessels which have been identified by immunohistochemistry against endothelial-cell specific antigens. The potential choice of primary antibody for endothelial cell immunohistochemistry covers several antibodies (Table 2.6). The anti-CD34 antibody was chosen for this study as it has been shown to give more reproducible immunostaining of microvessels than either the anti-CD31 or anti-Factor VIII monoclonal antibodies. This

Table 2.3: The streptavidin-biotin complex peroxidase technique of immunohistochemistry.
This was employed with the CD34, CD31 and EGFR primary antibodies.

Equilibrate in TBS pH7.4 for 5 mins
Block endogenous peroxidase with 0.3% H_2O_2 for 10 mins at RT
Wash in TBS pH7.4 for 5 mins
Incubate in 20% normal swine serum x 100 µl for 10 mins at RT
Blot excess and wash x 2 in TBS pH 7.4
Incubate with primary antibody x 100 µl for 1 hour at RT
Wash x 2 in TBS pH 7.4
Incubate with secondary antibody x 100 µl for 30 mins at RT
Immediately, mix ABC solution and allow to equilibrate for 30 mins at RT
Wash x 2 in TBS pH 7.4
Incubate with ABC for 30 mins at RT
Wash x 2 in TBS pH 7.4
Mix DAB
Incubate with 100µl or DAB for 5 mins at RT
Rinse in TBS then wash in running tap water for 5 mins
Counterstain with Mayer's Haematoxylin for 5 secs
Wash in running tap water for 5 mins
Dehydrate through graded alcohols (30 secs) to xylene (1 min) then fresh xylene
Mount in resinous mountant with coverslips

Table 2.4: Summary of the antibodies and conditions used for immunohistochemistry on formalin-fixed, paraffin-embedded (FFPE) and snap-frozen tissue sections.

	CD34	CD31	EGFR	COX-2
Tissue	FFPE	FFPE	FFPE	Snap-frozen, acetone-fixed
Antigen retrieval	nil	trypsin	pressure cooking, 2 minutes in 10mM citrate buffer	0.1% TritonX-100
Blocking serum	rabbit	rabbit	rabbit	rabbit
Primary antibody	Novocastra QB End/10	Dako M0283, Clone JC/70A	Novocastra EGFR.113	Santa-Cruz SC-1745
Dilution	1:50	1:50	1:20	1:80
Incubation	overnight 4°C	overnight 4°C	overnight 4°C	overnight 4°C
Secondary antibody	Biotinylated rabbit anti-mouse Ig's	Biotinylated rabbit anti-mouse Ig's	Biotinylated rabbit anti-mouse Ig's	FITC-labelled anti-goat
Dilution	1:400	1:400	1:400	1:50
Buffer	TBS	TBS	TBS	PBS

Table 2.5:   Details of the immunofluorescence technique, employed for COX-2 immunohistochemistry of snap-frozen tumour samples.

Wash x 3 changes of PBS in Coplin Jars
Permeabolise cells in PBS / 0.1% Triton X-100 for 30 mins at RT
Wash x 2 changes of PBS
Block with 100μl PBS / 10% NRS for 15 mins at RT
Incubate with primary antibody in PBS / 1% BSA overnight at RT
Wash x 4 changes PBS
Incubate with Secondary antibody in PBS / 1% BSA for 1 hour at RT
Wash x 3 changes PBS
Mount with coverslips
Store at 4°C once dry
Visualise with laser scanning confocal microscopy

Table 2.6: Endothelial cell antigens which may be used for assessment of MVD

Antibody	Sensitivity	Specificity	Frozen samples	FFPE	Lymphatic staining
Anti-FVIII-RAg	Large vessels Capillaries – focal and variable PolyAb more sensitive	MoAb – high PolyAb –stromal, tumour and inflammatory cell staining Requires antigen retrieval	+	+	Proportion
Anti-CD31	Large and small vessels Equal intensity	High (occasional plasma/inflammatory cell staining) Requires antigen retrieval	+	+	No
Anti-CD34	Large and small vessels Equal intensity	High (variable basement membrane staining) Reproducibility > CD31	+	+	Proportion
UEA	Large and small vessels Equal intensity	Low (tumour cell staining)	+	+	Yes
PAL-E	Large and small vessels Equal intensity	High	+	-	No

antibody has been proposed as having the best balance of reliability, specificity and sensitivity (Martin *et al.* 1997). In an early report of angiogenesis assessment on MM, an anti-CD 34 antibody was used (Kumar-Singh *et al.* 1997).

Early methods of microvessel counting involved the identification of areas within tumour sections with relative increase in vascular density and counting of the number of microvessels within these 'hot spots' using a 1mm square eyepiece graticule (Weidner *et al.* 1991). Areas from the tumour periphery, which are usually the most vascular and display least necrosis, were used. Only hotspots within viable tumour islets, or within one high power (x250) field distance from them, were counted.

Weidner described any distinct immunostained endothelial cell or cluster as a countable microvessel. It was not necessary for the microvessel to display a lumen containing red blood cells and there was no cut-off for the size of the microvessel. Microvessels that appeared to be transected by the plane of the tissue section more than once were counted as separate microvessels. This inter-observer error using this technique was assessed and it was found that the degree of experience of the observer was important in hot-spot selection but that vessel counting after agreement on the choice of hotspot was strong (Vermeulen *et al.* 1997).

More recently the Chalkley eyepiece graticule (Graticule, Surrey, UK) has been used to facilitate the measurement of tumour angiogenesis (Fox *et al.* 1995). This graticule, which consists of a circle which covers an area of $0.155mm^2$ at x 250 magnification and contains 25 randomly positioned dots, was originally proposed for morphimetric analysis of tissue areas (Chalkley 1943). After hot spot selection at x40 and x100 magnification, the

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graticule is orientated at x250 power so that the maximum number of dots overlie the vessels of the 'hot spot'. The dots are then counted. The Chalkley method of counting appears to have less inter-observer variation than the method of Weidner (Hansen *et al.* 1998).

In this study, the method used for microvessel assessment was that proposed by an international consensus on the methodology and criteria of evaluation for the quantification of angiogenesis in solid human tumours (Vermeulen *et al.* 1996). I was taught 'hot spot' identification, use of the Chalkley graticule and microvessel counting by Dr Giles Cox, who had in turn received training from Dr Russell Leek at the ICRF Molecular Oncology Laboratory, Institute of Molecular Medicine, John Radcliffe Hospital in Oxford. The most intense hot spots of immunostaining on the section were examined first and counted with the Chalkley graticule: further hot spots were counted until no change occurred in the greatest three vessel counts which were obtained. The sum of the three counts was designated as the microvessel density (MVD). The observers were blinded to clinicopathological data and outcome.

2.3.3.5.2. Assessment of Necrosis

For each case, all the routinely processed, formalin fixed, paraffin embedded, haematoxylin and eosin (H&E) stained tumor sections, which had been used for the original diagnostic purposes, were reviewed. The section displaying the most prominent TN (if present) was selected for scoring and interpretation. Sections were inspected using light microscopy at between x40 and x250 magnification by two independent observers, blinded to clinicopathologic data and outcome. A consensus was determined using a dual headed microscope where the observers differed in the allocated TN score. Two consultant pathologists (Dr. Louise Jones and Dr Salli Muller) assisted in the derivation of the scoring

system, the assessment of indeterminate cases and adjudicated unresolved differences. The

degree of TN on the section was scored subjectively as follows: grade 0, no necrosis; grade

1, 1 focus of necrosis per low power field (LPF), occupying <10% per LPF; grade 2, >1 foci

each occupying <10% or 1 focus occupying 10 to 30% per LPF; grade 3, single or multiple

foci >30% per LPF.

2.3.3.5.3. Assessment of EGFR Immunohistochemistry

Sections were inspected at up to x400 magnification using light microscopy by two

independent observers, blinded to clinicopathological data and outcome. The presence of

cytoplasmic or membranous EGFR expression was recorded separately. The criterion for

positive EGFR expression was immunostaining in greater than 5% of tumour cells on the

section. The distribution of staining between different cell type elements in the biphasic

cases was recorded. Results were compared between observers and in those cases where the

assessment of EGFR expression differed, a consensus was determined using a dual headed

microscope.

2.3.3.6. Laser Scanning Confocal Microscopy

For COX-2 immunohistochemistry, sections stained with the FITC-labelled

secondary antibody were inspected with the laser scanning confocal microscope at the

CMHT.

2.3.4. Snap-Frozen Sample Homogenisation

Snap-frozen samples were homogenised and the supernatants used for zymography,

Western blotting and EIAs.

2.3.4.1. Buffers

The homogenisation buffers used contained protease inhibitors to prevent proteolysis of the sample. The buffer used for zymography samples contained phenylmethylsulphonyl fluoride (PMSF), an inhibitor of serine proteases, whereas the broad spectrum protease inhibitor Complete™ (Roche Applied Science, Lewes, UK) was added for Western blotting and EIA samples.

2.3.4.2. Homogenisation Technique

The technique of homogenisation was adapted from that in routine use by Dr John McLaren in O+G for homogenisation of foetal membranes and subsequent matrix metalloproteinase zymography. All steps except centrifugation were performed in a Class II microbiological safety cabinet. Samples were kept on dry ice until use. Tissue blocks were removed from the cork with a scalpel, OCT mountant compound removed and samples inserted into 500μl – 1ml homogenisation buffer, which had been pre-cooled to 4°C. Tissue was minced into as small pieces as possible with sharp pointed scissors. Samples were homogenised with an Ultra Turrax T25 bench mounted mechanical homogeniser for about 15 seconds. The homogenate was pipetted into a 1.5ml eppendorf tube and centrifuged in a cold room at 4°C for 3 minutes at 13 000G in a MicroCentaur centrifuge. The supernatant was pipetted into 50μl aliquots and re-frozen at –80°C.

Homogenisation performed at the CMHT was different in two respects. Firstly, a hand held Ultra Turrax mechanical homogeniser was used. Secondly, the samples were subject to ultrasound sonication before centrifuging. This step is routine practice for the

lysis of cells in the CMHT but was not available at O+G. A Sanyo Soniprep 150 ultrasonic

disintegrator was programmed to give three cycles of 10 seconds off, 10 seconds on at 5μm

amplitude and a 3mm exponential microprobe tip was inserted just below the surface of the

homogenate in the eppendorf. The samples were kept on ice throughout sonication and then

centrifuged as above.

2.3.4.3. Bradford Protein Assays

Bradford Assays were used to determine the protein content of each sample, to allow

standard protein loading into electrophoresis gels and EIA wells. Sample dilutions were

determined to give spectrophotometer readings within the linear part of the standard curve.

A fresh 1mg/ml solution of bovine serum albumin (BSA) was used to produce known

standard concentrations. The two techniques recommended by the Bradford reagent

manufacturer (BioRad, Hemel Hempstead, UK), the "standard" assay and "microassay"

have been used. The "standard" technique was that in general use in O+G and the latter was

used at the CMHT. A comparison was made between the two techniques on using the latter

for the first time at CMHT, to test the variation between the two tests (Figure 2.1).

2.3.4.3.1. Standard Bradford Assay (O+G)

Neat supernatant samples were diluted in duplicate or triplicate to 1:10 and 1:20 with

H_2O. These dilutions were ascertained from a run incorporating serial dilutions, to ensure

that spectrophotometer readings were in the flat part of the standard curve. A fresh solution

of 1% BSA was mixed. Eight standards of between 0 and 600μg/ml protein were made up

in duplicate using the 1% BSA. To 100μl of dilute sample or standard, 2ml of Bradford

reagent (BioRad), which had been diluted 1:5 and filtered, was added. The samples were

Figure 2.1:   Comparison of Bradford "Standard" assay and Microassay on 18 samples. The tumour sample homogenised supernatant protein concentrations are shown (r=0.914, p<0.0001).
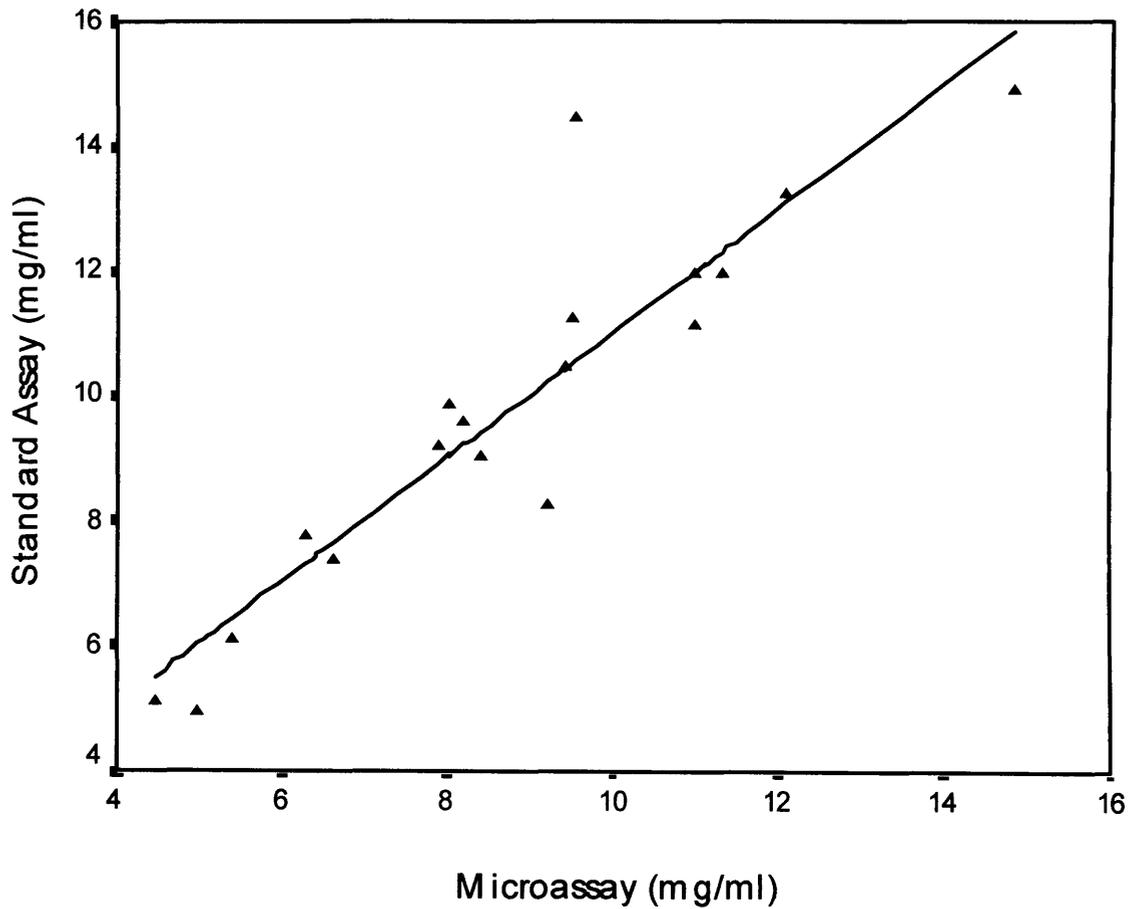
vortex mixed and transferred into 1.4ml cuvettes. The OD 595 was read on a spectrophotometer, at least 5 minutes and before 30 minutes after mixing.

2.3.4.3.2. Bradford Microassay (CMHT)

The Bradford Microassay is suitable for a smaller sample volume and higher protein concentrations. In addition, less Bradford reagent is used. Standards were made up in duplicate from 1% BSA. To 4µl of neat sample, 796µl of H_2O was added, followed by 200µl of neat Bradford reagent. The OD 595 was read as before but on a Perkin-Elmer UV/VIS Lambda 2S multi-channel spectrophotometer.

2.3.5. Western Blotting

2.3.5.1. Gel Preparation

The choice of acrylamide concentration depends on the molecular weight of the protein to be studied. A 10% acrylamide gel was used, which is appropriate for proteins in the 45-80kDa range. Hoefer mini-gel apparatus was used to cast 5 x 8cm gels and SE600 apparatus for 11 x 14cm gels, with 1.5mm thickness for both sized gels. Glass plates were first cleaned with ethanol, before assembly with 1.5mm spacers in the casting apparatus. Water-tightness was checked whilst the resolving gel was mixed. Safety glasses were worn throughout the gel-casting procedure. The resolving gel was pipetted slowly with a 1ml pipette, avoiding bubbles. After 2 minutes, the resolving gel was overlaid with 1-2ml of the bottom phase of sec-butanol to ensure an even interface between resolving and stacking gels. The resolving gel was left to set for 1 hour at room temperature, or overnight at 4°C. The stacking gel was then mixed and pipetted into the apparatus, the well comb having been partially inserted. A 10 well comb was used for 5 x 8cm gels and a 15 well comb for the 11

x 14cm gels. The comb was fully inserted and the stacking gel also allowed to set for 1 hour at room temperature, or overnight at 4°C. When gels were prepared the night before electrophoresis, damp paper towels were placed over the casting apparatus, which was wrapped in clingfilm, to prevent the gels drying out.

2.3.5.2. Sample and Standard Preparation

Samples were selected and thawed, together with sample buffer and dithiothreitol (DTT). Samples were aliquotted into screw cap 500µl Eppendorf tubes to give a standard amount of protein. 25 µg of protein was taken for 5 x 8cm gels and 150 µg for the 11 x 14cm gels. The volume of each sample was equilibrated by adding distilled water. 2x loading buffer solution was prepared from the thawed sample buffer and one volume added to each sample. A molecular weight marker (5µl Prestained SDS/PAGE Markers - Low Range, BioRad) and a COX-2 positive standard (ovine PGHS-2 NP04, Oxford Biomedical) were prepared in the same way. Samples and standards were each mixed with the sample buffer, spun to a pellet in a microfuge for a few seconds and boiled for 10 mins on a hotplate. Gels were loaded with 40 µl and 150µl per well for 5 x 8cm and 11 x 14cm gels respectively. Three samples of strong, moderate and weak COX-2 band intensity were chosen to run as internal controls on each gel.

2.3.5.3. Electrophoresis

Electrophoresis tanks were assembled with the gels in place and primed with reservoir buffer. Gels were run at 80V (voltage constant) with a BioRad PowerPak 300 until the blue marker line of the samples reached the resolving gel, then at 150V until the marker line reached the base of the gel.

2.3.5.4. Electroblotting

A BioRad Semi-Dry Electroblotter was used. Six pieces of blotting paper and one nitrocellulose membrane were cut to match the gel size. These were washed with the gel in blotting buffer for 10-20 mins. The electroblotter plates were wetted with blotting buffer, before loading (from cathode to anode) with three pieces of blotting paper, the nitrocellulose membrane, the gel, and the last three pieces of blotting paper. Any air bubbles were carefully ironed out at each layer, using a 10ml pipette as rolling pin. The electroblotter was run with a BioRad 1000/500 power supply, set at a maximum of $5.5mA/cm^2$, for 30 and 90 mins, for small and large gels respectively. For example, for 11 x 14cm gels, 500mA at 15V were used. Protein transfer was confirmed by staining with Panceau pink, followed by destaining with TBST.

2.3.5.5. Processing of Nitrocellulose Membrane

The blotted nitrocellulose membrane was blocked in blocking buffer at 4°C on a shaker overnight. The membrane was washed in TBST with 3 changes at RT on a shaking tray for 30 minutes. The primary antibody was diluted in antibody buffer, added to the membrane and incubated at RT on 3D rocking platform for 1½ hours. Use of the minimum volume of primary antibody required checking of the membrane regularly for complete coverage. The membrane was washed in TBST with 3 changes at RT on a shaking tray for 30 minutes. The secondary antibody was mixed with antibody buffer, added to the membrane and incubated at RT on a shaking tray for 1 hour. The membrane was washed in TBST at RT on a shaking tray for 30 minutes, with 5 changes to avoid unbound secondary antibody.

2.3.5.6. ECL Exposure

Equal volumes of the two ECL Solutions (Amersham Life Sciences) were measured. The solutions were mixed and poured evenly over the nitrocellulose membrane. After one minute, the excess was poured off and the membrane was wrapped in clingfilm, taking care to eliminate air bubbles, before being placed in a film cassette. The membrane was exposed to Hyperfilm ECL (Amersham Life Sciences) in a dark room, initially for 30 seconds and then as appropriate for the protein being probed. Once optimised, the same exposure times were used for each run.

2.3.5.7. Western Blot Densitometry

Developed films were scanned and band densitometry calculated on a densitometer (Kodak Digital Science Image Station 440CF camera and Kodak 1D Image Analysis Software, Kodak Scientific Imaging Systems, UK). A horizontal grid of uniform width and height cells was aligned with the COX-2 bands, as identified by the standard lane. The densitometry volume was calculated for each cell. The background activity for each band was calculated from the mean densitometry value of the perimeter of each cell. The net densitometry volume, corrected for each individual cell, was used in statistical analysis.

2.3.5.8. Nitrocellulose Stripping and Reprobing

Following ECL exposure and photography of the membrane probed with the COX-2 primary antibody, membranes were stripped and reprobed with an α-tubulin antibody, to confirm that protein loading in each well was equal. After washing the membrane in TBST, it was placed in stripping buffer in shaking water bath at 60°C for one hour. The stripping buffer and TBST were discarded in a fume cupboard sink. The membrane was blocked in

10% milk/TBST overnight at 4°C on a shaker, as before, and probed with either the α-tubulin primary antibody, or the COX-2 primary antibody which had been preadsorbed by the blocking peptide, following the procedures as described above.

2.3.6. Enzyme Immunoassay

PGE_2 concentrations of tumour sample homogenate supernatants were also derived using an enzyme immunoassay kit (Cayman Chemical). Since the half-life of PGE_2 in blood is approximately 30 seconds, determination of *in vivo* biosynthesis of PGE_2 is better achieved by the measurement of PGE_2 metabolites. The Cayman Chemical kit achieves this by the conversion of all major PGE_2 metabolites into the stable derivative, bicyclo-PGE_2, which is then measured by enzyme immunoassay. Conversion of PGE_2 metabolites to bicyclo-PGE_2 is performed by incubation with a bicarbonate buffer overnight at 4°C.

The assay is based upon the competition between the sample PGE_2 and a PGE_2-acetylcholinesterase conjugate for a limited amount of PGE_2 monoclonal antibody, bound within the wells of a 96 well plate. After washing to remove unbound reagents, Ellman's Reagent (which contains the substrate to acetylcholinesterase) is added to the wells, resulting in the formation of a yellow product, which is evaluated with a spectrophotometer. The amount of bicyclo-PGE_2 present is inversely proportional to the optical density at 412nm of the well.

2.3.7. Gelatin Zymography

Gelatin zymography and semi-quantitative computer-assisted image analysis was performed in O+G according to techniques developed by Dr John McLaren. The buffers were used were purchased from Novex (Frankfurt, Germany) in concentrated form. Gelatin

pre-impregnated, 8cm x 8cm, 12 well, 1.0mm thick, 10% Tris-Glycine gels (Novex, San

Diego, USA) were loaded with samples (10μg protein, pre-mixed with Novex Tris-Glycine

SDS Sample Buffer) in a mini XCell II apparatus (Novex) containing Tris-Glycine SDS

running buffer (Novex). Proteins were separated by electrophoresis at 125mV for 100

minutes, at which time the bromophenol blue stained front reached the bottom of the gel.

Positive controls included the Type I Collagenases AG770 (Chemicon) and C-0130 (Sigma);

the molecular weight marker SeeBlue Pre-Stained Standards (Novex) was also used. Gels

were renatured in Zymogram Renaturing Buffer (Novex) for 45 minutes and incubated

overnight at 37°C in Zymogram Developing Buffer (Novex). After staining with 0.5%

Coomassie Blue G-250, destaining with a 30% methanol, 10% acetic acid solution revealed

clear bands of gelatinolytic activity. Latent gelatinase isoforms are activated by SDS, which

enables assessment of both latent (pro) and active bands of enzymatic activity. Within each

assay, all samples were assessed simultaneously using the same reagents and batch of gels.

2.3.7.1. Gelatin Zymogram Band Densitometry

Gelatin zymograms were transilluminated on an ultra-violet light box (Ultra-Violet

Products, Cambridge, UK) and photographed with a computerised digital camera system

(UV Products VisionWorks 3.1). Band densitometry was assessed using a computer image

analysis system (Scion Image). Densitometry of each band was assessed blind to the

diagnostic group three times and the mean score used for statistical analysis. In order to

validate the zymography and densitometry techniques, assays were repeated for all samples

and three internal controls run on each gel.

2.4. Statistical Methods

2.4.1. Survival Analysis

In cases where the diagnostic biopsy yielded material suitable for experimentation, survival was calculated from the date of the diagnostic biopsy and pre-diagnostic variables, such as performance status and haematological indices, were taken from immediately before this time. In some instances, the diagnostic biopsy was performed at a peripheral hospital if the patient was referred for radical surgery, for example. In these cases, the reference date taken was that of the definitive surgical procedure and clinicopathological indices recorded accordingly.

The cut-points chosen for the categorisation of continuous variables were either based on those from previously published series, or on median values. For example, the cut-point for white blood cell count was $8.3 \times 10^9/l$, which was that used to assign patients into the EORTC prognostic groups. For MVD, COX-2 protein levels and MMP gelatinolytic activity, median values were used. The presence or absence of tumour necrosis and EGFR expression formed the categories for these factors.

Cancer-specific survival curves were estimated using the Kaplan-Meier method and the log-rank test was used to assess the statistical significance of differences between groups. Univariate Cox proportional hazards regression models (Cox 1972) were used to estimate hazard ratios and 95% confidence intervals (CIs). For the Cox proportional hazards models to be valid, the hazard ratios should be constant over time - i.e. the proportionality of hazards from one group to another should not vary with time. With the assistance of Dr Keith Abrams, Senior Lecturer in Medical Statistics, Department of Epidemiology and

Public Health, the assumption of proportional hazards was assessed graphically by plotting

log[-log(survivor)] against log(time) for each of the prognostic groups. Statistically

significant variables identified by univariate analysis were analysed in Cox multivariate

models. A forward, stepwise selection procedure was used, with variables being added to

the model according to a partial likelihood ratio test, using an entry criterion of $p<0.05$.

2.4.2. Assessment of Immunohistochemistry, Necrosis Scores and Angiogenesis

The level of agreement of categorical immunohistochemistry and tumour necrosis

scores between observers was assessed by calculating Cohen's kappa statistic. Linear

regression analysis and Cohen's kappa statistic were used to examine the correlation of

microvessel counts for inter- and intra-observer variation.

2.4.3. Correlations between Clinicopathological and Prognostic Factors

The Chi-squared test was used to analyse the correlation between two categorical

variables and Student's t-test, the Mann-Whitney U or the Kruskal-Wallis test for one

continuous and one categorical variable, as appropriate. For two continuous variables, linear

regression analysis was used. Haematological indices were analysed as both continuous and

categorical variables, the cut-points for the latter were based on the CALGB and EORTC

series.

2.5. Reagents

2.5.1. General

1% BSA	Bovine Serum Albumin	10mg/ml
Tris buffered saline (TBS 20x)	NaCl	2.6mM
	Tris	50mM
phosphate buffered saline (PBS 20x)	NaCl	2.6M
	Na_2HPO_4	60mM
	NaH_2PO_4	140mM

2.5.2. Immunohistochemistry

citric acid buffer	citric acid, pH 6.0	10mM
Trypsin pretreatment buffer	Trypsin	0.1% (w/v)
	$CaCl_2$	8.2mM
	NaOH	to pH 7.8
ABC solution	streptavidin	1μl
	biotin-peroxidase	1μl
	TBS pH7.4	1000μl
DAB solution	de-ionised water	9ml
	diaminobenzidine tetrahydrochloride solution	500μl
	TBS 20x	500μl
	3% H_2O_2	100μl

Mayers Haematoxylin	aluminium potassium sulphate (AlK(SO₄)₂.12H₂O)	105mM
	citric acid (C₆H₃Cl₃O₂)	5mM
	chloral hydrate (C₂H₃Cl₃O₂)	303mM
	sodium iodate (NaIO₃)	1mM

| *Eosin solution* | aqueous water soluble eosin | 1% (w/v) |

2.5.3. Snap-frozen sample homogenisation

Zymography homogensiation buffer	Urea	2mM
	Tris – HCl	50mM
	Brij35	0.1% (v/v)
	phenylmethylsulphonyl fluoride (PMSF)	0.1mM
	NaCl	17.1mM

Western blot / EIA homogenisation buffer	*(volume)*	
	NaCl	150mM
	Tris pH8	0.1M
	Tween-20	1% (v/v)
	diethyldithiocarbamic acid	50mM
	EDTA pH8	1mM
	Complete™ 25x (1 tab in 2ml)	4% (v/v)

70

2.5.4. Western Blotting

Resolving Buffer (5 x)	Tris-HCl	0.35M
	Tris-base	1.65M

Resolving Gel (40mls)	30% acrylamide/0.8% Bis (Anachem, Luton, UK)	13.3ml
	5x resolving gel buffer	8ml
	10% sodium dodecyl sulphate (SDS)	400µl
	H_2O	17.9ml
	10% ammonium persulphate (APS)	300µl
	N,N,N',N'-tetramethylenediamine (TEMED)	20µl

Stacking Buffer (5 x)	Tris-HCl	0.24M
	Tris-base	12.4mM

Stacking Gel (20mls)	30% acrylamide/0.8% Bis	2.5ml
	5x stacking gel buffer	4.0ml
	10% SDS	0.2ml
	H_2O	13.1ml
	10 % APS	0.15ml
	TEMED	15µl

Sample Buffer	Glycerol	22.2% (v/v)
	SDS	0.15M
	Tris-base	0.55M

Loading Buffer (2 x)	Sample buffer	81% (v/v)
	Dithiothreitol	0.09M
	bromophenol blue	0.0027%
	β-mercaptoethanol	4.5% (v/v)

Reservoir Buffer (10x)	Glycine	1.92M
	Tris-base	0.24M
	SDS	34.7mM
Blotting Buffer (1 litre)	Tris-HCl	19.2mM
	Glycine	0.192M
	Methanol	20% (v/v)
Wash Buffer - TBST (10 litres)	Tris pH7.5	0.05M
	NaCl	0.15M
	Tween 20	0.1% (v/v)
Blocking Buffer (10%)	milk powder (Marvel)	5g
	TBST	50ml
Antibody Buffer (1%)	milk powder (Marvel)	0.5g
	TBST	50ml
Stripping Buffer	SDS	69mM
	Tris - HCl	48mM
		Adjust to pH 6.7
	β-mercaptoethanol	0.8% (v/v)
Panceau Pink	Panceau S	2% (w/v)
	Tricholoroacetic acid	1.84M
	Sulphosalicylic acid	1.18M

2.5.5. Gelatinase Zymography

Sample Buffer (2 x)	Tris HCl	0.125M
	SDS	0.139M
	Bromophenol blue (w/v)	0.005%
Running Buffer (10x)	Tris base	0.239M
	Glycine	1.92M
	SDS	34.7mM
Renaturing Buffer (10x)	Triton X-100	25% (v/v)
Developing Buffer (10x)	Tris base	0.1M
	Tris HCl	0.4M
	NaCl	2M
	CaCl$_2$	0.05M
	Brij 35	0.2% (w/v)

Chapter Three

Prognostic Factors

3.1.   Introduction

The purpose of deriving the stage of a tumour is to provide both prognostic information and guidance towards the correct management protocol. There have been several efforts to devise accurate staging systems for MM in recent years (Sugarbaker *et al.* 1997). The first widely used staging system was devised by Butchart in 1976 and based on a historical series of patients who underwent radical surgery for MM (Butchart *et al.* 1976), with assessment of the extent and resectability of the tumour. In 1983, Chahinian proposed a TNM based system, which was modified by the International Union Against Cancer (UICC) in 1990. The International Mesothelioma Interest Group (IMIG) proposed further revisions in 1994 (Rusch 1995). Although the IMIG system was validated by Rusch and Venkatraman in a separate series of patients, (Rusch and Venkatraman 1996), there are limitations both with regards to the accuracy of the system and also its applicability in clinical practice. The IMIG system did not stratify for survival in a series of 183 patients who underwent radical multimodality treatment presented by Sugarbaker et al (Sugarbaker *et al.* 1999). Sugarbaker has proposed the Brigham staging system which is only relevant to patients who have undergone extrapleural pneumonectomy as it includes the assessment of tumour resection margins and mediastinal lymph nodes. However, the overwhelming majority of patients in the UK do not undergo radical surgery. Furthermore, there is little firm evidence that radiological assessment by CT or magnetic resonance imaging (MRI) correlates with the pathological TNM stage, as confirmed by radical surgery protocols. We found, for example, that although contrast-enhanced MRI is a useful tool in predicting resectability by radical surgery, there was concordance between radiological and pathological stage in only 38% of patients (Edwards *et al.* 2000).

As a result of this, prognostic scoring systems based on simple clinicopathological factors have been proposed by the European Organisation for Research and Treatment of Cancer (EORTC) (Curran *et al.* 1998) and United States based Cancer and Leukaemia Group B (CALGB) (Herndon *et al.* 1998). These systems were derived from statistical analysis of large series of patients within chemotherapy trials.

Two EORTC risk groups were identified after multivariate analysis of pre-treatment prognostic variables from 204 patients entered into five consecutive trials. The factors identified for the model were: white blood cell (WBC) count >8.3 x 10^9/L, Eastern Co-operative Oncology Group (ECOG) performance status (PS) ≥ 1, sarcomatoid tumour cell type, probable or possible histological diagnosis and male gender. The high risk group was defined by the presence of three or more of these factors (Table 3.1).

The CALGB system is more complex and derives from the analysis of 337 patients. Assessment of performance status, age, weight loss, chest pain, haemoglobin (Hb) and WBC were incorporated into a regression tree with eleven terminal groups. Those with similar survival characteristics were combined to form six prognostic groups (Figure 3.1).

3.2.   Aims

1   To assess prognostic factors in the Leicester series

2   To validate the EORTC and CALGB prognostic scoring systems

3   To compare Leicester survival data to the latter two series

Table 3.1:   EORTC Prognostic Scoring System for Malignant Mesothelioma

Factor	Group	Risk score
WBC	> 8.3 x10^9/L	+0.55
Performance Status	1 or 2	+0.60
Histology	"probable" or "possible"	+0.52
	sarcomatoid	+0.67
gender	male	+0.60
Prognostic Group	Low Risk	Total =< 1.27
	High Risk	Total >1.27

Figure 3.1:   Flowchart showing the derivation of the CALGB Prognostic Groups for Malignant Mesothelioma
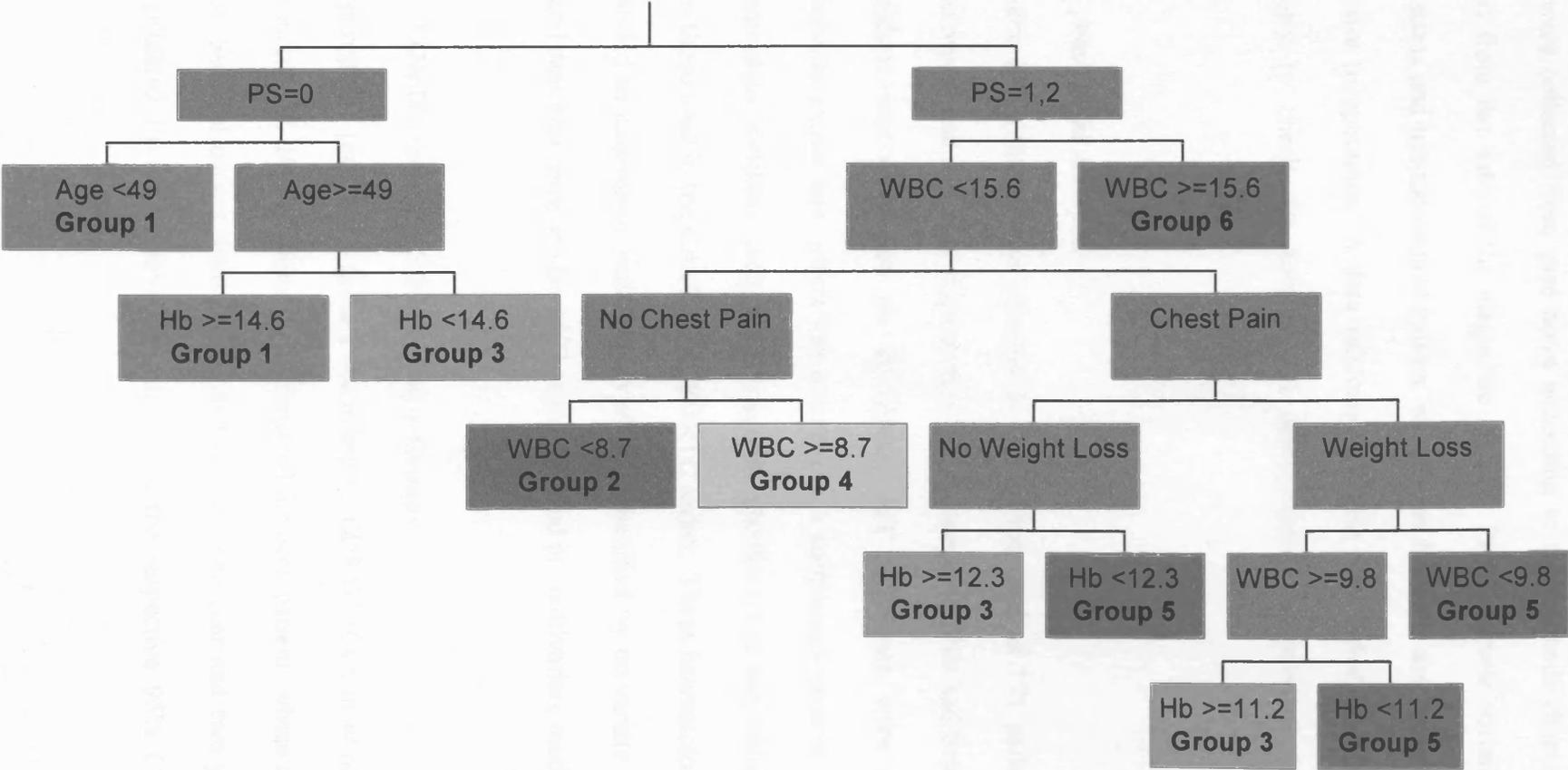
3.3. Methods

3.3.1. Data Collection

Data were collected from case notes according to the methods chapter. Survival was calculated from the date of the diagnostic biopsy. Pre-diagnostic variables, such as performance status and haematological indices, were taken from the assessment carried out immediately prior to operation. A data validation exercise was carried out with the Trent Cancer Registry to check the accuracy of demographic and survival data between databases.

3.3.2. Statistical Analysis

Complete data retrieval was possible in 152 of the total of 171 patients, due to missing or destroyed case notes, or missing data within case notes that had been inspected. Univariate analysis was performed on all cases. All parameters were analysed as categorical variables except age, which was assessed as a continuous variable. Cut-points chosen for continuous variables, such as haemoglobin, platelet count and white cell count, were based on those used in the CALGB and EORTC series. These haematological indices were also analysed as continuous variables. Variables identified by univariate analysis, for which $p < 0.1$ and data was complete (n=152), were analysed in multivariate models.

3.3.3. EORTC and CALGB Prognostic Groups

The appropriate prognostic groups according to EORTC (Curran *et al.* 1998) and CALGB (Herndon *et al.* 1998) criteria were identified for each patient, where relevant data was complete. Survival curves were plotted and median, one year and two year survival rates were calculated from life tables, together with the respective 95% CI. Survival

differences between, and hazard ratios for, each prognostic group were calculated using a

univariate Cox model, as above.

3.4. Results

3.4.1. Data Validation

The first 150 records (by date of diagnosis) were submitted to the Trent Cancer

Registry, of which 123 (82%) cases were already registered. A further 22 cases related to

non-Trent residents at the time of diagnosis and 4 cases had not been coded correctly by

GGH staff. Of 127 cases registrable in Trent, 116 were registered with a matching

diagnosis, 6 cases had been notified to the Registry with a less-specific diagnosis, 4 cases

had not been notified by hospital coders and 1 had been registered with an incorrect

diagnosis. There were a number of discrepancies between databases, with respect to date of

death (13 cases), spelling of surname (2 cases) and date of birth (1 case).

The Trent Cancer Registry checked these with the Office of National Statistics and 8 errors

in GGH data were corrected and passed on to the GGH Clinical Coding team.

3.4.2. Demographic and Diagnostic Data

There were 153 male and 18 female patients, of median age 62 (range 41 – 86)

years. The median time from onset of symptoms to obtaining a diagnostic biopsy, amongst

the 156 patients for whom a date of onset of symptoms was recorded, was 16.6 (range 0.7 –

135) weeks. The time from onset of symptoms to hospital referral by the general

practitioner (GP) in 81 patients was 5.0 (1 – 100) weeks and in 79 patients the interval

between GP referral and surgical referral was 3.7 (0.1-58) weeks. From the surgical

referral to the date of diagnostic biopsy the interval was 3.6 (0.1 – 122) weeks in 135

patients. Although closed pleural biopsy was performed in 47 patients, there was material

from surgical biopsies available in all cases (Table 3.2). In the 13 cases for which the

operative details were unobtainable, it was clear from inspection of the blocks that

thoracoscopic biopsy had been performed in 3 and open surgery in 10 cases. Since it was

not possible to determine whether these 10 underwent biopsy, debulking or palliative

surgery, they were excluded from any subsequent multivariate analyses which included

operative details. Immunohistochemistry was utilised in 54% of cases to confirm the

diagnosis (Table 3.3).

3.4.3. Survival

The reference date for calculation of survival was 10th June 2002. At this time, 155

patients had died and 16 were still alive. Overall median survival was 193 days (6.3

months). One, two and three year survival rates were 28%, 13% and 5% respectively

(Table 3.4). The longest survivor was still alive 4 years and 7 months following the

diagnostic biopsy. There was no significant change in survival between sequential cohorts

of patients over the twelve year period (data not shown). There were 17 deaths within 30

days of surgery. It was not possible to ascertain the precise mode of death for all these

patients, but it was noted during prospective data collection that there were a number of

early deaths which were not directly cancer related, but were associated with post-operative

complications. For example, one patient, who developed an oesophageal perforation

following an open decortication (which was treated successfully by an oesophageal stent),

suffered a fatal acute intrathoracic haemorrhage 11 days after surgery. Another died from

pulmonary embolism on day 25. There were at least two deaths attributable to

Table 3.2:   Type of operation performed. In 13 cases, it was not possible to ascertain the

type of operation from the case notes, although it was clear that open or thoracoscopic biopsy

or tumour resection had been performed.

Type of Operation	n		n
Not known	13		
Biopsy	53		
		Open biopsy	28
		Open biopsy, pleuroperitoneal shunt	1
		Open biopsy, rib resection	1
		Thoracoscopic or VATS biopsy	23
Debulking surgery	70		
		Open pleurectomy	13
		VATS pleurectomy	14
		Open decortication	38
		VATS decortication	3
		Chest wall resection of mass	2
Radical surgery	35		
		Radical decortication	7
		Extrapleural pneumonectomy	28

Table 3.3:   Immunohistochemical stains used to aid diagnosis. Immunohistochemistry was used in 93 cases (54%).

Antibody	First Used	n	n (positive)	n (negative)
CEA	1987	79	11 (focal)	68
Cytokeratins	1987	53	53	0
BerEP4	1990	75	12 (focal)	63
CAM 5.2	1990	42	42	0
S100	1993	10	0	1
AUA-1	1994	49	8 (weak focal)	41
Vimentin	1994	15	12	3
HBME-1	1995	44	42	2
Thrombomodulin	1997	55	34	21
Desmin	1997	2	1	1
SM Actin	1998	2	2	0
p53	1998	1	1	0
EMA	2000	2	2	0
Calretinin	2001	11	11	0

Table 3.4: Overall survival rates for the patients in the study

		Median survival (days)	One year survival (%)	Two year survival (%)	Three year survival (%)
All patients		193	28.1%	12.5%	4.6%
(n=171)	95% CI	151 – 235	16.2 – 34.9%	7.1 – 17.9%	0.6 – 8.5%
30 day survivors		229	31.2%	13.9%	5.1%
(n=154)	95% CI	182 - 276	23.9 – 38.6%	8.8 – 18.9%	0.7 – 9.4%

postoperative Acute Respiratory Distress Syndrome. Therefore, to avoid possible bias from postoperative deaths, all deaths within the 30 day period were excluded from subsequent survival analyses in this study, as is standard practice in surgical series (Table 3.4). The patients were followed up until death or for a minimum of 320 days (10.5 months).

3.4.4. Prognostic Factors – Univariate Analysis

All 154 patients who survived more than 30 days following surgical biopsy were examined for prognostic factors in univariate analysis, despite missing data fields in some cases. Univariate analysis is presented in Table 3.5 and Table 3.6. Selected Kaplan-Meier plots are shown in Figure 3.2. Poor clinical prognostic factors that were statistically significant were weight loss of >5%, presence of pleuritic chest pain and ECOG PS > 0. A history of exposure (occupational or domestic) to asbestos was given in 82% of patients but was not a poor prognostic factor. Low Hb and thrombocytosis were significantly associated with poor prognosis, either when assessed as categorical or continuous variables. High WBC was not a poor prognostic factor, although a trend towards this was seen when analysed as a continuous variable (p=0.07). Epithelioid cell type had a better prognosis than biphasic and sarcomatoid types (p<0.0001, Figure 3.3). The degree of certainty of histological diagnosis was not associated with survival (data not shown). The hazard ratios for weight loss, PS > 0 and non-epithelioid cell type, the variables for which the differences in survival were greatest, were 2.1, 1.9 and 2.5 respectively (Table 3.5).

There were differences in survival according to the type of operation performed (Table 3.7, Figure 3.4). On the Kaplan Meier plot, patients who underwent radical surgery by extrapleural pneumonectomy (EPP) or radical decortication survived longer than those

Table 3.5: Univariate Log Rank and Cox regression analysis of categorical variables

* = median survival not reached

Variable	Groups	n	Median Survival (days)	Log Rank p	Hazard Ratio	Hazard Ratio 95% Confidence Intervals		Cox p
Gender	Female	17	253	0.25	1			0.25
	Male	137	216		1.37	0.80	2.35	
Weight Loss	No	79	302	<0.0001	1			0.0001
	Yes	67	172		2.07	1.46	2.95	
Asbestos	No exposure	23	200	0.78	1			0.78
	Exposed	108	248		0.93	0.57	1.51	
Chest Pain	No	47	398	0.0009	1			0.0011
	Yes	100	185		1.91	1.30	2.82	
ECOG PS	0	76	286	0.0004	1			0.0005
	1 or 2	72	157		1.86	1.31	2.63	
WBC	=< 8.3 x10^9/l	53	286	0.1975	1			0.20
	> 8.3 x10^9/l	90	172		1.27	0.88	1.84	
Platelets	=<400 x 10^9/l	90	266	0.0124	1			0.013
	>400 x 10^9/l	53	175		1.58	1.10	2.26	
Haemoglobin	> 14 g/dl	90	358	0.0001	1			0.0002
	=< 14 g/dl	54	184		2.07	1.41	3.02	
Cell Type	Epithelioid	92	292	<0.0001	1			<0.0001
	Biphasic	34	196		1.70	1.11	2.60	
	Sarcomatoid	25	95		6.36	3.80	10.65	
	Desmoplastic	3	60		8.63	2.62	28.37	
Cell Type	Epithelioid	92	292	<0.0001	1			<0.0001
	Non-epithelioid	62	129		2.48	1.74	3.54	
IMIG Stage	Stage I	2	*	0.0284				
	Stage II	2	722					
	Stage III	22	361					
	Stage IV	22	196					
IMIG Stage	Stages I to III	26	388	0.0044	1			0.006
	Stage IV	22	196		2.56	1.31	5.02	

Table 3.6: Univariate Cox regression analysis of continuous variables

Variable	n	Hazard Ratio	Hazard Ratio 95% Confidence Intervals		Cox p
Age	154	1.01	1.00	1.03	0.15
WCC	143	1.06	0.99	1.13	0.0748
Platelets	143	1.0014	1.0003	1.0026	0.0132
Haemoglobin	144	0.87	0.80	0.94	0.0006

Table 3.7:   Associations between the type of surgery performed and outcome

Variable	Categories		Median Survival (days)	Log Rank p	Hazard Ratio	Hazard Ratio 95% Confidence Intervals		Cox p
		n						
Operation	Biopsy alone	44	172	0.08	1			0.08
	Palliative debulking	66	215		0.83	0.56	1.24	
	Radical surgery	33	382		0.56	0.34	0.93	
Radical Surgery	Biopsy / Palliative	110	200	0.0407	1			0.0428
	Radical Surgery	33	382		0.63	0.40	0.99	
Surgical Resection	Biopsy	44	172	0.1043	1			
	Palliative / Radical	99	254		0.73	0.50	1.07	0.1062

Figure 3.2 Kaplan-Meier plot showing the associations with poor survival of a) weight loss greater than 5%, b) the presence of chest pain, c) Eastern Co-operative Group Performance Status >0, d) platelet count greater than 400 x10^9/l.

a)
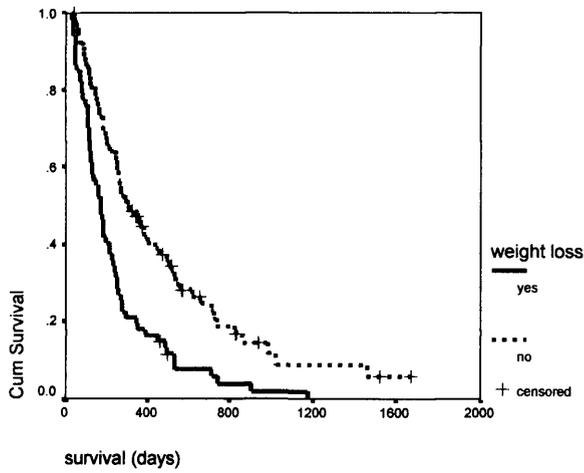
b)
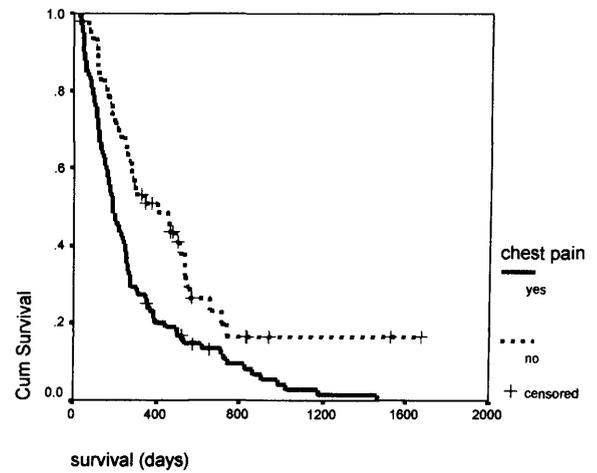
c)
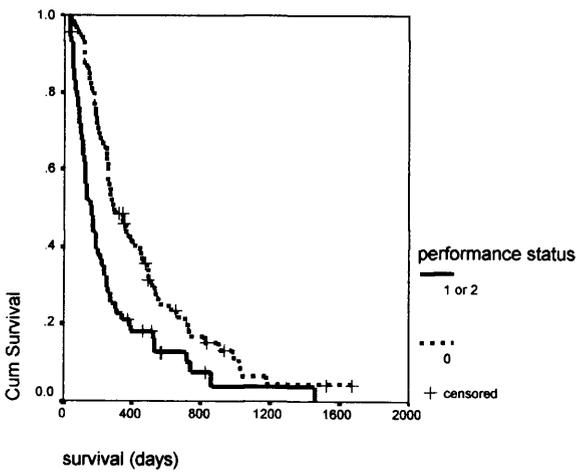
d)
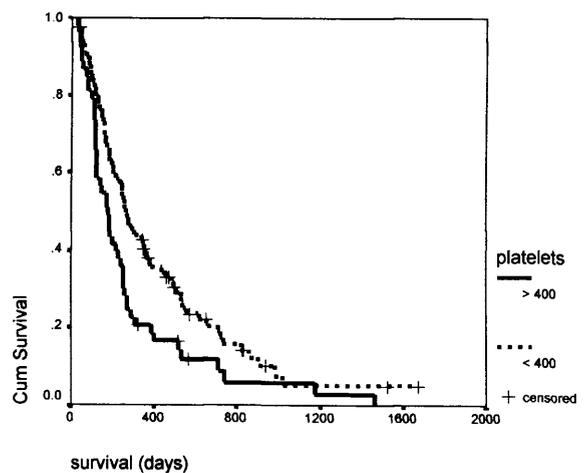
Figure 3.3: Kaplan-Meier plots showing the difference in survival according to the cell type. The 34 biphasic, 25 sarcomatoid and 3 desmoblastic cases in a) were combined into one group in b). There were 92 patients with epithelioid MM who survived longer than 30 days.

a)
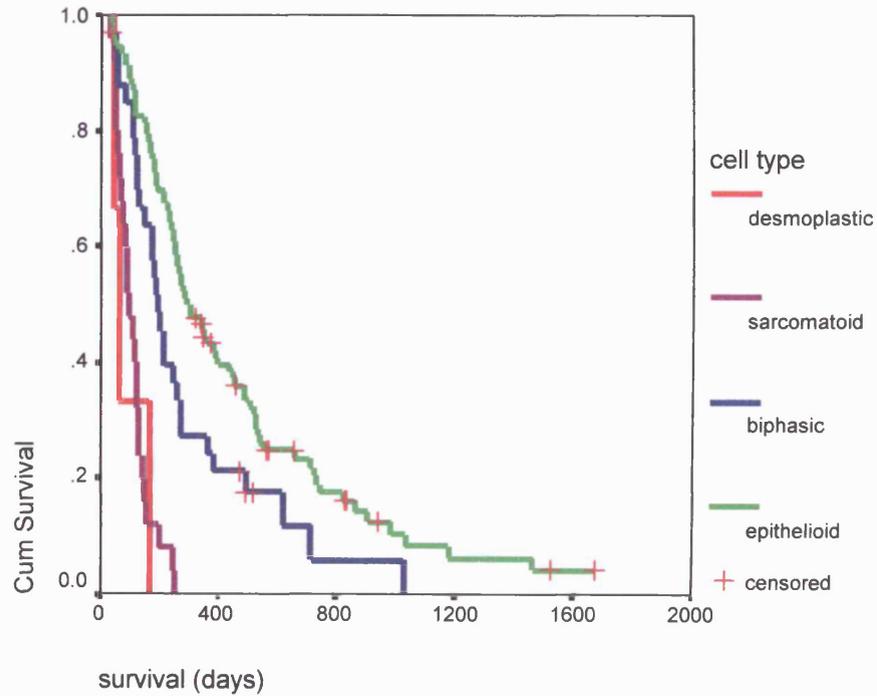
b)
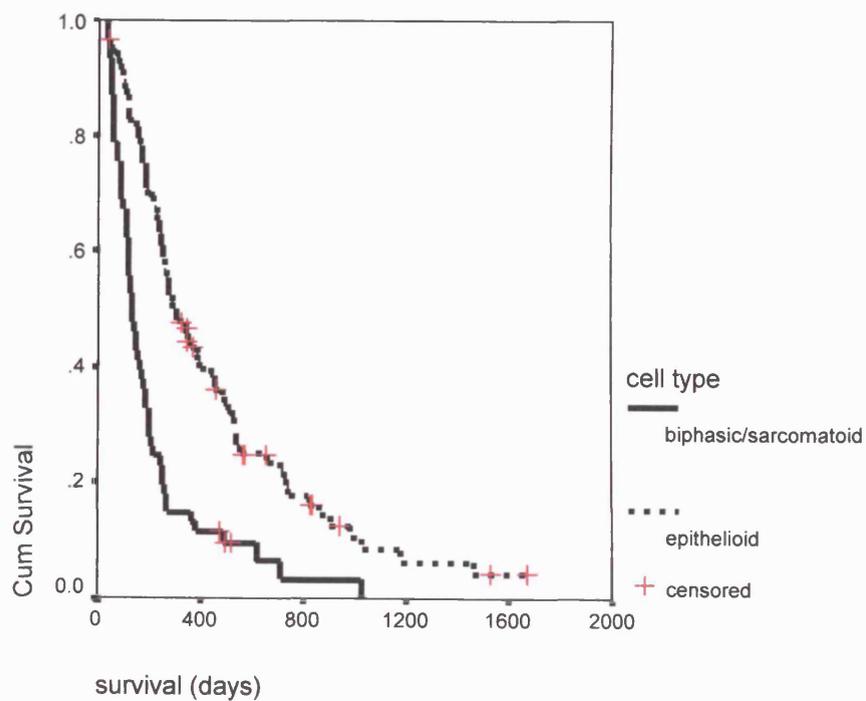
Figure 3.4: Kaplan-Meier plot showing the survival curves according to the surgical procedure performed. Comparing the three groups in a), p=0.08, whereas in b), p=0.04.

a)

b)
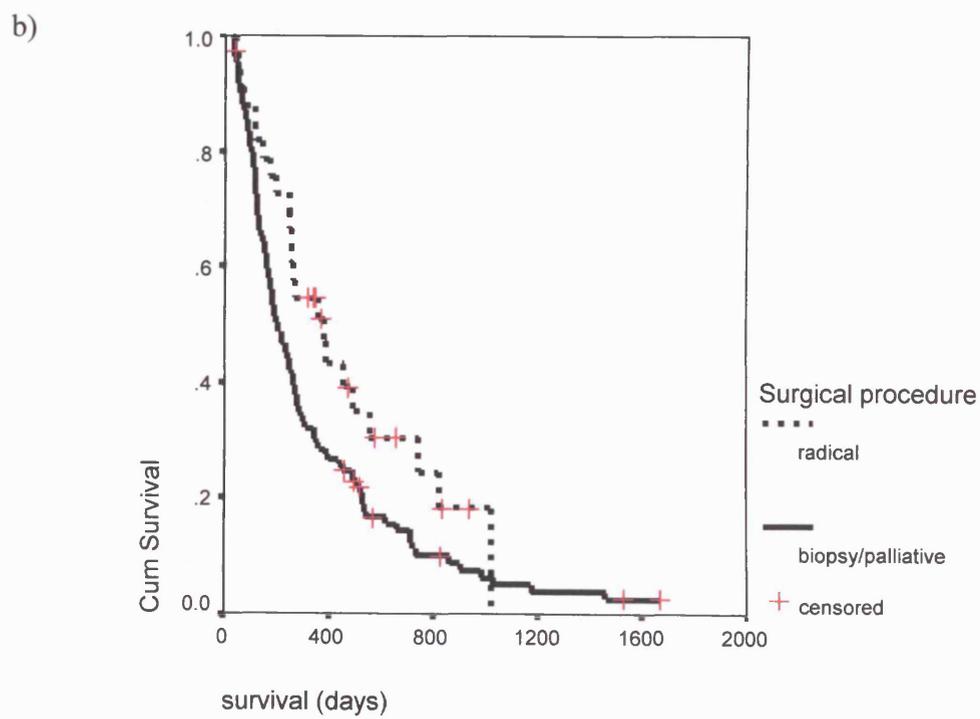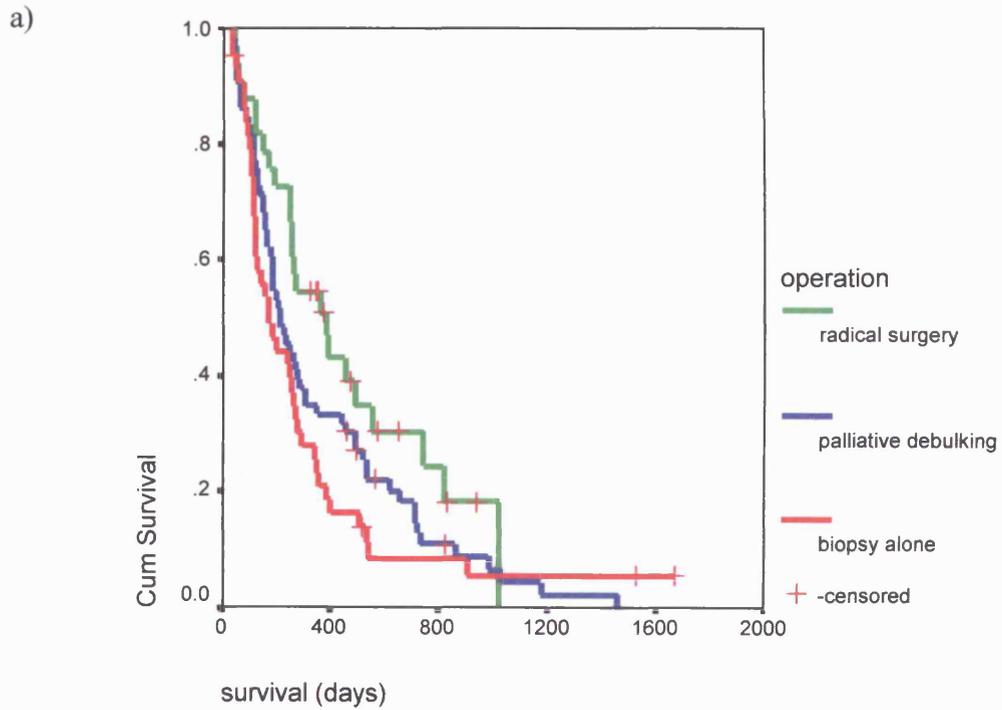
who did not (p=0.04). It was possible to derive an IMIG TNM stage in 53 patients who

underwent either radical surgery or staging surgical biopsy. Of these, 48 survived more

than 30 days. Two patients had stage I, 2 stage II, 22 stage III and 22 stage IV disease.

Survival was worse in those with stage IV mesothelioma than those with stage I to III

(Table 3.5).

3.4.5. Prognostic Factors – Multivariate Analysis

Entered into multivariate analysis were 138 cases who had complete data for the

prognostic factors identified in univariate analysis. Three variables emerged as statistically

significant independent predictors of prognosis from the multivariate Cox model (Table

3.8). In order of decreasing hazard ratio, these were histological cell type (HR 2.37),

weight loss (1.87) and performance status (1.57). Pre-operative haemoglobin and platelet

count, the presence of chest pain and not undergoing radical surgery were not retained as

independent prognostic factors in the model.

3.4.6. EORTC and CALGB Prognostic Groups

There was a significantly greater proportion of patients in the EORTC high-risk

group in the Leicester series than in the EORTC series (54% vs. 42%, p=0.025, χ^2).

However, χ^2 for trend analysis revealed that there was not a statistically significant shift

towards the high risk CALGB groups in our series (p=0.96).

Survival was correctly stratified according to the EORTC risk groups in this series

of patients (Table 3.9, Figure 3.5). The same was broadly true for the CALGB groups, but

there were only 5, 6 and 7 cases in-groups 2, 4 and 6 respectively (Table 3.9, Figure 3.6a).

Table 3.8: Cox multivariate regression analysis. Variables identified in univariate analysis were entered into a forward, stepwise logistic regression model. Cases for which data was incomplete for any of these variables were excluded – 138 cases were thus included.

Variable	p value	Hazard Ratio	Hazard Ratio 95% Confidence Interval	
Non-epithelioid cell type	<0.0001	2.37	1.62	3.46
Weight loss > 5%	0.0011	1.87	1.29	2.72
ECOG Performance Status >0	0.0169	1.57	1.08	2.27
Haemoglobin <14g/dl	0.0756			
Chest Pain	0.3328			
No radical surgery	0.3860			
Platelets > 400 x10^9/l	0.7467			

Table 3.9: Prognostic significance of EORTC and CALGB groups. Since the size of the even-numbered CALGB groups was small, they were combined with the preceding group.

* = median survival not reached

Variable	Group		Median Survival (days)	Log Rank p	Hazard Ratio	Hazard Ratio 95% Confidence Intervals		Cox p
		n						
EORTC	Low Risk	66	345	0.0002	1			0.0003
	High Risk	79	149		1.93	1.36	2.75	
CALGB	Group 1	27	540	<0.0001	1			<0.0001
	Group 2	5	*		0.68	0.16	2.93	
	Group 3	69	246		2.10	1.28	3.46	
	Group 4	6	157		1.90	0.72	5.07	
	Group 5	31	131		3.68	2.07	6.52	
	Group 6	7	75		9.54	3.94	23.10	
CALGB	Groups 1 and 2	32	540	<0.0001	1			<0.0001
	Groups 3 and 4	75	215		2.16	1.34	3.48	
	Groups 5 and 6	38	111		4.30	2.51	7.35	

Figure 3.5:   Kaplan-Meier survival plot of the EORTC prognostic groups (p=0.0002, log

rank).
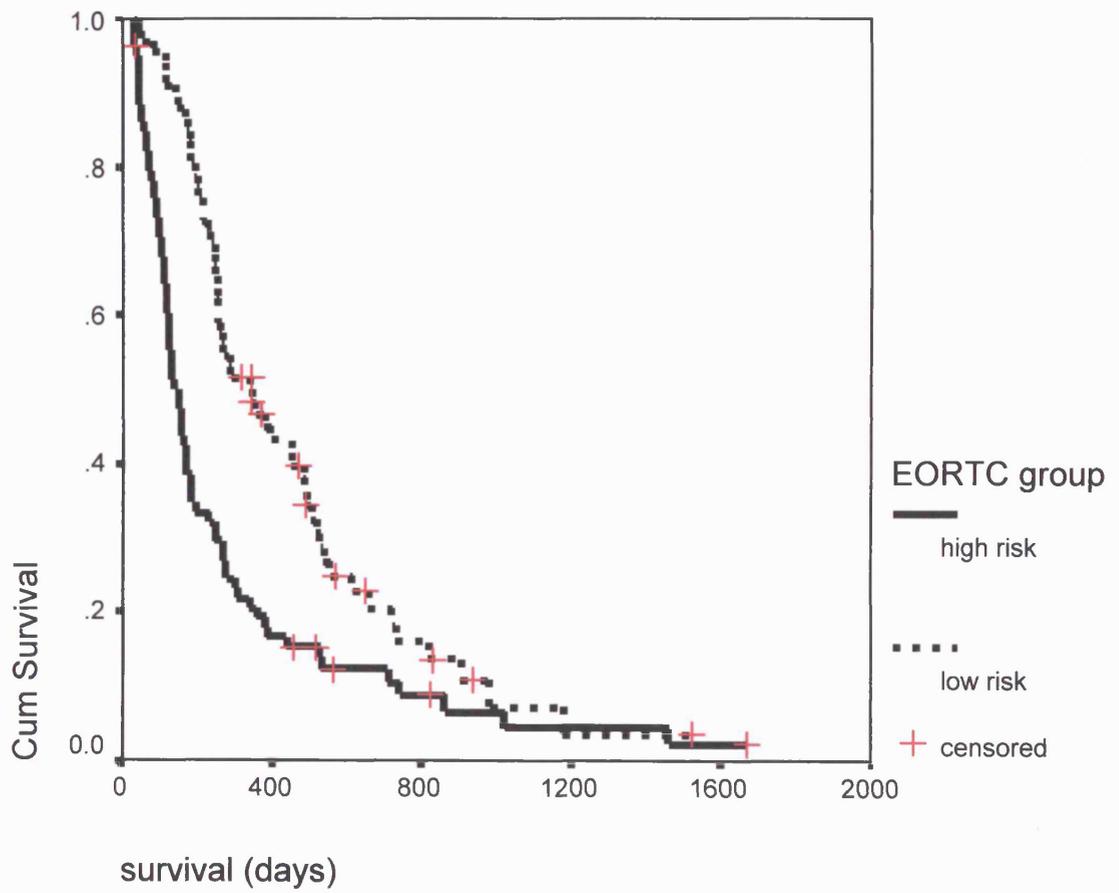
Figure 3.6: Kaplan-Meier survival plots of: a) the CALGB prognostic groups. The cases with an even-numbered CALGB group were combined (b) with the preceding odd-numbered group as there were relatively few of the former.

a)

CALGB group
6
5
4
3
2
1
+ censored

Cum Survival

survival (days)

b)

CALGB groups
5 and 6
3 and 4
1 and 2
+ censored

Cum Survival

survival (days)
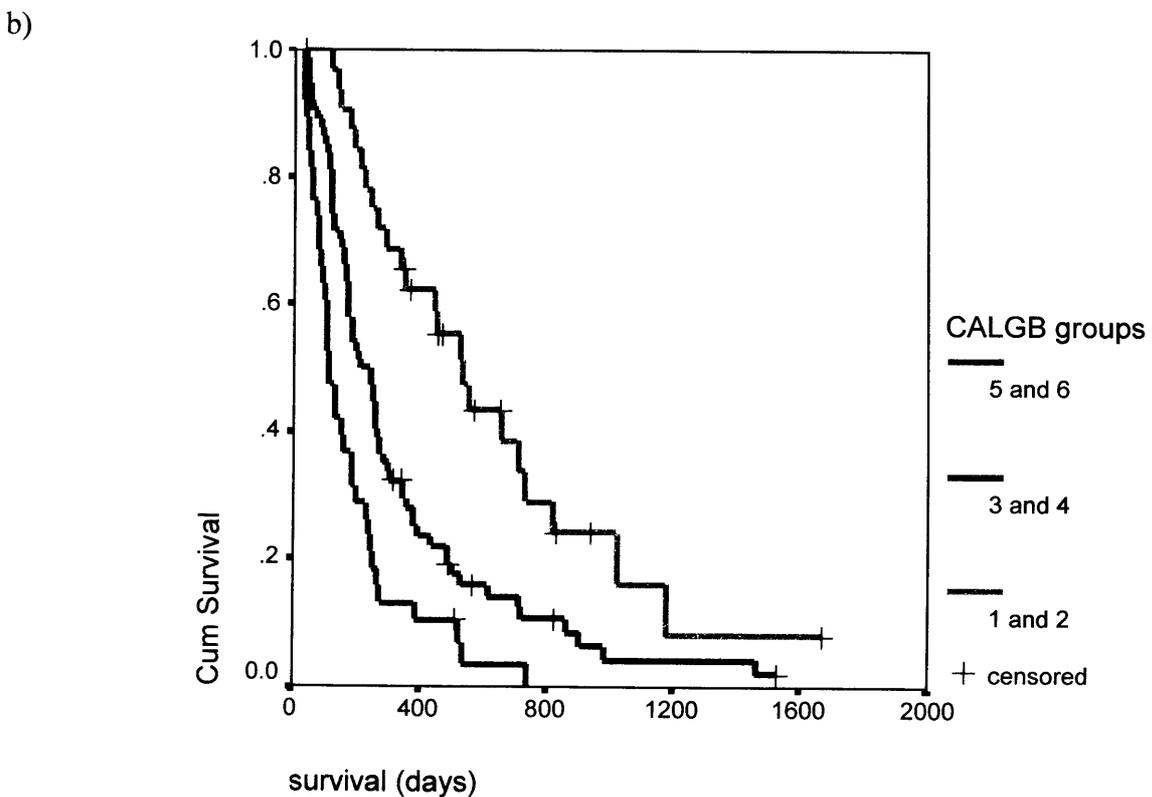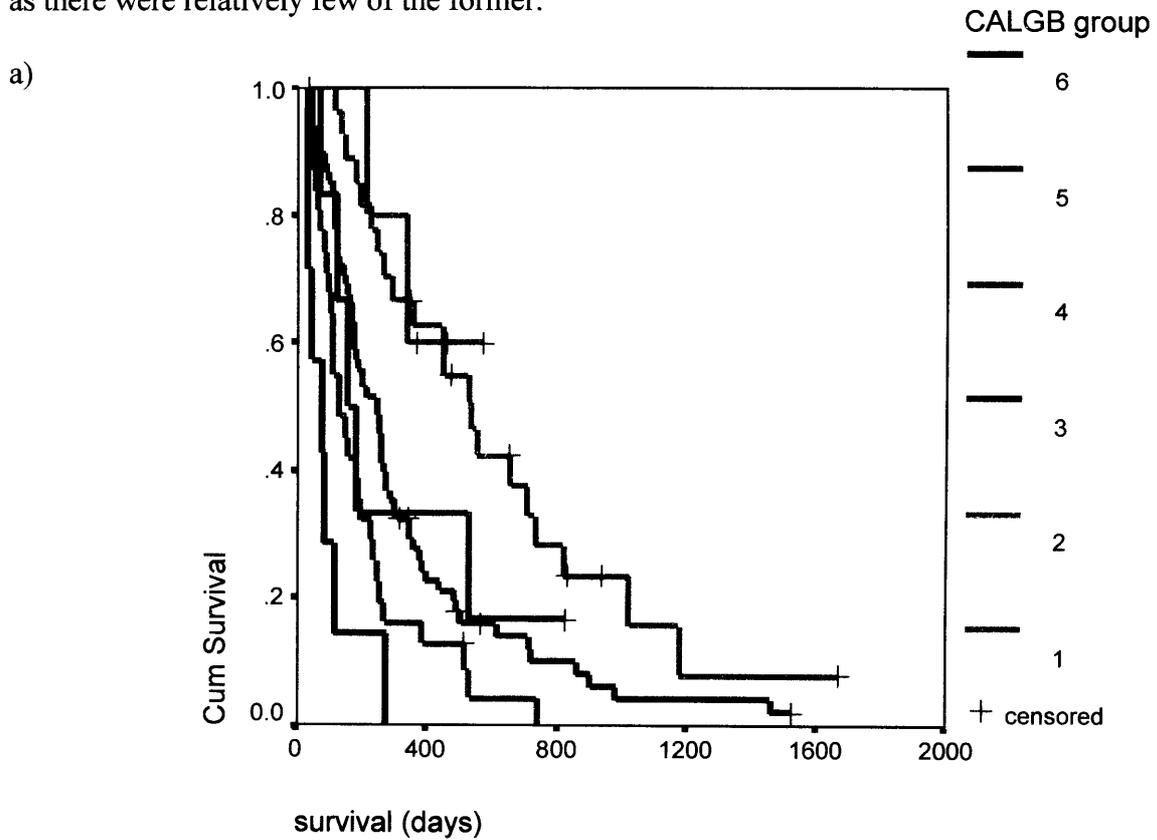
When groups 1 and 2, 3 and 4, 5 and 6 were combined, good stratification in the Kaplan-Meier plot was achieved (Figure 3.6b). Median, one and two year survival rates were generally comparable to the EORTC (Table 3.10) data, with the EORTC data well within the 95% CI of our cohorts. Survival rates were slightly, but consistently higher in the Leicester series. Similarly, median, one and two year survival of our series, when grouped according to the CALGB criteria, were comparable to the published series (Table 3.11). There were no significant differences between the series, although there were fewer long term survivors in the higher risk groups among the CALGB patients.

3.5. Discussion

The poor prognostic factors identified in univariate analysis were consistent with those of other studies. Specifically, it was confirmed that weight loss, chest pain, poor ECOG performance status, thrombocytosis, low haemoglobin and non-epithelial cell type were poor prognostic factors in univariate analysis. Similarly, stepwise multivariate analysis yielded as significant prognostic factors cell type, weight loss and performance status. These results are similar to other studies in which multivariate models have been used (Table 3.12). A history of exposure to asbestos was present in 82% of the patients, but was not a prognostic factor in this series, which included patients with a mixture of occupational and domestic exposure. Precise circumstances of exposure were not documented in all cases. Other studies have found that a history of occupational exposure was related to poor prognosis (Law et al. 1983; Ruffie et al. 1989). It is possible to speculate that this negative prognostic value is related to a higher asbestos burden which might be expected with occupational exposure. In keeping with this, a high burden of

Table 3.10: Survival of patients in the Leicester series compared to the EORTC series, when classified by prognostic group. Survival in this study was calculated from the date of diagnostic biopsy, whereas that of the EORTC series was from the date of registration into the relevant chemotherapy trial. Patients dying within 30 days of surgery have been omitted from the Leicester data.

Group	*n*	*n*	Median Survival (months)		One Year Survival %		Two Year Survival %	
	Leicester	*EORTC*	Leicester	EORTC	Leicester	EORTC	Leicester	EORTC
Low Risk	*66*	*105*	**11.3**	**10.8**	**46.6**	**40**	**18.2**	**14**
95% CI			7.2 – 15.5		34.5 – 58.7	30 - 50	7.8 – 28.6	6 - 22
High Risk	*79*	*76*	**4.9**	**5.5**	**19.2**	**12**	**10.6**	**0**
95% CI			3.7 – 6.1		10.5 – 27.9	4 - 20	3.4 – 17.8	
Total *n*	*145*	*182*						

Table 3.11:   Survival of patients in the Leicester series compared to the CALGB series, when classified by prognostic group. Survival in this study was calculated from the date of diagnostic biopsy, whereas that of the CALGB series was from the date of registration into the relevant chemotherapy trial. Patients dying within 30 days of surgery have been omitted from the Leicester data.

* = median survival not reached

Group	*n*	*n*	Median Survival (months)		One Year Survival %		Two Year Survival %	
	Leicester	*CALGB*	Leicester	CALGB	Leicester	CALGB	Leicester	CALGB
1	*27*	*36*	**17.7**	**13.9**	**62.3**	**63**	**32.3**	**38**
95% CI			13.8 – 21.7	11.1 - 31.4	44.4 – 81.1	46 - 77	14.0 – 51.7	23 - 55
2	*5*	*36*	*	**9.5**	**60**	**41**	**0**	**21**
95% CI				6.9 - 14.7	17.1 - 100	26 - 57		10 - 37
3	*69*	*146*	**8.1**	**9.2**	**27.5**	**30**	**10.0**	**10**
95% CI			5.9 – 10.3	7.5 - 10.5	16.8 – 38.2	23 - 37	2.2 – 17.8	6 - 16
4	*6*	*33*	**5.2**	**6.5**	**33.3**	**25**	**16.7**	**6**
95% CI			2.7 – 7.7	3.7 - 9.4	0 – 71.1	14 - 42	0 – 46.5	2 - 17
5	*31*	*73*	**4.3**	**4.4**	**16.1**	**7**	**4.3**	**0**
95% CI			2.5 – 6.1	3.4 - 5.1	3.2 – 29.1	3 - 15	0 – 83.5	
6	*7*	*13*	**2.5**	**1.4**	**0**	**0**	**0**	**0**
95% CI			0.03 – 4.9	0.5 - 3.6				
Total *n*	*145*	*337*						

Table 3.12:   Comparison of published series with multivariate analyses of prognostic factors

	Country	n	Median Survival (months)	Age	Gender	Chest Pain	Weight Loss	PS	Cell Type	Certainty of Histological Diagnosis	Asbestos exposure	WBC	Hb	Platelets	Stage
Chahinian et al. 1982	USA	69	12.9	+	-				+		-				
Samson et al. 1987		76		-	-			-	-						
Alberts et al. 1988	South Africa	262	9.6		-			+	-						
Antman et al. 1988	USA	180	15			+		+	+						
Chailleux et al. 1988	France	167	13	+	-										
Spiritas et al. 1988	USA	1475	7	+	+				-						
Calavrazos et al. 1988	Germany	132	9	+	-	+		+	+		-				
Ruffie et al. 1989	Canada	332	9	+	-		-				+			+	+
Achatzy et al. 1989	Germany	245	9.2												
Tammilehto et al. 1992	Finland	98	9	-	+	-		+	+						
Boutin et al. 1993	France	188		-	-		+		+						+
De Pangher Manzini et al. 1993	Italy	80	13	+	-		-	-	+					-	-
Fusco et al. 1993	Italy	113	10	-	-	-			+						
Curran et al. 1998	Europe	204	12.6	-	+			+	+	+		+	-		-
Herndon et al. 1998	USA	337	3.9 – 9.8*	+		+	+	+	+		-	-		+	
Pass et al. 1998	USA	48	14.4		+				-					+	
Rusch et al. 1999	USA	231			+				+					-	+
Leicester 1987 - 2001	UK	138	6.3	-	-	-	+	+	+	-	-	-	-	-	

+   Significant (p<0.05)
-    Not Significant
*   overall median survival not quoted

asbestos in lung tissue has been proposed as a prognostic factor in MM (Kayser *et al.* 1999;

Leigh *et al.* 1991).

Clinical features, such as the presence of pleuritic chest pain or weight loss and

haematological indices have been examined in fewer studies than cell type and performance

status. We found that all these factors were significant poor prognostic factors in univariate

analysis. Both the EORTC and CALGB scoring systems incorporated assessment of WBC

into the group allocations, but in this series increasing WBC was not a significant poor

prognostic factor (p=0.07).

One of the initial aims of this study was to evaluate the IMIG stage retrospectively.

Despite reviewing operation notes and computed tomography (CT) scans, it was felt that

retrospective TNM staging would not be sufficiently accurate to derive a reliable TNM

stage, unless either staging surgical biopsy or radical surgery had been performed. Despite

the low numbers in Stage I and II, there was a significant difference between Stage IV and

the earlier stages. Due to the small numbers, IMIG stage was not assessed in multivariate

analysis. The prognostic importance and correlations of IMIG Stage is considered more

carefully in later chapters.

3.5.1. EORTC and CALGB Prognostic Groups

When compared to the EORTC and CALGB series, the median survival time and

one and two year survival rates for appropriately allocated Leicester patients were

comparable. Therefore, whereas overall survival from MM in the UK is poor in

comparison to other European series, this is not the case when analysis by prognostic

groups is performed based on prognostic variables. It is important to note, however, that in both the CALGB and EORTC studies, survival was calculated from the date of registration into the relevant chemotherapy trial, rather than from the date of histological diagnosis. In the EORTC series, median survival was 12.6 months from diagnosis and 8.4 from trial entry. The interval was not quoted for the CALGB series. It is likely that there would be a bias towards longer survival in the Leicester series because of this difference in survival analysis.

3.6. Conclusions

In this series, the prognostic importance of known clinicopathological factors was confirmed. Furthermore, the effectiveness of the EORTC and CALGB prognostic scoring systems was validated in a surgical series of patients in a tertiary referral centre in the UK. When patients were stratified into appropriate EORTC and CALGB prognostic groups, survival was no worse in Leicester than in the two international chemotherapy-based series. This work provided the foundation for investigation of novel prognostic factors presented in subsequent chapters.

Chapter Four

Assessment of Angiogenesis

4.1. Introduction

Angiogenesis is the formation of new blood vessels from existing vasculature, during which quiescent endothelial cells proliferate and gain invasive characteristics. Basement membrane degradation and remodelling of the extracellular matrix are essential parts of this process. Angiogenesis is necessary for tumour growth of greater than 1 to 2mm in diameter (Hanahan and Folkman 1996). High intratumoural microvessel density (MVD), an indirect measure of the intensity of angiogenesis, are associated with a poor prognosis in solid tumours (Fox *et al.* 2001; Giatromanolaki 2001; Cox *et al.* 2000a; O'Byrne *et al.* 2000).

There have been preliminary reports of the prognostic value of MVD in malignant mesothelioma (MM) (Kumar-Singh *et al.* 1997; Ohta et al. 1999a). These relatively small studies, of 25 and 54 cases, have suggested a relationship between increased MVD and poor prognosis. There have been other studies published evaluating relationships of MVD with expression of angiogenic growth factors. However, methodology, the mean vessel count obtained and correlations obtained varied significantly between the studies (Table 4.1).

The conclusions of a consensus paper on the evaluation of tumour angiogenesis were incorporated into the methodology of assessment of angiogenesis for this study (Vermeulen *et al.* 1996). This suggested that manual vessel counting in hot spots was the appropriate method for assessing angiogenesis objectively in tumours and that either anti-CD34 or CD31 antibodies should be used. The anti-CD34 monoclonal antibody was chosen for this study as it had been shown to give more reproducible immunostaining of

Table 4.1:   Published reports of assessment of microvessel density (MVD) in malignant mesothelioma

Reference	Year	n	Antigen	Method of MVD evaluation	Other factors assessed	Increasing MVD associated with:
Kumar-Singh *et al.* 1997	1997	25	CD34 (CD31)	1. x200 field 2. Computerised image analysis	none	Poor survival
Kumar-Singh *et al.* 1998	1998	?25*	CD34	As above	Syndecan-1	No correlation
Kumar-Singh *et al.* 1999	1999	?25*	CD34	As above	VEGF, aFGF, bFGF, TGF-β IHC	Co-expression of all four cytokines, but no correlations individually
Konig *et al.* 1999	1999	103	FVIII	3 fields at x100	VEGF IHC	Increasing VEGF, survival not assessed
Ohta *et al.* 1999	1999	54	FVIII	3 fields at x400	VEGF RT-PCR	Increasing VEGF, poor survival (multivariate analysis only)
Tolnay *et al.* 1998	1998	39	FVIII	3 fields at x200	HGF/SF IHC and FISH, c-met IHC	HGF expression
Strizzi *et al.* 2001a	2001	12$^+$	FVIII	3 fields at x400	Pleural fluid VEGF Serum VEGF	No correlations
Strizzi *et al.* 2001b	2001	15$^+$	FVIII	3 fields at x400	Pleural fluid bFGF	No correlation
Soini *et al.* 2001	2001	36	FVIII	10 fields x high power	eNOS, VEGF, FLK1, FLT1	No correlations

* = presumed same cases as 1997 series, number of cases with MVD assessment in 1998 and 1999 publications not stated

$^+$ = presumed same cases

microvessels than either the anti-CD31 or anti-Factor VIII monoclonal antibodies in breast cancer (Martin *et al.* 1997). The Chalkey counting method was chosen because it had been shown to be a rapid and objective method of assessing MVD in breast cancer (Fox *et al.* 1995; Gasparini *et al.* 1996; Tokes *et al.* 1999; Hansen *et al.* 2000), bladder cancer (Dickinson *et al.* 1994; Chaudhary *et al.* 1999) and non-small cell lung cancer (Giatromanolaki *et al.* 1996; Decaussin *et al.* 1999; Cox *et al.* 2000b). Hansen made a direct comparison, in terms of inter- and intra-observer variations, of MVD quantification in breast cancer using techniques of Chalkley counting and three methods of microvessel count per high power field (one hot spot, mean of three hot spots, highest of three hotspots). The investigators found that the Chalkley method had less inter- and intra-observer variation (Hansen *et al.* 1998). Both the anti-CD34 antibody and the Chalkley counting method were in routine use at the ICRF laboratories in Oxford, where members of our group received training in these techniques.

4.2. Aims

1   To evaluate the prognostic significance of MVD, assessed by Chalkley microvessel counts, in a large series of MM patients, in univariate and multivariate models

2   To determine the correlations between MVD and clinicopathological factors

3   To examine the contribution of MVD to the CALGB and EORTC prognostic groups

4.3. Methods

4.3.1. Patients

Cases were identified as described in Section 2.2.1. The haematoxylin and eosin stained diagnostic histopathological slides were reviewed to assess the presence of suitable tumour for microvessel quantification.

4.3.2. Anti-CD34 Immunohistochemistry

Immunohistochemistry was performed with an anti-CD34 antibody (NCL-END, Novocastra, Newcastle, UK) as previously described Section 2.3.3.4. The conditions for immunohistochemistry had been optimised previously by a colleague for non-small cell lung cancer (Cox, *et al.* 2000b) and were found to work well for the MM samples.

4.3.3. Microvessel Quantification

Angiogenesis was assessed indirectly with the aid of a Chalkley eyepiece graticule (Cox, *et al.* 2000b). Each section was examined under low power (x40 and x100) to identify intratumoural microvessel 'hot spots.' Only hotspots within viable tumour islets, or within one high power (x250) field distance from them, were counted. These areas were then examined at x250 magnification using a 25-point Chalkley eyepiece graticule (Chalkley 1943; Fox, *et al.* 1995). The Chalkley graticule was orientated so that the maximum number of points coincided with immunostained structures. In line with the previous studies of this technique, structures stained with the chromogen that had the morphological features of microvessels were counted whether a lumen was present or not. The most intense hot spots of immunostaining were examined first and counted: further hot spots were counted until no change in the vessel counts obtained occurred. The MVD was

defined in this study as the sum of the number of points thus counted from the three

"hottest" hot spots. A sample of 51 sections was analysed by two investigators blinded to

clinicopathological factors and outcome: where disagreement occurred, a consensus was

reached. Tumour sections were counted twice by one observer, 6 months apart. Inter- and

intra-observer variability was assessed.

It was noted that, in some cases, a pattern of immunostaining of stromal staining

occurred with the anti-CD34 antibody. The morphological features were distinct from what

could be considered to be microvessels and, after expert pathological review, appeared to

be myofibroblasts. Accurate Chalkley counting was felt to be possible in a proportion of

those cases in which the morphological pattern of stromal staining was clearly distinct from

that of microvessels.

4.3.4. Anti-CD31 Immunohistochemistry

In order to resolve the difficulties of vessel counting in cases with stromal

immunostaining with the anti-CD34 antibody, selected cases were stained with an anti-

CD31 antibody.

4.3.5. Statistical Analysis

The statistical analysis performed was as for the prognostic factors above.

Associations with clinicopathological data were explored. Differences in MVD within

categorical variables were assessed with Student's t-test. Linear regression analysis was

used to assess correlations with continuous variables. Survival curves were estimated using

the Kaplan-Meier method and the log-rank test was used to assess the statistical

significance of differences between groups. The impact of MVD on survival was analysed

further in univariate and multivariate Cox proportional hazards regression models.

4.4. Results

4.4.1. Immunohistochemistry and Microvessel Quantification

Of the 171 cases which were stained with the anti-CD34 antibody, 20 had

insufficient material for microvessel counting (i.e. tumour cells occupying less than three

high power (x100) fields). Amongst the remaining 151 cases, immunostaining of stromal

elements was present in 38 (25%) cases (Figure 4.1). Microvessel quantification was

carried out in 15 of these cases in which the morphological pattern of stromal staining was

clearly distinct from that of microvessels. Stromal staining therefore precluded microvessel

quantification in 23 (15%) cases. Using the anti-CD34 antibody, the median MVD (sum of

three hotspots) in the resulting 128 cases was 23 (range 13 – 59). Examples of a high and

low MVD are shown in Figures 4.2.

4.4.2. Correlations with Clinical and Pathological Factors

There was no correlation between MVD and cell type or other categorical

prognostic variables (Table 4.2). The MVD for epithelioid cases was mean 24.4 (SD 7.7),

median 23 (range 13 - 59) vessels and for non-epithelioid was mean 25.7 (SD 7.7), median

25 (range 13 – 55) vessels (p=0.322, Figure 4.3). With regard to continuous variables,

MVD greater than the median was associated with an increased platelet count (p=0.05,

Table 4.3), but not with age, white blood cell count or haemoglobin. By linear regression

analysis, a weak but statistically significant correlation was seen between increasing MVD

and platelet count (r = 0.19 p=0.037, Figure 4.4). The platelet count was significantly

Table 4.2: Correlations between the MVD and categorical clinicopathological and biological variables

Factor	Categories	MVD		χ^2 p value
		< median	>= median	
Gender	Male	49	67	0.35
	female	4	8	
Chest pain	No	14	20	0.99
	Yes	37	53	
Weight loss	No	30	33	0.083
	Yes	19	40	
Cell type	Epithelioid	31	36	0.24
	Non-epithelioid	22	39	
Performance status	0	27	25	0.038
	1, 2	24	48	
CALGB prognostic group	Groups 1 and 2	12	12	0.19
	Groups 3 and 4	27	32	
	Groups 5 and 6	12	28	
EORTC prognostic group	Low risk	19	27	0.98
	High risk	32	45	
Radical Surgery	No	39	59	0.92
	Yes	9	13	

Table 4.3: Correlations between the MVD and continuous clinicopathological and biological variables using Student's t-test.

Factor	Units	MVD		p
		< median	>= median	
		Mean value (SD)		
Age	years	63.7 (9.8)	63.9 (10.4)	0.92
WBC	x 10^9/l	9.8 (3.3)	10.7 (4.7)	0.25
Platelets	x 10^9/l	365 (135)	424 (181)	0.05
Haemoglobin	g/dl	13.2 (2.1)	13.1 (2.0)	0.87

Table 4.4: Univariate log rank and Cox regression analysis of angiogenesis, with derivation of the MVD Chalkley microvessel counting (n=113). Results as a categorical variable (with the cut-point at the median value) and as a continuous variable are shown.

Variable	Categories	n	Median Survival (days)	Log Rank p	Hazard Ratio	Hazard Ratio 95% Confidence Intervals		Cox p
MVD	< median	47	270	0.0141	1			0.0153
	>=median	66	158		1.68	1.10	2.55	
MVD	Continuous	113			1.04	1.01	1.06	0.0049

Figure 4.1: Photomicrograph of a tumour section displaying stromal anti-CD34 immunostaining, which prevented accurate assessment of microvessel density
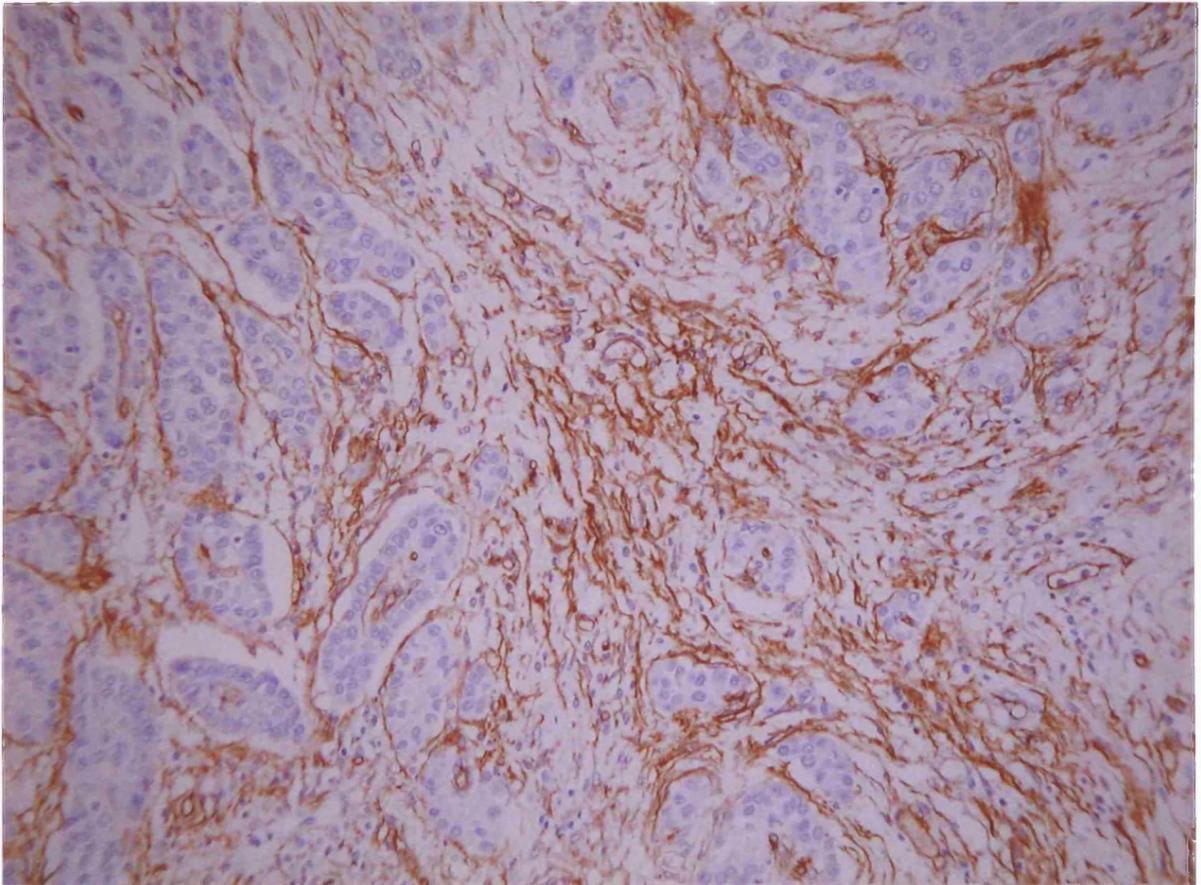
Figure 4.2: Photomicrographs of tumour sections with a) a high and b) a low microvessel density after CD34 immunohistochemistry

a)
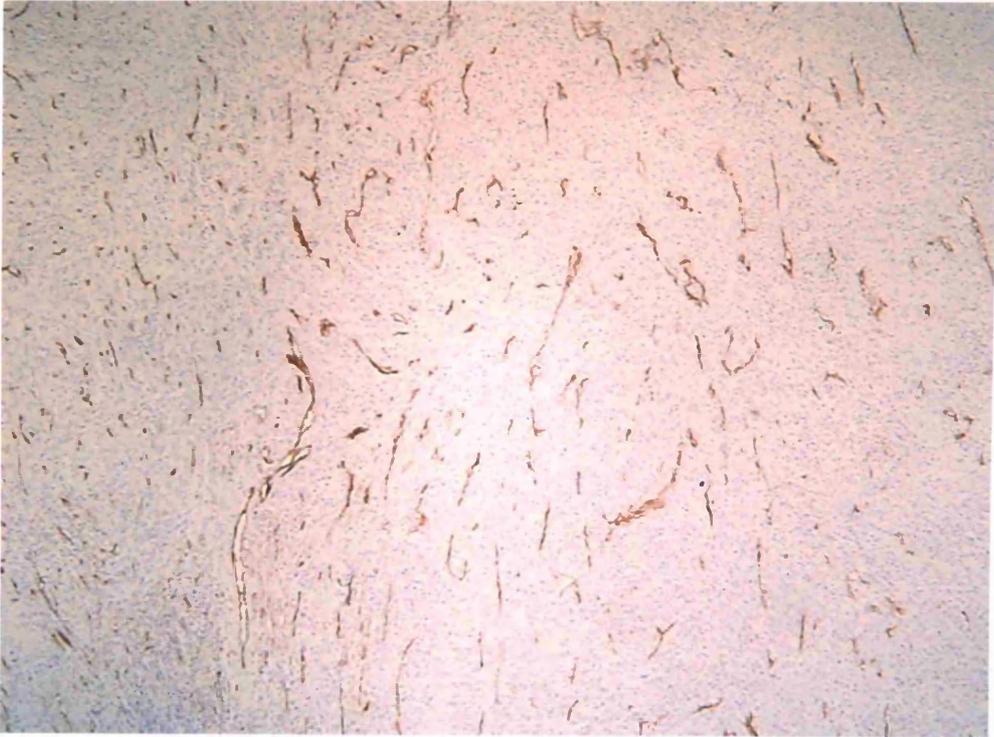
b)
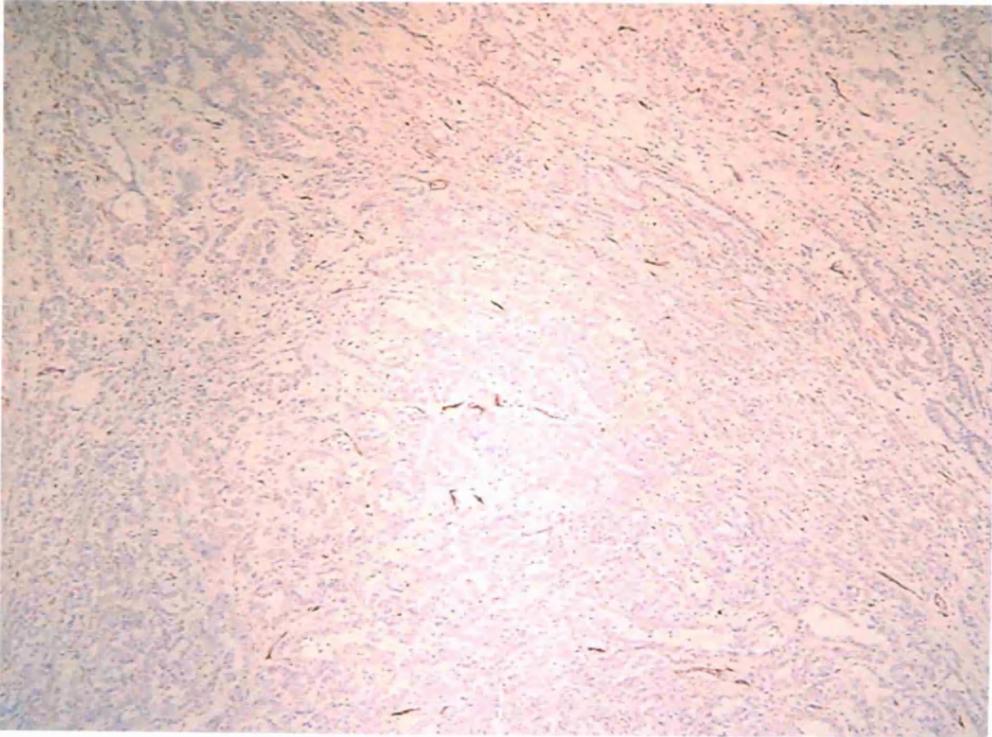
Figure 4.3: Boxplot showing the distributions of MVD by cell type. There was no significant difference between groups (p=0.59, Kruskal Wallis Test). The horizontal bars represent the median values, boxes the interquartile range, vertical bars the 10 – 90 % range, solid circles the outliers and solid triangles the extremes.
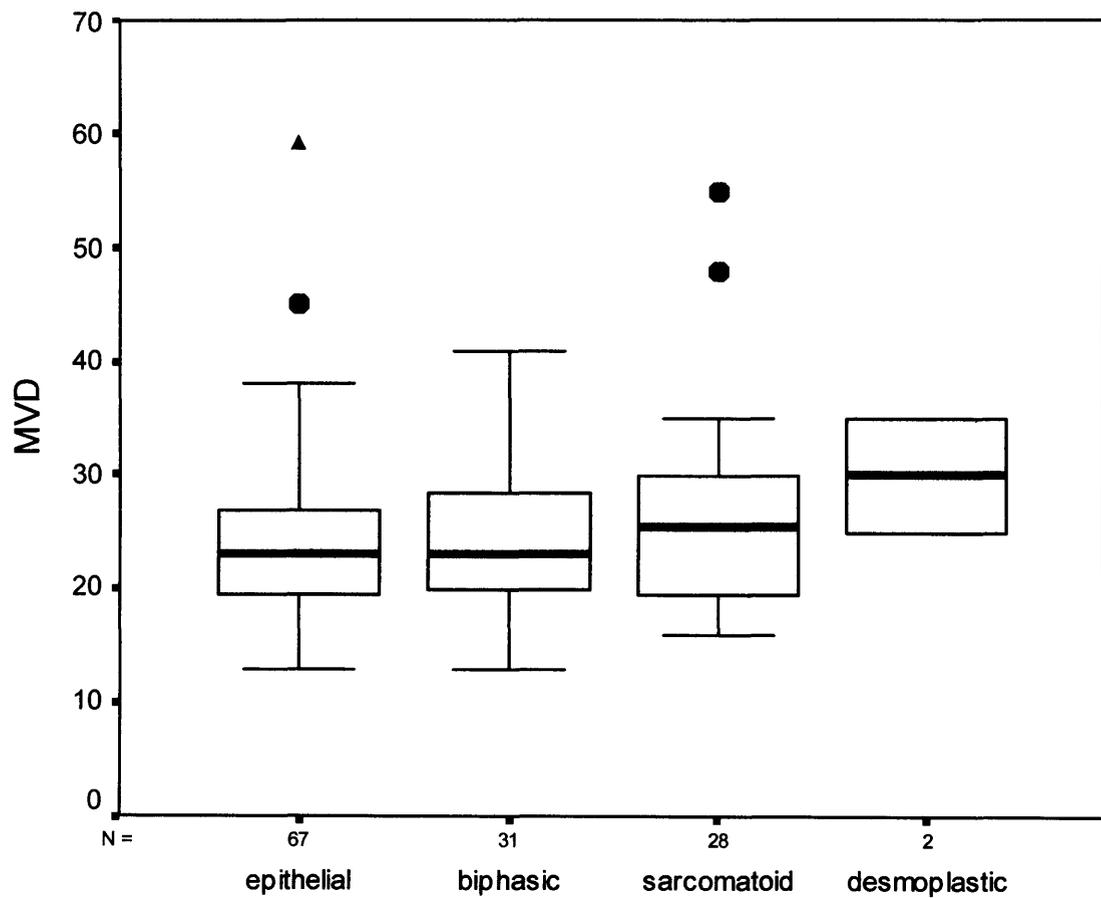
Figure 4.4: Scatterplot showing the correlation between MVD and platelet count (r=0.19, p=0.037)
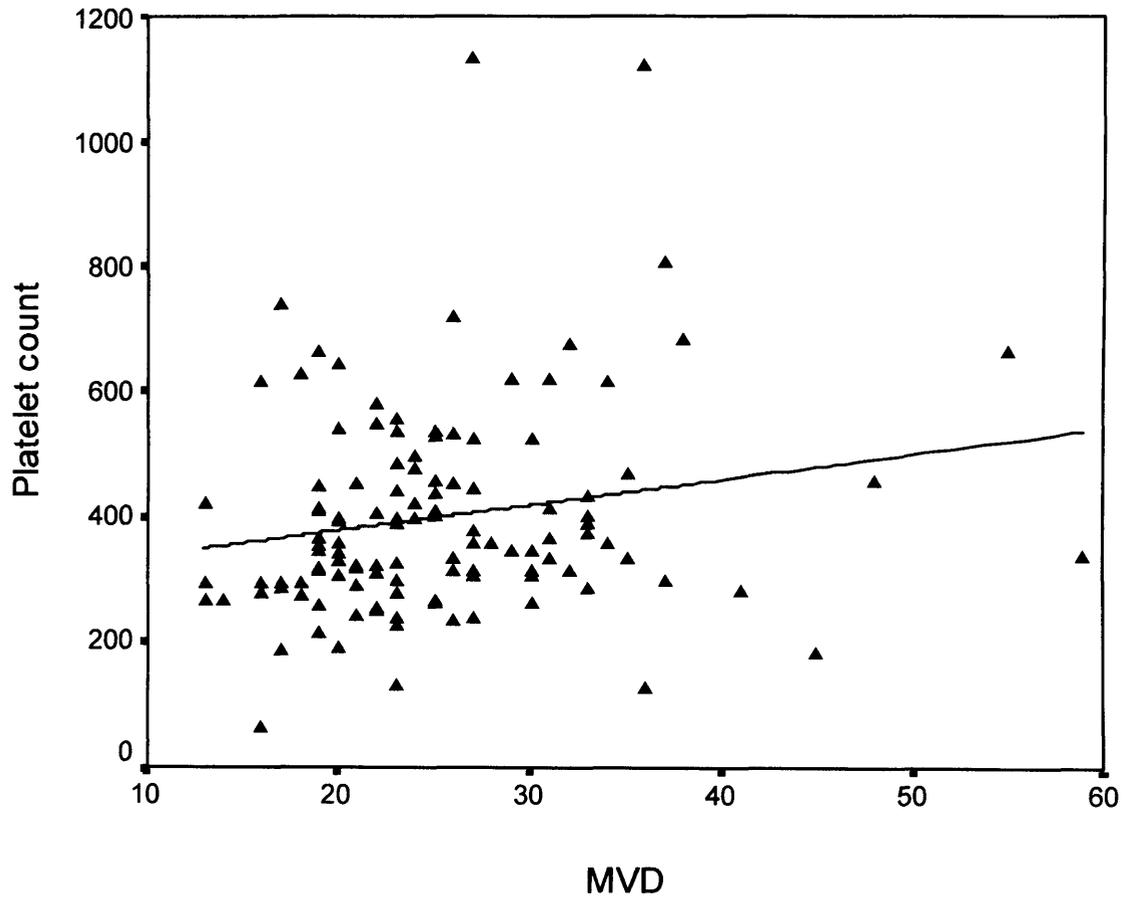
higher in patients with weight loss than without (mean 443 (SD 185) $\times 10^9$/ml compared to

348 (112) $\times 10^9$/ml, p<0.001). There was no significant difference in MVD between the

high and low risk groups of the EORTC prognostic scoring system. With regard to the

CALGB system, although there were a greater number of patients with a high vessel count

in Groups 5 and 6, this failed to reach significance.

4.4.3. Survival – univariate analysis

Fifteen cases died within 30 days and were excluded from further survival analyses.

The MVD was no higher in these cases (p=0.91). A MVD greater than the median was

associated poor prognosis with Kaplan-Meier analysis and the Log Rank Test (p=0.0141,

Figure 4.5, Table 4.4). When analysed with Cox proportional hazards models, high MVD

was a poor prognostic factor as a continuous variable (p=0.0049) and as a categorical

variable (p=0.0153, Table 4.4).

4.4.4. Survival – multivariate analysis

MVD was entered into the multivariate Cox proportional hazards model derived in

Chapter 3, together with weight loss, chest pain, performance status, haemoglobin, platelet

count, radical surgery and cell type. In multivariate analysis, non-epithelial cell type

remained the strongest independent risk factor followed by high MVD, performance status

>0 and weight loss (Table 4.5.) When tested against the prognostic scoring systems in Cox

multivariate analysis, MVD greater than the median contributed independently to the

EORTC system (p=0.0157) but not the CALGB system (p=0.1335, Table 4.6.)

Table 4.5: Cox multivariate regression analysis. The prognostic variables identified in univariate analysis in Section 3.4.4 were entered into a forward, stepwise logistic regression model together with the MVD (< or >= median). With the exclusion of cases with missing data, n=105.

Variable	p value	Hazard Ratio	Hazard Ratio 95% Confidence Interval	
Non-epithelioid cell type	0.0007	2.14	1.38	3.31
MVD > median	0.0294	1.67	1.05	2.66
ECOG Performance Status >0	0.0376	1.58	1.03	2.44
Weight loss > 5%	0.0390	1.58	1.02	2.43
Haemoglobin <14g/dL	0.1216			
Chest Pain	0.1672			
No radical surgery	0.2011			
Platelets > 400 $\times 10^9$/mL	0.5867			

Table 4.6: Cox multivariate regression analysis with the CALGB or EORTC groups. The MVD (< or >= median) was tested, in turn, against the CALGB and EORTC prognostic scoring systems.

Variable	Category	n	p value	Hazard Ratio	Hazard Ratio 95% Confidence Interval	
MVD	< median	47	0.1335			
	>= median	63				
CALGB groups	Groups 1 and 2	23	<0.0001	1		
	Groups 3 and 4	55		2.99	1.63	5.48
	Groups 5 and 6	32		6.57	3.35	12.91

Variable	Category	n	p value	Hazard Ratio	Hazard Ratio 95% Confidence Interval	
MVD	< median	47	0.0157	1		
	>= median	63		1.67	1.10	2.58
EORTC groups	Low risk	44	0.0003	1		
	High risk	66		2.14	1.42	3.25

Figure 4.5: Kaplan-Meier plot showing that an MVD greater than the median value is associated with poor survival (p=0.01)
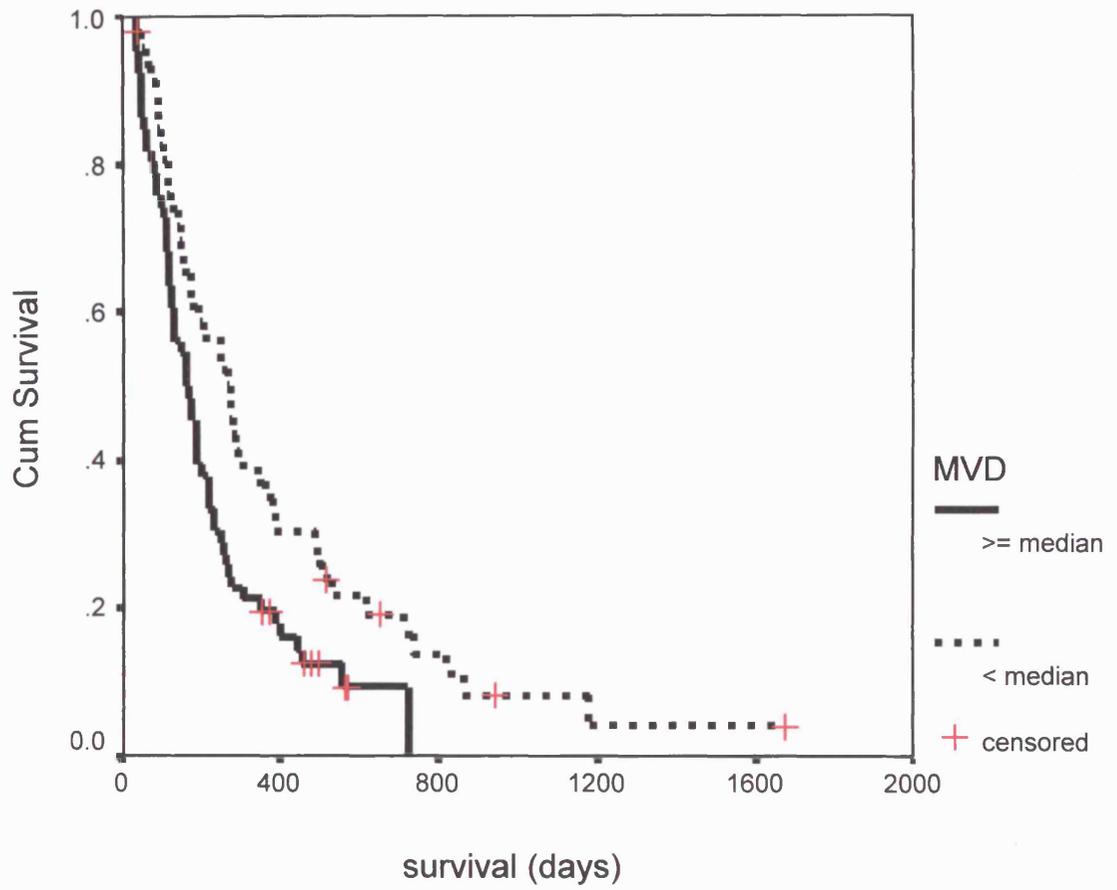
4.4.5. Inter- and intra-observer variation

The 51 initial cases stained for CD34 were counted independently once by Dr Giles Cox (Observer B) and twice by myself (Observer A), to assess inter- and intra-observer variation. There were significant differences between the median MVD reported by Observer B, which was 19, and those obtained by Observer A (23 on both occasions, $p<0.001$). However, statistically significant correlations were seen between all three sets of data (each $p<0.0001$, Figure 4.6). Spearman's correlation coefficients were as follows: Observer A-1 vs. Observer A-2, $r = 0.75$; Observer A-1 vs. Observer B, $r = 0.50$, Observer A-2 vs. Observer B, $r = 0.54$.

Before gaining a consensus, agreement between observers according to whether samples were above or below the median cut-point was reached in 34 cases (65%, Cohen's kappa $= 0.309$, $p=0.026$). Similarly, there was agreement between readings above and below the median value taken by myself on two separate occasions 6 months apart in 42 cases (82%, Cohen's kappa $= 0.645$, $p<0.001$).

When counts above and below median values of each observer were tested for prognostic significance, all three readings were found to be predictive of survival with both the log rank test and Cox proportional hazards analysis when assessed either as continuous or categorical variables (Table 4.7).

4.4.6. Assessment of CD31 immunohistochemistry

Immunohistochemistry for CD31 resulted in the abolishment of stromal staining in all cases examined. The intensity of staining was not quite as good as that for the CD34 immunohistochemistry, however. Within the limit of the CD34 and CD31 immunostaining

Table 4.7: Inter- and intra-observer variation. Log rank and Cox proportional hazards survival analyses are shown following Chalkley microvessel counting of the initial 51 cases immunostained against CD34. Five patients died within 30 days of surgery and were excluded from analysis. Results are given for the three assessments: two by observer A and one by observer B.

Observer	MVD Categories	n	Median Survival (days)	Log Rank p	Hazard Ratio	Hazard Ratio 95% Confidence Intervals		Cox p
Observer A-1	< median	23	272	<0.0001	1			0.0001
	>=median	23	111		4.06	1.99	8.26	
	Continuous				1.11	1.06	1.15	<0.0001
Observer A-2	< median	23	266	0.0165	1			0.0189
	>=median	23	128		2.11	1.13	3.93	
	Continuous				1.07	1.02	1.11	0.0039
Observer B	< median	25	266	0.0051	1			0.0064
	>=median	21	115		2.38	1.28	4.43	
	Continuous				1.07	1.03	1.12	0.0012

Figure 4.6: Variation in the MVD obtained between two observers. Observer A performed Chalkley counting on 51 specimens on two separate occasions (a). Each of these sets of data is plotted against the results produced by Observer B before a consensus was reached (b). The solid line indicates the regression line and the dashed line the line of quality. Spearman's correlation coefficients were as follows: Observer A-1 versus Observer A-2, r=0.75; Observer A-1 vs. Observer B, r=0.50, Observer A-2 vs. Observer B, r=0.54.

a
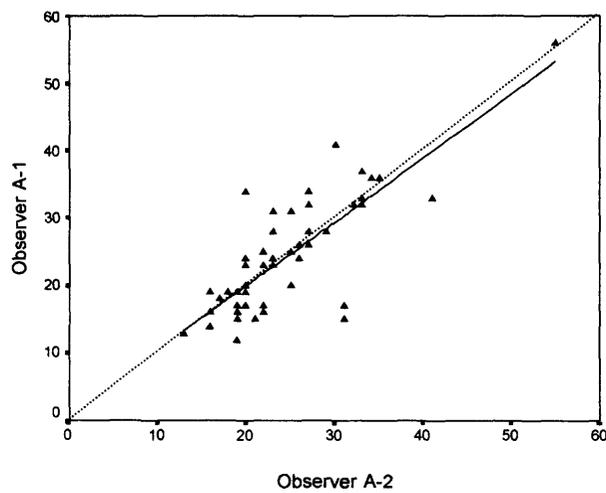
b
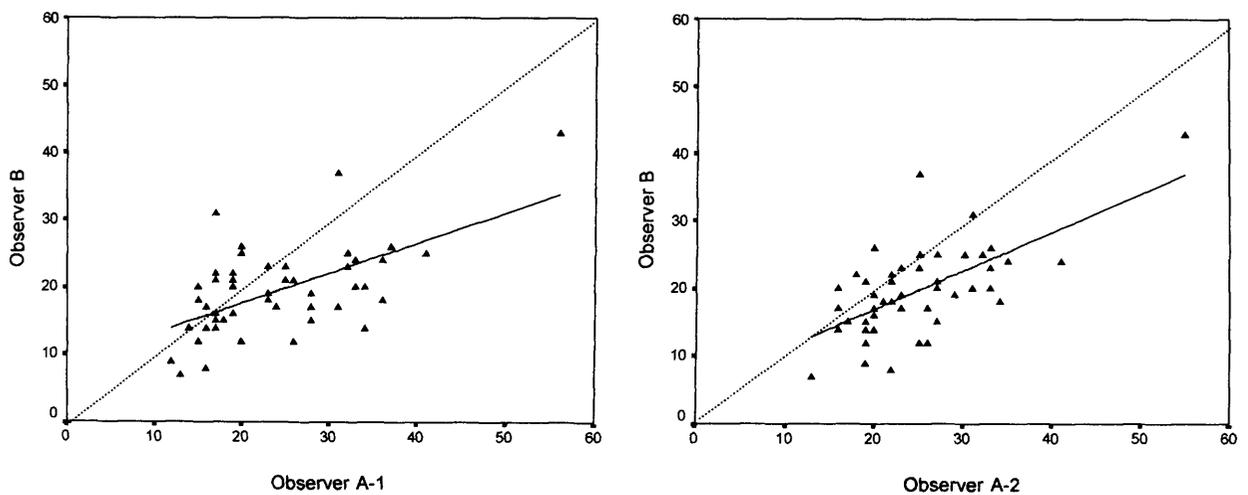
not being performed on sequential sections, the number of microvessels appeared to be comparable (Figure 4.7).

4.5. Discussion

This study, significantly larger than any of the other published series, demonstrates that increased MVD, as assessed by Chalkley counting, is an independent prognostic factor in MM. The median survival for cases with an MVD greater than the median value was 158 days (6.0 months) compared to 270 days (8.9 months) for those below. Although inter- and intra-observer correlations were low with regards to the median MVD values seen, a relatively close correlation was seen between assessments when (a) the counts were assessed as continuous variables and (b) the prognostic significance of MVD was analysed. Therefore whilst assessment of angiogenesis by Chalkley microvessel counting may be an effective research tool in a large patient series it may not be useful in providing prognostic information on the individual patient in routine clinical practice. However, a vascular grading method has been described which correlated well with Chalkley counting. This involved eye appraisal of immunostained microvessels in tumour sections by three observers using a conference microscope. The sections were then divided into high, medium and low vascular grades. A 100% correlation with a pre-selected MVD cut-off point value was obtained separating high from medium and low vascular grade tumours (Giatromanolaki *et al.* 1996).

The relationship between high MVD and worse survival is in agreement with findings in other solid tumours and confirms previous reports in MM (Kumar-Singh *et al.* 1997; Ohta et al. 1999a) (Table 4.1). Kumar Singh *et al* found that microvessel density was

Figure 4.7: Photomicrograph of a tumour section displaying a) stromal anti-CD34 immunostaining, which prevented accurate assessment of microvessel density. b) With the anti-CD31 immunohistochemistry, the stromal immunostaining was lost, allowing the microvessels to be seen more clearly.

a)
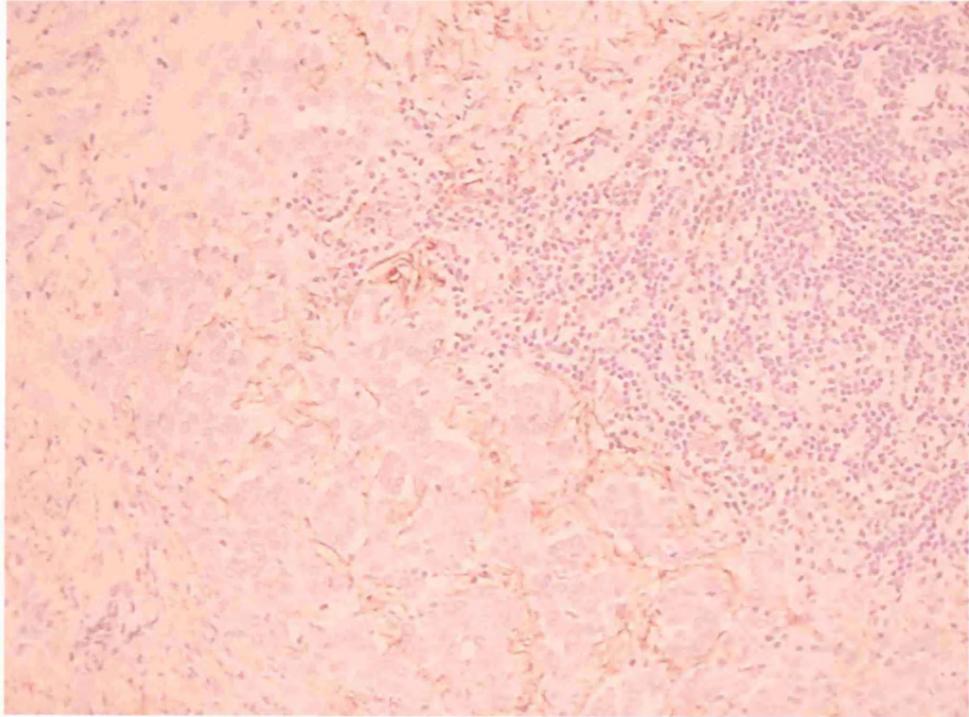
b)
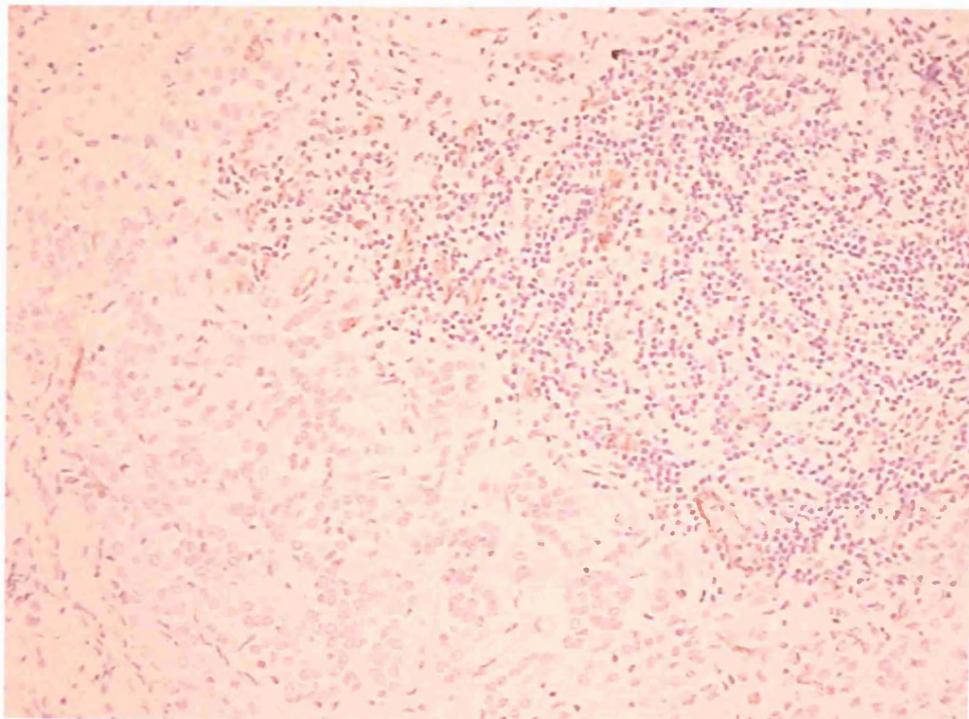
a significant prognostic factor in univariate analysis and was independent of MM cell type, tumour grade and patient age in multivariate analysis (Kumar-Singh *et al.* 1997). However, total microvessel area, when calculated from computer-aided image analysis, was not a significant prognostic factor, thus suggesting that the size of microvessels is not as important as their number. Ohta *et al* examined both microvessel density and lymphatic vessel density in 54 tumours and found statistically insignificant trends in univariate analyses between high microvessel density and both poor survival and positive lymph node status. In multivariate analysis, gender, IMIG Stage and high microvessel density were significant independent poor prognostic factors (Ohta et al. 1999a). The remaining studies did not assess prognostic significance but correlated microvessel counts with angiogenic growth factors in tumour, serum and pleural fluid samples (Tolnay *et al.* 1998; Konig *et al.* 1999; Strizzi *et al.* 2001a; Strizzi *et al.* 2001b; Soini *et al.* 2001).

The protocol for this study was based on the findings of an international consensus paper on the quantification of angiogenesis in solid tumours (Vermeulen *et al.* 1996). The choice of antibody was supported by Kumar-Singh *et al*, who found that CD34 staining was better delineated and easier to assess than CD31 in MM (Kumar-Singh *et al.* 1997). Stromal staining was seen in 25% of cases in our series. In 15 cases the pattern of stromal staining was clearly morphologically distinct from that seen in microvessels and these were included in analysis. Kumar-Singh reported a "perivascular wash" in some cases with anti-CD34 immunohistochemistry, but did not describe staining of specific stromal elements, nor report interference with microvessel counting.

No stromal staining occurred with the CD31 antibody. Initial experience with the anti-CD31 antibody suggests that it could be used to obtain microvessel counts from the

cases with stromal CD34 immunostaining. Ideally, this would require staining the whole cohort of patients with both antibodies on sequentially cut sections. This would allow the most accurate correlation between MVD obtained with each antibody. Unfortunately, it was not possible, within the time scale of this study, to complete staining and interpretation of the whole cohort of cases. Neither, due to intervening work, was it possible to compare appearances on sequentially cut sections. This is subject to ongoing work in the department. Interestingly, researchers in Cardiff have stained a series of 92 mesotheliomas for both CD34 and CD31, but the study was investigating endothelial tumour cell differentiation in MM in relation to malignant vascular tumours of the pleura, in patients with a heavy occupational exposure to asbestos. Unfortunately, no note of differing characteristics in endothelial cell immunostaining of the two antibodies was made (Attanoos *et al.* 2000).

With regard to correlations with clinicopathological factors, a significant correlation with the platelet count was seen. Trends were seen towards positive correlations with weight loss and poor performance status. The correlation with the preoperative platelet count provides supportive evidence that platelets may play an important role in tumour angiogenesis. Platelets are an important source of angiogenic growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Banks *et al.* 1998; Pinedo *et al.* 1998). Platelets are likely to adhere to the endothelium of the relatively fragile intratumoural microvessels, which can lead to platelet activation and the release and accumulation of high local concentrations of these growth factors (Verheul *et al.* 2000; Pinedo *et al.* 1998). A positive correlation has been established between serum VEGF and platelet count in cancer patients (Vermeulen *et al.* 1999) and these have been further correlated to prognosis in solid tumours (O'Byrne *et al.* 1999).

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Of further note is the trend towards high MVD and weight loss (p=0.08). In patients

with solid tumours, weight loss has been associated with high levels of Interleukin(IL)-6

(Scott *et al.* 1996; Blay *et al.* 1997; Tisdale 1999). IL-6 promotes thrombopoiesis (Kaser *et al.* 2001; Blay *et al.* 1997) and the expression of Vascular Endothelial Growth Factor

(VEGF) (Cohen *et al.* 1996). Both IL-6 and VEGF are angiogenic and have been

characterised as playing central roles in the pathogenesis of malignant mesothelioma

(Bielefeldt-Ohmann *et al.* 1995; Nakano *et al.* 1998; Ohta et al. 1999a; Kumar-Singh *et al.*

1999; Strizzi *et al.* 2001a). In this study, platelet count correlated significantly with both

MVD and weight loss. This observation suggests that cytokines such as IL-6, linking

thrombocytosis, weight loss and angiogenesis, play an important role in the pathogenesis of

MM.

Two of the other reports of microvessel density assessment in MM comment on a

relationship with cell type. Konig found that epithelioid tumours had a higher microvessel

density than either biphasic or sarcomatoid tumours (10.4, 7.5, 5.9 vessels/mm^2

respectively) (Konig *et al.* 1999). Tolnay also found a trend towards higher microvessel

density in epithelioid tumours, although this did not reach statistical significance in the 39

cases (Tolnay *et al.* 1998). Soini found no association between microvessel counts and cell

type (Soini *et al.* 2001). Kumar-Singh did not quantify the microvessel densities by cell

type, but noted that the prognostic value was independent of cell type (Kumar-Singh *et al.*

1997). Ohta et al did not assess microvessel density according to cell type in their 54 cases

(Ohta et al. 1999a). In this series, we found no difference in MVD between epithelioid and

non-epithelioid cases. Of the 14 cases which had to be rejected after

immunohistochemistry, due to the lack of sufficient tumour area to assess for Chalkley

counting, only one was of sarcomatoid cell type. The nature of biopsy specimens in the other series was not always clear. The specimens of Ohta et al were obtained at the time of thoracotomy for either radical or debulking surgery and snap frozen (Ohta et al. 1999a). Kumar-Singh insisted on the tissue size being at least 5mm x 5mm (Kumar-Singh *et al.* 1997). In this study, although no precise limitation on tumour size was applied, there had to be three fields at x100 magnification of tumour cells present on the section. Many paraffin-embedded blocks contained more than one piece of tumour. Only 11 of Konig's patients underwent surgical resection of tumour (Konig *et al.* 1999), whereas Tolnay did not specify the source of material (Tolnay *et al.* 1998). It is possible that a lack of biopsy material in the latter two series, which did find a relationship between MVD and cell type, may have led to a sampling error and a derivation of a false low microvessel density in the sarcomatoid group. Certainly, in the case of sarcomatoid MM, it is necessary to ignore large portions of the tumour, which are comprised of extracellular collagen surrounding fibroblasts, when locating *intratumoural* hotspots.

This study demonstrates that the Chalkley microvessel count is an independent prognostic factor which contributes significantly to the EORTC, but not to the CALGB, prognostic scoring system. A feasible explanation of this difference is that the CALGB system includes assessment of weight loss, which was found to correlate with MVD in this study and, as discussed earlier, there are logical reasons as to why this correlation may exist.

4.6. Conclusions

This was the first study published which considered the contribution of angiogenesis to the CALGB and EORTC systems in multivariate analysis. Assessment of angiogenesis may have an important role in the prognostic evaluation of MM and contribute to currently established prognostic scoring systems. Investigation of the mechanisms of angiogenesis in MM may provide further prognostic information and help to rationalise therapy. Such markers may be useful in the selection of patients for radical surgical and chemotherapeutic treatment protocols, including the use of anti-angiogenic agents.

Chapter Five

Tumour Necrosis

5.1. Introduction

Coagulative necrosis is a common feature of solid tumours. Tumour microvessels are fragile and susceptible to hypoxia (Ausprunk and Folkman 1976), which suggests that the degree of tumour necrosis (TN) may reflect the level of intra-tumoural hypoxia. Measured experimentally with a polarographic needle, intra-tumoural hypoxia correlates with poor prognosis and resistance to radiotherapy and chemotherapy in solid tumours (Vanselow *et al.* 2000; Brizel *et al.* 1997; Brizel *et al.* 1999; Stadler *et al.* 1999; Hockel *et al.* 1996). TN has been reported as an indicator of a poor prognosis in a number of solid tumours, including non-small cell lung cancer (NSCLC) (Swinson *et al.* 2002; Shahab *et al.* 1992; Kessler *et al.* 1996; Eerola *et al.* 1999), gastrointestinal stromal tumours (Muro-Cacho *et al.* 2000) and Ewing's sarcoma (Llombart-Bosch *et al.* 1986). In breast cancer, TN has been shown to correlate with increased tumour size, high-grade disease, negative oestrogen receptor status, high microvessel density and infiltrates of macrophages which express vascular endothelial growth factor (VEGF) (Leek *et al.* 1999; Jitsuiki *et al.* 1999; Lee *et al.* 1997; Lewis *et al.* 2000). These findings suggest that, in rapidly growing tumours, a hypoxic environment that results in TN stimulates angiogenesis due to the release of angiogenic growth factors from infiltrating macrophages.

TN has only been noted in MM as a possible indicator of malignancy in the differential diagnosis between MM and benign conditions (Churg *et al.* 2000). However, TN has not been characterised in terms of relations to clinical and pathological features and prognosis.

5.2. Aims

1   To evaluate the incidence of TN in MM

2   To explore correlations with known clinicopathological factors, the EORTC and

CALGB prognostic scoring systems and angiogenesis

3   To establish the prognostic significance of TN in univariate and multivariate models

5.3. Methods

5.3.1. Necrosis assessment

The methods for assessment of tumour necrosis were developed by Dr. Daniel

Swinson and Dr. Louise Jones in NSCLC (Swinson *et al.* 2002) and are similar to those

described elsewhere (Leek *et al.* 1996). All routinely processed, formalin fixed, paraffin

embedded, haematoxylin and eosin (H&E) stained tumour sections from each case, which

had been used for diagnostic purposes, were examined, as described in the Methods chapter

2.3.3.5.2

5.3.2. Statistical analysis

The Chi-squared test was used to analyse the correlation of TN with categorical

variables and Student's t-test for continuous variables. Survival curves were estimated using

the Kaplan-Meier method and the log-rank test was used to assess the statistical significance

of differences between groups. Cox proportional hazards regression models were used to

identify statistically significant differences in survival and estimate hazard ratios and 95%

Confidence Intervals (CIs).

5.4. Results

5.4.1. Tumour necrosis scores

TN was identified in 39 cases out of 171 (22.8%). TN was grade 1, 2 and 3 in 14, 14 and 11 cases respectively (Figure 5.1). Agreement between observers on the TN score, before obtaining a consensus, was reached in 161 patients (94.2%, Cohen's kappa = 0.845, p<0.001, Table 5.1). Scores were agreed for the remaining 10 cases with the aid of the conference microscope and the adjudication of Dr Louise Jones, Senior Lecturer in Pathology. Cohen's kappa for the agreement of the presence of any necrosis, before the consensus was reached, was 0.899 (p<0.001).

5.4.2. Correlation with clinicopathological factors and angiogenesis

Due to the relatively small numbers in each grade of necrosis, TN was defined as being either present or absent when assessing correlations with the clinicopathological variables analysed in the preceding chapters.

The associations between TN and clinicopathological variables are shown in Tables 5.2 and 5.3. The presence of TN correlated positively with a platelet count greater than $400 \times 10^9/l$ (p=0.02) and haemoglobin less than 14g/dl (p=0.01); when analysed as continuous variables, the trend remained with lower haemoglobin but statistical significance was lost (p=0.06, Table 5.3.) High Chalkley counts were also associated with TN when expressed both as a continuous variable (p=0.002) and when greater than the median value (p=0.03). In the cases with TN, the microvessel hotspots tended not to be situated immediately adjacent to the areas of TN (Figure 5.2). No relationships were seen with the other clinicopathological variables, such as performance status and the presence of weight loss or

Table 5.1: Level of agreement between the two observers scoring for Tumour Necrosis, before a consensus was reached with a conference microscope. Cohen's kappa = 0.845, p<0.001.

		Necrosis Score – Observer B				
		0	1	2	3	TOTAL
Necrosis	0	130	1	1	0	132
Score -	1	4	8	1	0	13
Observer	2	0	3	12	0	15
A	3	0	0	0	11	11
	TOTAL	134	12	14	11	171

Table 5.2: Correlations between tumour necrosis and categorical clinicopathological and biological variables

Factor	Categories	TN absent	TN present	χ^2 p value
Gender	Male	115	38	0.065
	female	17	1	
Chest pain	No	35	13	0.35
	Yes	90	23	
Weight loss	No	67	16	0.29
	Yes	56	20	
Cell type	Epithelioid	77	23	0.94
	Non-epithelioid	55	16	
Performance status	0	61	16	0.67
	1, 2	65	20	
White blood cells	<8.3 x 10^9/l	46	9	0.14
	>8.3 x 10^9/l	74	27	
Platelets	<400 x 10^9/l	80	17	0.023
	>400 x 10^9/l	40	20	
Haemoglobin	<14 g/dl	66	28	0.013
	>14 g/dl	55	8	
IMIG TNM stage	I	3	0	0.43
	II	2	0	
	III	13	8	
	IV	18	8	
CALGB prognostic group	Groups 1 and 2	26	7	0.24
	Groups 3 and 4	64	15	
	Groups 5 and 6	32	14	
EORTC prognostic group	Low risk	53	16	0.98
	High risk	70	21	
MVD	< median	44	9	0.027
	> median	49	26	

Table 5.3: Correlations between tumour necrosis and continuous clinicopathological and

biological variables (Student's t-test)

Factor	Units	Mean value (SD)		p
		TN absent	TN present	
Age	years	62.9 (9.7)	62.42 (9.9)	0.71
WBC	x 10^9/l	9.81 (4.0)	10.88 (3.68)	0.15
Platelets	x 10^9/l	378 (146)	439 (185)	0.038
Haemoglobin	g/dl	13.4 (2.1)	12.8 (2.0)	0.062
MVD	Number of vessels	23.8 (6.3)	28.3 (9.7)	0.002

Table 5.4: Univariate log rank and Cox regression analysis of tumour necrosis

Factor	Categories	n	Median Survival (days)	Log Rank p	Hazard Ratio	Hazard Ratio 95% Confidence Interval		p
Tumour Necrosis	Not present	120	249	0.0187	1			0.0201
	Present	34	158		1.63	1.08	2.45	
Necrosis Score	Grade 0	120	248	0.0318	1			0.0381
	Grade 1	13	120		1.84	1.03	3.31	
	Grade 2	12	183		1.17	0.61	2.25	
	Grade 3	9	114		2.31	1.11	4.81	
Necrosis Score	Grade 1	13	120	0.3432				0.3562
	Grade 2	12	183					
	Grade 3	9	114					

Figure 5.1: Hematoxylin and Eosin stained sections of epithelioid MM demonstrating: a) grade 1 TN; b) grade 2 TN; c) grade 3 TN (arrows)

a)
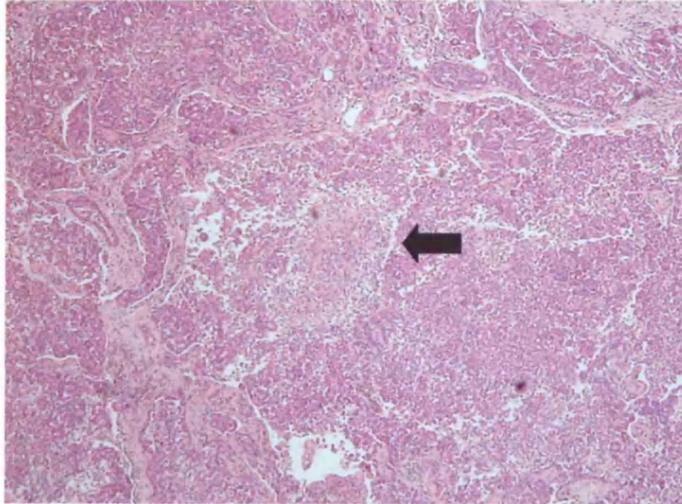
b)
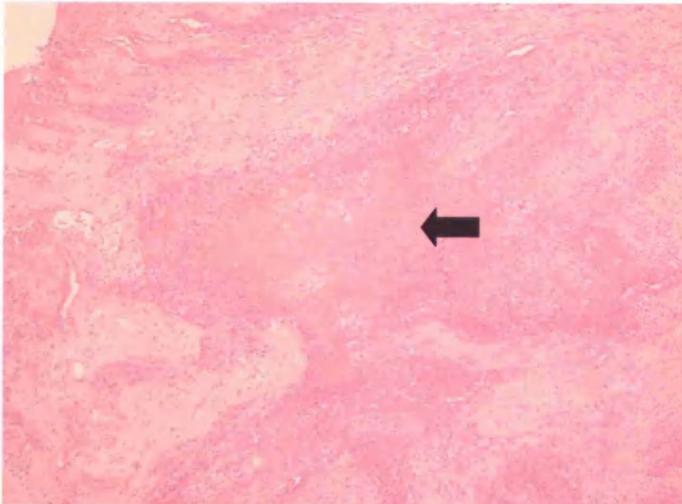
c)
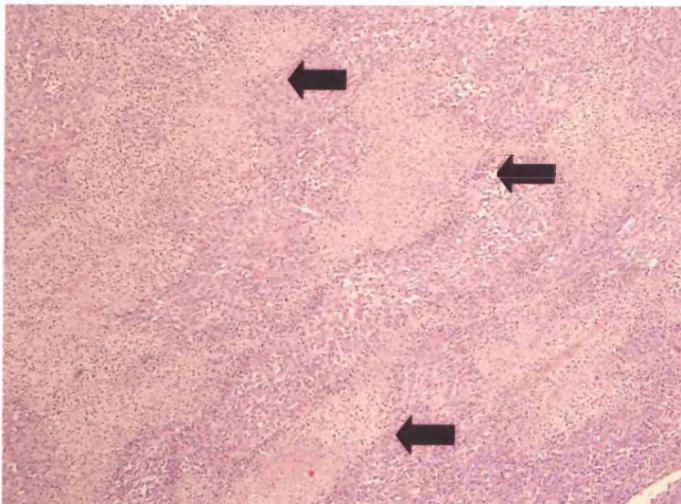
Figure 5.2: Correlation between angiogenesis and tumour necrosis. These photomicrographs are of anti-CD34 immunostained areas of the same tumour section.

a) At the border of the necrotic area (large arrow), there are few microvessels (small arrows).

b) However, away from the necrosis, there were many microvessels
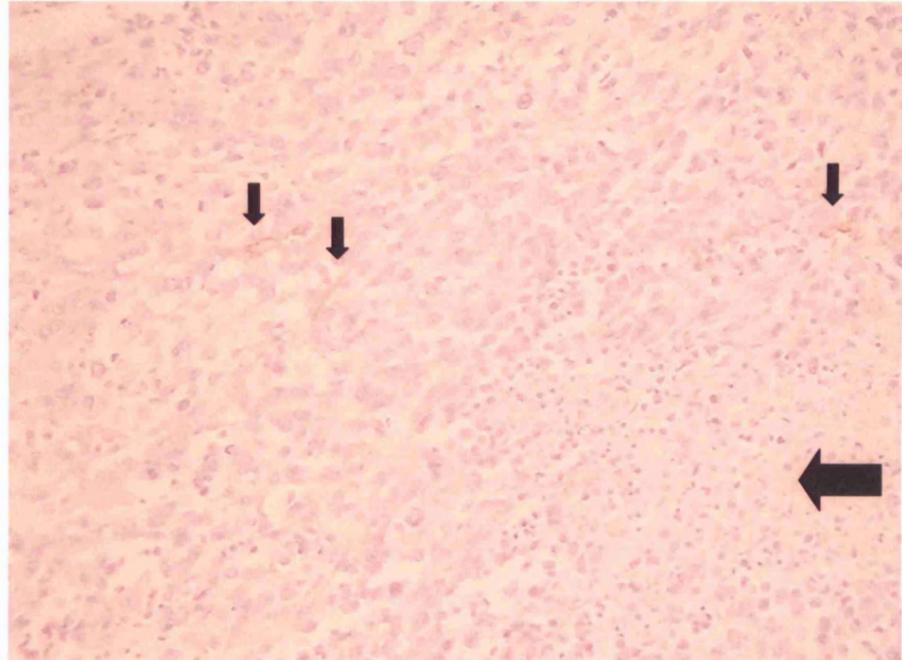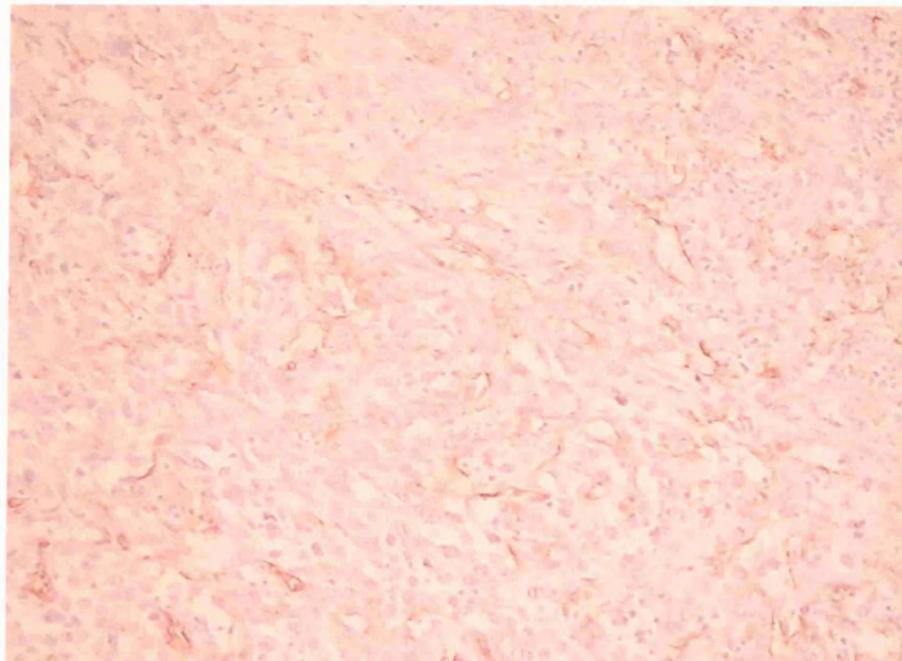
pleuritic chest pain. With regard to the cell type, 23 of the 100 epithelioid, 9 of the 38

(24%) biphasic and 7 of the 30 (23%) sarcomatoid tumours displayed TN (p=0.91). There

was no significant difference in the incidence of TN in patients undergoing surgical biopsy

only, compared to those who had resection of tumour by palliative debulking surgery or

radical surgery (p=0.6). There was no association between the presence of TN and the

IMIG TNM stage of the tumour (p=0.43). The grade of TN had no significant impact on

any of the above relationships.

5.4.3. Survival

As described previously, 17 cases who died within 30 days of surgery, were excluded

from survival analysis. The proportion of patients who had TN in this group was similar to

that in the other 154 patients (p=0.5). The presence of any grade of TN was associated with

a worse survival (p=0.0187, Log Rank, Figure 5.3, Table 5.4). Although, when assessing

the three grades of TN together with its absence with the Log Rank test, increasing grade

was associate with worse survival (p=0.0318, Figure 5.4a), there was no difference in

survival between the three grades of TN (p=0.34, Figure 5.4b, Table 5.4). The grades of

TN were thus combined into a single category for the following analyses. The overall

median survival for the 120 cases without TN living longer than 30 days was 249 days,

whereas it was 158 days for the 34 included cases with TN (p=0.0187, Log Rank). Six and

twelve month survival rates were 62.1% (95% confidence interval (CI) 53.4% - 70.9%) and

34.5% (25.8% - 43.3%) respectively for patients without TN, but 47.1% (30.3% - 63.8%)

and 15.9% (3.3% - 28.6%) for those with TN.

Figure 5.3: Kaplan-Meier plot showing that the presence of tumour necrosis was associated with a poor survival (p=0.0187)
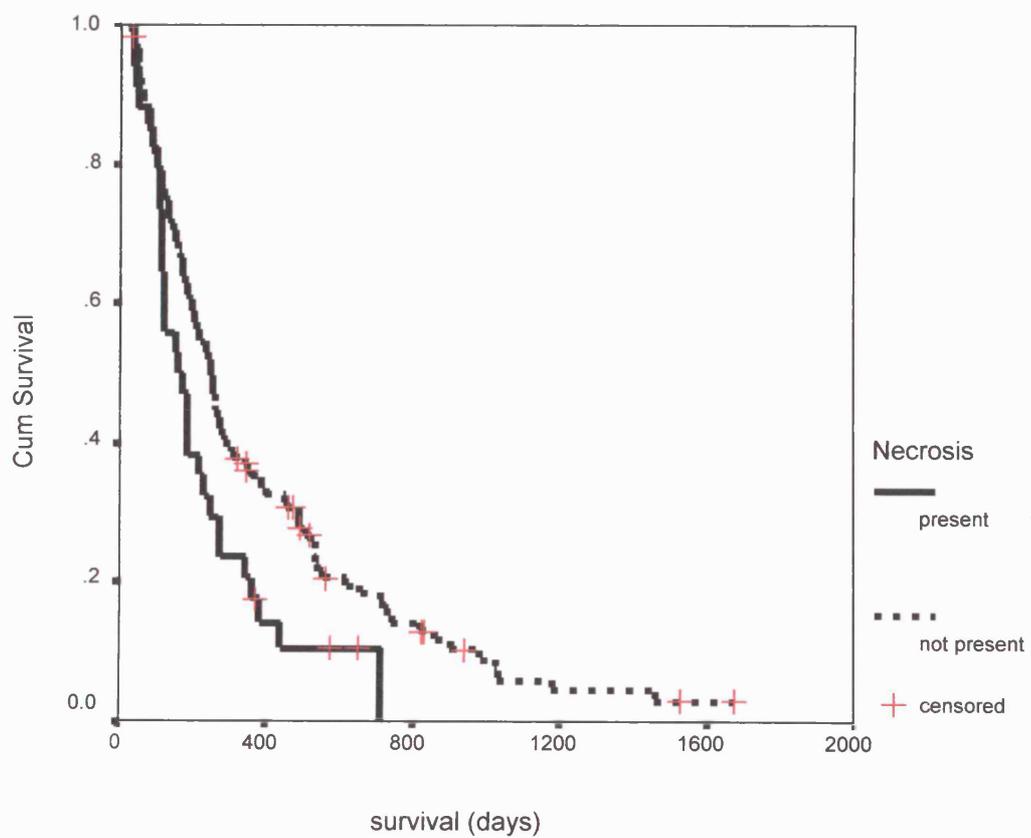
Figure 5.4: Kaplan-Meier plots showing the effects of grade of tumour necrosis on survival. Although a) when evaluating the absence of TN against all three TN grades combined, there was a significant difference in survival (p=0.03), there was no difference in survival b) between the three grades of necrosis alone (p=0.34)

a)

b)
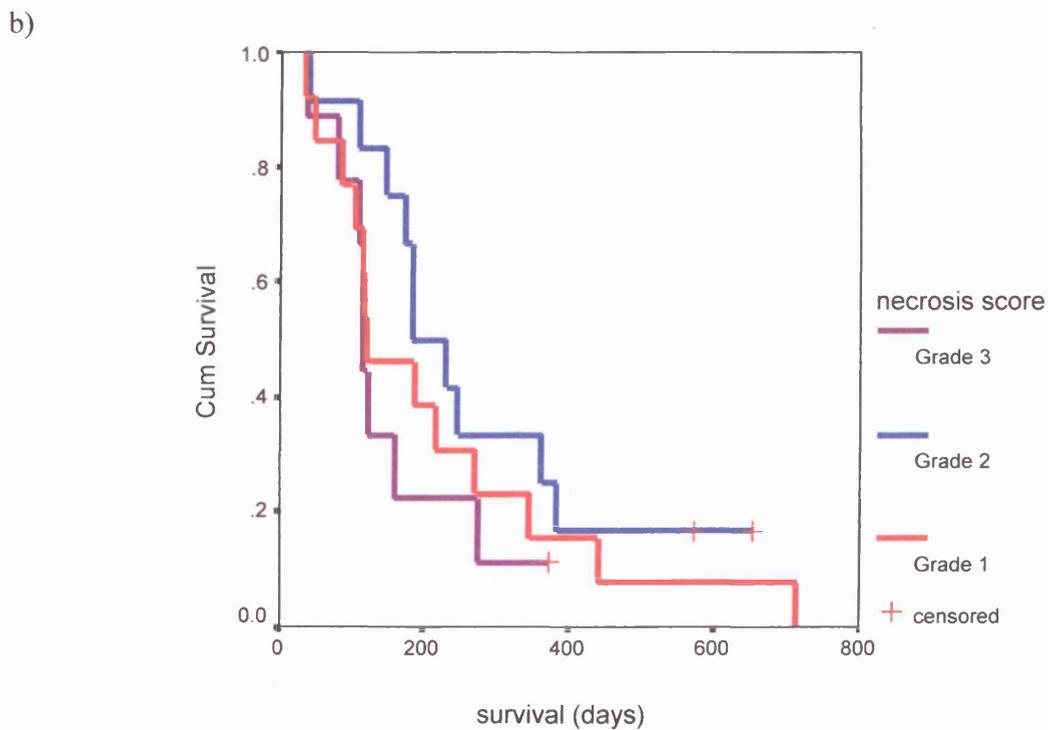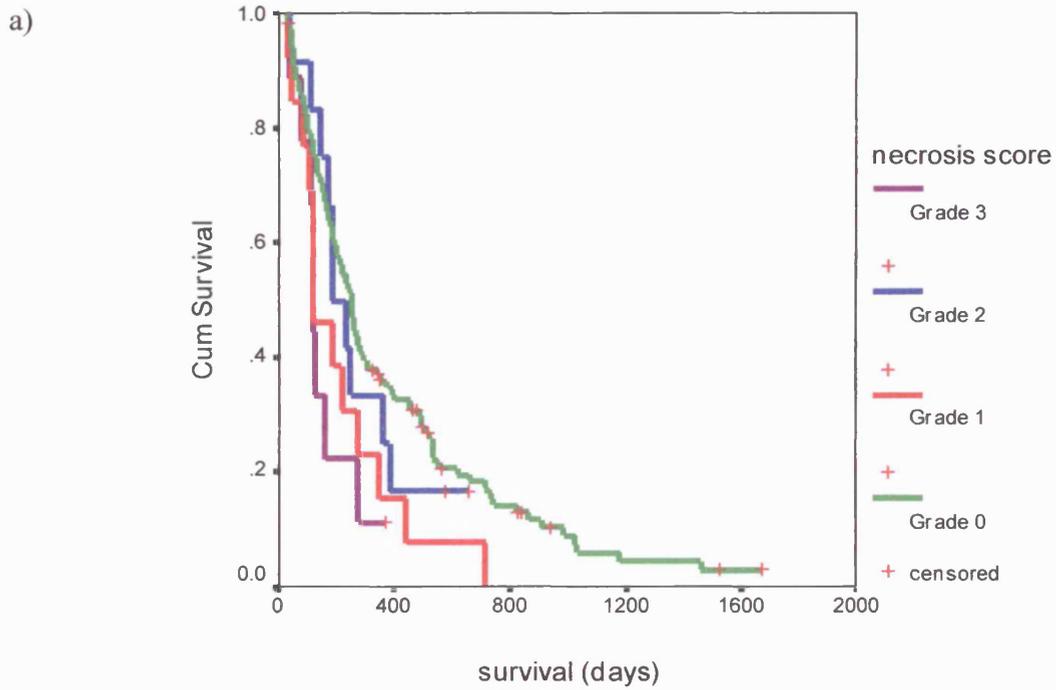
When analysing the epithelioid cases alone with the log rank test, there was a significant difference in survival between those with and without TN (p=0.0004, Figure 5.5). The median survival in the 70 epithelioid cases without TN surviving longer than 30 days was 384 days; it was 158 days in the 22 epithelioid cases with TN.

5.4.4. Multivariate analysis

In multivariate analysis, cell type, performance status, weight loss and the Chalkley microvessel count remained the independent predictors of poor survival (Table 5.5). TN was not retained in the statistical model, even when the Chalkley count was not analysed. However, the presence of TN contributed to both the CALGB and EORTC prognostic scoring systems, when analysed with each, in turn, in Cox multivariate analysis (Table 5.6).

5.5. Discussion

This study has characterised, for the first time, TN in a large series of MM and demonstrates that the presence of TN is associated with poor survival. The correlation between TN and survival is particularly marked in the epithelioid cases. This implies a relationship where increased tumour cell death indicates a more MM phenotype. It is possible to explain this, apparently paradoxical, relationship by rapid tumour growth outstripping the vascular supply, causing ischaemic damage to the microvasculature and thereby increased TN   (Shimizu *et al.* 1996; Ausprunk *et al.* 1999).

The association of TN and MVD is consistent with studies in breast cancer (Leek *et al.* 1999; Jitsuiki *et al* 1999). TN, and the surrounding zone of hypoxia, results in the attraction of an inflammatory infiltrate, in which macrophages are prominent (Crowther *et*

Table 5.5: Cox multivariate regression analysis. The prognostic variables identified in univariate analysis in Chapter 3 were entered into a forward, stepwise logistic regression model together with Tumour Necrosis (present or absent). The MVD has also been included in the second table. With the exclusion of cases with missing data, n=138 in the first analysis and n=105 in the second.

Variable	p value	Hazard Ratio	Hazard Ratio 95% Confidence Interval	
Non-epithelioid cell type	<0.0001	2.37	1.62	3.46
ECOG Performance Status >0	0.0169	1.57	1.08	2.27
Weight loss > 5%	0.0011	1.87	1.29	2.72
Haemoglobin <14g/dl	0.0731			
Tumour Necrosis	0.1767			
Chest Pain	0.3418			
No radical surgery	0.3869			
Platelets > 400 x10^9/l	0.5774			

Variable	p value	Hazard Ratio	Hazard Ratio 95% Confidence Interval	
Non-epithelioid cell type	0.0007	2.14	1.38	3.31
MVD > median	0.0294	1.67	1.05	2.66
ECOG performance status >0	0.0376	1.58	1.03	2.44
Weight loss > 5%	0.0390	1.58	1.02	2.43
Haemoglobin <14g/dl	0.1216			
Chest Pain	0.1672			
No radical surgery	0.2011			
Tumour Necrosis	0.5439			
Platelets > 400 x10^9/l	0.5867			

Table 5.6: Cox multivariate regression analysis. The presence of Tumour Necrosis was tested, in turn, against the CALGB and EORTC prognostic scoring systems.

Variable	Category	n	p	Hazard Ratio	Hazard Ratio 95% Confidence Interval	
Tumour Necrosis	Absent	112	0.0229	1		
	Present	33		1.63	1.07	2.48
CALGB groups	Groups 1 and 2	32	<0.0001	1		
	Groups 3 and 4	75		2.16	1.34	3.47
	Groups 5 and 6	38		4.31	2.52	7.38

Variable	Category	n	p	Hazard Ratio	Hazard Ratio 95% Confidence Interval	
Tumour Necrosis	Absent	112	0.0366	1		
	Present	33		1.56	1.03	2.37
EORTC groups	Low risk	66	0.0004	1		
	High risk	79		1.90	1.34	2.71

Figure 5.5: Kaplan-Meier plot showing the effects of grade of tumour necrosis on survival amongst the epithelioid cases only (p=0.0004).
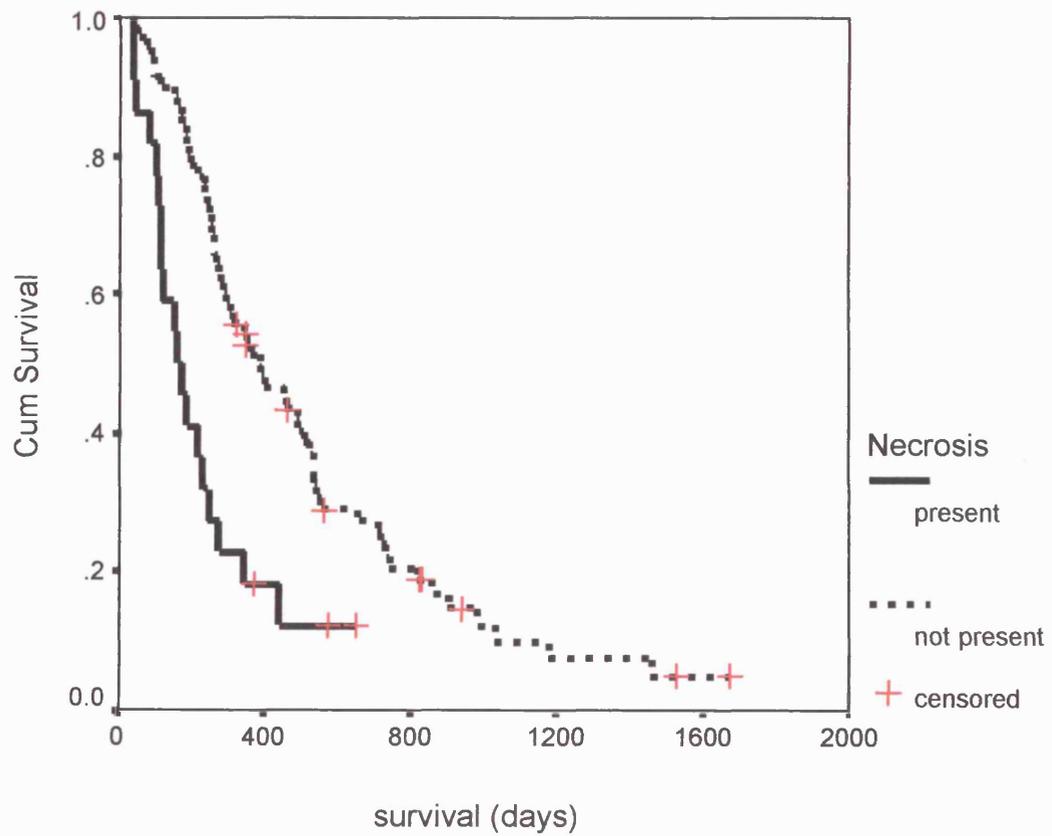
al. 2001; Lee *et al.* 1997; Leek *et al.* 1999). Macrophages may play a central role in

angiogenesis through the secretion of pro-angiogenic factors such as VEGF, TNF-α, IL-8,

and bFGF (Ono *et al.* 1999). These factors, following macrophage recruitment, stimulate

angiogenesis in the remaining viable tumour. In breast cancer, microvessel hotspots were

situated away from areas of TN (Leek *et al* 1999), as was found in this study. In keeping

with this, in a further study in breast cancer, MVD increased from the central though the

intermediate to the peripheral zone of the tumour (Jitsuiki *et al.* 1999).

There were also associations with high platelet counts and low haemoglobin

concentrations, both of which were poor prognostic factors in univariate analysis.

Thrombocytosis is associated with a poor outcome in other solid tumours, such as NSCLC

(Cox *et al.* 2000; O'Byrne *et al.* 2000), in which we have also found a correlation between

TN and platelet count (Swinson *et al.* 2002). The association between platelet count and

TN may simply reflect the fact that both are seen in tumours with an aggressive phenotype.

It is possible, however, to form a hypothesis that thrombocytosis, angiogenesis and tumour

necrosis are linked mechanistically. Vascular stasis, exposure of subendothelial collagen and

increased expression of platelet binding factors may each lead to increased platelet adhesion

and activation in malignant disease. Platelet adhesion and activation may result in the

release of angiogenic growth factors, such as VEGF and platelet-derived endothelial cell

growth factor, but also the formation of platelet plugs that cause coagulative necrosis. This

sequence of events may be more likely to occur if the platelet count is elevated (Pinedo *et al.*

1998; O'Byrne *et al.* 1999). TN may also result in thrombocytosis through the release of

cytokines such as IL-6 from activated infiltrating inflammatory cells. IL-6 is a potent

stimulator of platelet synthesis, is upregulated by hypoxia (Yan *et al.* 1995), is angiogenic

(Motro *et al.* 1990; Cohen *et al.* 1996) and is expressed in MM (Schmitter *et al.* 1992; Nakano *et al.* 1998).

TN was not an independent prognostic factor when compared in multivariate analysis, which may be due both to the relatively low proportion of cases with TN, when compared to the incidences of the other factors and to the associations between TN and other prognostic variables. However, TN did contribute to both the CALGB and EORTC prognostic scoring systems in multivariate analysis.

A possible confounding association between necrosis and tumour mass could not be assessed in this study. Increased tumour volumes are associated with worse survival in MM (Pass *et al.* 1998). More bulky tumours might be expected to demonstrate more extensive TN, but there was no correlation between TN and IMIG TNM stage. It should be noted, however, that the IMIG T stage does not take account of tumour bulk, but is based on invasion of intrathoracic tissues and structures (Rusch 1995). In NSCLC, we found that TN correlated with increasing T stage (Swinson *et al.* 2002). However, TN remained a prognostic factor in a large series of stage IA NSCLC (Goldstein *et al.* 1999). In breast cancer, TN correlated with increasing tumour size (Leek *et al.* 1999), whereas in other tumours, an association between T stage and necrosis remains unclear. Increasing mass was associated with hypoxia in an experimental murine allograft model (De Jaeger *et al.* 1998).

The subjective assessment of TN used resulted in a high agreement between the observers and expert adjudication was required only for a few cases. Similar subjective methods of TN assessment have been described and validated (Leek *et al.* 1999; Swinson *et*

al. 2002). In this study the grade of TN was not significant with respect to either clinicopathologic correlations or survival. It is therefore proposed that assessment of the presence of any TN is sufficient for prognostic purposes in MM, rather than evaluation of the extent of TN in the tumour. The proportion of patients with TN detected from biopsy specimens was similar to that from those who had underwent palliative or radical tumour resection. It is important to note that the biopsy specimens were not from closed (needle) procedures but were large surgical biopsies from either open or video assisted thoracoscopic surgery. Slides from all the pathological diagnostic tissue blocks were reviewed. These observations support the contention that sampling error in our assessment of the clinical relevance of TN is unlikely.

5.6. Conclusions

In conclusion, the presence of TN is a poor prognostic factor in MM. The methodology adopted does not require immunohistochemistry and is reproducible between observers. The positive correlations of TN with angiogenesis and thrombocytosis support evidence that these processes are related and confirm that TN is part of an aggressive tumour phenotype in MM. The presence of TN in patients with MM who are resistant to radiotherapy and chemotherapy deserves further investigation, which was beyond the scope of this initial study.

Chapter Six

Epidermal Growth Factor Receptor

6.1. Introduction

Epidermal Growth Factor Receptor (EGFR) is one of the ErbB family of receptor tyrosine kinases. These are cell membrane receptors which play a central role in cell proliferation, differentiation, migration, adhesion and survival (Yarden 2001). EGFR expression by tumours may contribute to the growth and invasive potential of malignant cells (Walker 1998). EGFR has been linked to angiogenesis via the induction of Vascular Endothelial Growth Factor (Petit *et al.* 1997). Targeting EGFR with selective tyrosine kinase inhibitors or anti-EGFR antibodies results in suppression of tumour growth not only through direct anti-proliferative effects on EGFR positive tumour cells but also by inhibiting angiogenesis. EGFR targeted therapies have been demonstrated to cause both a reduction of pro-angiogenic growth factor production and apoptosis of endothelial cells (Ciardiello *et al.* 2001; Bruns *et al.* 2000; Hirata *et al.* 2002; Bruns *et al.* 2000).

Three previous studies have evaluated EGFR expression in MM and included between 24 and 34 patients. EGFR immunoreactivity was seen in between 58 and 100% of cases, being detected in cytoplasmic and/or membranous compartments (Dazzi *et al.* 1990; Ramael *et al.* 1991; Govindan *et al.* 2001).

6.2. Aims

1   To evaluate EGFR immunostaining in MM

2   To correlate EGFR status with clinicopathological and biological variables

3   To evaluate the prognostic impact of EGFR status in univariate and multivariate models

6.3. Methods

6.3.1. Immunohistochemistry

Immunohistochemistry was performed with the anti-EGFR mouse monoclonal primary antibody EGFR.113 (Novocastra Laboratories Ltd, Newcastle, UK) as described in the Section 2.3.3.4. Positive controls included in each run were a specimen of non-small cell lung cancer which was known to be positive for EGFR (Cox *et al.* 2000) and five EGFR positive MM samples of varying intensity. Negative controls had the primary antibody omitted.

6.3.2. Interpretation

Sections were inspected at up to x400 magnification using light microscopy by two independent observers, blinded to clinicopathological data and outcome. Presence of cytoplasmic and/or membranous EGFR expression was noted, where present in at least 5% of tumour cells. In those cases where the observers differed in their assessment of EGFR expression, a consensus was determined using a dual headed microscope. The distribution of staining between different cell type elements in the biphasic cases was also noted.

6.3.3. Statistical Analysis

The Chi-squared test was used to analyse the correlation of EGFR expression with categorical variables and Student's t-test for continuous variables. Haematological indices were analysed as both continuous and categorical variables, the cut-points for the latter being based on the CALGB and EORTC series. Survival curves were estimated using the Kaplan-Meier method and the log-rank test was used to assess the statistical significance of differences between groups. Cox proportional hazards models were used to identify

statistically significant differences in survival and estimate hazard ratios and 95% Confidence

Intervals (CI).

6.4. Results

6.4.1. Immunohistochemistry

EGFR immunostaining (Figure 6.1) was identified in 74 cases out of 168 (44%).

The pattern was membranous staining only in 10 cases, cytoplasmic only in 28 and both

membranous and cytoplasmic in 36 cases (Table 6.1).

6.4.2. Correlation with clinicopathological and biological factors

The relationships of EGFR immunostaining and clinicopathological variables are seen

in Tables 6.2 and 6.3. EGFR immunostaining was associated with the absence of chest pain,

maintenance of weight, epithelioid cell type, and ECOG PS of 0. EGFR expression

correlated with the low risk CALGB and EORTC prognostic groups. Surgical biopsy alone

was performed in 62 patients and palliative debulking surgery, radical pleurectomy /

decortication and extrapleural pneumonectomy in 71, 7 and 28 patients respectively.

Amongst the patients who underwent radical surgery, there were no associations between

EGFR immunostaining and nodal metastasis. Of the 52 (31%) patients in whom it was

possible to derive a pathological IMIG TNM stage, 3 patients had Stage I, 2 Stage II, 21

Stage III and 26 Stage IV disease. Stage I-III disease was associated with cytoplasmic

EGFR expression (p=0.01) and EGFR positivity (cytoplasmic and/or membranous, p=0.05)

but not membranous EGFR immunostaining alone (p=0.6). There was no correlation

between EGFR status and MVD in the 128 cases for which it was possible to derive the

Table 6.1 : Distribution of cytoplasmic versus membranous EGFR staining in MM.

		Membranous		
		Negative	Positive	Total
Cytoplasmic	Negative	94	10	104
	Positive	28	36	64
	Total	122	46	168

Table 6.2: Correlations between EGFR immunostaining and categorical clinicopathological and biological variables

Variable	Categories	EGFR negative	EGFR positive	χ^2 p value
Gender	Male	85	66	0.792
	female	9	8	
Chest pain	No	16	32	<0.0001
	Yes	73	38	
Weight loss	No	40	42	0.055
	Yes	48	27	
Asbestos exposure	No	14	12	0.995
	Yes	62	53	
Cell type	Epithelioid	40	58	<0.0001
	Non-epithelioid	54	16	
	Epithelioid	40	58	<0.0001
	Biphasic	24	13	
	Sarcomatoid	27	3	
	Desmoplastic	3	0	
Performance status	0	28	46	<0.0001
	1, 2	62	24	
WBC	=< 8.3 x10^9/l	30	22	0.742
	> 8.3 x10^9/l	56	46	
Platelets	< 400 x10^9/l	50	44	0.407
	> 400 x10^9/l	36	24	
Haemoglobin	< 14 g/dl	58	37	0.12
	> 14 g/dl	29	31	
Necrosis	None	80	48	0.004
	Present	14	25	
MVD	< median	28	25	0.337
	> median	46	29	
CALGB prognostic group	Groups 1 and 2	10	23	0.001
	Groups 3 and 4	44	32	
	Groups 5 and 6	34	13	
EORTC prognostic group	Low risk	25	41	<0.0001
	High risk	63	28	

Table 6.3: Correlations between EGFR immunostaining and continuous clinicopathological

and biological variables using Student's t-test.

Variable	Units	Mean value (SD)		p
		EGFR negative	EGFR positive	
Age	years	62.8 (10.64)	62.8 (8.83)	0.97
WBC	x 10^9/l	10.19 (4.3)	10.04 (3.4)	0.82
Platelets	x 10^9/l	400 (156)	383 (156)	0.50
Haemoglobin	g/dl	12.98 (2.1)	13.63 (2.1)	0.061
MVD	number	25.1 (7.27)	24.4 (8.17)	0.64
COX-2	densitometry units	54800 (61900)	83900 (103900)	0.26

Figure 6.1: EGFR immunohistochemistry in MM. Strong membranous and weaker cytoplasmic immunostaining in epithelioid MM at a) high and b) low power. Figure 6.1b represents the same field as Figure 4.7)

a)
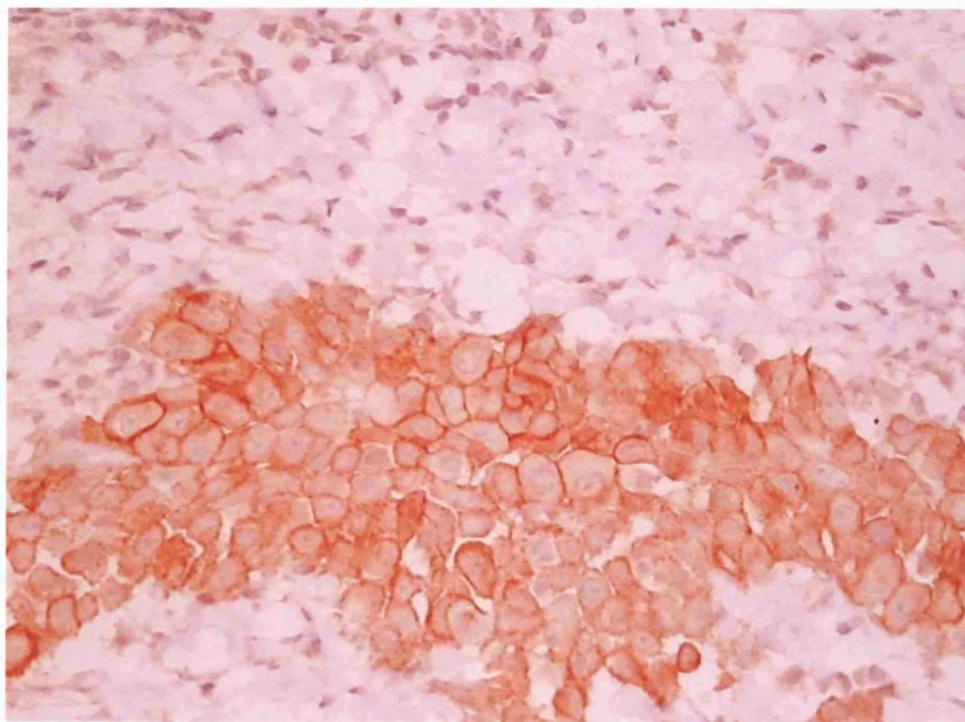
b)
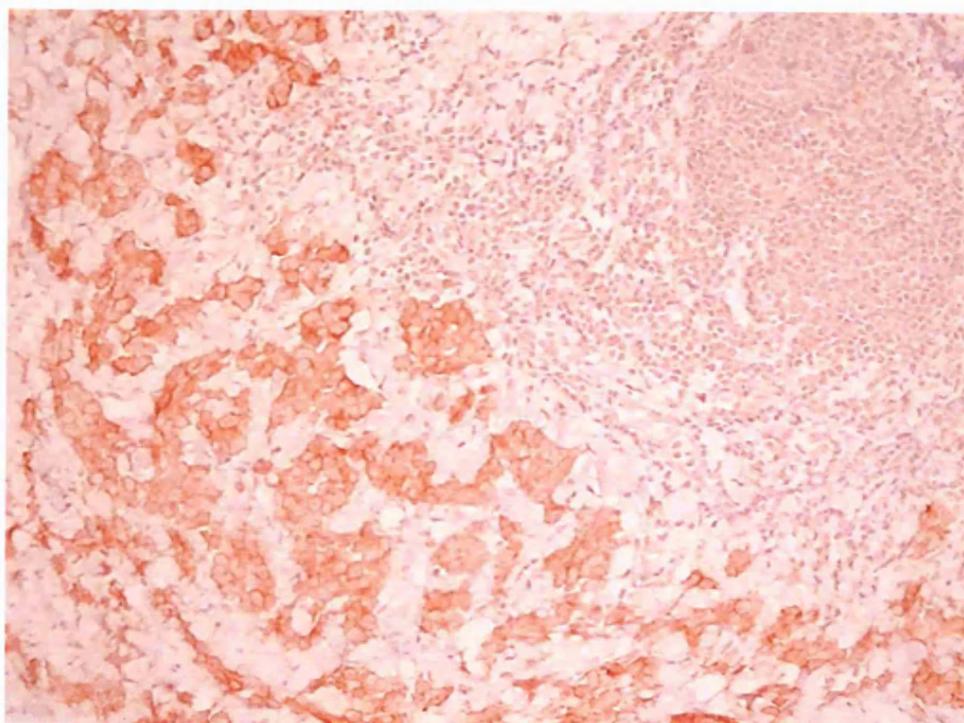
Figure 6.1: EGFR immunohistochemistry in MM:

c) weakly positive cytoplasmic and membranous EGFR immunostaining in epithelioid MM
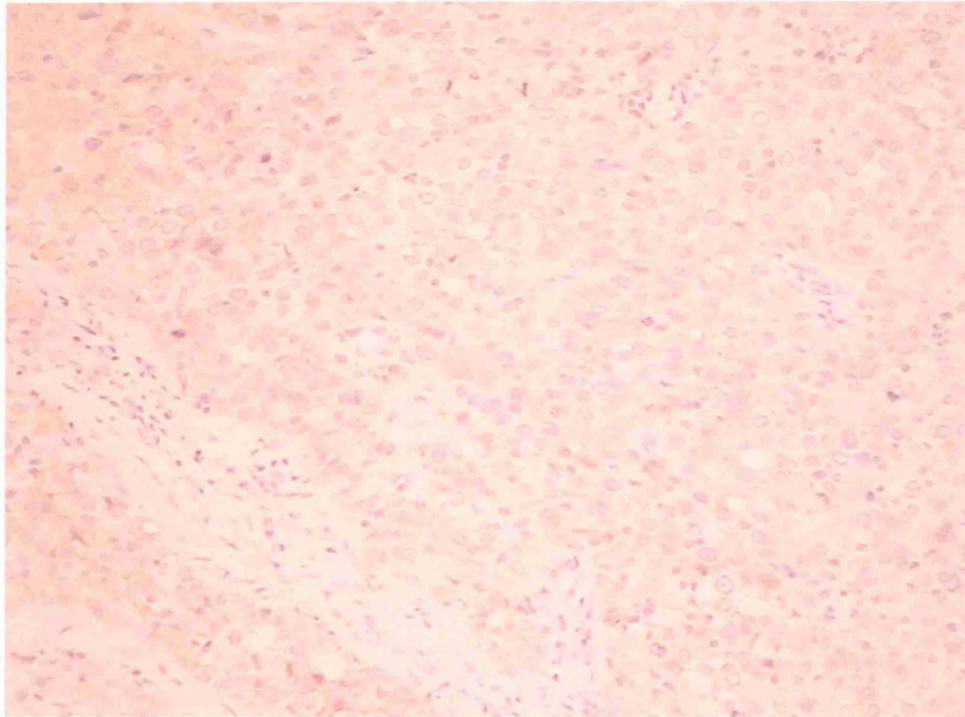
d) EGFR immunostaining in both epithelioid (large arrow) and sarcomatoid (small arrow) elements of a biphasic MM
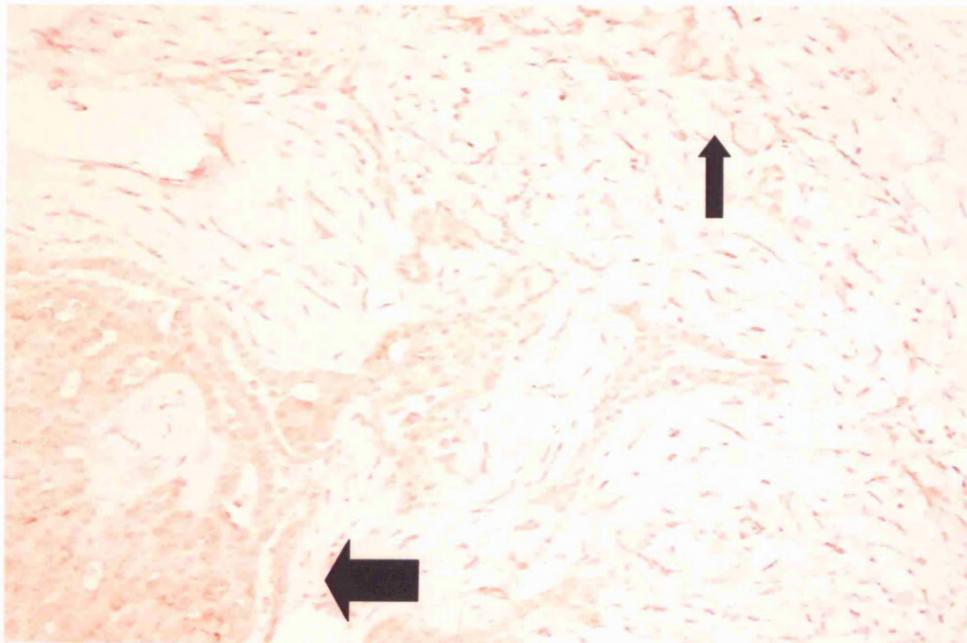
Figure 6.1e: Sarcomatoid MM with positive EGFR immunostaining
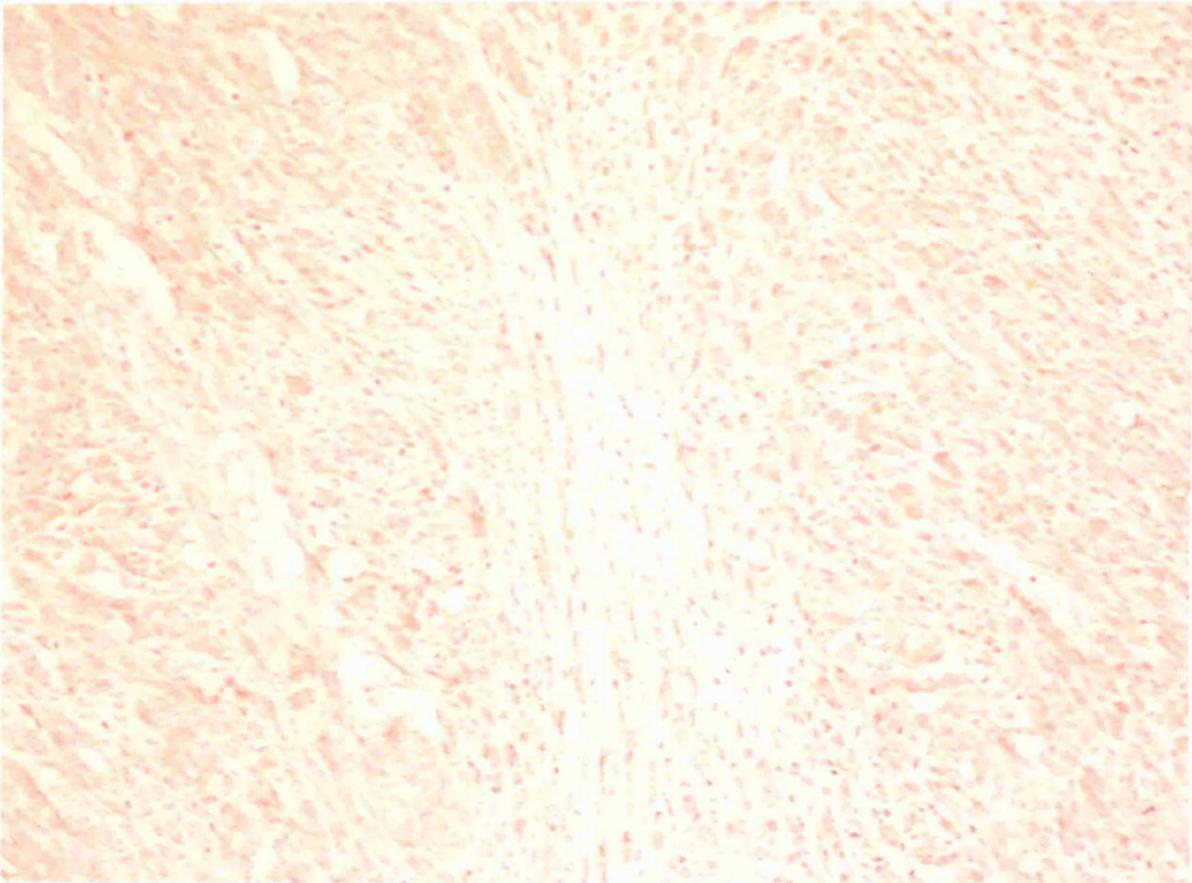
latter. EGFR expression was more likely in tumours with necrosis (p=0.004), although a

perinecrotic pattern of immunostaining was not seen.

6.4.3. Survival

The median survival for all 168 cases was 185 days. However, 17 cases died within

30 days of the date of diagnostic biopsy and were excluded from further survival analyses to

avoid bias from postoperative death.

EGFR immunostaining was associated with a favourable prognosis (p=0.001, log

rank, Figure 6.2, Table 6.4). The overall median survival for the 81 cases without EGFR

immunostaining was 172 days, whereas for the 70 cases with EGFR positivity it was 269

days. Six and twelve month survival rates were 71.0% (95% confidence interval 60.3% -

81.7%) and 41.9% (30.2% - 53.6%) for those with EGFR positivity compared to 47.5%

(36.6% - 58.5%) and 21.1% (12.1% - 30.0%) respectively for patients without EGFR

immunostaining. There were no differences in survival for EGFR positive cases according

to the microscopic pattern of staining (membranous vs. cytoplasmic vs. mixed, p=0.21). In

univariate Cox proportional hazards analysis, negative EGFR expression conferred a hazard

ratio of 1.8 (95% CIs 1.3 – 2.5, p=0.001).

When analysing the epithelioid cases alone with the log rank test, there was no

significant difference in survival between EGFR positive and negative cases (p=0.29). There

was, however, a trend towards epithelioid cases with *cytoplasmic* EGFR immunostaining

having a better outcome than the cases without cytoplasmic staining (p=0.06). Amongst the

non-epithelioid cases, EGFR status was prognostic but this was due to the higher rate of

Table 6.4: Univariate log rank and Cox regression analysis of EGFR expression (n=151).

Variable	Categories	n	Median Survival (days)	Log Rank p	Hazard Ratio	Hazard Ratio 95% CI		Cox p
EGFR	Positive	70	269	0.0012	1			0.0014
	Negative	81	172		1.77	1.25	2.51	

Table 6.5: Cox multivariate regression analysis. The prognostic variables identified in univariate analysis in Chapter 3 were entered into a forward, stepwise logistic regression model together with EGFR expression (present or absent). The MVD and Tumour Necrosis have also been included in the second table. With the exclusion of cases with missing data, n=135 in the first analysis and n=105 in the second.

Variable	p value	Hazard Ratio	Hazard Ratio 95% Confidence Interval	
Non-epithelioid cell type	<0.0001	2.32	1.58	3.40
Weight loss > 5%	0.0007	1.94	1.33	2.85
ECOG Performance Status >0	0.0465	1.46	1.01	2.13
Haemoglobin <14g/dl	0.0987			
EGFR	0.3837			
Chest Pain	0.2035			
No radical surgery	0.2976			
Platelets > 400 x10^9/l	0.6470			

Variable	p value	Hazard Ratio	Hazard Ratio 95% Confidence Interval	
Non-epithelioid cell type	0.0007	2.14	1.38	3.31
MVD > median	0.0294	1.67	1.05	2.66
ECOG performance status >0	0.0376	1.58	1.03	2.44
Weight loss > 5%	0.0390	1.58	1.02	2.43
Haemoglobin <14g/dl	0.1216			
Chest Pain	0.1672			
No radical surgery	0.2011			
Tumour Necrosis	0.5439			
Platelets > 400 x10^9/l	0.5867			
EGFR	0.5291			

Figure 6.2: Kaplan-Meier plot showing that EGFR expression was associated with a good prognosis (p=0.001)
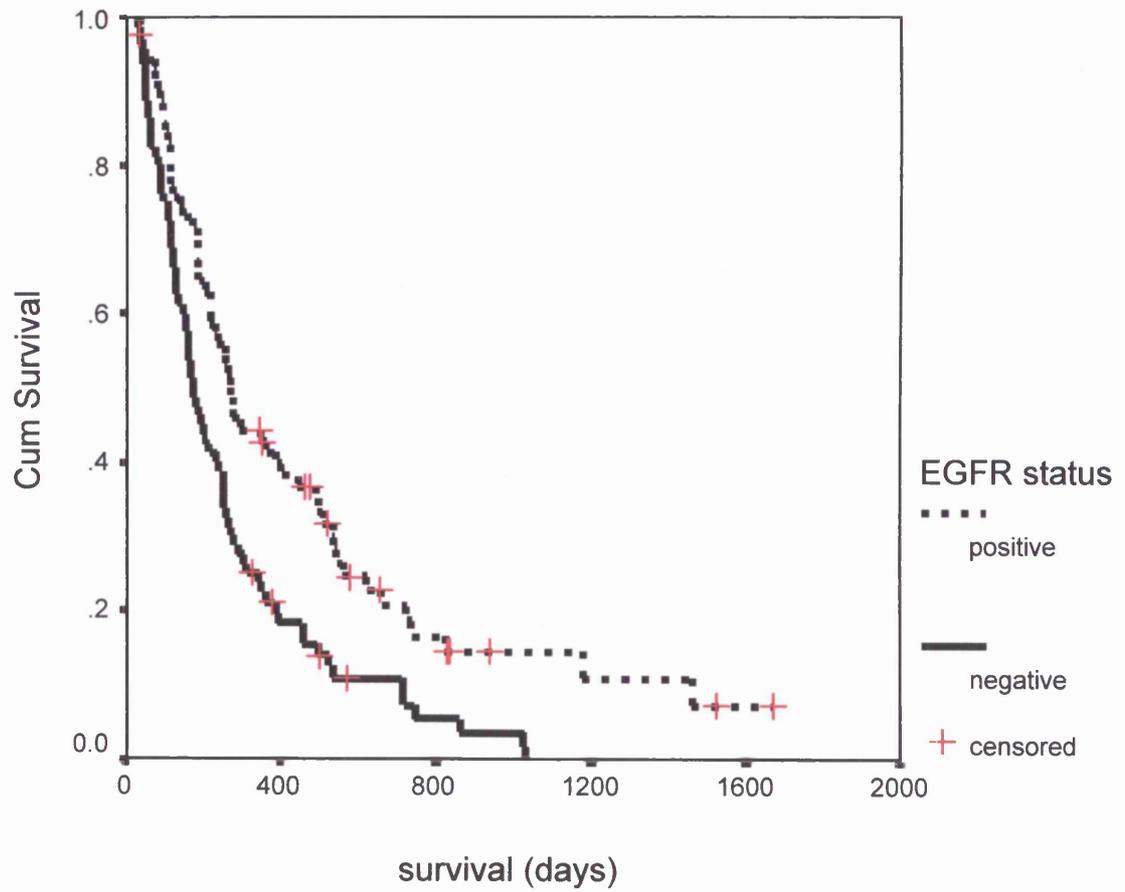
expression in biphasic than in sarcomatoid tumours. Separate multivariate analyses were not performed in the epithelioid and non-epithelioid cell cohorts due to the small numbers remaining.

In addition to the absence of EGFR immunostaining, non-epithelioid cell type, the presence of weight loss > 5% or chest pain, ECOG performance status > 0, haemoglobin < 14g/dl, platelet count > $400x10^9$/L, high MVD and the presence of TN were all poor prognostic factors in univariate analysis. Furthermore, both the CALGB and EORTC prognostic scoring systems stratified each prognostic group to survival.

6.4.4. Multivariate Analysis

The variables entered into multivariate analysis were non-epithelioid cell type, the presence of weight loss >5% or chest pain, ECOG performance status >0, haemoglobin <14g/dl and platelet count >$400x10^9$/l, as identified in section 3.4.4. In multivariate analysis, cell type, weight loss, performance status were the independent predictors of poor survival (Table 6.5). Although the selected patients who received radical surgery had a significantly longer survival in univariate analysis (p=0.04), this was not a significant factor in subsequent multivariate analyses. Likewise, EGFR status was not retained in the statistical model. When the MVD and presence of TN were added to the multivariate model, cell type, weight loss and performance status retained their independent status with MVD adding to the model (as characterised in Section 4.4.4). Neither EGFR expression nor the presence of TN were independent prognostic factors.

However, EGFR expression did contribute to both the CALGB and EORTC prognostic scoring systems, when analysed with each, in turn, in Cox multivariate analysis (Table 6.6).

6.5. Discussion

EGFR immunoreactivity may reflect gene amplification, upregulated transcription, increased translation and/or reduced degradation of the protein, all of which may be present in tumours (Arteaga 2001; Slichenmyer and Fry 2001; Yarden 2001; Gill *et al.* 1987). Assessment of EGFR protein expression by immunohistochemistry, rather than by Western blotting or receptor binding assays, is probably the most appropriate technique for clinical use. Immunohistochemical assessment of EGFR has been shown to correlate well with ligand-binding assays in breast cancer (Newby *et al.* 1995) and enzyme-linked immunosorbent assays in non-small cell lung cancer (NSCLC) (Pfeiffer *et al.* 1998). The antibody that was used in this study recognises a polypeptide epitope of the EGFR extracellular domain (Fox *et al.* 1994b). Whether possible alterations in structure of the EGFR gene, that have been noted in some cases of MM (Ramael *et al.* 1993), resulting in a variant EGFR protein, are recognised by this antibody is unknown.

The 44% of cases with positive immunostaining in this series is smaller than previous reports of EGFR immunohistochemistry in MM. Dazzi et al. found EGFR expression (>5% cells) in 23 of 34 (68%) cases examined, with positivity more common in the epithelioid cell type (Dazzi *et al.* 1990). In the study by Ramael et al., all 32 tumours examined were positive for EGFR, although the intensity of staining was greater in epithelioid tumours. Finally Govindan et al. examined paraffin embedded sections of 24 cases of MM (21 pleural

Table 6.6: Multivariate Cox Proportional Hazards models: the absence of EGFR expression was a poor prognostic factor independent of both the CALGB (a) and EORTC (b) prognostic scoring systems. The six CALGB prognostic groups were combined as the numbers in the even-numbered groups were small.

a

Variable	category	HR	95% CIs	p
EGFR	Positive	1		0.0150
	Negative	1.60	1.10 – 2.33	
CALGB Groups	Groups 1 and 2	1		<0.0001
	Groups 3 and 4	2.21	1.36 – 3.58	
	Groups 5 and 6	3.66	2.11 – 6.35	

b

Factor	category	HR	95% CIs	p
EGFR	Positive	1		0.0036
	Negative	1.76	1.20 – 2.58	
EORTC Groups	Low risk	1		0.0103
	High risk	1.64	1.12 – 2.38	

MM) by immunohistochemistry and found EGFR expression in 14 (58%) (Govindan *et al.* 2001). The nuclear localisation of EGFR that was seen by Govindan et al. was not confirmed by this study. With regard to the other erbB family members, one early report has found expression of HER-2 / erbB-2 in 28 of 29 MM tumour samples (Thirkettle *et al.* 2000) but this was not confirmed by a second study (Govindan *et al.* 2001).

In contrast with studies in many other solid tumours in which EGFR immunopositivity has been implicated as a poor prognostic factor, Dazzi found that EGFR immunostaining was associated with a favourable outcome in MM (Dazzi *et al.* 1990). Increasingly, however, it is being recognised that the correlation between EGFR overexpression and poor survival in solid tumours is either absent or inconsistent between studies (Nicholson *et al.* 2001). Despite this, reports of EGFR being a favourable prognostic factor are rare. In this regard a study in oral carcinoma that demonstrated membranous EGFR expression to be associated with a favourable outcome similar to MM is noteworthy (Maiorano *et al.* 1998).

In this study, in general, the lack of EGFR immunoreactivtity correlated with predictors of poor prognosis, such as the presence of chest pain, weight loss and poor performance status. Like the previous studies, the proportion of epithelioid tumours staining positive for EGFR was higher than for biphasic and sarcomatoid cell types. Dazzi et al. found that, after the cell type was taken into consideration, lack of EGFR immunostaining was no longer significant (Dazzi *et al.* 1990). The inverse correlation between EGFR status and survival may therefore be explained, at least partly, by the differences in expression between the epithelioid and sarcomatoid cell types, the association

of the latter with poor prognosis being well established. Although differences in expression

of growth factors and proliferation markers between MM cell types have been described,

still little is known about the differences in tumour biology of sarcomatoid and epithelioid

MM in terms of patterns of gene and protein expression.

With regard to angiogenesis, these results are in keeping with other studies in which

no correlation was found between EGFR and microvessel counts in breast cancer (Fox *et al.*

1994a) or NSCLC (Giatromanolaki *et al.* 1996). EGFR positive tumours were more likely

to display tumour necrosis, although there was no spatial relationship between areas of

necrosis and EGFR positive tumour cells. Our group did not find any such relationship in

NSCLC (Swinson *et al.* 2002). Reports linking EGFR expression with hypoxia and/or

necrosis in tumour samples are lacking, although increased EGFR expression by squamous

carcinoma cells was noted in hypoxic conditions *in vitro* (Laderoute *et al.* 1992).

Stage I-III MM (combined) had a significantly greater proportion of cases positive

for EGFR than Stage IV, this being particularly the case for cytoplasmic expression. This

contrasts with NSCLC where EGFR overexpression has been reported to be associated with

advanced tumour stage (Veale *et al.* 1987) although this is far from a consistent finding

(Fontanini *et al.* 1995; Fontanini *et al.* 1998; Dazzi *et al.* 1989). EGFR expression was also

not related to stage in breast cancer (Gasparini *et al.* 1992; Fox *et al.* 1994b).

That EGFR positivity is a good prognostic factor does not detract from the fact that

EGFR may be a novel therapeutic target in MM. In breast cancer, oestrogen receptor status

is well recognised as a good prognostic factor and yet the receptor is an effective target of

anti-hormone therapies such as tamoxifen (Early Breast Cancer Trialists' Group 1998).

EGFR has been the subject of much interest in the last few years as a target for selective

EGFR tyrosine kinase inhibitors (TKIs) and anti-EGFR monoclonal antibodies (Mendelsohn

and Baselga 2000; Huang and Harari 1999). EGFR expression has also been noted in MM

cell lines. There is accumulating evidence that, despite being a favourable prognostic

variable, EGFR forms part of an autocrine growth loop in MM cells (Morocz *et al.* 1994).

Furthermore, serum levels of the ligand EGF are known to be higher in patients with MM

than healthy controls (Betta *et al.* 2002). A recent report has demonstrated that treatment

of MM cell lines with the selective EGFR TKI ZD1839 (AstraZeneca) results in a significant

dose-dependent reduction of colony formation when the cells are grown in soft agarose

(Janne *et al.* 2002).

6.6. Conclusions

In the largest study to date, it was found that EGFR expression is a good prognostic

factor in MM that contributes to both the CALGB and EORTC prognostic scoring systems.

Given that ligand-bound EGFR induces receptor dimerisation (with either EGFR itself or

one of HER2-4) and cross-phosphorylation before activation of cell signalling (Yarden

2001), further study of the other EGFR family members, as well as their ligands, is

warranted in MM. The recent development of EGFR targeted therapies with demonstrable

in vitro anti-tumour activity in MM provides a potential new approach for the management

of patients afflicted by a tumour which remains relatively resistant to conventional treatment

modalities.

Chapter Seven

Cyclooxygenase-2

7.1. Introduction

Cyclooxygenases (COXs) catalyse the initial, rate-limiting steps of prostaglandin synthesis from arachidonic acid (Taketo 1998). There are two isoforms of COX. COX-1 is constitutively expressed and plays a key role in cytoprotection. In contrast, COX-2 is an inducible enzyme which is upregulated by inflammatory cytokines including IL-1β, IL-2, IFN-α, -β and -γ and TNF-α (Dalgleish and O'Byrne 2002). The particular prostaglandin synthesised depends on the prostaglandin synthase enzyme present in the cell (Brock *et al.* 1999) although, in malignant cells, COX-2 activity is associated with the formation of prostaglandin-E$_2$ (PGE$_2$). COX-2 has been implicated in carcinogenesis through the inhibition of apoptosis, downregulation of cell mediated immunity, promotion of angiogenesis and the formation of carcinogenic metabolites such as malondialdehyde (Tsujii and DuBois 1995; O'Byrne *et al.* 2000; Uefuji *et al.* 2000). COX-2 expressing cancer cell lines are associated with increased proliferative and invasive potential (Tsujii *et al.* 1997). COX-2 overexpression has been noted in a wide range of solid tumours, including colorectal (Sheehan *et al.* 1999), breast (Hwang *et al.* 1998), gastric (Murata *et al.* 1999), oesophageal (Zimmermann *et al.* 1999) and lung cancer (Hida *et al.* 1998a). Selective inhibition of COX-2 is a novel therapeutic approach under investigation in both the chemoprevention and treatment of solid tumours (Reddy *et al.* 2000; Ziegler 1999).

7.2. Aims

1   To evaluate COX-2 expression in MM samples by immunohistochemistry

2   To establish a reproducible, semi-quantitative Western blot assay of COX-2 expression in tumour tissue

3   To assess the correlations of COX-2 expression with clinicopathological variables

4   To evaluate the prognostic significance of COX-2 expression and its contribution to

the CALGB and EORTC prognostic scoring systems

5   To determine the presence of PGE_2 by enzyme immunoassay.

7.3.   Methods

7.3.1.   Immunohistochemistry

Immunohistochemistry was performed on tissue sections cut from 18 snap-frozen

tissue blocks, with a monoclonal COX-2 primary antibody and a fluorescein isothiocyanate

(FITC) labelled secondary antibody, as described in Section 2.3.3.4. COX-2

immunostaining was visualised with laser scanning confocal microscopy at low and high

power, and interpreted with the assistance of Prof. Rosemary Walker, Department of

Pathology, University of Leicester.

7.3.2.   Western blotting

7.3.2.1. Method 1

Snap-frozen MM tumour tissue samples were initially homogenised in a lysis buffer

containing PMSF as the sole protease inhibitor and, subsequently, in a buffer containing

Complete® (Roche Applied Science, Lewes, UK), a broad-spectrum protease inhibitor.

Samples were kept as cool as possible throughout: blocks were removed from dry ice and

placed straight into ice-cold homogenisation buffer, were homogenised, clarified and

sonicated on ice, with centrifugation at 4°C. Aliquots of the supernatant were run on 5cm

by 8cm SDS/polyacrylamide gels, and the PG27B primary antibody (Oxford Molecular

Research) was used to probe for COX-2.

7.3.2.2. Method 2

In order to increase the intensity of the COX-2 bands, it was necessary to move up in size from the 5cm x 8cm gel, which could be loaded with sufficient volume to equate to approximately 25µg protein per well, to the 11cm x 14 cm size. With a 15 well comb and a 1.5mm gel thickness, it was possible to load 150µl into the wells, allowing a protein load of over 300µg, if required. In addition, the deeper resolving gel allowed the gels to run for a longer period of time, gaining further separation between the bands. Finally, a change in primary antibody was made from the PG27B to the SC-1745 antibody (Santa-Cruz Biotechnology), to assess whether this would reduce the background activity of the blot.

7.3.2.3. Method 3

To ensure that a delay between surgical excision of the sample and freezing in liquid nitrogen did not affect the band, a time course experiment was performed. A large tumour sample was excised at the time of surgery and divided into equal blocks. These were then snap-frozen in the operating theatre, commencing at 1 minute from the moment of surgical excision, with further samples frozen at 2, 3, 5, 7, 10, 20, 30, 40 and 60 minutes from excision. Western blots were performed on three such collected cases using method 2 described above.

7.3.2.4. Method 4

Once the results were consistent with the Santa-Cruz primary antibody, the linearity of the COX-2 protein / densitometry value relationship was assessed running serial dilutions of the two MM samples with the strongest COX-2 bands, each in duplicate.

7.3.2.5. Method 5

As an additional control to confirm the specificity of the Santa-Cruz primary antibody, an antibody-blocking peptide (SC-1745p, Santa-Cruz Biotechnology) was used. After probing for COX-2, the blots were stripped and re-probed with the same primary antibody which had been incubated overnight with the blocking peptide.

7.3.2.6. Method 6

Gels were stripped of antibody and reprobed for α-tubulin, as a housekeeping protein, using standard techniques. Due to the high intensity of chemiluminescence obtained, short, automated film exposure times were necessary to obtain bands that were distinct.

7.3.3. Western blot densitometry

7.3.3.1. Method 7

At the time of the switch of primary antibody, it was necessary, due to equipment failure, to adopt a different densitometry system. With both systems, adjustment was made for local background by the median perimeter value subtraction method. The densitometry values obtained with the Oxford antibody and the ImageQuant v3.3 densitometer (Molecular Dynamics) were correlated with those using the Santa-Cruz antibody and the Kodak Digital Science Image Station.

7.3.3.2. Method 8

A key part of the study was to ensure standardisation of results and minimum variation between gels. Therefore the densitometry values of the same samples run on different gels were compared. When densitometry results from several gels were to be

pooled for statistical analysis, four positive samples of varying intensity (including the two

that were subject to the serial dilution study: method 4 above) were run in every gel, in

addition to a COX-2 protein positive control. It was possible to perform the semi-dry

blotting procedure with two gels adjacent to each other, transferring the proteins onto a

single nitrocellulose membrane. With two electrophoresis tanks each running two gels

simultaneously, both nitrocellulose membranes could be handled in the same wash tray

from the overnight block in milk/buffer solution onwards. Therefore, 60 samples

(including controls) could be incorporated into one day's experiment. With the use of the

COX-2 standard and the four samples run as internal controls, adjustment was made

between the different blots to allow for any variation between them. One blot was chosen

as a reference and, using linear regression analysis between the values for the internal and

positive controls, an adjustment factor was derived. The densitometry values for the other

blots were adjusted by multiplication of the gradient of the linear regression line. This was

performed for both the COX-2 bands and the α-tubulin. The unadjusted and adjusted COX-

2 densitometry values were correlated.

7.3.3.3. Method 9

The densitometry values obtained from COX-2 Western blotting performed on the

larger 11cm x 14cm gels with the SC-1745 antibody (Method 2) were correlated with

clinicopathological variables and survival.

7.3.4. Bicyclo-PGE₂ enzyme immunoassay

Owing its short half life, the manufacturers of the PGE_2 enzyme immunoassay

(EIA) kits recommended that, in homogenised tissue, levels of PGE_2 and its physiological

metabolites were measured by conversion to their stable moiety, bicyclo-PGE_2. A 96 well

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bicyclo-PGE$_2$ EIA kit was used to investigate PGE$_2$ levels in 29 cases of MM. Samples were run at 1:50 and 1:100 dilutions, rather than as corrected for protein content, to minimise the volume loaded. These dilutions were found to give optical density readings on the spectrophotometer within the limits of the standard curve set by the manufacturer.

7.3.5. Statistical analysis

The comparisons between i) the results of the two sets of primary antibodies and densitometry systems, ii) adjusted and unadjusted COX-2 densitometry values and iii) the COX-2 and α-tubulin values were made with linear regression analysis. The serial dilution study was assessed graphically plotting the mean value and 95% confidence intervals for each data point.

Non-parametric methods were used to correlate COX-2 and α-tubulin levels with clinicopathological factors. Kaplan-Meier analysis and the log rank test were used to identify prognostic factors in this cohort of patients. Cox proportional hazards were used to estimate hazard ratios and 95% confidence intervals in univariate and multivariate models.

7.4. Results

7.4.1. Immunohistochemistry

There was strong cytoplasmic tumour cell staining in all 18 cases (Figure 7.1). Both tumour cells from epithelioid and sarcomatoid tumours displayed strong immunoreactivity. There was no obvious variability in intensity or percentage of cells staining which could be exploited to correlate with clinicopathological variables or survival. Surrounding stroma stained with variable immunointensity.

Figure 7.1: COX-2 immunohistochemistry with a FITC-labelled secondary antibody, visualised with laser-scanning confocal microscopy

a) Epithelioid MM with strong cytoplasmic immunofluorescence (large arrow) and weak stromal staining (small arrow)
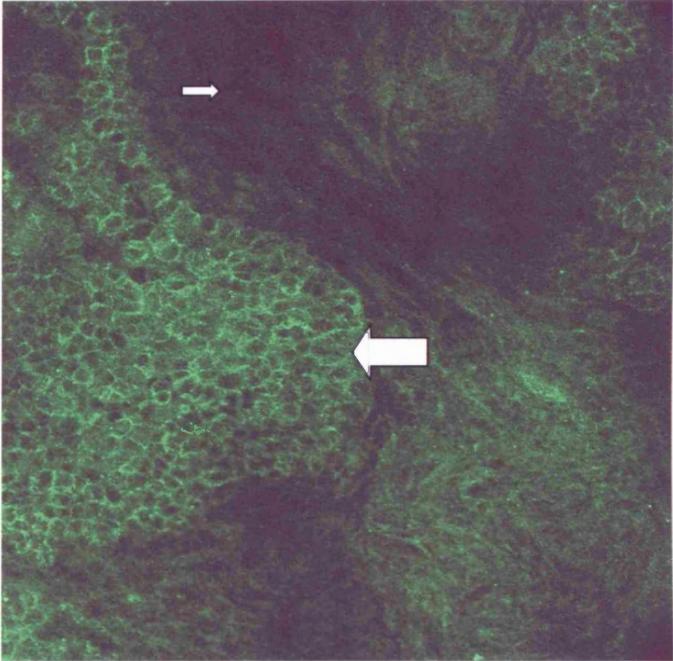
b) Islets of epithelioid MM tumour cells with weaker staining stroma
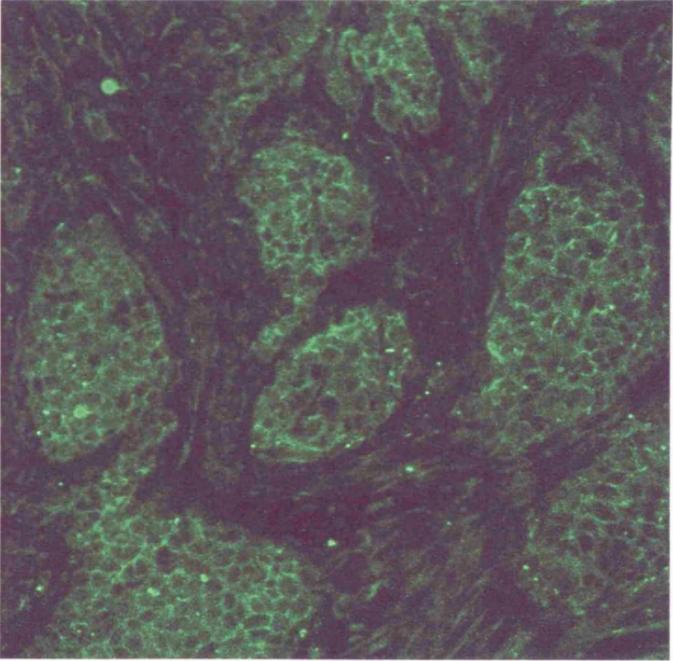
c) Epithelioid MM with strong stromal immunofluorescence
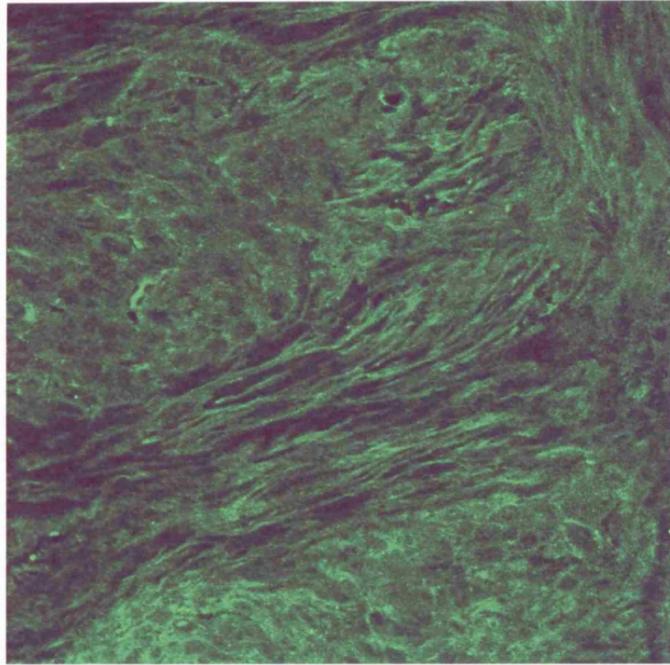
d) Sarcomatoid MM with strong cytoplasmic immunofluorescence
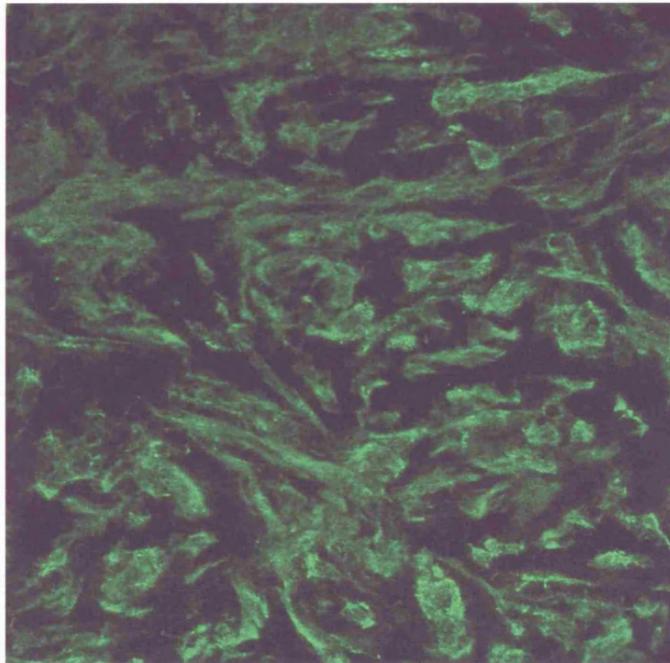
7.4.2. Western blotting

7.4.2.1. Method 1

An example of the Western blot obtained with the 5cm by 8cm gels and the PG27B antibody is shown in Figure 7.2. The predominant immunoreactivity was at approximately 50-60kDa, in three sharp bands that were consistent between cases. Faint bands at 72 and 74 kDa, consistent with COX-2, were seen in some of the MM samples. It was suggested that the three prominent bands could represent either non-specific binding of proteins completely unrelated to COX-2 or COX-2 after partial proteolysis.

7.4.2.2. Method 2

The early blots of the larger gel size showed much stronger bands consistent with COX-2, which improved further with optimisation of the both primary and secondary antibody concentrations and the concentration of the milk buffer solution. The three dense bands at 50 – 60 kDa persisted. The change of primary antibody allowed the film exposure time to be reduced from typically 20 minutes to 1 minute, resulting in a much cleaner blot, with a marked reduction in background reactivity. Furthermore, the density of the three persistent bands at lower molecular weight were also significantly reduced (Figure 7.3). Immunoreactive bands consistent with COX-2 were visible in 44 (94%) of the 47 cases analysed. Two bands in close proximity were seen once again, corresponding with the COX-2 standard at 72 and 74 kDa, indicating different glycosylation states (Figure 7.4).

7.4.2.3. Method 3

With the time course analysis, faint bands were seen once again in line with the control ban; the densitometry values were consistent across the time course (Figure 7.5).

Figure 7.2: Early COX-2 Western blot, showing the three bands at 50-60kDa, with a faint band present in some samples at the level of the COX-2 positive control (arrow)

Figure 7.3: Comparison between the COX-2 Western blots obtained with the Oxford PG27B primary antibody (a) and the Santa Cruz SC-1745 primary antibody (b). The blot in (a) was exposed to film for 20 minutes, whereas in (b) the exposure was 1 minute. The MM cases do not correspond between the two blots. (PC = positive control, arrow = COX-2 bands)

a)

PC
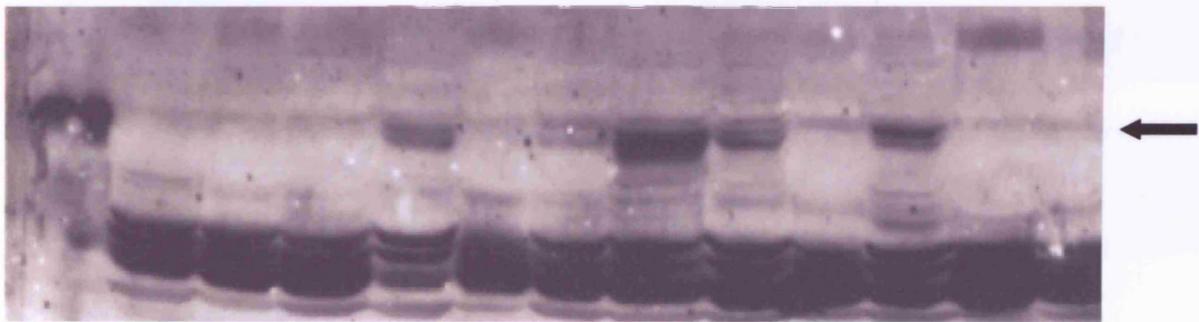
b)

PC
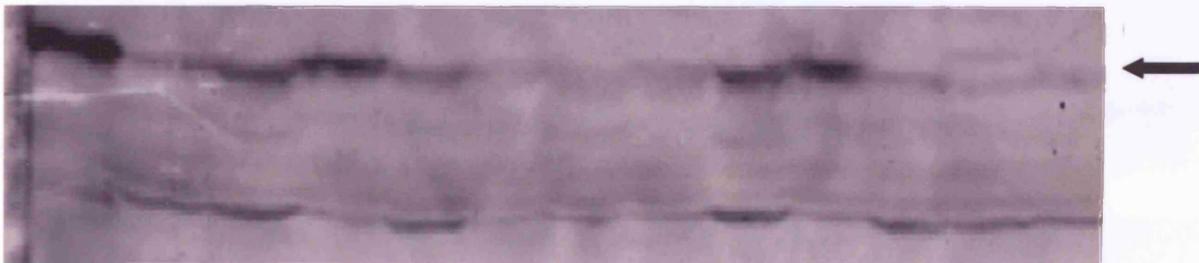
Figure 7.4: Malignant Mesothelioma tumour sample COX-2 and α-Tubulin Western blots. The upper of each pair shows the membrane probed for COX-2, the lower for α-Tubulin. The left-most lane is the COX-2 standard and the next four represent the internal control samples (up to the vertical line).
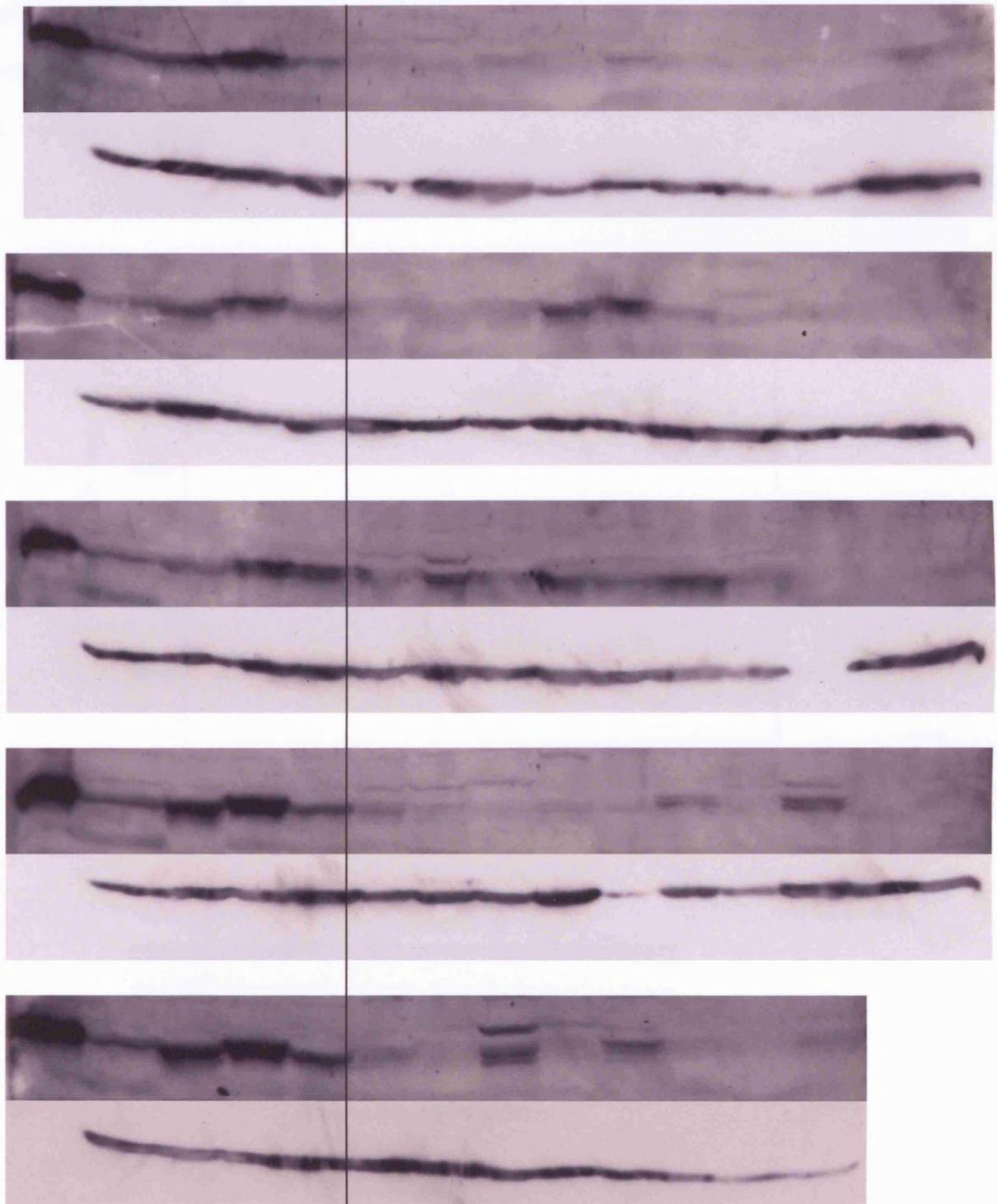
Figure 7.5: COX-2 Western blot time course analysis. A large MM tumour sample was excised in the operating theatre and divided into smaller pieces. At time points of 1, 3, 5, 7, 10, 20, 30, 40 and 60 minutes from the time of excision, samples were snap-frozen in liquid nitrogen. a) Western blot densitometry values for one of the cases (six time points, each run in duplicate) are shown. There was no reduction in COX-2 protein level according to the time interval between excision and snap-freezing. b) A photograph of a corresponding Western blot (PC = positive control)

a)
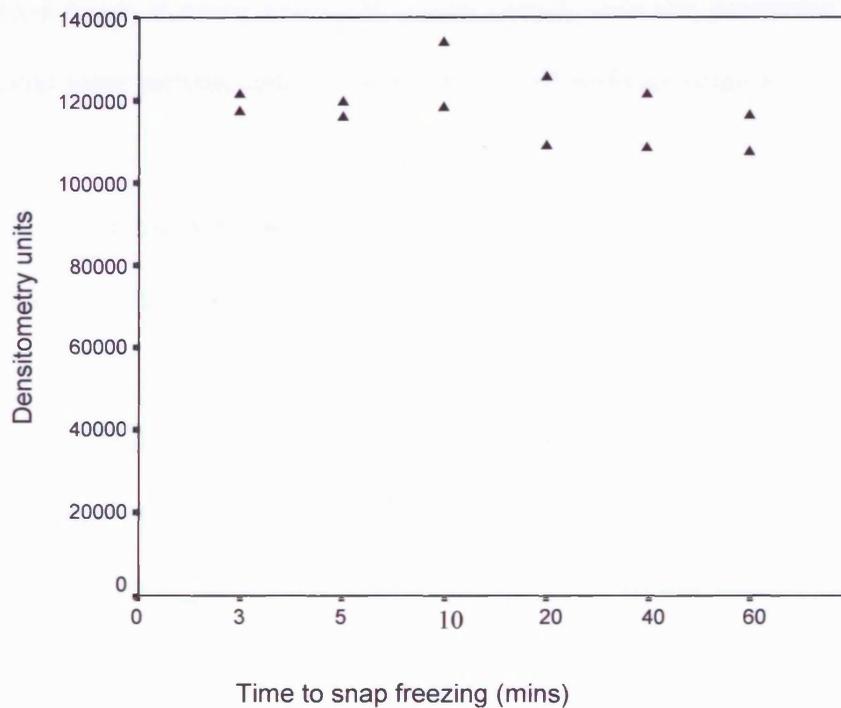
b)
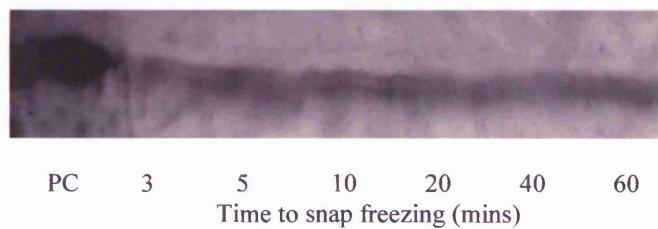
7.4.2.4. Method 4

The serial dilution experiment confirmed the semi-quantitative nature of the assay (Figure 7.6). There was a linear relationship between the protein load and the densitometry values obtained ($r=0.94$, $p<0.0001$).

7.4.2.5. Method 5

Preadsorption of the COX-2 primary antibody with the blocking peptide confirmed that the bands seen in the MM samples were indeed COX-2. Interestingly, the three persistent bands at lower molecular weight (which were less prominent with the Santa-Cruz antibody) were partially reduced with the blocked antibody (Figure 7.7).

7.4.2.6. Method 6

α-Tubulin bands were visible in all samples (Figure 7.4)

7.4.2.7. Method 7

Negative densitometry values were obtained in 4 cases for COX-2 and 1 for α-tubulin. In two of these COX-2 samples and the α-tubulin sample, immunoreactive bands were visible to the naked eye, but, with the densitometer subtracting the greater perimeter background non-specific chemiluminescence, negative values occurred. In one further specimen with a densitometry value just above zero, no band was visible to the eye. COX-2 bands were identified in all other samples. There was a close relationship between the two sets of results obtained with the different primary antibodies and densitometry systems ($r=0.89$, $p<0.0005$, Figure 7.8).

Figure 7.6:   Densitometry values obtained from serial dilution of the two strongest

MM samples (each in duplicate). The protein load for each dilution is plotted against

these 4 densitometry values, expressed as a percentage of the values of the 150µg load.

The bars represent the 95% confidence intervals of each mean, which is depicted by the

solid square. For the main Western blotting experiments, 150µg protein of each samples

was added to each well of the gel.
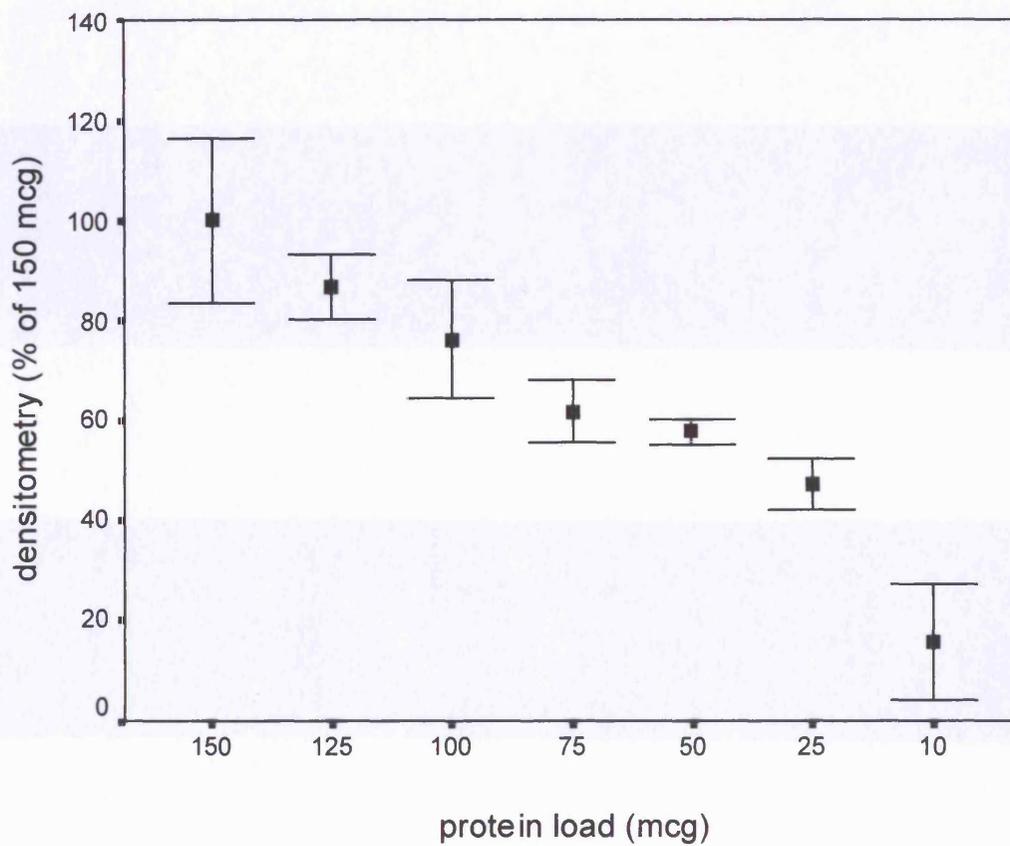
Figure 7.7: The effect of the adsorbing the Santa-Cruz SC-1745 primary antibody with a synthetic COX-2 blocking peptide (SC-1745 P). The primary antibody binding site was blocked by incubation with the blocking peptide overnight at 4°C. After probing with the unblocked antibody (a), the membrane was stripped and processed normally thereafter with the preadsorbed primary antibody (b). The thin band seen in the positive control (PC) lane in (b) is at the level of the positive control in (a)

a)
   PC
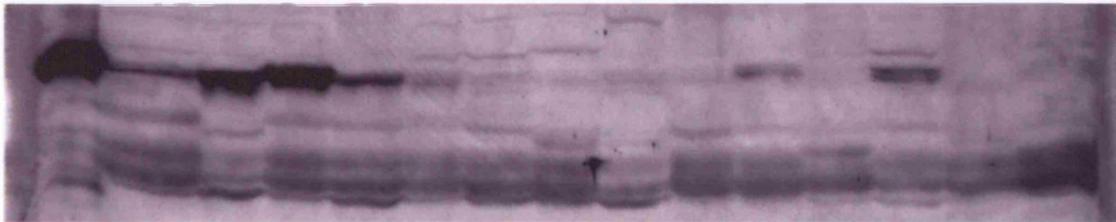
b)
   PC
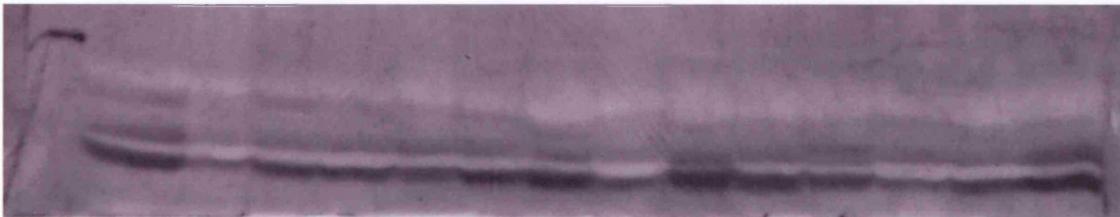
Figure 7.8:   Relationship between densitometry values obtained with the Oxford

PG27B antibody and the ImageQuant densitometer, compared to the Santa Cruz SC-

1745 and the Kodak Digital Science Image Station (r=0.89, p<0.0005).
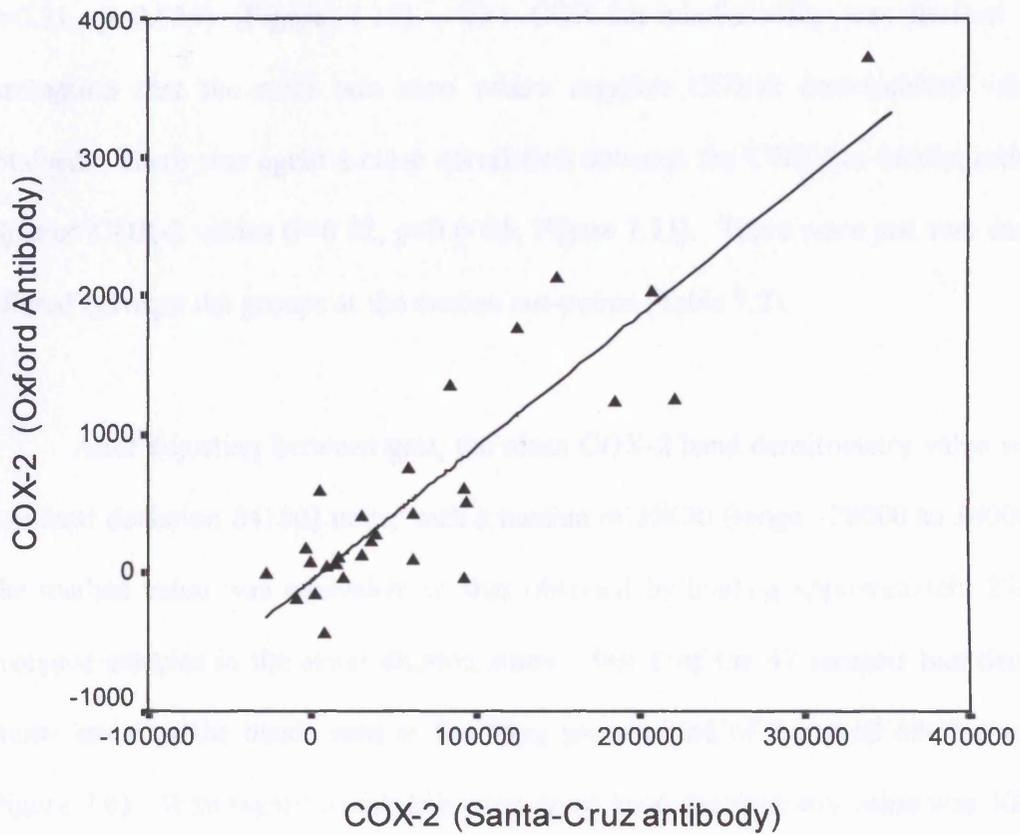
7.4.2.8. Method 8

There was excellent correlation between the unadjusted and adjusted COX-2 values ($r=0.986$, $p<0.0005$, Figure 7.9). Indeed, there was complete agreement between the groups when divided above and below the median value (Table 7.1). There was a weak, but statistically significant, positive correlation between α-tubulin and COX-2 values ($r=0.31$, $p=0.036$) (Figure 7.10). The COX-2:α-tubulin ratio was derived with the assumption that the ratio was zero where negative COX-2 densitometry values were obtained. There was again a close correlation between the COX-2:α-tubulin ratio and the adjusted COX-2 values ($r=0.92$, $p<0.0005$, Figure 7.11). There were just two cases which differed between the groups at the median cut-points (Table 7.2).

After adjusting between gels, the mean COX-2 band densitometry value was 66900 (standard deviation 84100) units, with a median of 35400 (range –28000 to 340000) units. The median value was equivalent to that obtained by loading approximately 25µg of the strongest samples in the serial dilution study. Just 6 of the 47 samples had densitometry values less than the bands seen at the 10µg protein load of the serial dilution experiment (Figure 7.6). With regard to α-tubulin, the mean band densitometry value was 303000 (SD 155000), median 295000 (-5700 to 715000) units. The mean COX-2:α-tubulin ratio was 0.22 (SD 0.25), median 0.15 (0 – 1.09).

7.4.2.9. Method 9

7.4.2.9.1. Correlation with clinicopathological variables

High COX-2 band densitometry values correlated with weight loss ($p=0.003$) and tumour necrosis ($p=0.03$, Table 7.3, Figure 7.12), but not with cell type or a history of asbestos exposure. There was a trend towards a positive correlation with the white blood

Table 7.1:   Contingency table showing the distribution of cases above and below the respective median values for the unadjusted COX-2 and adjusted COX-2 densitometry values.

		Adjusted COX-2		
		< median	>= median	Total
Unadjusted COX-2	< median	23	0	23
	>=median	0	24	24
	Total	23	24	47

Table 7.2:   Contingency table to show level of agreement between COX-2 densitometry units (adjusted between blots) and COX-2:Tubulin ratio (adjusted between blots).   Cohen's kappa = 0.915, p<0.001.

		COX-2 : Tubulin Ratio		
		< median	> median	TOTAL
COX-2 (corrected)	< median	22	1	23
	> median	1	23	24
	TOTAL	23	24	47

Table 7.3:   Correlation between COX-2 expression and categorical clinicopathological variables. The mean densitometry value and the SD are quoted. The Mann-Whitney U test was used for the comparison of two groups and the Kruskal-Wallis Test for greater than two groups.

Variable	Category	n	COX-2 densitometry units $(x10^3)$		p
			Mean	SD	
Gender	Male	41	72.7	88.0	0.13
	female	6	27.1	32.0	
Chest pain	Absent	16	73.0	89.7	0.65
	Present	31	63.7	82.4	
Weight loss	Absent	32	40.6	52.9	0.003
	Present	15	122.8	110.1	
Asbestos exposure	No	9	51.5	67.0	0.34
	Yes	37	72.4	88.5	
Cell type	Epithelioid	29	75.9	93.3	0.46
	Non-epithelioid	18	53.4	66.6	
ECOG Performance status	0	30	60.7	77.0	0.77
	1 or 2	17	77.7	97.0	
CALGB prognostic group	Groups 1, 2	13	67.0	91.8	0.54
	Groups 3,4	23	61.6	85.5	
	Groups 5,6	11	77.7	78.6	
EORTC prognostic group	Low risk	29	70.9	93.5	0.83
	High risk	18	60.3	68.5	
Radical Surgery	Yes	22	64.0	82.9	0.95
	No	25	69.4	86.8	
Tumour Necrosis	No	33	50.2	73.8	0.029
	Yes	14	106.1	96.4	
EGFR	Negative	23	54.8	61.9	0.55
	Positive	23	80.6	102.7	

Figure 7.9:   Relationship between the raw COX-2 densitometry values and those after
adjusting the values for variation between different blots, according to the internal and
positive controls (r=0.986, p<0.0005).
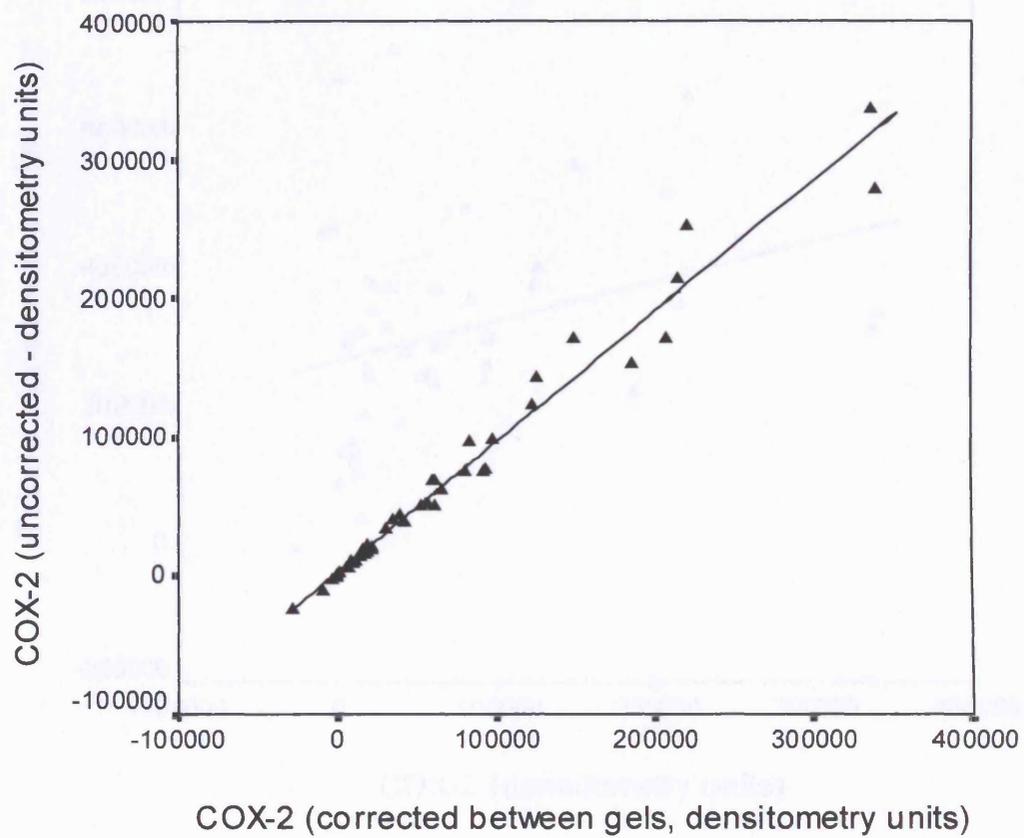
Figure 7.10: Relationship between the COX-2 and α-Tubulin densitometry values, after correcting the values for variation between different blots (r=0.31, p=0.036).
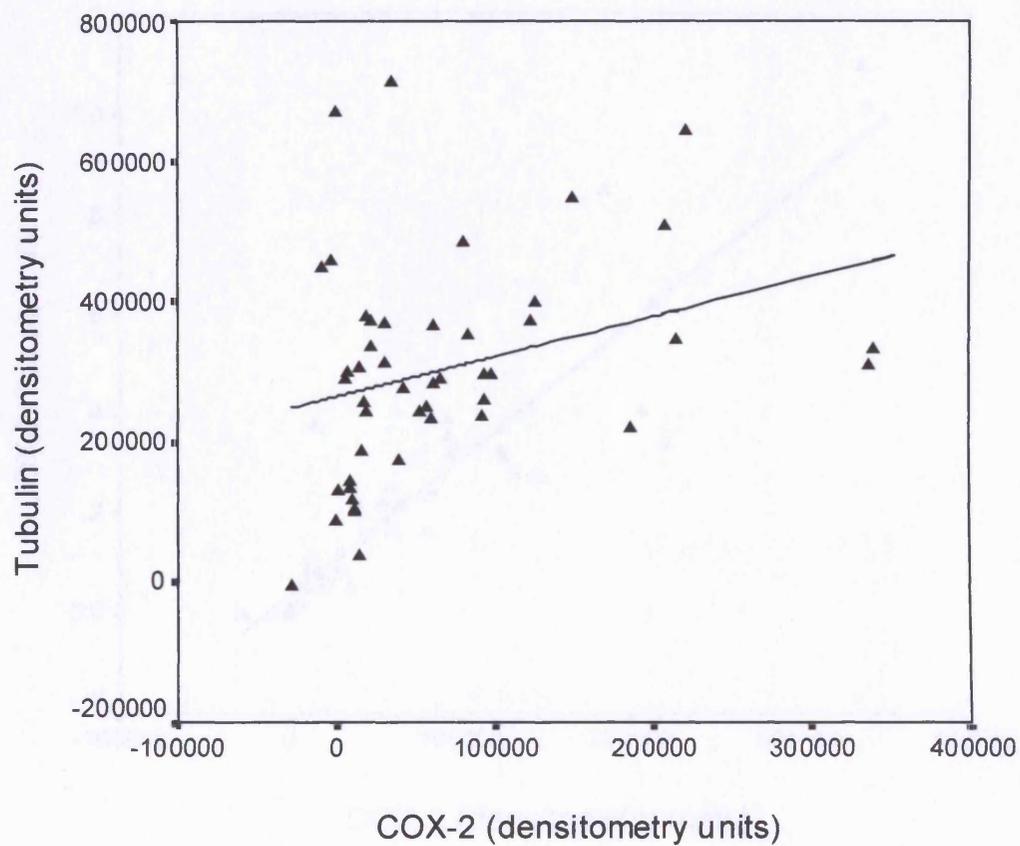
Figure 7.11: Relationship between the COX-2 densitometry values and the ratio of COX-2 : α-Tubulin, after adjusting the values for variation between different blots (r=0.92, p<0.0005).
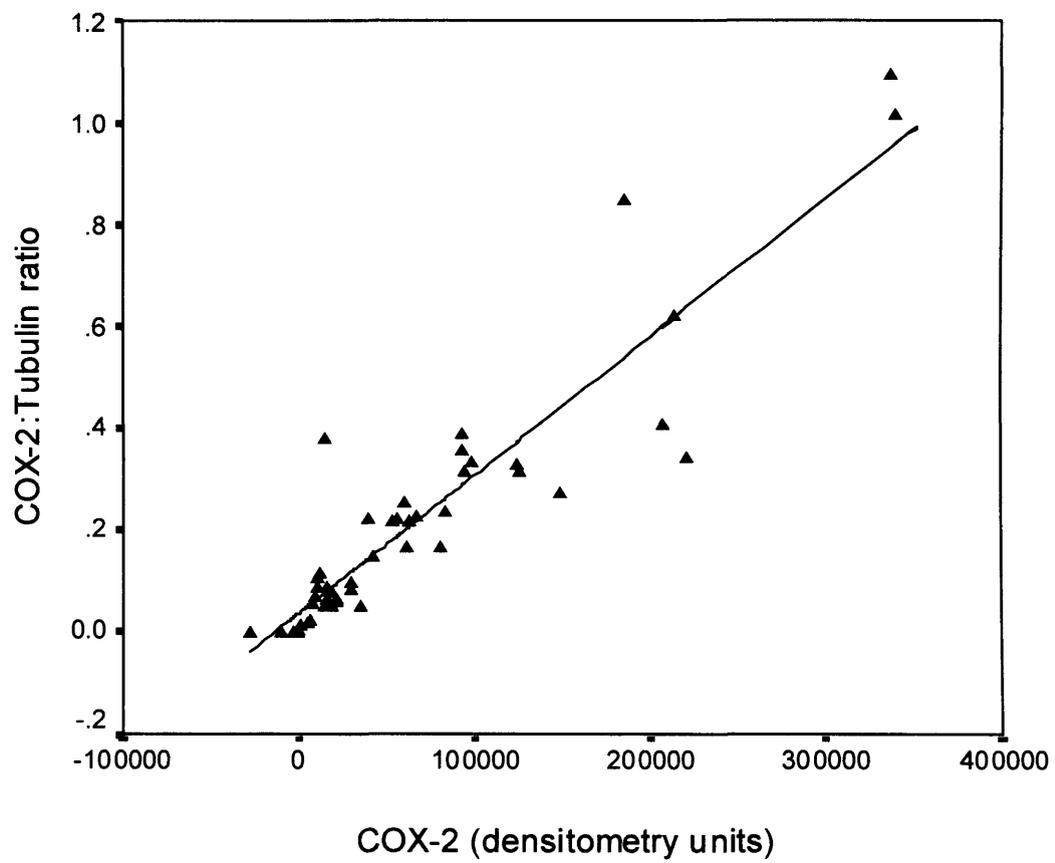
Figure 7.12: Boxplot showing the distributions of COX-2 densitometry values with and without the presence of tumour necrosis. COX-2 levels were significantly higher in the presence of tumour necrosis (p=0.03, Mann-Whitney U Test). The horizontal bars represent the median values, boxes the interquartile range, vertical bars the 10 – 90 % range, solid circles the outliers and solid triangles the extremes.
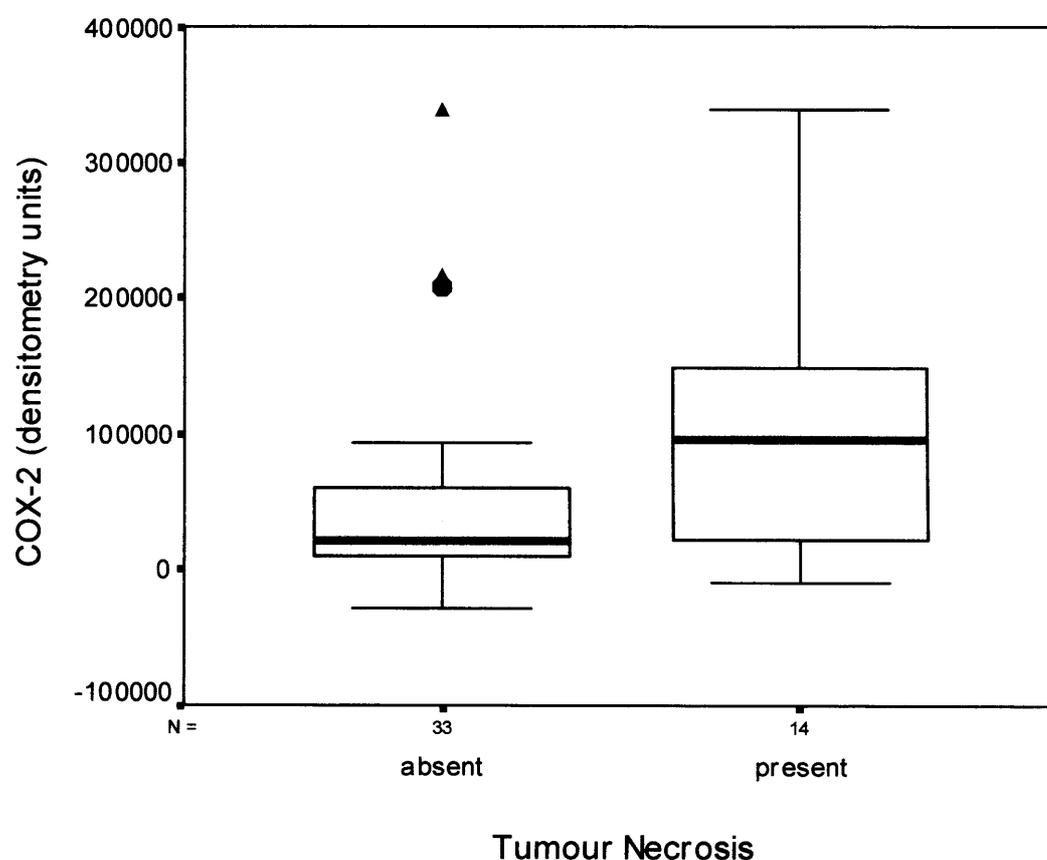
cell count, which did not reach statistical significance (p=0.097, Table 7.4). Neither MVD

or EGFR expression correlated with COX-2.

The α-tubulin densitometry values were significantly higher in the non-epithelioid

cases (p=0.038, Table 7.5, Figure 7.13) and in those with weight loss (p=0.036). There was

a positive correlation between α-tubulin densitometry values and the white blood cell count

and a trend towards the same with platelet count (Table 7.6). α-tubulin values did not

correlate with TN.

7.4.2.9.2. Survival – univariate analysis

Due to the presence of the correlations between α-tubulin and cell type and WBC,

the COX-2 levels (adjusted between blots) were used for survival analysis, rather than the

COX-2:α-tubulin ratio.

Two patients died from surgical complications within 30 days of surgery and were

therefore excluded from survival analysis. COX-2 levels were significant predictors of

poor survival, expressed either as a categorical (with a median cut-point) or a continuous

variable (Figure 7.14, Table 7.7). Of note is the fact that when the densitometry results of

the 27 cases probed with the PG-27B antibody were analysed, levels of COX-2 greater than

the median value were associated with a worse prognosis (p=0.0127, Figure 7.15). The

other clinicopathological prognostic factors for this cohort of patients in univariate analysis

are shown in Table 7.8. Weight loss, chest pain, high platelet count, low haemoglobin,

non-epithelioid cell type and having not undergone radical surgery were all poor prognostic

factors.

Table 7.4: Correlations of COX-2 expression and continuous variables, assessed by linear regression.

Variable	r	p
Age	0.03	0.86
WBC	0.28	0.097
Platelets	0.24	0.17
Haemoglobin	0.20	0.24
MVD	0.09	0.62

Table 7.5: Correlation between α-Tubulin expression and categorical clinicopathological variables. The mean densitometry value and the SD are quoted. The Mann-Whitney U test was used for the comparison of two groups and the Kruskal-Wallis Test for greater than two groups.

Variable	Category	n	α-Tubulin densitometry units (x10^3)		p
			Mean	SD	
Gender	Male	41	295	151	0.50
	female	6	357	192	
Chest pain	Absent	16	273	119	0.36
	Present	31	318	171	
Weight loss	Absent	32	266	146	0.036
	Present	15	381	150	
Asbestos exposure	No	9	321	203	0.99
	Yes	37	304	143	
Cell type	Epithelioid	29	260	126	0.038
	Non-epithelioid	18	372	176	
ECOG Performance status	0	30	303	153	0.91
	1 or 2	17	303	164	
CALGB prognostic group	Groups 1, 2	13	239	118	0.24
	Groups 3,4	23	317	161	
	Groups 5,6	11	349	171	
EORTC prognostic group	Low risk	29	286	134	0.39
	High risk	18	331	186	
Radical Surgery	Yes	22	316	183	0.95
	No	25	288	120	
Tumour Necrosis	No	33	287	162	0.29
	Yes	14	340	138	
EGFR	Negative	23	345	178	0.13
	Positive	23	260	122	

Table 7.6: Correlations of α-Tubulin expression and continuous variables, assessed by linear regression.

Variable	r	p
Age	0.04	0.80
WBC	0.49	0.003
Platelets	0.31	0.069
Haemoglobin	0.20	0.24
MVD	0.11	0.53

Table 7.7: Univariate log rank and Cox regression analysis of COX-2 expression, as assessed by semi-quantitative western blotting (n=45).

Variable	Category	n	Median Survival (days)	Log Rank p	Hazard Ratio	Hazard Ratio 95%CI		Cox p
COX-2	< median	22	382	0.0127	1			0.0157
	>=median	23	183		2.47	1.19	5.15	
COX-2	Continuous	45			1.0000	1.0000	1.0000	0.0138

Table 7.8: Univariate log rank and Cox regression analysis of categorical variables (n=45)

Variable	Category	n	Median Survival (days)	Log Rank p	Hazard Ratio	Hazard Ratio 95% Confidence Intervals		Cox p
Gender	Female	6	193	0.50	1			0.50
	Male	39	254		1.39	0.53	3.61	
Weight Loss	No	31	382	0.0003	1			0.0007
	Yes	14	114		3.66	1.73	7.74	
Asbestos Exposure	No	9	200	0.78	1			0.78
	Yes	35	252		1.13	0.47	2.74	
Chest Pain	No	16	555	0.0025	1			0.0044
	Yes	29	215		3.41	1.47	7.94	
ECOG PS	0	30	254	0.64	1			0.64
	1 or 2	15	215		1.19	0.57	2.44	
WBC	=< 8.3 x10^9/l	20	382	0.31	1			0.31
	> 8.3 x10^9/l	25	193		1.42	0.72	2.83	
Platelets	=<400 x 10^9/l	28	260	0.038	1			0.042
	>400 x 10^9/l	17	215		2.08	1.03	4.21	
Haemoglobin	> 14 g/dl	30	200	0.0078	1			0.011
	=< 14 g/dl	15	555		2.87	1.28	6.45	
Cell Type	Epithelioid	29	307	0.012	1			0.015
	Non-epithelioid	16	145		2.46	1.19	5.08	
Radical Surgery	Yes	22	382	0.032	1			0.037
	No	23	200		2.09	1.05	4.16	
MVD	< median	15	193	0.25	1			0.26
	>= median	18	215		1.62	0.71	3.69	
Tumour Necrosis	Absent	32	254	0.42	1			0.42
	Present	13	215		1.36	0.64	2.87	
EGFR	Positive	21	200	0.13	1			0.13
	Negative	23	254		0.58	0.28	1.18	
CALGB groups	Groups 1 and 2	13	820	0.0001	1			0.0005
	Groups 3 and 4	22	254		2.29	0.93	5.63	
	Groups 5 and 6	10	88		7.21	2.60	20.00	
EORTC groups	Low risk	29	382	0.01	1			0.013
	High risk	16	114		2.41	1.21	4.80	

Figure 7.13: Boxplot showing the distributions of α-Tubulin densitometry according to the cell type. α-Tubulin levels were significantly higher in the non-epithelioid cases (p=0.04, Mann-Whitney U Test). The horizontal bars represent the median values, boxes the interquartile range, vertical bars the 10 – 90 % range, solid circles the outliers and solid triangles the extremes.
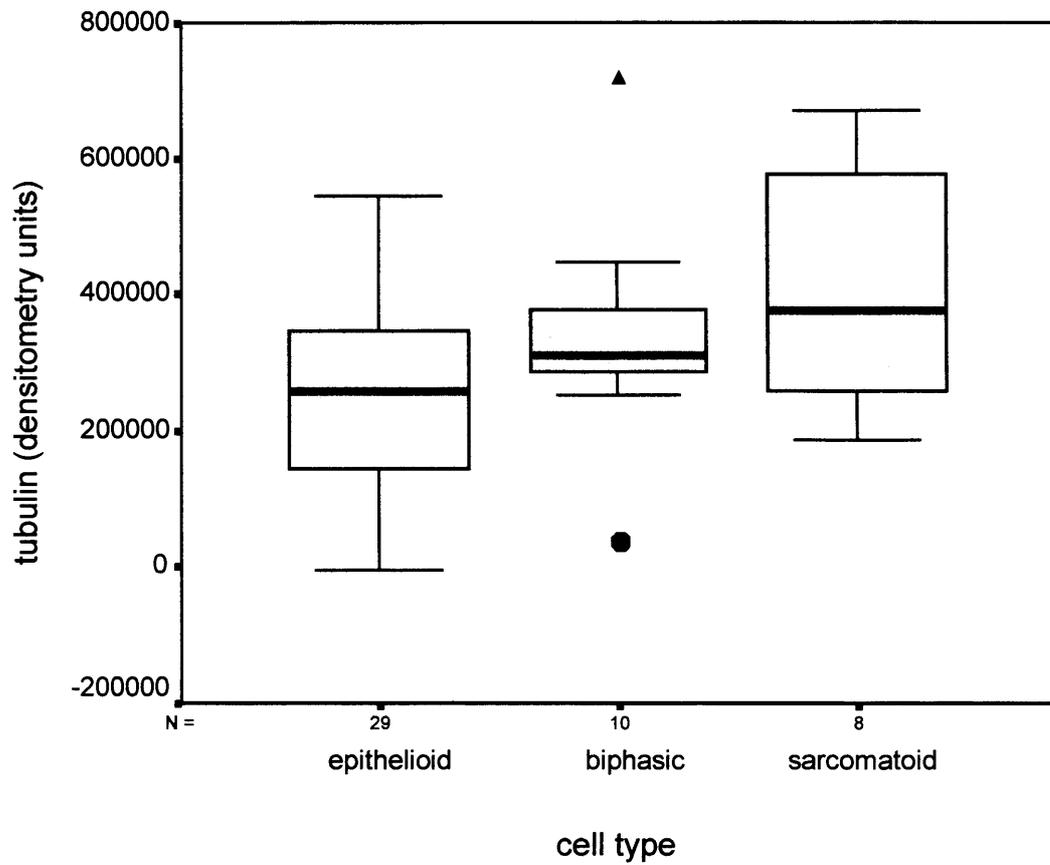
Figure 7.14: Kaplan-Meier plot showing that high COX-2 levels were associated with a

worse survival (p=0.01, log rank). In this experiment, COX-2 was probed with the

Santa-Cruz SC-1745 anti-COX-2 primary antibody (n=45).
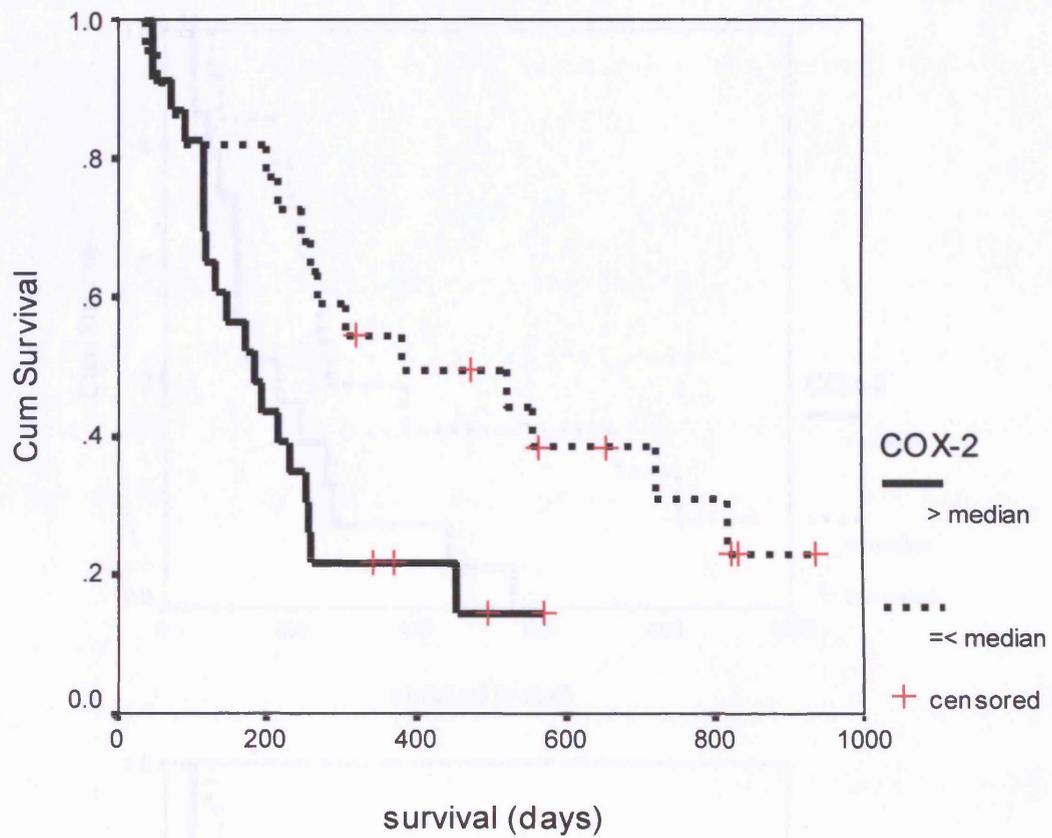
Figure 7.15: Kaplan-Meier plot showing the association between COX-2 levels, as assessed by Western blotting. In (a), the PG27B primary antibody (Oxford Biomedical Research) was used (p=0.03) whereas in (b), the plot shows the same 27 cases (p=0.0016) probed with the SC-1745 primary antibody (Santa-Cruz Biotechnology).

a)
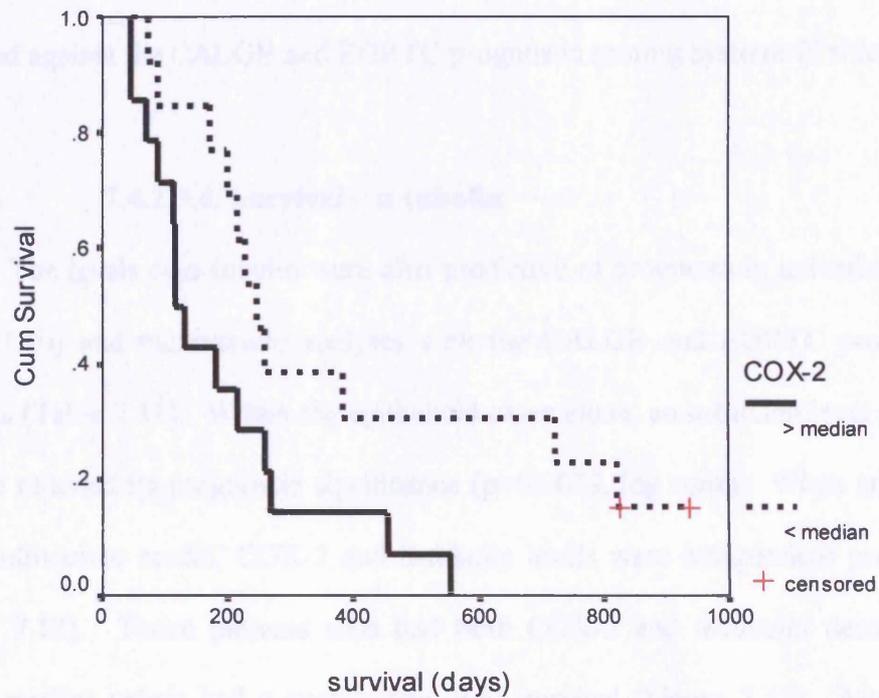
b)
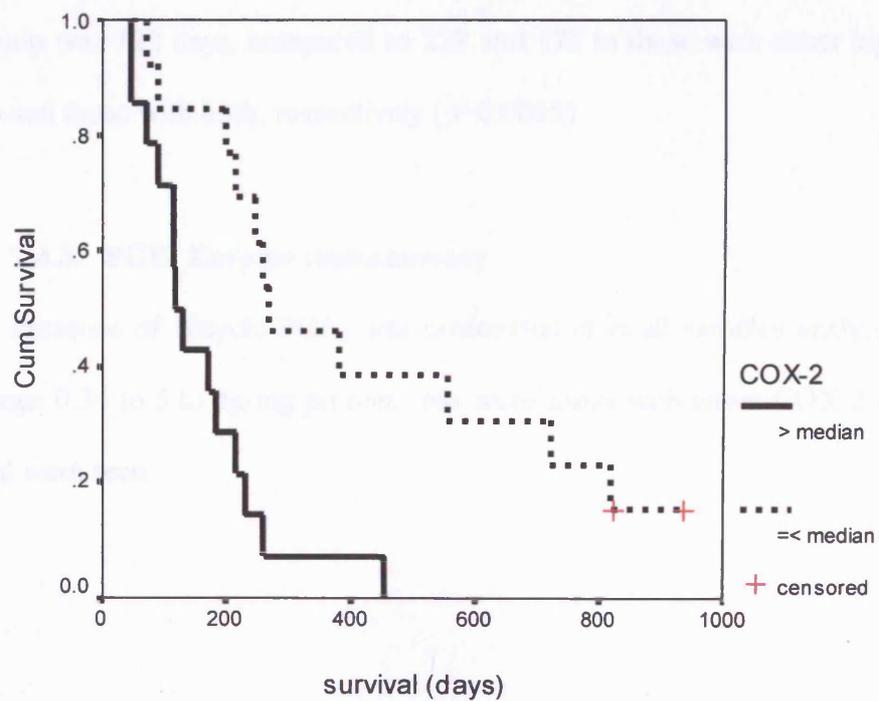
7.4.2.9.3. Survival – multivariate analysis

COX-2 levels were analysed as a categorical variable in multivariate analysis. Multivariate analysis with weight loss, chest pain, high platelet count, low haemoglobin, non-epithelioid cell type and having not undergone radical surgery was not performed due to the high ratio of the number of variables to be entered compared to the number of events (deaths) which occurred. However, COX-2 was an independent prognostic factor when analysed against the CALGB and EORTC prognostic scoring systems (Table 7.9).

7.4.2.9.4. Survival – α-tubulin

The levels of α-tubulin were also predictive of prognosis in univariate (Figure 7.16, Table 7.10) and multivariate analyses with the CALGB and EORTC prognostic scoring systems (Table 7.11). Within the epithelioid cases alone, an α-tubulin level greater than the median retained its prognostic significance (p=0.0073, log rank). When analysed together in a multivariate model, COX-2 and α-tubulin levels were independent prognostic factors (Table 7.12). Those patients who had both COX-2 and α-tubulin densitometry values below median values had a particularly good survival (Figure 7.17). Median survival in this group was 722 days, compared to 229 and 172 in those with either high COX-2 or α-tubulin and those with both, respectively (p=0.0015).

7.4.3. PGE$_2$ Enzyme immunoassay

Presence of Bicyclo-PGE$_2$ was demonstrated in all samples analysed, with median 1.8 (range 0.34 to 5.6) pg/mg protein. No correlations with either COX-2 protein levels or survival were seen.

Table 7.9: Cox multivariate regression analysis. COX-2 expression (< or >= median densitometry units) was tested, in turn, against the CALGB and EORTC prognostic scoring systems.

Variable	Category	n	p	Hazard Ratio	Hazard Ratio 95% Confidence Interval	
COX-2	< median	22	0.0036	1		
	>= median	23		3.14	1.45	6.79
CALGB groups	Groups 1 and 2	13	0.0002	1		
	Groups 3 and 4	22		2.53	1.02	6.23
	Groups 5 and 6	10		9.34	3.24	29.93

Variable	Category	n	p	Hazard Ratio	Hazard Ratio 95% Confidence Interval	
COX-2	< median	22	0.0027	1		
	>= median	23		3.37	1.52	7.46
EORTC groups	Low risk	29	0.0017	1		
	High risk	16		3.31	1.56	7.00

Table 7.10: Univariate log rank and Cox regression analysis of α-tubulin expression, as assessed by semi-quantitative western blotting (n=45).

Variable	Category	n	Median Survival (days)	Log Rank p	Hazard Ratio	Hazard Ratio 95% CI		Cox p
α-Tubulin	=< median	23	454	0.005	1			0.007
	> median	22	183		2.72	1.31	5.63	
α-Tubulin	Continuous	45			1.0000	1.0000	1.0000	0.0008

Table 7.11: Cox multivariate regression analysis. α-Tubulin expression (< or >= median densitometry units) was tested, in turn, against the CALGB and EORTC prognostic scoring systems.

Variable	Category	n	p	Hazard Ratio	Hazard Ratio 95% CI	
α-Tubulin	=< median	23	0.0025	1		
	> median	22		3.28	1.52	70.08
CALGB groups	Groups 1 and 2	13	0.0003	1		
	Groups 3 and 4	22		2.21	0.89	5.59
	Groups 5 and 6	10		9.07	3.12	26.43

Variable	Category	n	p	Hazard Ratio	Hazard Ratio 95% CI	
α-Tubulin	=< median	23	0.0011	1		
	> median	22		3.69	1.68	8.08
EORTC groups	Low risk	29	0.0015	1		
	High risk	16		3.39	1.59	7.22

Table 7.12: Cox multivariate regression analysis. COX-2 and α-tubulin expression (< or >
median densitometry units) were tested in a multivariate Cox proportional hazards model. No
other clinicopathological prognostic factors were examined in this model.

Variable	Category	n	p	Hazard Ratio	Hazard Ratio 95% Confidence Interval	
α-Tubulin	=< median	23	0.0128	1		
	> median	22		2.56	1.22	5.36
COX-2	< median	22	0.0290	1		
	>= median	23		2.30	1.09	4.87

Figure 7.16: Kaplan-Meier plot showing that high α-tubulin levels were associated with

a worse survival (p=0.005, log rank).
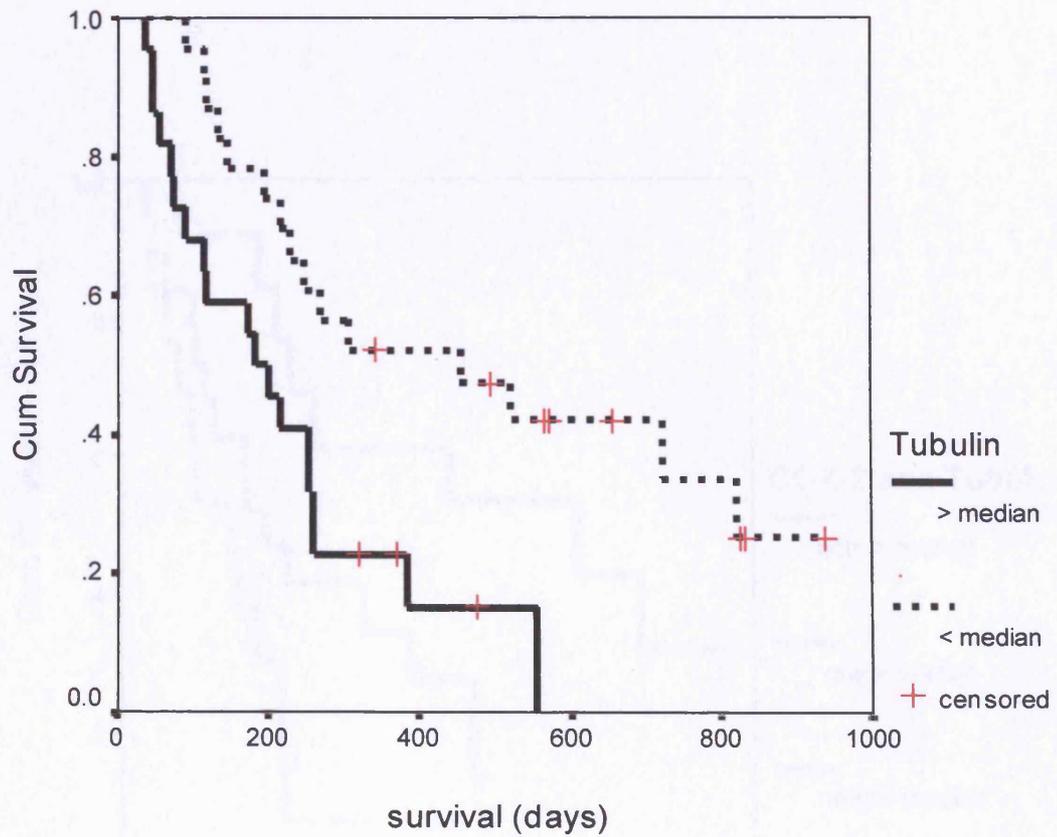
Figure 7.17: Kaplan-Meier plot showing that patients with levels of COX-2 and α-tubulin less than the median values were associated with better survival (p=0.0015, log rank). All analyses between the three groups in pairs were also statistically significant (none > median and one > median, p=0.037; one > median and both > median, p=0.048; none > median and both > median, p=0.0006).
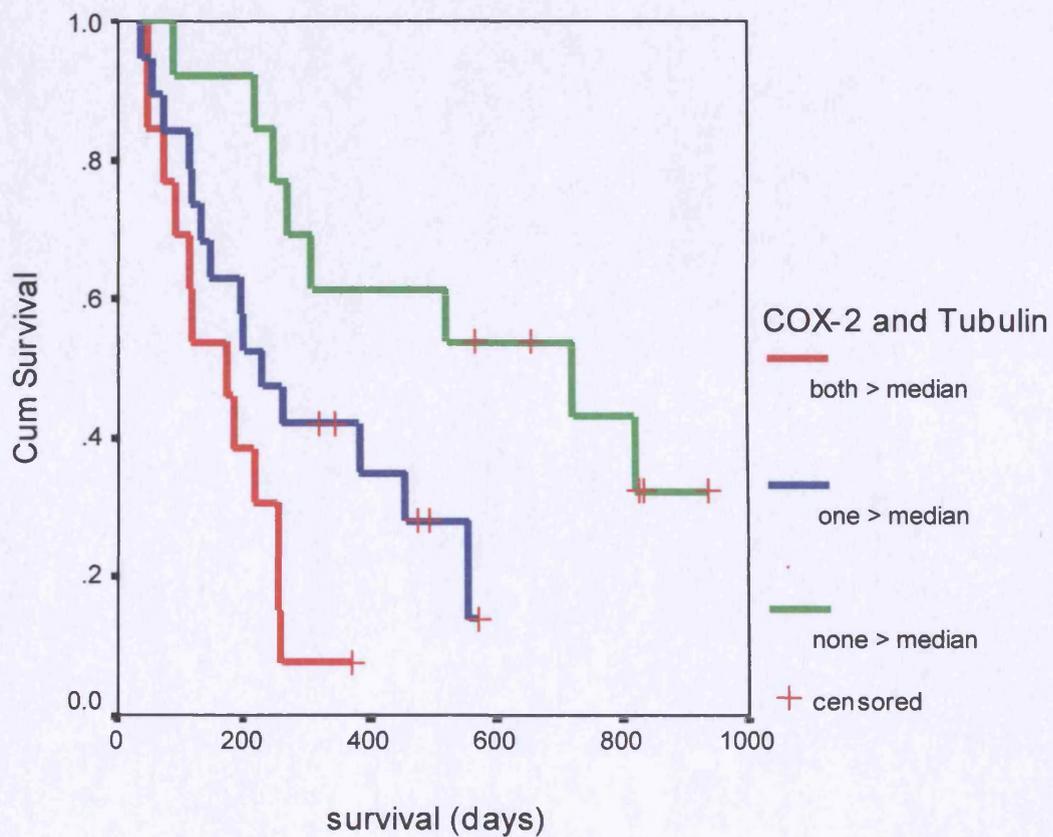
7.5. Discussion

An initial report of COX-2 immunohistochemistry in MM failed to demonstrate COX-2 protein in either tumour samples or cell lines by Western blotting (Marrogi *et al.* 2000a). This current study confirms that COX-2 is expressed in MM and can be detected not only using immunohistochemistry and but also with Western blotting. Furthermore, PGE_2 was also detected in all samples. Although there was little variability in MM tumour cell immunofluorescence using immunohistochemistry, there was variation in the levels of COX-2 protein levels between different MM samples by Western blotting.

With Western blotting, presence of COX-2 protein was detected in 97% of cases. The immunoreactive bands at the level of the COX-2 standard were abolished by the use of the blocking peptide, showing that the primary antibody was acting by its anti-COX-2 binding site and confirming that these were COX-2 protein bands. The results were consistent between the different gels run. Adjustment of the COX-2 bands, according to the internal controls run on each gel, had minimal effect on the results, as did expressing the results as the COX-2:α-tubulin ratio rather than the COX-2 bands alone. The non-specific bands at lower molecular weight, which were partially blocked by the action of the peptide, remain unidentified. With the serial dilution experiment (Method 4), there was a linear relationship between the protein load and COX-2 densitometry values across the range within which the majority of samples lay, demonstrating the semi-quantitative nature of the assay. Although the Santa-Cruz primary antibody gave a much cleaner blot than that from Oxford Biomedical Research, there was a close correlation between them. Therefore the results of these experiments validated the technique of COX-2 Western blotting in snap-frozen MM samples by demonstrating low variability between different gels, runs,

antibodies, running and densitometry conditions. Furthermore, these data show that it is possible to run samples on separate gels under different conditions and yet achieve closely comparable results. As such the data indicating that high levels of COX-2 correlated with a worse prognosis in univariate analysis can be stated with a relatively high degree of confidence. High COX-2 levels also contributed to the CALGB and EORTC groups in multivariate analysis.

High levels of COX-2 were found in association with tumour necrosis. The relationship of COX-2 levels and necrosis is in keeping with suggestions that COX-2 is upregulated in response to hypoxia (Ji *et al.* 1998; Schmedtje *et al.* 1997; Fosslien 2000; Gately 2000). COX-2 is a pro-angiogenic factor (Marrogi *et al.* 2000b; Uefuji *et al.* 2000; Masunaga *et al.* 2000; Williams *et al.* 1999; Fosslien 2001) and associated with cell survival (Saha *et al.* 1999; Fosslien 2000). Therefore COX-2 expression may well be a central part of the survival response of MM to hypoxic conditions induced, perhaps, by a high proliferative rate. Evidence of hypoxic tumour surrounding the areas of necrosis (for example, through immunohistochemistry for carbonic anhydrase(CA)-IX (Giatromanolaki *et al.* 2001a) or hypoxia-inducible factor (HIF) (Giatromanolaki *et al.* 2001b)) has not been sought in this study. EGFR may exert some of its effects through the induction of COX-2 (Coffey *et al.* 1997; Mestre *et al.* 1997; Kulkarni *et al.* 2001; Yoon *et al.* 2002) and vice versa (Pai *et al.* 2002; Kinoshita *et al.* 1999). However, no correlation was found between COX-2 and EGFR expression in this patient series (although EGFR expression also correlated with necrosis). A similar result has been reported for ovarian cancer, where there was no correlation between COX-2 and EGFR expression by immunohistochemistry (Ferrandina *et al.* 2002).

The prognostic significance of COX-2 tumour cell expression has been examined in other cancers. With immunohistochemistry, COX-2 over-expression has been correlated with colorectal tumour growth (Fujita *et al.* 1998), lymph node metastasis (Sheehan *et al.* 1999) and recurrence (Tomozawa *et al.* 2000), although results were not consistent between studies. COX-2 expression has been proposed as a significant poor prognostic factor in colorectal and gastric cancer, but only with univariate analysis (Chen *et al.* 2001a; Masunaga *et al.* 2000). In non-small-cell lung cancer, COX-2 immunostaining has been found to be associated with a significantly worse prognosis in Stage I adenocarcinomas (Achiwa *et al.* 1999).

Clinicopathological correlations with COX-2 protein levels, as assessed by Western blotting and semi-quantitative densitometry, have been less well characterised. COX-2 over-expression, according to this method, has been described in 15 gastric tumour samples, compared to paired normal mucosa. Samples with a COX-2 densitometry ratio between tumour and normal mucosa greater than 2 were associated with lymphatic invasion, lymph node metastasis and TNM stage (Murata *et al.* 1999). Similarly COX-2 Western blot densitometry values were greater in Barrett's oesophagus and oesophageal adenocarcinoma than in normal tissues (Shirvani *et al.* 2000).

This study was one of the first to demonstrate that COX-2 protein levels are a prognostic factor in malignant disease, with high expression correlating with a poor outcome. COX-2 protein levels also contributed to both the CALGB and EORTC prognostic scoring systems in multivariate analysis, suggesting that this is an important factor in MM.

COX-2 inhibitors have a number of beneficial effects in solid tumours. COX-2 inhibition has been shown to increase tumour cell apoptosis (Elder *et al.* 2000; Hida *et al.* 2000) and reduce proliferation (Goldman *et al.* 1998), invasion (Tsujii *et al.* 1997) and angiogenesis (Tsujii *et al.* 1998; Masferrer *et al.* 2000; Skopinska-Rozewska *et al.* 1998). COX-2 may be implicated in the T-cell anergy which is seen in MM (Valle *et al.* 1998). For example it is well established that asbestos causes inhibition of cell mediated cytotoxicity through the release of PGE_2 from alveolar macrophages (Bissonnette *et al.* 1990; Leikauf *et al.* 1995). Therefore COX-2 inhibition may play a role in reversing the immune anergy seen in solid tumours such as MM (Stolina *et al.* 2000). This contention is supported by the observation that indomethacin, an inhibitor of both COX-1 and COX-2 activity, restored the depressed lymphokine-activated killer cell activity in patients with MM in an ex-vivo model (Manning *et al.* 1989). A number of growth factors known to play a role in the pathogenesis of MM, including transforming growth factor-β (Marzo *et al.* 1997; Sheng *et al.* 2000), platelet-derived growth factor (Langerak *et al.* 1996; Xie and Herschman 1996) and HGF/SF (Harvey *et al.* 1998; Tolnay *et al.* 1998), upregulate COX-2 expression (Jones *et al.* 1999; Chen *et al.* 2001b). Collectively these results suggest that selective COX-2 inhibitors may be an effective anti-cancer treatment alone or in combination with cytotoxic chemotherapeutic and other biological agents.

COX-2 inhibitors enhance tumour cell line chemosensitivity to cytotoxic agents (Hida *et al.* 2000) and radiotherapy (Petersen *et al.* 2000). Recent evidence in NSCLC suggests that these effects may translate to the clinical setting. A ongoing phase II trial combining the specific COX-2 inhibitor celecoxib with 2 cycles of paclitaxel and carboplatin prior to surgery for early stage NSCLC showed encouraging results. Of 16 patients who have completed the combined treatment to date, a major clinical response was

achieved in 12 (8 partial, 4 complete) and 4 had stable disease. The combination resulted in

>95% tumour cell death in 37% of tumours (Alktorki *et al.* 2002).

COX-2 inhibitors are also an effective means of chemoprevention in a number of

carcinogenic models reducing, for example, the number and size of intestinal polyps in

$Apc^{\Delta716}$ mice (Kawamori *et al.* 1998; Harris *et al.* 2000; Grubbs *et al.* 2000; Reddy *et al.*

1999; Tomozawa *et al.* 1999; Oshima *et al.* 2001). Further data suggest that COX-2 plays

an important role in MM carcinogenesis. This effect may be mediated through the EGFR

(Hida *et al.* 1998b; Coffey *et al.* 1997). We have previously observed increased expression

and activation of EGFR in pleural mesothelial cells after exposure to carcinogenic asbestos

fibres (Faux *et al.* 2000). Furthermore, exposure of mesothelial cells to asbestos results in

activation of the transcription factor nuclear factor-κB (NF-κB) after autophosphorylation

of the EGFR (Faux *et al.* 2001). An NF-κB binding site is present in the promoter region

of the COX-2 gene (Kosaka *et al.* 1994).

Of further interest was the unexpected correlation of high α-tubulin levels and poor

prognosis, which was independent from COX-2 expression in multivariate analysis and

contributed to both the EORTC and CALGB prognostic scoring systems. Tubulin is the

component protein of microtubules, which form a key part of the cytoskeleton and play an

important part in cell motility. A relationship to sarcomatoid MM was not anticipated but

could explain the morphology and increased invasive potential of these tumours compared

to the epithelioid phenotype. The cell of origin of MM is the subject of debate (Suzuki *et*

al. 1987). The phenotypic change from epithelioid to sarcomatoid cell type (epithelial -

mesenchymal transformation) occurs as a result of up- or down-regulation of genes,

including thioredoxin (Sun *et al.* 2000), proteoglycans and syndecans (Kumar-Singh *et al.* 1998). That α-tubulin levels were prognostic within the epithelioid tumour group might suggest that epithelioid tumour cells with high levels of α-tubulin represent cells undergoing early stages of epithelial-mesenchymal transformation, without yet having changed morphology. α-Tubulin deserves further investigation to confirm whether indeed it is also a marker of the sarcomatoid phenotype. Furthermore, tubulin forms the microtubules of the nuclear spindle which divides the replicated chromosome during mitosis. It would be interesting, therefore, to examine markers of cell proliferation and mitosis to explore possible correlations with α-tubulin expression. Assuming that necrosis is associated with a high proliferative state, the fact that there was no correlation between α-tubulin and necrosis in this study may not support the contention that α-tubulin levels, assessed by this method, reflect cell proliferation. Formalin-fixed, paraffin-embedded tumour samples need to be examined for α-tubulin expression to explore whether these findings are applicable to routine histopathological specimens.

7.6. Conclusions

Increasing levels of both COX-2 and α-tubulin protein, as assessed by Western blot analysis, are poor prognostic factors in MM, which contribute independently to the CALGB and EORTC prognostic scoring systems. These data support an important role of COX-2 in pathogenesis of this malignancy. The cell signalling pathways involved in the regulation of this important immunomodulatory and tumour-promoting factor require further investigation. COX-2 inhibitors may have a role to play in the chemoprevention of the disease. The association of α-tubulin with poor survival deserves further examination, for example, through correlation with tumour cell morphology and proliferation markers.

Chapter Eight

Matrix Metalloproteinases

8.1. Introduction

Proteolysis of the extracellular matrix (ECM) and basement membrane, by proteases such as matrix metalloproteinases (MMPs), is a central part of tumour growth and metastasis. The stromal remodelling mediated by these enzymes also facilitates tumour angiogenesis. MMPs are a family of zinc dependent enzymes, which are implicated in the growth of primary and secondary tumours (Cox *et al.* 1999). Over-expression of MMPs, in particular gelatinase A (MMP-2), gelatinase B (MMP-9) and stromelysin-3 (MMP-11), is related to tumour progression and metastasis in gastric cancer (Schwartz 1996), colon cancer (Karakiulakis *et al.* 1997) and NSCLC (Cox *et al.* 1999). The expression or activity of MMPs has not been described in MM.

8.2. Aims

1   To evaluate the activities of the gelatinases MMP-2 and MMP-9 in snap-frozen specimens of MM using gelatin zymography

2   To compare expression of MMP-2 and MMP-9 in MM with that seen in benign uninflamed (UP) and inflamed pleura (IP)

3   To correlate gelatinase expression with the other clinicopathological and biological variables analysed in the project

4   To establish the prognostic significance of gelatinases in MM

8.3. Methods

8.3.1. Method 1

Samples were prepared for gelatin zymography as described in section 2.3.7..

8.3.1.1. Experiment 1

Samples from 22 MM, 16 UP (from patients undergoing surgery for pneumothorax) and 12 IP (from patients undergoing surgery predominantly for empyema) were run simultaneously on 6 gels in three electrophoresis tanks. Densitometry values obtained were compared between the groups. Three samples (of high and medium band intensity) included on each gel as internal controls. With the use of these samples, adjustment was made between the different blots to allow for any variation between them. Linear regression analysis was used to derive a multiplication factor for each gel from the pro- and active MMP-2 and -9 bands (compared to mean values) and this was used to adjust the values between gels. The correlation between the densitometry values of all samples thus adjusted and unadjusted was assessed.

8.3.1.2. Experiment 2

After experiment 1, a $-80^{\circ}C$ freezer failure resulted in the loss of the homogenised specimens stored. Therefore in *experiment 2* snap-frozen blocks were available only for the last 35 MM cases of the 49 collected. Furthermore, the digital photography system was also updated from the ImageStore 5000 Version 7.11 Gel Documentation System (Ultra-Violet Products) to the VisionWorks 3.1 system (Ultra-Violet Products) between experiments. As a result no comparison was possible between these and the MM cases analysed earlier against the benign pleural specimens akin to the work conducted for COX-

2. In all other aspects the methodology used in *experiment 2* was identical to that in *experiment 1*.

8.3.2. Method 2

In *experiment 1* the variation between identical runs was assessed, with the 22 MM and 26 benign cases run on different days and the inter-run results correlated.

8.3.3. Method 3

Two samples, each loaded into 6 wells, in 4 separate gels (12 wells per gel) were run simultaneously. The 2 samples studied were those with the strongest gelatinase bands. The variation in densitometry values obtained for each of these two samples within 1 gel and between the 4 gels was assessed.

8.3.4. Method 4

The relationship between the amount of gelatinases present and the densitometry value obtained was determined by running gels with serial dilutions of the 2 samples above. A protein load of between 1 and 30μg per well was used.

8.3.5. Statistical Analysis

Differences in densitometry readings between groups were assessed with the Mann-Whitney U and Kruskal-Wallis tests, where appropriate. The analysis of survival with the Log rank test and Cox proportional hazards models was performed as previously described in section 3.3.2. The results analysed were prospectively.

8.4. Results

8.4.1. Method 1

8.4.1.1. Experiment 1

It was possible to determine densitometry values for all gelatinase isoforms in each of the MM, UP and IP cases (Table 8.1). MMP-2 was the predominant gelatinase seen in MM (Figure 8.1). Active MMP-2 was significantly elevated in MM compared with UP specimens (p=0.04, Table 8.1, Figure 8.2). Densitometry values for all gelatinase isoforms were no higher in sarcomatoid than in epithelial MM. There were no differences between MM and UP groups with regard to MMP-9 activity. There was significantly greater pro- and active MMP-9 gelatinolytic activity in IP specimens compared to the UP (p=0.05, p=0.01 respectively) and MM (p=0.02, p=0.009 respectively) groups. However, unlike MM, there was no significant difference in pro- and active MMP-2 gelatinolytic activity between IP and UP specimens.

8.4.1.2. Experiment 2

Gelatinolytic bands corresponding with pro-MMP-9 and pro- and active MMP-2 were seen in all 35 cases of MM. No detectable active MMP-9 was seen in 9 of the 35 MM cases (26%). The adjustment factors for the four gels were 0.96, 1.00, 0.89 and 1.15. Dividing the variables above and below the median values for each isoform before and after adjustment, there was agreement in all but 2 cases (5.7%) for each of pro- and active MMP-9 and pro-MMP-2. Just 1 case (2.9%) was reallocated into the greater than the median group for active MMP-2 following the adjustment procedure. There was a close correlation between the unadjusted and adjusted values (Figure 8.3). Adjusted pro-MMP-2 and -9

Table 8.1: Zymogram densitometry values in MM and benign pleura. Data are displayed as mean arbitrary densitometry units (95% CI).

	n	Pro-MMP-9	Active MMP-9	Pro-MMP-2	Active MMP-2
MM	22	1996 (1268-2724)	1400 (953-1848)	2743 (2102-3385)	1936 (1183-2689)
UP	16	1934 (1466-2403)	1423 (1063-1783)	2297 (1543-3051)	1038 (648-1428)
IP	12	3642 (2324 – 4960)	2380 (1591 – 3170)	3485 (2132 – 4837)	2499 (1005 – 3994)
Mann-Whitney p values:					
MM vs UP	0.5	0.6	0.4	0.04	
IP vs MM	0.02	0.009	0.7	0.7	
IP vs UP	0.05	0.01	0.1	0.07	

Figure 8.1: Gelatin zymogram of malignant mesothelioma (MM), inflamed pleura (IP) and uninflamed pleura (UP). Bands of gelatinolysis, correlating with the latent, pro- and active isoforms of MMP-2 and MMP-9. The gelatinolytic bands at the top of the gel (at higher molecular weight) represent MMP-9 dimerisation products (a=MMP-9 heterodimer at 220kDa, b=MMP-9 homodimer at 135 kDa).
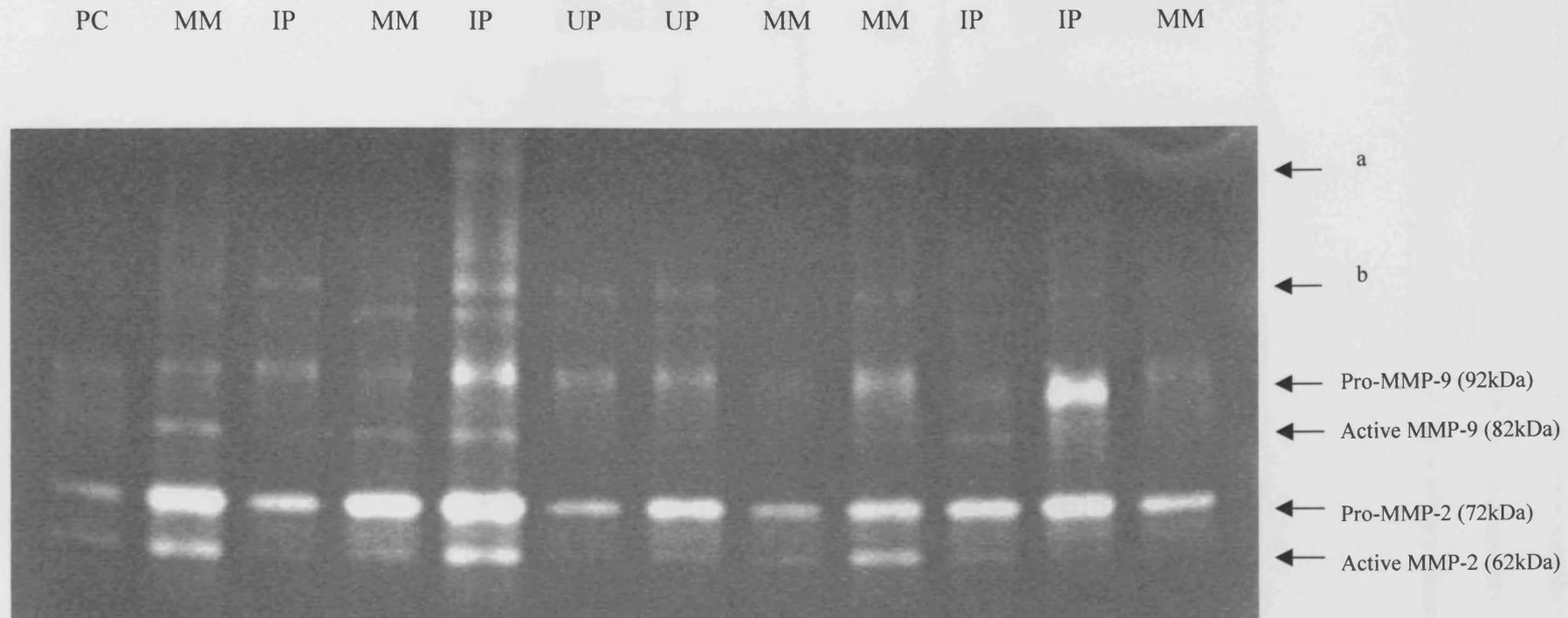
Figure 8.2: Boxplots showing the differences in MMP activity between MM (n=22), UP (n=12) and IP (n=16). Data are shown for pro-MMP-9, active MMP-9, pro-MMP-2 and active MMP-2. Horizontal bars represent the median values, boxes the interquartile range, vertical bars the 10 – 90 % range and solid circles the outliers. MMP enzyme activity was measured with semi-quantitative gelatin zymography and is expressed by arbitrary densitometry units per milligram protein.
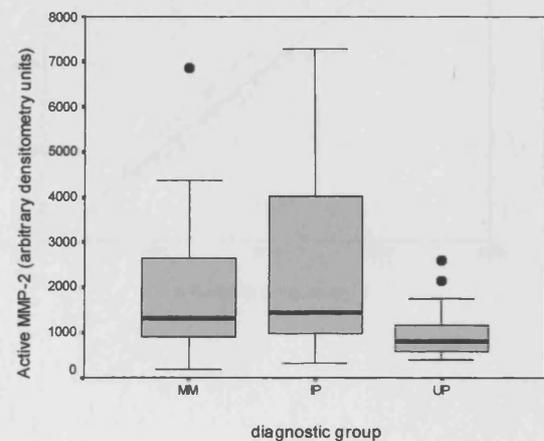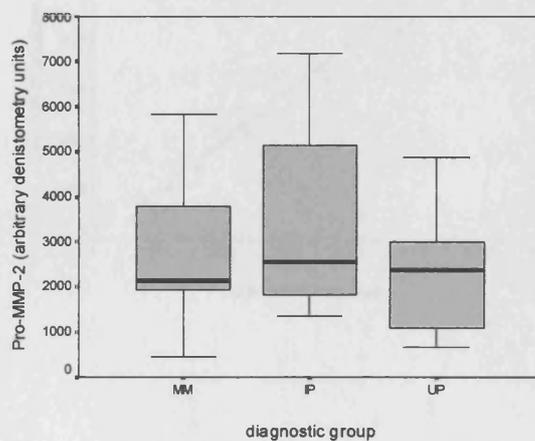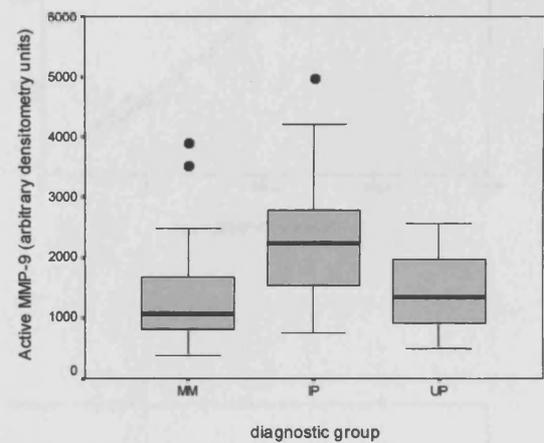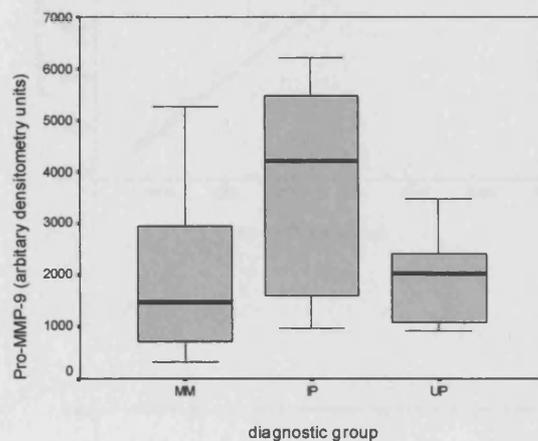
Figure 8.3: The relationships between unadjusted and adjusted MMP densitometry values for

the 35 mesothelioma cases. Pearson's correlation coefficients were 0.989, 0.995, 0.977 and

0.970 for pro-MMP-9, active MMP-9, pro MMP-2 and active MMP-2 respectively.
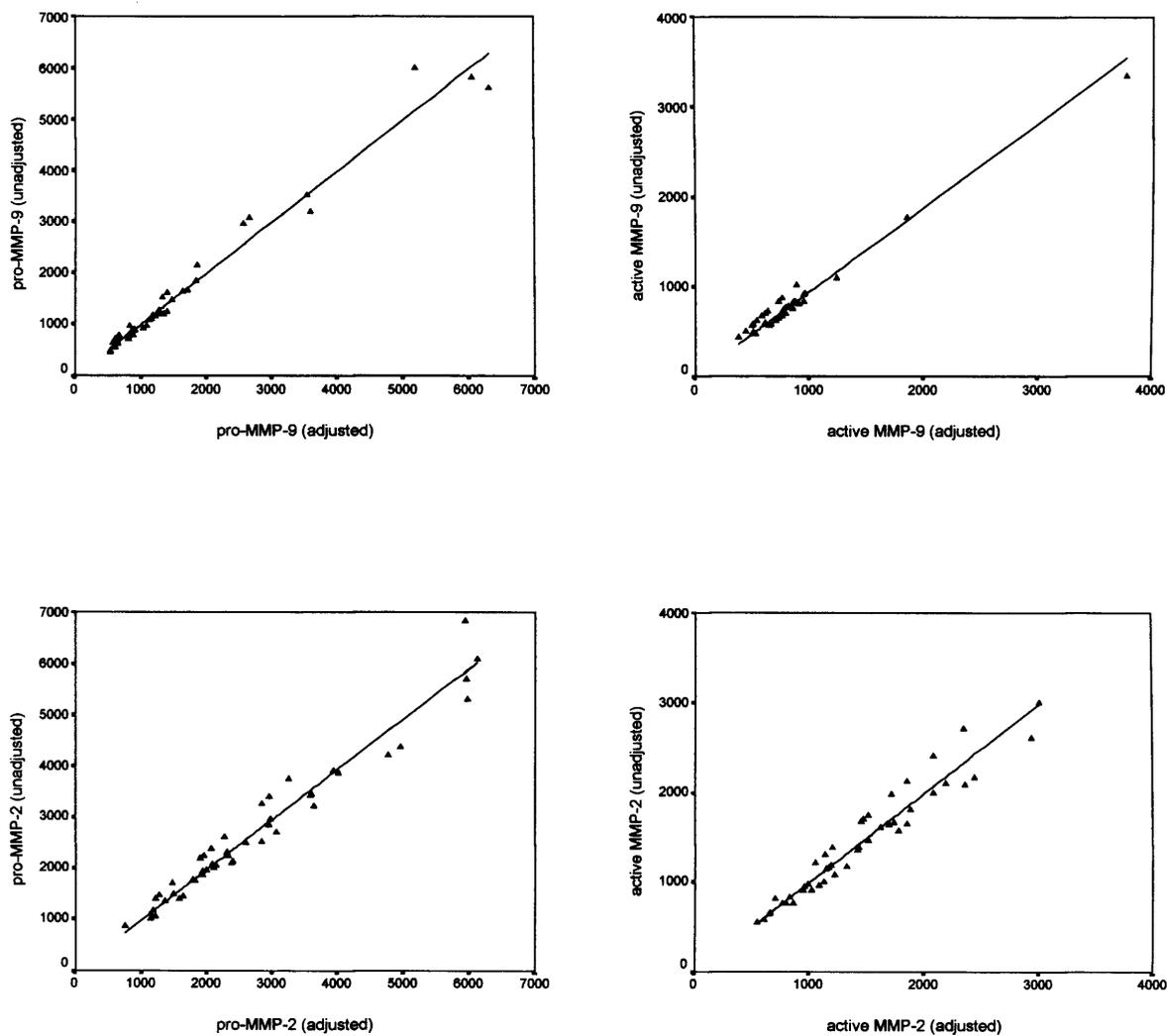
levels were mean 2600 (95%CI 2130 - 3070) and 1640 (960 – 2330) respectively

(p=0.006). Values were 1540 (1300 - 1770) and 850 (580 - 1110) for the active isoforms

(p<0.001). The adjusted values were used for subsequent analyses, although the use of the

unadjusted values had no effect on outcomes.

8.4.1.3. Correlation with clinicopathological factors

There were no significant correlations between gelatinases and gender, age,

presence of chest pain or weight loss, cell type or performance status. There was no

correlation between any MMP isoform and either the EORTC or CALGB prognostic

scores. Pathological staging was performed only for the 13 patients who underwent radical

surgery. Nodal stage was N0 in 7, N1 in 1 and N2 in 5 patients. Pro-, active and total

MMP-2 activities were greater in those with extrapleural nodal metastasis than without

(Table 8.2). There were no associations seen between gelatinases and pathological T or

overall IMIG TNM stages. Preoperative white cell count was associated with greater total

MMP-9 (sum of pro-MMP-9 and active MMP-9, p=0.0006) and a trend towards a similar

result was seen with thrombocytosis (p=0.06). There were no significant correlations with

the MVD, presence of tumour necrosis, EGFR expression, or COX-2 protein levels (Table

8.3).

8.4.1.4. Survival

In univariate Cox proportional hazards analysis, trends towards increasing pro-

MMP-2 and total MMP-2 activities (as continuous variables) and poor prognosis were seen

(p=0.08, HR 1.0003, 95%CI 1.0000 – 1.0006 and p=0.08, HR 1.0002, 95%CI 1.0000 –

1.0005 respectively, Figures 8.4 and 8.5). However, although trends towards poor

prognosis were seen with the categorical analyses, these did not reach statistical

Table 8.2: Correlation between MMP-2 and MMP-9 activities and clinicopathological variables. The median densitometry value for each isoform is quoted. The Mann-Whitney U test was used for the comparison of two groups and the Kruskal-Wallis Test for greater than two groups. Total MMP activity equals the sum of pro- and active enzyme values.

Variable		n	Pro-MMP-9	Active MMP-9	Total MMP-9	Pro-MMP-2	Active MMP-2	Total MMP-2
Cell type	Epithelioid	23	1176	608	1693	2104	1199	3418
	Non-epithelioid	12	816	633	1419	2422	1491	4087
	p		0.082	0.344	0.237	0.281	0.889	0.314
White blood cells	<8.3 x 10^9/l	13	788	511	1279	1988	1193	3426
	>= 8.3 x 10^9/l	22	1167	638	1796	2468	1559	3895
	p		0.065	0.109	0.006	0.585	0.824	0.539
Platelets	<400 x 10^9/l	22	824	582	1419	2279	1449	3547
	>=400 x 10^9/l	13	1176	608	1917	2105	1199	3771
	p		0.172	0.757	0.060	0.864	0.670	0.811
Haemoglobin	<14 g/dl	22	824	629	1412	2279	1491	3814
	>=14 g/dl	13	1367	502	1764	1955	1193	3012
	p		0.037	0.158	0.142	0.517	0.597	0.375
CALGB prognostic group	Groups 1, 2	10	1247	562	1567	2218	1090	3265
	Groups 3,4	17	820	662	1693	2241	1622	3485
	Groups 5,6	8	1036	608	1353	2446	1113	4087
	p		0.598	0.363	0.999	0.890	0.187	0.592
EORTC prognostic group	Low risk	20	895	611	1448	2279	1440	3547
	High risk	15	1157	608	1764	2092	1199	4018
	p		0.463	0.534	0.217	0.714	0.841	0.790
IMIG nodal status	N0, N1	8	1091	189	1353	1093	1086	3069
	N2	5	1042	662	1727	3643	1852	6097
	p		0.884	0.075	0.107	0.019	0.013	0.008
IMIG Stage	II	2	962	383	1345	1566	1317	2883
	III	11	1043	625	1483	2847	1725	4677
	IV	8	1000	409	1437	1923	1184	3207
	p		0.840	0.689	0.755	0.173	0.797	0.353

Table 8.3: Correlation between MMP-2 and MMP-9 activities and biological variables. The median densitometry value for each isoform is quoted. The Mann-Whitney U test was used for the comparison of two groups and the Kruskal-Wallis Test for greater than two groups. Total MMP activity equals the sum of pro- and active enzyme values.

Variable		n	Pro-MMP-9	Active MMP-9	Total MMP-9	Pro-MMP-2	Active MMP-2	Total MMP-2
MVD	< median	11	820	511	1425	1652	1523	4677
	> median	17	1043	634	1652	2952	1090	3485
	p		0.832	0.225	0.832	0.249	0.196	0.196
Tumour Necrosis	Absent	24	856	521	1417	1917	1196	3401
	Present	11	117	662	1829	2952	1677	4204
	p		0.286	0.062	0.110	0.102	0.631	0.127
EGFR	Absent	17	805	608	1413	2092	1185	3426
	Present	17	1176	634	1693	2331	1440	3623
	p		0.221	0.862	0.617	0.293	0.850	0.459
COX-2	< median	18	1097	535	1454	1971	1189	3422
	> median	16	862	648	1699	2584	1599	4111
	p		0.448	0.103	1.0	0.285	0.293	0.178

Figure 8.4:   Scatterplot showing the relationship between survival and total MMP-2

activity (sum of pro-MMP-2 and active MMP-2 band arbitrary densitometry units). There

was a trend towards worse survival in patients with increasing values (p=0.08, Cox

proportional hazards).
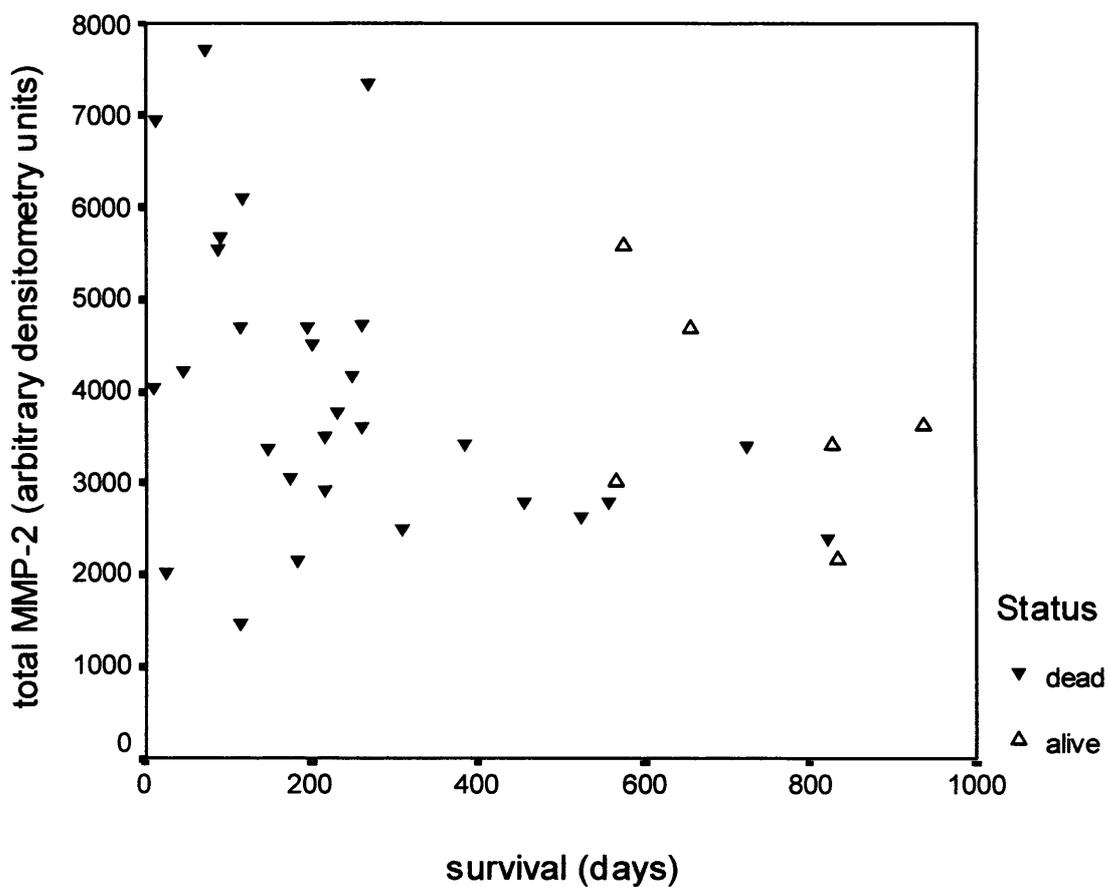
Figure 8.5: Kaplan-Meier plot showing survival curves for cases with total MMP-2 (sum of pro-MMP-2 and active MMP-2 isoforms) greater and less than the median value (p=0.187).
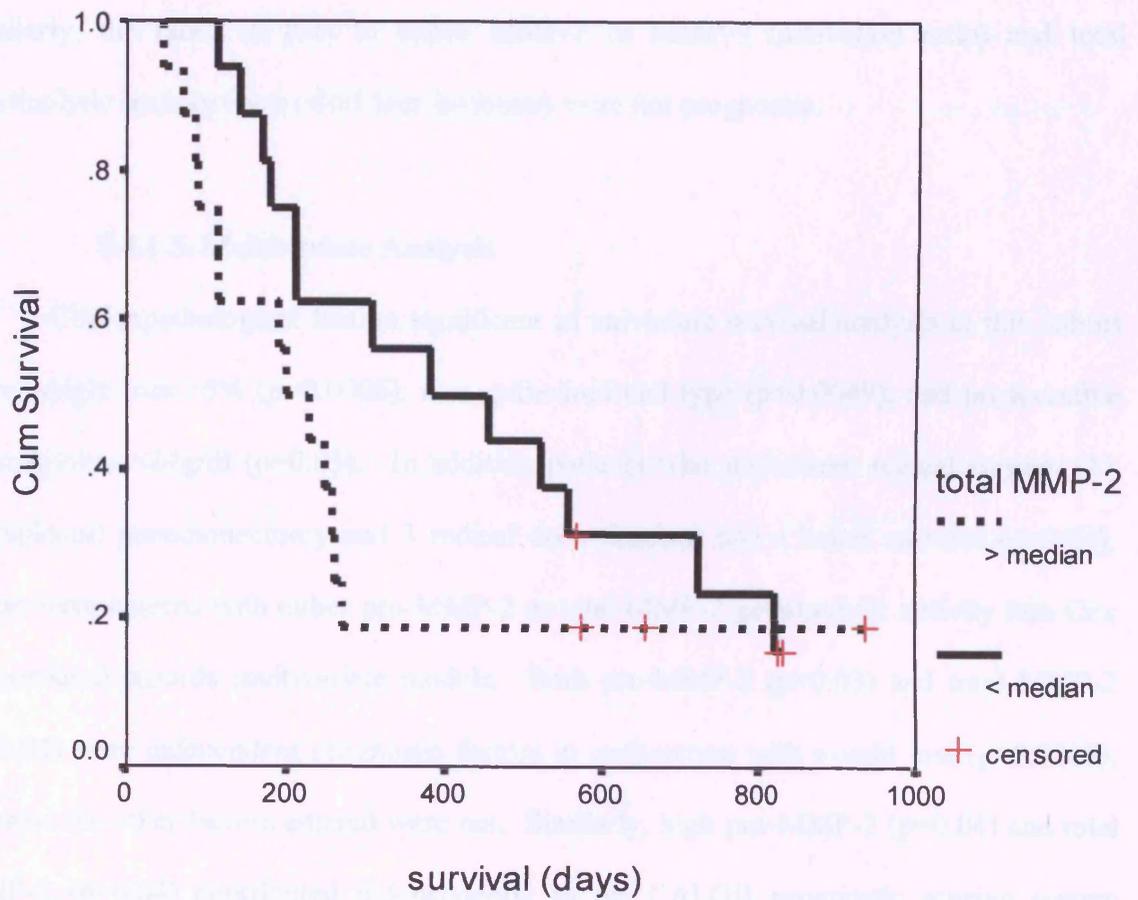
significance in either univariate Cox proportional hazards models (p=0.16 and p=0.19 respectively) or the log rank test (p=0.15 and p=0.19). There were no significant relationships between poor prognosis and active MMP-2, or total, pro-, or active MMP-9. Similarly, the ratios of pro- to active MMP-2 or MMP-9 (activation ratio) and total gelatinolytic activity (sum of all four isoforms) were not prognostic.

8.4.1.5. Multivariate Analysis

Clinicopathological factors significant in univariate survival analysis in this cohort were weight loss >5% (p=0.0006), non-epithelioid cell type (p=0.0049), and preoperative haemoglobin <14g/dl (p=0.03). In addition patients who underwent radical surgery (11 extrapleural pneumonectomy and 3 radical decortication) had a better survival (p=0.04). These were entered with either pro-MMP-2 or total MMP-2 gelatinolytic activity into Cox proportional hazards multivariate models. Both pro-MMP-2 (p=0.03) and total MMP-2 (p=0.04) were independent prognostic factors in conjunction with weight loss (p=0.0008), whereas the other factors entered were not. Similarly, high pro-MMP-2 (p=0.04) and total MMP-2 (p=0.04) contributed independently to the CALGB prognostic scoring system (p=0.005 and p=0.02 respectively). The EORTC risk groups were not prognostic in univariate analysis in this series (p=0.16, log rank) and therefore were not tested in multivariate analysis.

8.4.2. Method 2

Correlations between runs on different days were as follows: r = 0.936, 0.863, 0.973 and 0.946 for pro-MMP-9, active MMP-9, pro-MMP-2 and active MMP-2 bands respectively, Figure 8.6).

Figure 8.6: Scatterplots showing the correlation between gelatin zymography runs performed on different days. r=0.936, 0.863, 0.973 and 0.946 for pro-MMP-9, active MMP-9, pro-MMP-2 and active MMP-2 bands respectively.
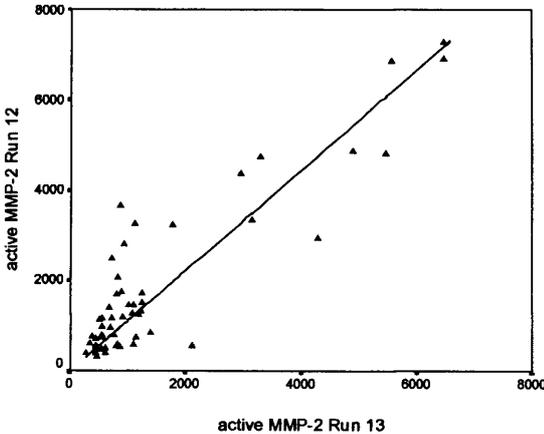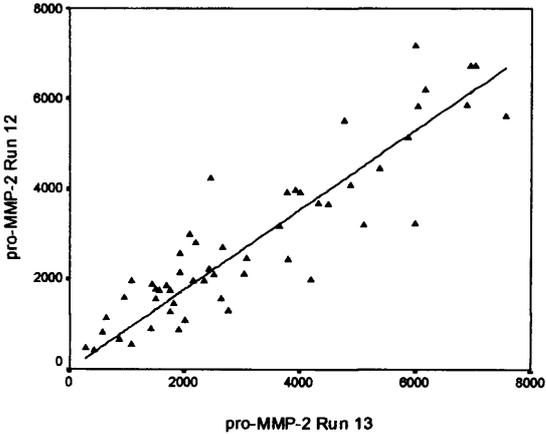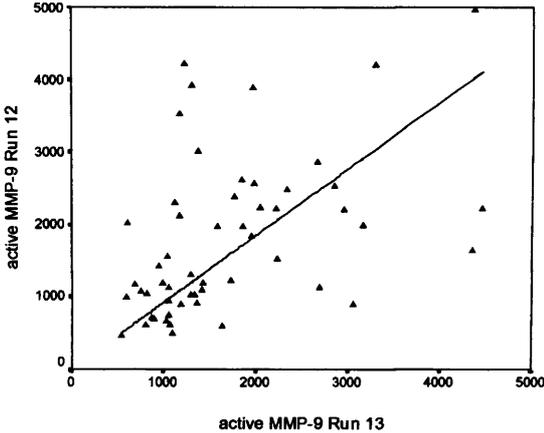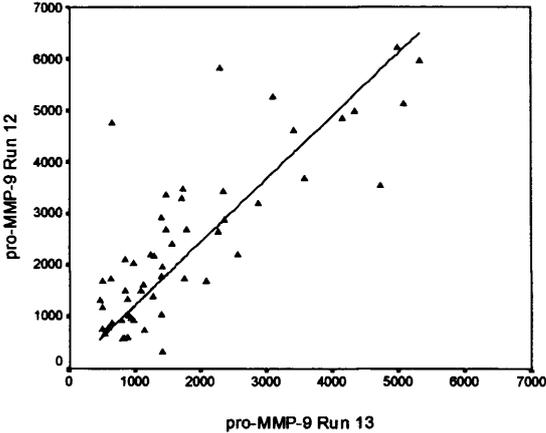
8.4.3. Method 3

Neither of the two samples examined displayed visible bands of active MMP-9. The variation between gels is depicted in Figure 8.7, both by each gelatinase isoform individually and with all three detected combined. Evaluating the gels for differences in mean band densitometry with the Kruskal-Wallis test, there was a near significant difference between the gels (p=0.054).

Therefore it was decided that, in order to allow comparison between samples run on different gels, a correction factor would need to be applied according to the densitometry values of internal control samples run on each gel. This was performed by running three internal control samples on each gel. An adjustment factor was derived for each gel by linear regression analysis of the densitometry values of all gelatinase isoforms for the internal controls on a particular gel compared to mean values.

8.4.4. Method 4

At protein loads of greater than 10 μg per well, the densitometry values reached a plateau (Figure 8.8). The relationship of protein load to densitometry value appeared linear at < 10 μg per well.

Figure 8.7: Variation in band densitometry within and between gels, depicted as the percentage of the overall mean for each isoform. Two samples were run in sextuplicate on four gels. The bars represent the 95% confidence intervals of each mean, which is depicted by the solid square.
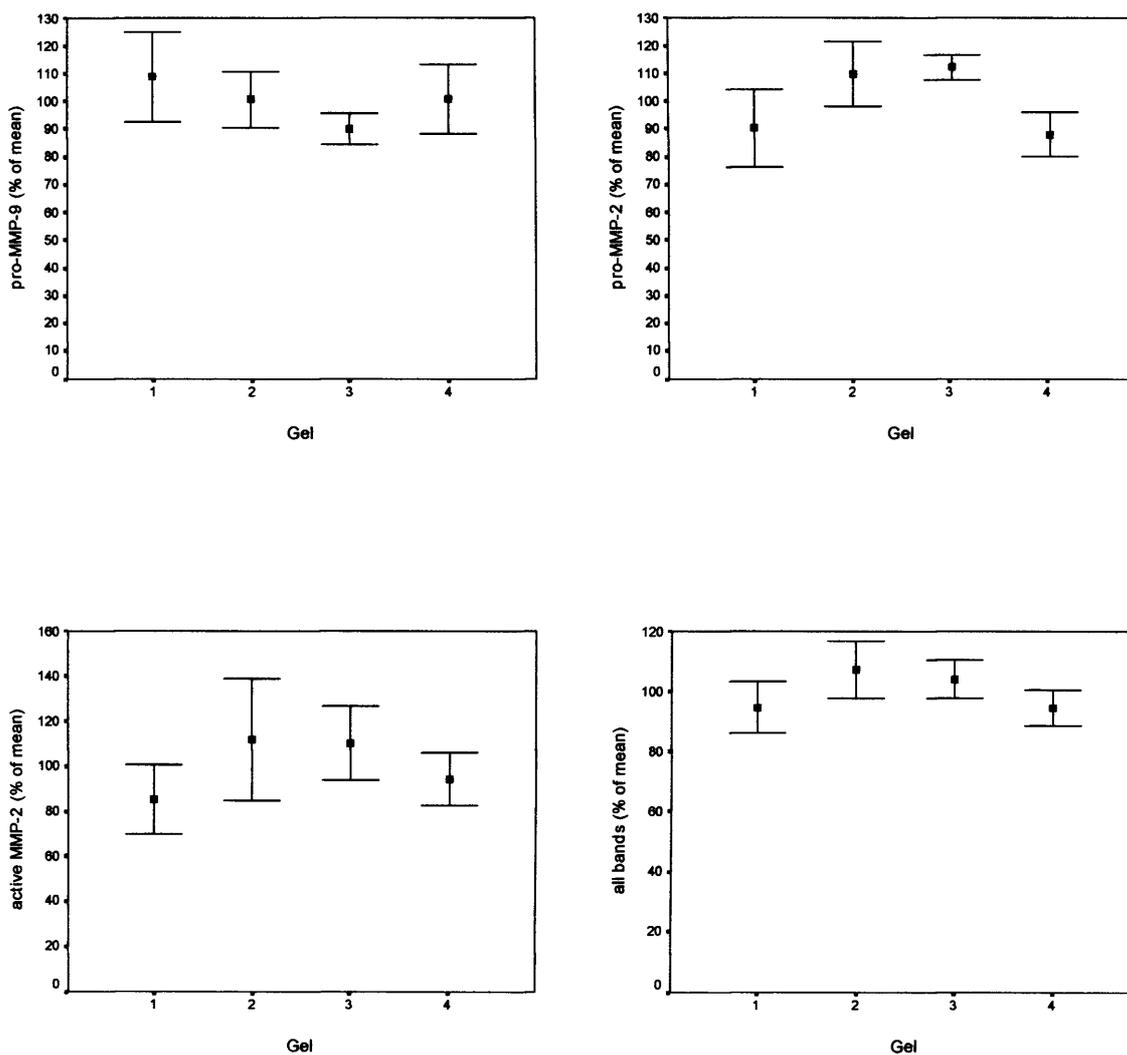
Figure 8.8:   Densitometry values obtained from serial dilution of the two strongest

MM samples (in duplicate). The protein load for each dilution is plotted against the

densitometry values obtained. Values for pro-MMP-9, pro-MMP-2, and active MMP-2

have been combined on this plot. The bars represent the 95% confidence intervals of

each mean, which is depicted by the solid square. For the main gelatin zymography

experiments, 10µg of protein of was added to each well of the gel.

protein (mcg)
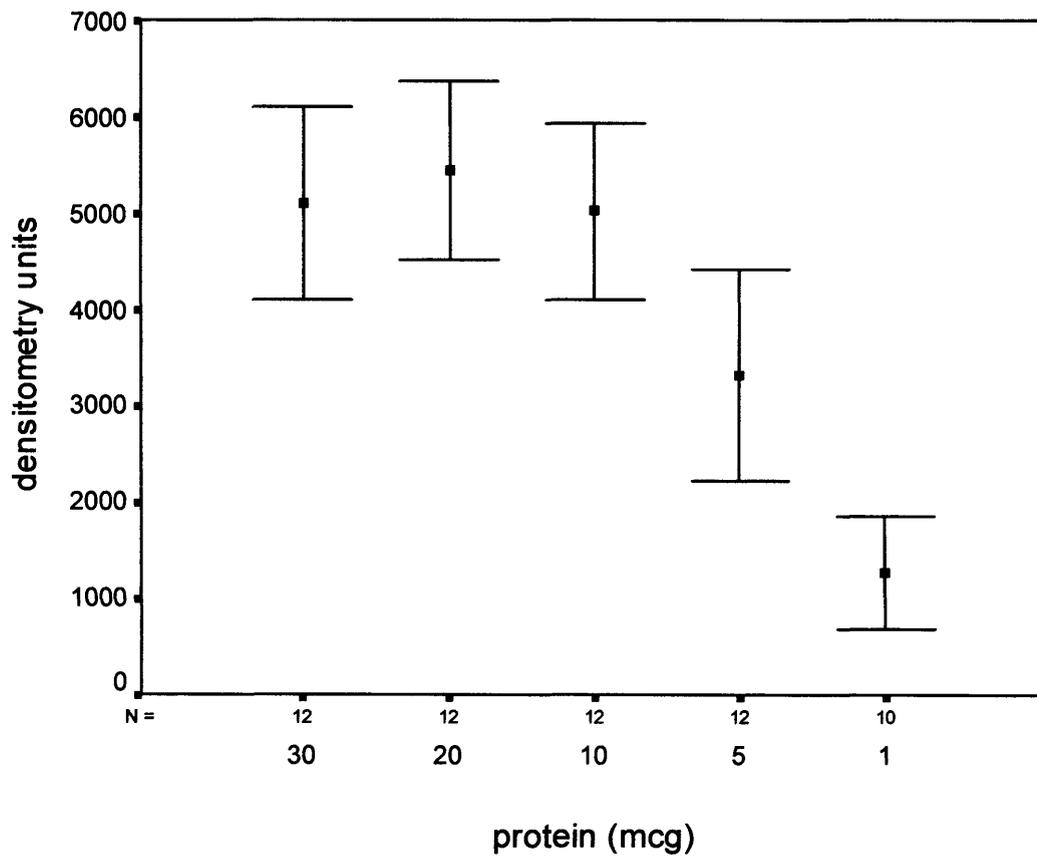
8.5. Discussion

MM is characterised by extensive local growth and invasion of intrathoracic organs. This pattern of tumour development suggests an important role for proteases, including the matrix metalloproteinases (MMPs), in the evolution of the disease. This study demonstrates consistent expression of active and latent forms of MMP-2 and MMP-9 in MM tumour samples by gelatin zymography. Whole tumour extracts were used rather than microdissected extracts since recent evidence has suggested that stromal expression of MMPs is important in tumour progression (DeClerck 2000; Kikuchi *et al.* 2000; Himelstein *et al.* 1994; Polette and Birembaut 1998; Nawrocki *et al.* 1997).

Gelatin zymography was found to be reproducible, with a low variation between samples run on different days. The variation between gels was also acceptable and the adjustment of densitometry values according to the internal control samples had little effect. Serial dilutions of the strongest two samples displayed a linear relationship between the densitometry values and protein load at less than $10\,\mu g$ per well. This confirmed the semi-quantitative nature of the assay when $10\,\mu g$ protein was loaded to each well.

The benign UP control group specimens were obtained from patients undergoing pleurectomy for primary spontaneous pneumothorax. However, even this group may not display true baseline expression of MMPs. Both acute and chronic inflammatory changes were noted in the histopathology reports of the diagnostic tissue blocks from the 'UP' specimens that accompanied the snap-frozen samples used in this study. It is possible to speculate, therefore, that the degree of upregulation of MMPs in MM, and indeed in IP, has been underestimated.

The patterns of gelatinolytic activity seen in MM, IP and UP are likely to reflect both the nature and extent of the inflammatory cell infiltrate in these conditions, as well as between benign and malignant mesothelial cells. The predominant gelatinase expressed by macrophages and neutrophils in inflammatory conditions such as emphysema, asthma, sepsis and inflammatory bowel disease is MMP-9 (Finlay *et al.* 1997; Mautino *et al.* 1997; Pugin *et al.* 1999), which is in keeping with the significantly elevated activity of this enzyme seen in IP specimens compared to either MM or UP samples. Furthermore the observation that total MMP-9 was significantly greater in MM patients with a WBC > 8.3×10^9/l supports this contention. The levels of active MMP-2 were found in this study to be significantly higher in MM than UP. This finding suggests that the MMP-2 activity seen in MM may be more specific than MMP-9 to the carcinogenic process.

Activity of MMP-2 and –9 isoforms has been investigated by gelatin zymography in other solid tumours. Up-regulation of MMP-2 is seen in solid tumours including hepatocellular cancer (Maatta *et al.* 2000), colorectal cancer (Liabakk *et al.* 1996; Baker *et al.* 2000; Waas *et al.* 2002) and ovarian cancer (Lengyel *et al.* 2001). With regard to the relationship of MMP activity, as assessed by gelatin zymography, with prognosis, results differ between studies and tumour types. In oral squamous cell carcinoma, activities of MMP-2 and –9 correlate with disease free survival (Yorioka *et al.* 2002). In colorectal cancer, the relationships between MMP activities and tumour progression remain unclear. Waas *et al.* (Waas *et al.* 2002) found no association between MMP activity and nodal metastasis but did note significantly lower tumour extract MMP-2 activity in patients with distant metastasis. Baker *et al.* (Baker and Leaper 2002) found that total MMP-2 and –9 levels in colorectal tumour samples, as assessed by gelatinase activity assays, correlated

positively with Dukes' stage. Liabakk *et al.*, however, demonstrated higher levels of MMP-9 in Dukes' stage A and C (Liabakk *et al.* 1996). Brown *et al.* found that active MMP-2 correlated with stage in NSCLC (Brown *et al.* 1993a) but not breast cancer (Brown *et al.* 1993b). Pro-MMP-9, but neither MMP-2 isoform, was associated with poor survival in both univariate and multivariate analyses in ovarian cancer (Lengyel *et al.* 2001). No relationships were seen in bladder cancer (Papathoma *et al.* 2000), hepatocellular carcinoma (Maatta *et al.* 2000) or soft tissue sarcomas (Maguire *et al.* 2000).

The numbers in this study are relatively small, particularly with respect to those with an accurate pathological TNM stage. The accuracy of radiological TNM staging in MM remains unclear and therefore we made no attempt to assess the stage of patients who did not undergo radical surgery and detailed pathological assessment. Nonetheless the positive correlation between any MMP-2 activity and nodal status noted here is in keeping with a number of the studies in other tumours cited above. Similarly, although our multivariate survival analyses may lack statistical rigor, the results are provocative and justify confirmatory studies in a larger series of patients.

The regulation of MMP expression (Jones and Walker 1997) in MM remains to be characterised. Nonetheless many growth factors known to induce MMP expression are detectable in MM. These include bFGF (Kumar-Singh *et al.* 1999), HGF/SF (Tolnay *et al.* 1998), VEGF (Ohta *et al.* 1999), IGF-1, TNF-α, TGF-α and -β (Bielefeldt-Ohmann *et al.* 1996) and IL-8 (Antony *et al.* 1996). The inter-relationships between these growth factors and MMP expression in MM tumour samples need to be investigated. Furthermore, gelatin zymography does not discriminate between free MMPs and those complexed with TIMPs, mRNAs for which have been identified in MM cells in vitro (Liu *et al.* 2001). Further

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study is required to address the balance of MMPs with respect to TIMPs in MM tumour samples.

The detection of active gelatinase isoforms in MM samples, in particular MMP-2, is important and suggests that MMP inhibitors may be therapeutic in this disease. Evidence to support this contention comes from both experimental and clinical studies. Inhibition of MMPs reduces tumour growth, invasion and angiogenesis *in vivo* (Maekawa *et al.* 1999). Initial phase III trials of MMP inhibitors in other solid tumours alone or in combination with cytotoxic agents have overall been disappointing. In unresectable pancreatic cancer, there was no difference in survival between patients who received gemcitabine and marimastat versus gemcitabine alone (Bramhall *et al.* 2002a). However, in unresectable gastric and gastro-oesophageal cancer, the marimastat alone led to longer progression-free and overall survival in those who had received prior chemotherapy (Bramhall *et al.* 2002b). In the whole study group, there was a trend towards increased survival amongst those who received marimastat. A phase III study of gemcitabine and cisplatin, with or without prinomastat, did not show any benefit of the MMPI in advanced NSCLC (Bissett *et al.* 2002). Nonetheless inhibition of MMP activity, with synthetic MMP inhibitors (Drummond *et al.* 1999; Steward 1999) or with biological agents that downregulate the synthesis and activation of the enzymes such as the selective COX-2 inhibitors (Pan *et al.* 2001; Abiru *et al.* 2002; Attiga *et al.* 2000; Reich and Martin 1996) deserves clinical investigation as a novel approach to the management of MM. This is supported by a recent study from Macaulay *et al.* which demonstrated that administration of the MMP inhibitor batimastat into the pleural cavity resulted in a reduction in the rate of reaccumulation of pleural fluid in patients with malignant effusions (Macaulay *et al.* 1999).

8.6. Conclusions

This study supports a role for MMPs in the pathogenesis of MM. This particularly applies to MMP-2 where the results indicate that elevated activities of the enzyme correlate with a poor outcome. Selective inhibitors of gelatinases, or inhibitors of their synthesis and/or activation, require evaluation in the management of the disease.

Chapter Nine

Concluding Remarks

This study examined, using immunohistochemistry, Western blotting, enzyme immunoassay and gelatin zymography, five major factors which are central to tumour growth, survival, invasion and metastasis. Their correlations with clinical and pathological variables were explored. The impact of each of these factors on survival was evaluated in univariate and multivariate models.

Prognostic factors and CALGB and EORTC prognostic groups were derived from retrospective case note data. The prognostic factors which were significant in multivariate analysis (histological cell type, weight loss and performance status) were consistent with other studies. When patients were placed into the appropriate groups of the two prognostic scoring systems, the median survival and one and two year survival rates were closely comparable. This validated the two systems and provided the first independent report of their potential use. Furthermore, it also demonstrated that the survival figures for MM in the Leicester, which may be felt to be poor by international standards, are comparable when appropriate adjustment is made for the prognostic group.

The assessment of angiogenesis as a prognostic factor confirmed, in a much larger series than those previously published, that MVD is an independent prognostic factor in MM. Although there was a degree of inter- and intra-observer variability both observers found that high MVD was predictive of poor prognosis. The importance of MVD as a prognostic factor is supported by its persistence as an independent prognostic factor in multivariate analyses to which other variables, such as TN and EGFR status, were added. Stromal staining with the anti-CD34 antibody precluded assessment of MVD in 25% of cases studied. Early work suggests that the anti-CD31 antibody may allow MVD assessment in these cases in the future.

TN, which had only previously been noted as a diagnostic marker, was found to correlate with poor prognostic factors such as thrombocytosis and low haemoglobin. TN was itself a marker of poor prognosis, but it was not an independent variable in multivariate analysis. However, TN did contribute to the CALGB and EORTC systems in multivariate models. The correlation of TN with high MVD adds to the existing evidence suggesting that tumour hypoxia (and necrosis) stimulates intratumoural angiogenesis. Further investigation is now underway in the laboratory to characterise factors central to hypoxia and the induction of angiogenesis. In preliminary work, we have recently discovered expression of CA-IX by immunohistochemistry in MM (Figure 9.1). Hypoxia is emerging as a novel target in solid tumours. For example tirapazamine is a hypoxic cytotoxin, which is activated in hypoxic cells and has been shown to improve the chemosensitivity and radiosensitivity of hypoxic tumours (Gandara *et al.* 2002).

The favourable prognostic impact of EGFR expression, which had been described in an earlier study (Dazzi *et al.* 1990), was confirmed. It was possible, with the size of this series, to clarify the correlation with cell type seen by Dazzi *et al.* Analysis of the pure epithelioid cases alone did not demonstrate a survival advantage for the EGFR positive cases, although a trend was seen for cases with cytoplasmic EGFR positivity. However, EGFR expression did contribute as a prognostic factor independent of the CALGB and EORCT prognostic groups in multivariate analyses. The EGFR TKI ZD1839 (Iressa, AstraZeneca) is subject to clinical trials as a therapeutic target in MM as described previously (Nowak *et al.* 2002b). Knowledge of the function and correlations of EGFR in MM will be important in the interpretation of such trial results.

Figure 9.1: Immunohistochemistry for Carbonic anhydrase-IX, with both membranous and cytoplasmic immunostaining seen in this section of epithelioid MM
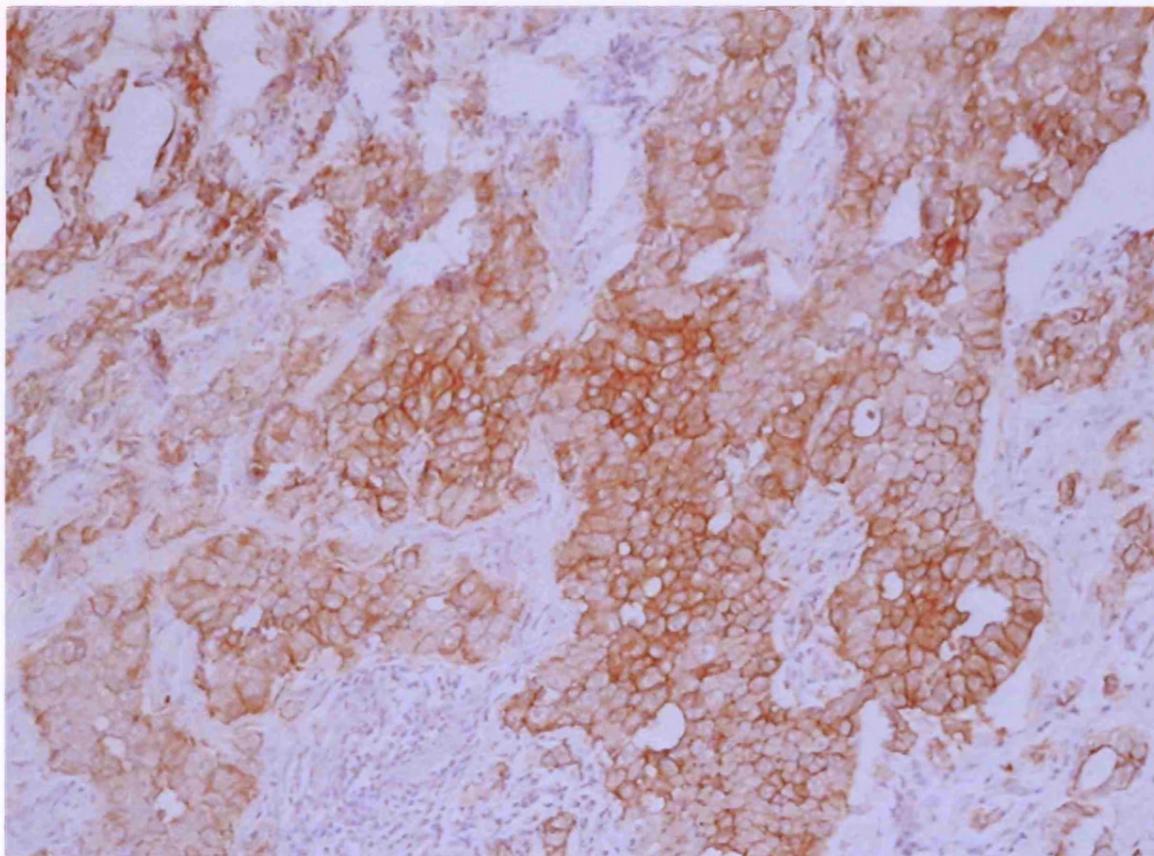
This study led to the first publication of the description of COX-2 protein expression, as assessed by Western blotting, in MM. With the starting point of a working method for COX-2 Western blotting of cell line and human blood sample lysates, a method was developed for semi-quantitative COX-2 Western blotting of homogenised tumour sample supernatants. It was demonstrated that it was possible to run several gels at once, processing the nitrocellulose membranes simultaneously. There was little variation between the different blots, when appropriate adjustment was made according to the densitometry values of the samples run as internal controls on each gel. Furthermore, even with the same samples run several months apart, with both different primary and secondary antibodies and also different densitometry equipment, a close correlation was found between the sets of results. This shows that it is possible to make comparisons between samples run on different gels at the same time, and also that the technique is robust and reproducible with different techniques of membrane probing and densitometry. That the COX-2 bands were abrogated by the action of the blocking peptide confirms that it was COX-2 protein being detected. The significance of the bands at lower molecular weight, which were seen most prominently with the Oxford PB27B antibody, remains unknown.

High COX-2 protein levels were found to be prognostic in MM by this semi-quantitative method. This is in keeping with studies in a growing number of other solid tumours. It is apparent that COX-2 plays a central role in the pathogenesis of MM, and it is an attractive therapeutic target. There is emerging evidence that the addition of COX-2 inhibition may enhance the chemosensitivity of NSCLC (Alktorki *et al.* 2002). Early experience with concomitant COX-2 inhibition and palliative chemotherapy for MM in Leicester suggests that selective COX-2 inhibition may lead to a higher than expected

response rate [Dr. K.J. O'Byrne, personal communication]. Protocols for trials of COX-2

inhibition in combination with chemotherapy are under development in Leicester.

COX-2 inhibition may also have a role in the chemoprevention of MM. There may

be roles for COX-2 inhibitors as maintenance therapy after radical resection and possibly

amongst patients who are at risk of MM, such as those with a history of asbestos exposure.

However, little is known about the pre-malignant lesions of MM and, given the late

presentation of MM in many cases, it is unlikely that pre-malignant lesions would be

detectable clinically. It would be difficult, therefore, to target chemopreventative COX-2

inhibition to patients with pre-malignant lesions, rather than to those generally at risk due to

a prior exposure.

The identification of α-tubulin as a prognostic factor was unexpected. It is hoped to

characterise this further, by investigating the expression of α-tubulin with

immunohistochemistry and correlating the results with markers of proliferation (see below).

Matrix metalloproteinases had not been described in MM at the beginning of this

project. Recently, reports of MMPs in MM cell lines have been published (Harvey *et al.*

2000; Liu *et al.* 2001; Rubins *et al.* 2001) (Liu and Klominek 2002). Gelatin zymography

was chosen for this project, since it allows evaluation of both pro- and active MMP-2 and –

9 isoforms. The detection of active gelatinase enzymes, especially MMP-2, is important.

This suggests, firstly, that further work should be carried out to ascertain whether the active

isoforms are free and functioning, or whether they are bound with TIMPs and are therefore

functionally inactive. Assessment by immunohistochemistry, in-situ hybridisation and in-

situ gelatin zymography may generate useful data in this regard. No correlations were

found between MMP isoform expression and other prognostic variables evaluated in this

project. The prognostic significance of MMP-2 expression, which is suggested by this

study, needs clarification in a larger series.

These findings add to existing "biological" prognostic factors in MM (Table 9.1).

This study has a considerably larger number of cases than all previous series of novel

biological prognostic factors in MM. The median number of cases in the 36 published

studies is 37. Few of the factors investigated were subject to multivariate analysis with

clinical and pathological variables. The most commonly studied biological prognostic

factors are those concerning proliferation and apoptosis. These have been assessed by the

MIB 1 (using the Ki67 antibody), mitotic and apoptotic indices. It is planned to investigate

the prognostic significance of the MIB 1 index in this series of patients. Assessment of

proliferation in this series of patients would be interesting further work, allowing

correlations with angiogenesis, hypoxia / necrosis, EGFR and COX-2 to be evaluated.

The expanding program of radical surgery for MM will allow the accrual of snap-

frozen tumour samples. Tumour biology plays an important part in the outcome of MM

and further work in this cohort will allow assessment as to whether it is possible to generate

a biological prognostic model which is independent of pathological stage, as our group has

found in NSCLC (O'Byrne *et al.* 2001).

In conclusion, this work has led to the clarification of existing clinical, pathological

and biological, and establishment of novel, prognostic factors. New therapeutic targets

have been identified some of which are now under investigation in translational clinical

trials. Inevitably, many new avenues of research have been suggested. Collectively, this

work has lead to an increased understanding of the biology of MM and, it is hoped, will

now bring forward novel therapies which could lead to an improvement in the outcome for

patients suffering from this disease.

Appendix I

Publications

Original articles relating to thesis

1. **Edwards JG**, Abrams KR, Leverment JN, Spyt TJ, Waller DA, O'Byrne KJ (2000): Prognostic factors for malignant mesothelioma in 142 patients: validation of CALGB and EORTC prognostic scoring systems.
Thorax 2000;55(9):731-735

2. **Edwards JG**, Cox G, Andi A, Jones JL, Walker RA, Waller DA, O'Byrne KJ (2001): Angiogenesis is an independent prognostic factor in malignant mesothelioma.
British Journal of Cancer 2001;85(6):865-868

3. **Edwards JG**, Faux SP, Plummer SM, Walker RA, Waller DA, O'Byrne KJ (2002): Cyclooxygenase-2 expression is a novel prognostic factor in malignant mesothelioma.
Clinical Cancer Research 2002;8(6):1857-62

4. **Edwards JG**, Swinson DEB, Jones JL, Waller DA, O'Byrne KJ (2003): Tumour necrosis is a predictor of poor outcome and correlates with angiogenesis in malignant mesothelioma *(accepted, subject to revision, by CHEST.)*

5. **Edwards JG**, McLaren J, Jones JL, Waller DA, O'Byrne KJ (2003): Expression of matrix metalloproteinases by benign and inflamed pleura and malignant mesothelioma.
British Journal of Cancer *(in press.)*

6. **Edwards JG**, Swinson DEB, Jones JL, Waller DA, O'Byrne KJ (2002): EGFR expression is a favourable prognostic factor in malignant mesothelioma: correlation with clinical, pathological and biological variables *(research paper in preparation.)*

Associated original articles

7. Martin-Ucar AE, **Edwards JG**, Rengarajan A, Muller S, Waller DA (2001): Palliative debulking surgery in malignant mesothelioma - predictors of survival and symptom control.
European Journal of Cardiothoracic Surgery 2001;20(6):1117-21

8. Martin-Ucar AE, **Edwards JG**, Rengarajan A, Muller S, Waller DA (2002): Palliative debulking surgery in malignant mesothelioma
European Journal of Cardiothoracic Surgery 2002;21 (6): 1128-9

9. O'Byrne KJ, Cox G, Swinson D, Richardson D, **Edwards JG**, Lolljee J, Andi A, Koukourakis MI, Giatromanolaki A, Gatter K, Harris AL, Waller DA, Jones JL (2001): Towards a biological staging model for operable non-small cell lung cancer.
Lung Cancer 2001;34:S83-S89

10. Swinson DEB, Jones JL, Richardson D, **Edwards JG**, O'Byrne KJ (2002): Tumour necrosis is an independent poor prognostic variable in non-small cell lung cancer: correlation with biological variables.
Lung Cancer 2002;37(3):235-240

11. Andi A, Cox G, **Edwards JG**, Jones JL, O'Byrne KJ (2002). Angiogenesis, Integrin $\alpha_v\beta_3$ expression and macrophage infiltration in operable non-small cell lung cancer.
Journal of Pathology *(in press)*

12. Faux SP, Houghton CE, Swain WA, **Edwards JG**, Plummer SM, O'Byrne KJ (2001): The epidermal growth factor receptor activates NFκB following asbestos exposure and provides a signal for mesothelial cell survival *(submitted to Carcinogenesis.)*

13. **Edwards JG**, Entwisle JJ, Jeyapalan K, Waller DA (2002): Contrast enhanced magnetic resonance imaging in the selection for radical surgery in malignant mesothelioma *(submitted to Annals of Thoracic Surgery.)*

14. **Edwards JG**, Waller DA, O'Byrne KJ (2002): Malignant pleural mesothelioma: current management and the impact of molecular biology on novel treatment strategies *(review article in preparation.)*

15. Andi A, Cox G, **Edwards JG**, Jones JL, O'Byrne KJ (2002): Alpha-v beta-3 integrin, angiogenesis and macrophage infiltration in non-small cell lung cancer *(research paper in preparation.)*

16. Lollijee J, Richardson D, **Edwards JG**, Jones JL, O'Byrne KJ (2002): β-Catenin expression in non-small cell lung cancer: loss of tumour cell staining and nuclear expression confer poor prognosis *(research paper in preparation.)*

17. Swain WA, O'Byrne KJ, Houghton CE, **Edwards JG**, Faux SP (2002): Epidermal growth factor receptor activation of the PI3K/Akt survival pathway in human mesothelial cells exposed to asbestos *(research paper in preparation.)*

Unrelated publications during the period of registration

1. Waller DA, **Edwards JG**, Rajesh PB (1999): Physiological comparison of flutter valve drainage bags and underwater seal systems for postoperative air leaks.
Thorax 1999;54 (5):442-443

2. Waller DA, **Edwards JG**, Rajesh PB (1999): Postoperative air leaks.
Thorax 54 (12); 1141

3. **Edwards JG**, Waller DA (2001): The evidence base for surgical intervention in lung cancer.
In: **The Effective Management Of Lung Cancer**, Aesculapius Medical Press, London.

4. **Edwards JG**, Duthie DJR, Waller DA (2001): "Lobar volume reduction surgery" - a method of increasing the lung cancer resection rate in patients with emphysema.
Thorax 2001;56(10):791-795

5. Martin-Ucar AE, Chaudhuri N, **Edwards JG**, Waller DA (2002): Can pneumonectomy for non-small cell lung cancer be avoided? An audit of parenchymal sparing lung surgery.
European Journal of Cardiothoracic Surgery 2002;21(4):601-5

6. Waller DA, **Edwards JG**, Martin-Ucar AE (2002): Video-assisted thoracoscopic surgery. In: **Pleural Disease: An International Textbook**. Ed: Light RW and Lee YCG, Arnold, London.

Original articles

Prognostic factors for malignant mesothelioma in 142 patients: validation of CALGB and EORTC prognostic scoring systems

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Abstract

Background—The incidence of malignant mesothelioma is increasing. There is the perception that survival is worse in the UK than in other countries. However, it is important to compare survival in different series based on accurate prognostic data. The European Organisation for Research and Treatment of Cancer (EORTC) and the Cancer and Leukaemia Group B (CALGB) have recently published prognostic scoring systems. We have assessed the prognostic variables, validated the EORTC and CALGB prognostic groups, and evaluated survival in a series of 142 patients.

Methods—Case notes of 142 consecutive patients presenting in Leicester since 1988 were reviewed. Univariate analysis of prognostic variables was performed using a Cox proportional hazards regression model. Statistically significant variables were analysed further in a forward, stepwise multivariate model. EORTC and CALGB prognostic groups were derived, Kaplan-Meier survival curves plotted, and survival rates were calculated from life tables.

Results—Significant poor prognostic factors in univariate analysis included male sex, older age, weight loss, chest pain, poor performance status, low haemoglobin, leukocytosis, thrombocytosis, and non-epithelial cell type (p<0.05). The prognostic significance of cell type, haemoglobin, white cell count, performance status, and sex were retained in the multivariate model. Overall median survival was 5.9 (range 0–34.3) months. One and two year survival rates were 21.3% (95% CI 13.9 to 28.7) and 3.5% (0 to 8.5), respectively. Median, one, and two year survival data within prognostic groups in Leicester were equivalent to the EORTC and CALGB series. Survival curves were successfully stratified by the prognostic groups.

Conclusions—This study validates the EORTC and CALGB prognostic scoring systems which should be used both in the assessment of survival data of series in different countries and in the stratification of patients into randomised clinical studies.

(*Thorax* 2000;55:731–735)

The incidence of malignant mesothelioma is increasing in the UK and the number of cases may treble over the next 20 years.[1] [2] Management in the UK generally involves little more than a closed or surgical pleurodesis, typically with talc. Surgical intervention has fallen in the UK over the last 10 years despite the increasing incidence of malignant mesothelioma. The UK Thoracic Surgical Register of the Society of Cardiothoracic Surgeons of Great Britain and Ireland recorded eight extrapleural pneumonectomies and 19 pleurectomies in 1998 compared with 35 and 88, respectively, in 1989. Chemotherapy for malignant mesothelioma is not common in the UK, although phase II studies indicate good symptom control[3] [4] and a phase III British Thoracic Society (BTS)/Medical Research Council (MRC) trial is planned. Radiotherapy is usually given to intercostal drain or thoracoscopy sites only, rather than as radical treatment to the hemithorax. Despite encouraging results for multimodality approaches combining surgery, radiotherapy and chemotherapy, no randomised studies have been performed and a survival benefit has not been established.[5]

The current nihilism towards malignant mesothelioma in the UK is due to the perception that therapeutic interventions have little to offer. Survival from time of diagnosis is typically 7–10 months[6] [7] which appears to be worse than the 12–19 months typically quoted in European and American series.[8] [9] It is not clear whether the apparent poor survival in the UK is a reflection of less aggressive management or later tumour stage at the time of diagnosis.

There have been increased efforts to devise accurate staging systems for malignant mesothelioma in recent years.[10] The International Mesothelioma Interest Group (IMIG) staging system was proposed in 1995[11] and validated by Rusch and Venkatraman.[12] However, the IMIG

Table 1 Prognostic factors for malignant mesothelioma (MM) analysed in a univariate Cox proportional hazards model (n = 142)

Variable	n	Hazard ratio	95% Confidence intervals		p value
Sex					
Female	12				
Male	126	2.24	1.10	4.52	0.013
Age	142	1.02	1.00	1.04	0.015
Weight loss					
No	58				
Yes	65	1.89	1.27	2.82	0.002
Asbestos exposure					
No	20				
Yes	87	1.10	0.65	1.84	0.73
Chest pain					
No	32				
Yes	95	1.70	1.08	2.68	0.017
ECOG performance status					
0	56				
1 or 2	73	3.33	1.19	5.07	<0.0001
White cell count					
<8.3 × 10⁹/l	42				
>8.3 × 10⁹/l	81	1.52	1.00	2.32	0.04
Platelets					
<400 × 10⁹/l	73				
>400 × 10⁹/l	50	1.67	1.12	2.48	0.01
Haemoglobin					
>14 g/dl	79				
<14 g/dl	45	1.66	1.10	2.52	0.01
Cell type					
Epithelial	65				
Mixed/sarcomatoid	55	2.71	1.80	4.08	<0.0001
Diagnostic certainty					
Definite MM	87				
Probable/possible	32	1.10	0.70	1.75	0.67
Surgical resection					
Yes	71				
No	47	1.43	0.95	2.13	0.09
EORTC					
Low risk	49				
High risk	75	2.39	1.59	3.62	0.0001
CALGB					
Group 1	22				
Group 2	2	3.03	0.67	13.70	<0.0001
Group 3	55	1.87	1.02	3.44	
Group 4	5	2.04	0.72	5.73	
Group 5	30	5.60	2.82	11.10	
Group 6	9	10.94	4.53	26.42	

For each categorical variable the reference category is given in the first row and the hazard ratio, 95% confidence intervals, and p value are given in the next row. Analysis of the EORTC and CALGB groups is also included. Age was assessed as a continuous variable.

system was not successful as a survival predictor in the radical multimodality treatment series of Sugarbaker et al.[9] They amended the Brigham staging system to include assessment of resection margins and revised the nodal variable, but this surgical staging system is only applicable after extrapleural pneumonectomy. It is therefore difficult to assess the survival of the vast majority of patients with malignant mesothelioma in the UK by a Tumour/Nodes/Metastasis (TNM) staging system alone, although an accurate assessment of prognosis must be a central part of entry into future phase III trials.

Prognostic scoring systems have been proposed by the European Organisation for Research and Treatment of Cancer (EORTC)[8] and by the Cancer and Leukaemia Group B (CALGB).[13] These systems were derived from statistical analysis of large series of patients within chemotherapy trials. Two EORTC risk groups were identified after multivariate analysis of prognostic variables from 204 patients entered into five consecutive trials. The factors included in the model were: white blood cell count >8.3 × 10⁹/l, Eastern Cooperative Oncology Group (ECOG) performance status ≥1, sarcomatoid tumour cell type, probable or possible histological diagnosis, and male sex.

The high risk group was defined by the presence of three or more of these factors. The CALGB system is more complex and derives from the analysis of 337 patients. A regression tree leads to 11 groups of which those with similar survival characteristics are combined to form six prognostic groups.

We have analysed data from 142 patients with malignant mesothelioma presenting to the Cardiothoracic Surgical Service in Leicester since 1988. The aims of this study were to assess prognostic variables, to validate the EORTC and CALGB prognostic scoring systems, and to evaluate the survival data of our series when split into relevant prognostic risk groups.

Methods

DATA COLLECTION

The Department of Pathology at Glenfield Hospital provided a list of all pathological specimens with a diagnosis of malignant mesothelioma since 1988. Most of these cases represented patients passing through the Department of Cardiothoracic Surgery at Groby Road Hospital, and latterly Glenfield Hospital, Leicester. Patients were referred for surgical biopsy, management of pleural effusion or empyema, or radical surgery. Case notes were identified and original notes or stored microfilm copies were reviewed. Case notes from referring hospitals were also reviewed, where possible. Relevant demographic, clinical, and pathological data, as well as management and survival data, were retrieved and entered into a computer database (Microsoft Access). The detailed histopathological report was reviewed, but tissue sections were not re-examined as part of this study. From August 1998 data have been collected prospectively. Survival was calculated from the date of the diagnostic biopsy and pre-diagnostic variables such as performance status and haematological indices were taken from immediately before this time.

STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS software (SPSS Inc, USA). Complete data retrieval was only possible in 101 of the total of 142 patients because of missing or destroyed case notes, or missing data within case notes that had been inspected. Univariate analysis was performed on all 142 cases. All parameters were analysed as categorical variables except age which was assessed as a continuous variable. The cut off points chosen were based on those used in the CALGB and EORTC series. Haemoglobin and white cell count were also analysed as continuous variables. Survival curves were estimated using the Kaplan-Meier method. A Cox proportional hazards regression model[14] was used to identify statistically significant differences in survival and to estimate hazard ratios and 95% confidence intervals (CI). The assumption of proportional hazards was assessed graphically by plotting log[–log(survivor)] against log(time) for each of the prognostic groups. In the case of the multivariate models the final model was re-estimated, stratifying for each variable

Table 2 Analysis of prognostic variables in a forward, stepwise multivariate Cox proportional hazards model. Only cases with complete data for all prognostic variables were included in this model (n = 101)

Variable	p value	Hazard ratio	95% Confidence intervals	
Cell type	0.0004	2.33	1.46	3.72
Haemoglobin	0.0004	1.27	1.11	1.43
White cell count	0.001	1.11	1.04	1.19
Performance status	0.005	2.01	1.24	3.28
Sex	0.008	3.13	1.33	7.14

in turn. Statistically significant variables identified by the univariate analysis, and for which data were complete (n = 101), were analysed in a multivariate model. A forward stepwise selection procedure was used, with variables being added to the model according to a partial likelihood ratio test using an entry criterion of p<0.05.

EORTC AND CALGB PROGNOSTIC SCORING GROUPS

The appropriate prognostic groups according to EORTC[8] and CALGB[13] criteria were identified for each patient where relevant data were complete. Survival curves were plotted and median, one year, and two year survival rates were calculated from life tables, together with the respective 95% CI. Survival differences between, and hazard ratios for, each prognostic group were calculated using a univariate Cox model, as above.

Results

One hundred and forty two patients were identified (13 women, 129 men); survival data were available for 138 patients. The median age at the time of diagnosis was 64 years (range 42–86). The median time from onset of symptoms to hospital referral by the general practitioner (GP) was 4.3 weeks (range 1–74) and from GP referral to surgical referral was 3.7 weeks (range 0.1–58). Although closed pleural biopsy specimens were taken in 47 patients, this was the sole histological material in only eight cases. Immunohistochemistry was required in 56% of cases to confirm the diagnosis. The most commonly used markers were CEA, BerEP4, AUA-1, HBME-1, cytokeratin, thrombomodulin, and CAM 5.2 in 58, 54, 37, 29, 29, 29, and 23 cases, respectively. The time from surgical referral to the date of diagnostic biopsy was 3.0 weeks (range 0.1–122). Overall median survival from the date of histological diagnosis was 5.9 months (range 0–34.3). One and two year survival rates were 21.3% (95% CI 13.9 to 28.7) and 3.5% (0 to 8.5), respectively. There was no significant change in survival between sequential cohorts of patients over the 10 year period (data not shown).

Univariate analysis of the 142 cases is presented in table 1. Poor clinical prognostic
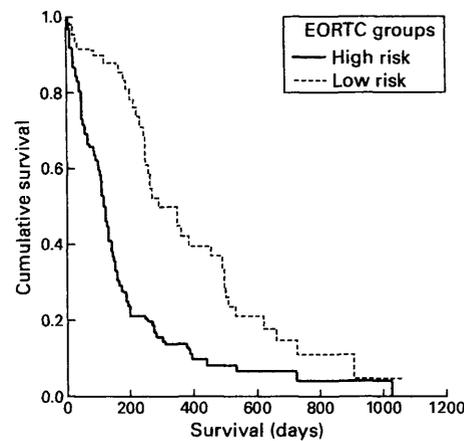
Figure 1 Kaplan-Meier survival plot showing the difference in survival between patients within EORTC high and low risk groups (p = 0.0001, Cox's proportional hazards).
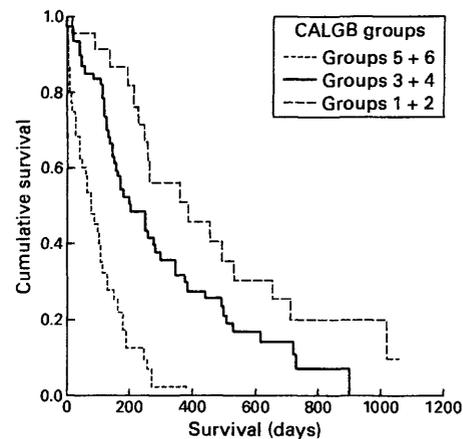
Figure 2 Kaplan-Meier survival plot showing the difference in survival between patients within CALGB prognostic groups (p <0.0001, Cox's proportional hazards). Groups have been combined because of the small numbers in the even numbered groups.

factors that were statistically significant were male sex, old age, weight loss of >5%, presence of pleuritic chest pain, and ECOG performance status of >0. A history of exposure to asbestos was given in 81% of patients but was not a poor prognostic factor. Low haemoglobin, high white blood cell count, and thrombocytosis were significantly associated with poor prognosis. Epithelial cell type had a better prognosis than mixed cellularity and sarcomatoid types (p<0.0001). The degree of certainty of histological diagnosis was not associated with survival. The hazard ratios for performance status >0 and for non-epithelial cell type, the variables for which the differences in survival were greatest, were 3.33 and 2.7,

Table 3 Survival of patients in the Leicester series compared with the EORTC series when classified by prognostic group

Group	No. of patients		Median survival (months)		One year survival (%)		Two year survival (%)	
	Leicester	EORTC	Leicester	EORTC	Leicester	EORTC	Leicester	EORTC
Low risk	49	105	9.4	10.8	40.6	40	9.9	14
95% CI			5.9 to 12.9		25.6 to 55.7	30 to 50	0 to 21.3	6 to 22
High risk	75	76	3.8	5.5	14.1	12	3.5	0
95% CI			3.2 to 4.4		6.0 to 22.2	4 to 20	0 to 9.2	

Table 4   Survival of patients in the Leicester series compared with the CALGB series when classified by prognostic group

Group	No. of patients		Median survival (months)		One year survival (%)		Two year survival (%)	
	Leicester	CALGB	Leicester	CALGB	Leicester	CALGB	Leicester	CALGB
1	22	36	14.8	13.9	55.9	63	16.8	38
95% CI			8.5 to 21.2	11.1 to 31.4	33 to 78.8	46 to 77	0 to 36.5	23 to 55
2	2	36	6.4	9.5	0	41	0	21
95% CI				6.9 to 14.7		26 to 57		10 to 37
3	55	146	6.6	9.2	29.0	30	5.3	10
95% CI			3.0 to 10.1	7.5 to 10.5	16.1 to 41.9	23 to 37	0 to 14.6	6 to 16
4	5	33	8.1	6.5	40	25	0	6
95% CI			3.7 to 12.5	3.7 to 9.4	0 to 82.9	14 to 42		2 to 17
5	30	73	3.4	4.4	3.5	7	0	0
95% CI			2.5 to 4.2	3.4 to 5.1	0 to 10.2	3 to 15		
6	9	13	1.1	1.4	0	0	0	0
95% CI			0.4 to 1.8	0.5 to 3.6				
Total	133	337						

respectively. Biopsy alone was performed in 47 patients, whereas surgical resection of tumour was carried out in 72 patients (parietal pleurectomy in 22, visceral decortication in 42, and extrapleural pneumonectomy in eight patients). It was not possible to determine the exact surgical procedure in 23 patients. Although there was a trend towards longer survival in patients who underwent surgical resection, this did not reach statistical significance (p = 0.09).

Five variables emerged as statistically significant from the multivariate Cox model (table 2). In order of decreasing significance these were histological cell type, haemoglobin, white blood cell count, performance status, and sex. Non-epithelial cell type was associated with a hazard ratio of 2.33 (95% CI 1.46 to 3.72). For each rise in haemoglobin of 1 g/dl there was a fall in risk of 21%, whereas each rise in white blood cell count of $1 \times 10^9/l$ increased the risk by 11%. An ECOG performance status of 1 or 2 was associated with a hazard ratio of 2.01 (95% CI 1.24 to 3.28) in the multivariate model.

There was a significantly greater proportion of patients in the EORTC high risk group in the Leicester series than in the EORTC series (60% versus 42%, p = 0.002, χ^2 test). However, χ^2 for trend analysis revealed that there was not a statistically significant shift towards the high CALGB groups in our series (p = 0.27).

EORTC risk groups were correctly correlated with survival in this series of patients (fig

1). The same was broadly true for the CALGB groups, but there were only two, five and nine cases in groups 2, 4 and 6, respectively. When groups 1 and 2, 3 and 4, and 5 and 6 were combined, good stratification in the Kaplan-Meier plot was achieved (fig 2). Median survival rates were generally comparable to the EORTC series (table 3), with the EORTC data within the 95% CI of our series for the low risk group and just outside for the high risk group. The one and two year survival rates were equivalent in Leicester and the EORTC series in both high and low risk groups. Similarly, median, one, and two year survival of our series, when grouped according to the CALGB criteria, were comparable to the published series (table 4).

Discussion

The poor prognostic factors identified in univariate analysis are consistent with those of other studies. Specifically, we confirm that male sex, older age, weight loss, chest pain, poor ECOG performance status, abnormal haematological indices, and non-epithelial cell type are poor prognostic factors. Similarly, stepwise multivariate analysis yielded as significant prognostic factors cell type, haemoglobin, white cell count, performance status and sex. These results are similar to other studies in which multivariate models have been used (table 5). Clinical features such as the presence of pleuritic chest pain or weight loss and

Table 5   Review of previous multivariate analyses of prognostic factors in malignant mesothelioma and comparison with this series

	n	Age	Sex	Chest pain	Weight loss	PS	Cell type	Certainty of histological diagnosis	WBC count	Hb	Platelet count
Chahinian et al [15]	69	+	–				+				
Samson et al [16]	76	–	–		–	–					
Alberts et al [17]	262		–		+	–					
Antman et al [18]	180			+	+	+					
Chailleux et al [19]	167	+	–								
Spiritas et al [20]	1475	+	+			–					
Ruffie et al [21]	332	+	–		–						+
Tammilehto et al [22]	98	–	+	–		+	+				
Boutin et al [23]	188	–	–		+		+				
De Pangher Manzini et al [24]	80	+	–			–	+				–
Fusco et al [25]	113	–	–	–			+				
Curran et al [8]	204	–	+			+	+	+	+	–	–
Herndon et al [13]	337	+	–	+	+	+	+		–	–	+
Pass et al [26]	48		+				–				+
Leicester 1988–1999	101	–	+	–	–	+	+	–	+	+	–

PS = performance status.
+ indicates p = 0.05, – indicates p>0.05.

haematological indices have been examined in fewer studies than cell type and performance status. However, these variables were incorporated into the EORTC and CALGB prognostic systems.

One of the initial aims of this study was to evaluate the IMIG stage in our patients. Despite reviewing operation notes and computed tomographic (CT) scans, it was felt that retrospective TNM staging would not be sufficiently accurate. We therefore rejected the possibility of assessing stage-specific survival in this series. We decided to analyse survival according to EORTC and CALGB prognostic criteria. Figures 1 and 2 show the success of both systems in stratifying survival. There were relatively few patients in the even numbered CALGB groups and so groups were combined to aid clarity.

Analysis of the results revealed that the median survival time and one and two year survival rates for the Leicester patients, when grouped appropriately into the EORTC and CALGB groups, were comparable. Therefore, whereas this study confirms that overall survival from malignant mesothelioma in the UK is poor in comparison with other European series, this is not the case when analysis by prognostic groups is performed. This may also be of relevance in other solid tumours when survival in the UK is compared with other countries.

Recent work has identified angiogenesis and other biological markers to be of prognostic value in malignant mesothelioma.[27-29] The value of new molecular markers should be analysed with respect to existing factors in order to assess their prognostic relevance.

In conclusion, this study is the first independent series to validate the effectiveness of the EORTC and CALGB systems. Survival in our series is no worse than in the two chemotherapy based series in which patients are stratified into prognostic groups. In view of the difficulty of accurate TNM staging, assessment of these prognostic groups should be incorporated into the planning of future clinical trials, including the proposed BTS/MRC trial of chemotherapy and radiotherapy.

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Angiogenesis is an independent prognostic factor in malignant mesothelioma

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Summary Angiogenesis is essential for tumour growth beyond 1 to 2 mm in diameter. The clinical relevance of angiogenesis, as assessed by microvessel density (MVD), is unclear in malignant mesothelioma (MM). Immunohistochemistry was performed on 104 archival, paraffin-embedded, surgically resected MM samples with an anti-CD34 monoclonal antibody, using the Streptavidin–biotin complex immunoperoxidase technique. 93 cases were suitable for microvessel quantification. MVD was obtained from 3 intratumoural hotspots, using a Chalkley eyepiece graticule at × 250 power. MVD was correlated with survival by Kaplan–Meier and log-rank analysis. A stepwise, multivariate Cox model was used to compare MVD with known prognostic factors and the EORTC and CALGB prognostic scoring systems. Overall median survival from the date of diagnosis was 5.0 months. Increasing MVD was a poor prognostic factor in univariate analysis ($P = 0.02$). Independent indicators of poor prognosis in multivariate analysis were non-epithelial cell type ($P = 0.002$), performance status > 0 ($P = 0.003$) and increasing MVD ($P = 0.01$). In multivariate Cox analysis, MVD contributed independently to the EORTC ($P = 0.006$), but not to the CALGB ($P = 0.1$), prognostic groups. Angiogenesis, as assessed by MVD, is a poor prognostic factor in MM, independent of other clinicopathological variables and the EORTC prognostic scoring system. Further work is required to assess the prognostic importance of angiogenic regulatory factors in this disease. © 2001 Cancer Research Campaign http://www.bjcancer.com

Keywords: malignant mesothelioma; angiogenesis; prognosis; staging

Malignant mesothelioma (MM) is a fatal cancer of increasing incidence associated with asbestos exposure (Peto et al, 1999). MM responds poorly to aggressive conventional therapy (Sterman et al, 1999) and has an appalling prognosis. Median survival in the United Kingdom, where management has been typically palliative, is between 6 and 12 months from the time of onset of symptoms (Law et al, 1984; McLean and Patel, 1997; Edwards et al, 2000). Pathological tumour, nodes and metastasis (TNM) staging is difficult to achieve. In a large series of patients undergoing extrapleural pneumonectomy and adjuvant chemoradiotherapy, International Mesothelioma Interest Group (IMIG) TNM staging (Rusch, 1995) failed to stratify survival (Sugarbaker et al, 1999), questioning the value of this approach in predicting outcome. Biological markers of prognosis have attracted interest in other solid tumours and may provide prognostic information independent from TNM stage (Cox et al, 2000a, 2001).

Angiogenesis is the formation of new blood vessels from existing vasculature, during which normally quiescent endothelial cells proliferate and gain invasive characteristics. Angiogenesis is necessary for tumour growth of greater than 1 to 2 mm in diameter (Hanahan and Folkman, 1996). High intratumoural microvessel counts, and indirect measure of the intensity of angiogenesis, are associated with a poor prognosis in solid tumours (Fox et al, 1995; Giatromanolaki et al, 1996; Cox et al, 2000b). There have been

preliminary reports of the prognostic value of microvessel counts in malignant mesothelioma (Kumar-Singh et al, 1997; Ohta et al, 1999). These relatively small studies, of 25 and 54 cases, have suggested a relationship between increased microvessel counts and poor prognosis. However methodology, the mean vessel count obtained and statistical significance varied greatly between the studies.

This study incorporated the conclusions of a consensus paper on the evaluation of tumour angiogenesis into our methodology (Vermeulen et al, 1996) and evaluated microvessel density (MVD) in 104 cases of MM. The prognostic significance of MVD was examined in a multivariate model, incorporating clinical and pathological factors. The contribution of MVD to the Cancer and Leukemia Group B (CALGB) (Herndon et al, 1998) and European Organisation for the Research and Treatment of Cancer (EORTC) (Curran et al, 1998) prognostic scoring systems, which we have validated previously in this cohort of patients (Edwards et al, 2000), was analysed.

MATERIALS AND METHODS

Patients

All cases of MM presenting to our institution since 1988 were identified and case notes reviewed. Relevant demographic, clinical and pathological data were retrieved. Clinicopathological prognostic factors, including CALGB (Herndon et al, 1998) and EORTC (Curran et al, 1998) prognostic groups were assessed, as described previously (Edwards et al, 2000). The majority of patients were referred to the regional Department of

Cardiothoracic Surgery for surgical biopsy, management of pleural effusion or empyema, or for radical surgery. The detailed histopathological report was obtained for each case and the slides reviewed by a pathologist to both confirm the diagnosis and to assess the most suitable block for microvessel quantification. One block was selected and a single histological section stained, as this has been shown to be representative of tumour angiogenesis as a whole in breast cancer (Martin et al, 1997b). Cancer-specific survival was calculated from the date of the diagnostic biopsy. Pre-diagnostic variables, such as performance status and haematological indices, were taken from immediately before this time.

Immunohistochemistry

Representative formalin-fixed paraffin-embedded blocks of tumour were chosen for each case. 4 μm sections were cut onto glass slides previously treated with 2% 3-aminopropylethoxysilane. Sections were dewaxed in xylene and rehydrated through graded alcohols. Endogenous peroxidase activity was blocked by immersion in 2% hydrogen peroxide for 30 minutes. Sections were rinsed in deionised water followed by tris-buffered saline (TBS) containing 0.1% bovine serum albumin. Non-specific staining was blocked by incubation with 20% normal rabbit serum for 10 minutes. Sections were incubated overnight at 4°C with CD34 antibody (NCL-END, Novocastra, Newcastle, UK) at a dilution of 1 in 50. Following washing in TBS, sections were incubated for 30 minutes with a biotinylated rabbit anti-mouse whole immunoglobulin secondary antibody (E0354, Dako, Ely, UK). Sections were rinsed in TBS before incubation with streptavidin–biotin peroxidase complex (K0377, Dako) for 30 minutes. Finally, sections were rinsed in TBS and incubated with the chromogen diaminobenzidine tetrahydrochloride for 10 minutes before counterstaining with haematoxylin. Sections were dehydrated through graded alcohols and mounted in resinous mountant. Negative controls had the primary antibody omitted, whilst microvessels from surrounding normal lung were used as an internal positive control.

Microvessel quantification

Angiogenesis was assessed indirectly with the aid of a Chalkley eyepiece graticule, as previously described (Cox et al, 2000b). Each section was examined under low power to identify 3 intratumoural microvessel 'hot spots'. These areas were then examined at × 250 magnification using a 25-point Chalkley eyepiece graticule. The Chalkley graticule was orientated so that the maximum number of points coincided with immunostained structures. Structures with the morphological features of microvessels that stained with the chromogen, irrespective of whether a lumen was present, were counted. MVD was defined in this study as the sum of the number of points thus counted from 3 hot spots. The Chalkley graticule covers an area of 0.115 mm² at × 250 magnification. Sections were analysed by 2 investigators blinded to clinicopathological factors and outcome.

Statistical analysis

Statistical analysis was performed using the SPSS software system (SPSS for Windows Version 9.0, SPSS Inc, Chicago, USA). Differences in total Chalkley count within categorical prognostic factors were assessed with Student's t-test. Linear regression

analysis was used to assess correlations with continuous prognostic variables. Cancer-specific survival curves were estimated using the Kaplan–Meier method and the log-rank test was used to assess the statistical significance of differences between groups. A Cox proportional hazards regression model was used to identify statistically significant differences in survival and estimate hazard ratios and 95% confidence intervals (CI) (Cox, 1972). The assumption of proportional hazards was assessed graphically by plotting log[-log(survivor)] against log(time) for each of the prognostic groups. Prognostic variables identified by univariate analysis, with $P < 0.1$, were analysed in a multivariate Cox model. Cases in which complete prognostic data retrieval was not possible (due to missing or destroyed case notes, or missing data within case notes that had been inspected) were excluded from multivariate analysis. A forward, stepwise selection procedure was used, with variables being added to the model according to a partial likelihood ratio test, using an entry criterion of $P < 0.05$.

RESULTS

Patient characteristics and immunohistochemistry results

In all cases, microvessels stained for CD34 but tumour cells did not. Figures 1 and 2 show examples of anti-CD34 immunostaining with a high and low MVD. Of the 140 cases of MM presenting to our department, 36 had insufficient material for microvessel counting (i.e. less than 3 full high power fields of tumour). In the remaining 104 cases, immunostaining of stromal elements, which had the morphological features of myofibroblasts, was present in 18 (17%) cases (Figure 3). Microvessel quantification was only carried out in 7 of these cases in which the morphological pattern of stromal staining was clearly distinct from that of microvessels. Stromal staining therefore precluded microvessel quantification in 11 (11%) cases. In the 93 cases successfully assessed with microvessel counting the surgical procedures performed were biopsy alone (54 cases), parietal pleurectomy (18), decortication (33) and extrapleural pneumonectomy (8). Immunohistochemistry was required in 52% of these cases to confirm the diagnosis. The most commonly used markers were CEA, BerEP4, AUA-1, cytokeratin, thrombomodulin, HBME-1 and CAM 5.2 in 41, 38, 27, 22, 19 and 16 cases respectively. Of the 93 cases in which MVD was derived, cell type was epithelioid in 48, mixed cellularity in 18 and sarcomatoid in 27. It did not prove possible to derive an accurate IMIG TNM stage from this cohort of patients: 58% of patients underwent computed tomography, but pathological verification of stage was only available in 20 (21.5%) patients.

Microvessel quantification

The median Chalkley count (sum of 3 hotspots) in the 93 cases was 23 (range 13–29). There was no significant difference in Chalkley count between epithelial and non-epithelial cell types, nor within other categorical prognostic factors (data not shown). However, a trend towards a positive correlation between cases with vessel counts ≥ median and weight loss of greater than 5% ($P = 0.055$) was noted. With linear regression analysis, a significant correlation was seen between increasing MVD and platelet count ($P = 0.048$, Table 1), but not with white cell count, haemoglobin or age. Furthermore a positive correlation was seen between high platelet counts and weight loss > 5% ($P < 0.0001$). There was
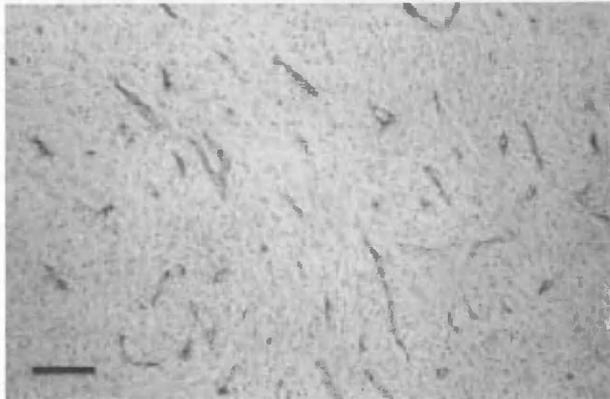
**Figure 1** Photomicrograph of a tumour section with a high microvessel density (× 200, bar = 50 μm)

Figure 2 Photomicrograph of a tumour section with a low microvessel density (× 200, bar = 50 μm)
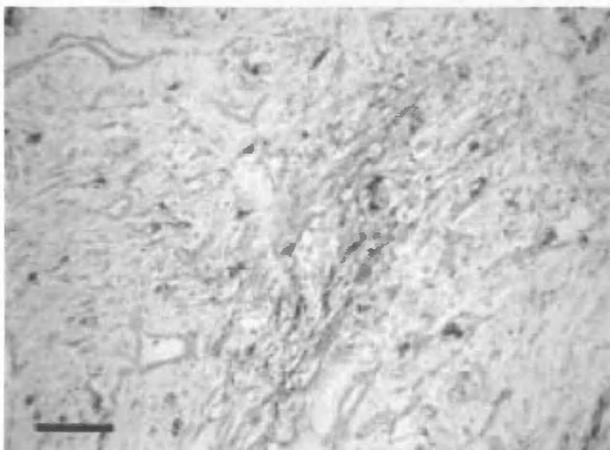

Figure 3 Photomicrograph of a tumour section displaying stromal anti-CD34 immunostaining, which prevented accurate assessment of microvessel density (× 250, bar = 50 μm)

no significant difference in MVD between the high and low-risk groups of the EORTC prognostic scoring system. With regard to the CALGB system, even numbered groups displayed small numbers and so groups were combined for statistical analysis. Although a significant difference in MVD was noted between

Table 1 Correlation of clinicopathological prognostic factors with MVD by linear regression analysis

	r	P
WBC	0.06	0.57
Platelets	0.21	0.048
Haemoglobin	0.04	0.71
Age	0.06	0.53

Groups 3 and 4 compared to Groups 5 and 6, there was no significant overall trend towards increased MVD in the higher risk groups.

Survival

Overall median survival from the date of histological diagnosis for the 93 cases was 5.0 months. 12 cases died within 30 days. Excluding these cases did not have a significant effect on any of the survival analyses: therefore all 93 cases were included. When entered into a Cox proportional hazards model, high MVD was a poor prognostic factor as a continuous variable ($P = 0.02$) and as a categorical variable at cut points from the 20th centile up to the median ($P = 0.007$) (Figure 4). Other statistically significant poor prognostic factors by univariate Cox proportional hazards analysis were increasing age ($P = 0.03$), weight loss > 5% ($P = 0.002$), presence of pleuritic chest pain ($P = 0.03$), Eastern Co-operative Oncology Group (ECOG) performance status > 0 ($P < 0.0001$), white blood count (WBC) > 8.3×10^9 l⁻¹ ($P = 0.02$) and cell type ($P = 0.0001$, Table 2). 10 cases were excluded from multivariate analysis due to missing prognostic data in case notes. In multivariate analysis, non-epithelial cell type was the strongest independent risk factor followed by performance status > 0 and increasing MVD (Table 3). When tested against the prognostic scoring systems in Cox multivariate analysis, increasing MVD contributed independently to the EORTC system ($P = 0.006$) but not the CALGB system ($P = 0.1$, Table 4).

DISCUSSION

This study demonstrates that increased MVD, as assessed by Chalkley counting, is an independent prognostic factor in MM. This is in agreement with other solid tumours and with 2 previous reports in MM (Kumar-Singh et al, 1997; Ohta et al, 1999). Kumar Singh et al found that MVD was a significant prognostic factor in univariate analysis and was independent of MM cell type, tumour grade and patient age in multivariate analysis (Kumar-Singh et al, 1997). However, total microvessel area, when calculated from computer-aided image analysis, was not a significant prognostic factor, thus suggesting that the size of microvessels is not as important as their number. Ohta et al examined both MVD and lymphatic vessel density in 54 tumours and found statistically insignificant trends between high MVD and both poor survival and positive lymph node status. In multivariate analysis, gender, IMIG stage and high MVD were significant independent poor prognostic factors. In a further study, Tolnay et al noted that MVD correlated with expression of the angiogenic growth factor hepatocyte growth factor/scatter factor (HGF/SF) but did not comment on any relationship to prognosis (Tolnay et al, 1998).

The protocol for this study was based on the findings of an international consensus paper on the quantification of angiogenesis in solid tumours (Vermeulen et al, 1996). This suggested that manual

Table 2 Prognostic factors analysed in a univariate Cox proportional hazards model, for cases with satisfactory MVD assessment ($n = 93$)

Variable		n	Hazard ratio	Hazard ratio 95% confidence intervals		P
Gender	Female	8				
	Male	85	2.19	0.95	5.07	0.07
Age		93	1.03	1.00	1.05	0.03
Weight loss	No	38				
	Yes	47	2.02	1.28	3.19	0.002
Asbestos exposure	No	14				
	Yes	61	0.70	0.38	1.26	0.2
Chest pain	No	22				
	Yes	66	1.79	1.07	3.00	0.03
ECOG performance status	0	37				
	1 or 2	52	2.61	1.66	4.11	< 0.001
WBC	$< 8.3 \times 10^9 \, l^{-1}$	25				
	$> 8.3 \times 10^9 \, l^{-1}$	61	1.77	1.08	2.90	0.02
Platelets	$< 400 \times 10^9 \, l^{-1}$	51				
	$> 400 \times 10^9 \, l^{-1}$	35	1.48	0.95	2.31	0.09
Haemoglobin	$> 14 \, g \, dl^{-1}$	56				
	$< 14 \, g \, dl^{-1}$	31	1.39	0.87	2.22	0.2
Cell Type	Epithelial	47				
	Mixed or sarcomatoid	46	2.46	1.57	3.85	0.0001
Surgical resection	Yes	55				
	No	38	1.33	0.82	2.16	0.2
EORTC	Low risk	31				
	High risk	56	2.33	1.45	3.74	0.0005
CALGB	Groups 1 and 2	14				
	Groups 3 and 4	41	2.39	1.14	5.00	0.02
	Groups 5 and 6	31	6.51	2.94	14.39	< 0.0001
Microvessel density		93	1.03	1.01	1.05	0.02

Table 3 Significant prognostic variables identified in a forward, stepwise, multivariate Cox proportional hazards model (MVD was analysed as a continuous variable)

Factor	Hazard ratio	95% Confidence interval	P
Non-epithelial cell type	2.23	1.35–3.67	0.002
Performance status > 0	2.10	1.29–3.43	0.003
MVD	1.04	1.01–1.06	0.01

Table 4 Contribution of MVD to CALGB and EORTC prognostic scoring systems in multivariate Cox proportional hazards analysis

Factor		Hazard ratio	95% Confidence Interval	P
CALGB Groups 1/2		1		
	Groups 3/4	2.39	1.14–5.00	
	Groups 5/6	6.50	2.94–14.39	< 0.0001
MVD				0.1
EORTC Low-risk group		1		
	High-risk group	2.42	1.50–3.90	0.0003
MVD	1.04	1.01–1.06	0.006	

vessel counting in hot spots was the appropriate method for assessing angiogenesis objectively in tumours and that either anti-CD34 or CD31 antibodies should be used. The anti-CD34 monoclonal antibody was chosen for this study as it has been shown to give more reproducible immunostaining of microvessels than either the anti-CD31 or anti-Factor VIII monoclonal antibodies in breast cancer (Martin et al, 1997a). This choice was supported by Kumar-Singh et al who found that CD34 staining was better delineated and easier to assess than CD31 in MM (Kumar-Singh et al, 1997). Stromal staining was seen in 18 cases in our series. In 7 cases the pattern of stromal staining was clearly morphologically distinct from that seen in microvessels and these were included in the analysis. Kumar-Singh described a 'perivascular wash' in some cases with anti-CD34 immuno-histochemistry, but did not report staining of specific stromal elements. In no case was MM tumour-cell CD 34 positivity seen, in keeping with a recent report (Attanoos et al, 2000). The Chalkley counting method of MVD assessment was chosen because it has been shown to be a rapid and objective method of MVD assessment in breast (Fox et al, 1995; Hansen et al, 2000), bladder (Dickinson et al, 1994; Chaudhary et al, 1999) and non-small-cell lung cancers (Giatromanolaki et al, 1996; Cox et al, 2000b).

A significant correlation with the platelet count was seen in this study. This supports the hypothesis that platelets may play an important role in tumour angiogenesis. Platelets are an important source of angiogenic growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Banks et al, 1998; Pinedo et al, 1998). Platelets are likely to adhere to intratumoural endothelium, which can lead to platelet activation and the release and accumulation of high local concentrations of these growth factors (Pinedo et al, 1998; Verheul et al, 2000). A positive correlation has been established between serum VEGF and platelet count in cancer patients (Vermeulen et al,

1999) and these have been further correlated to prognosis in solid tumours (O'Byrne et al, 1999).

Accurate TNM staging is difficult to achieve in the majority of patients with MM. Only a small proportion of patients are suitable for radical surgery, which does allow accurate pathological TNM staging, but even the validity of the International Mesothelioma Interest Group TNM staging system has been questioned (Sugarbaker et al, 1999). Therefore, biological markers may have an important role in providing prognostic information, which is not only of use in individual cases, but also crucial to the design and interpretation of clinical trials. This study clearly demonstrates that MVD is an independent prognostic factor in MM, which also contributes significantly to the EORTC prognostic scoring system. It is possible that the lack of contribution to the CALGB prognostic groups is due to the inclusion of weight loss as a parameter in this prognostic system. We found a near significant correlation between high MVD and weight loss. Weight loss in cancer patients is associated with raised inflammatory cytokine levels, including interleukin (IL)-6 (Scott et al, 1996). IL-6 is an angiogenic growth factor (Motro et al, 1990). Serum IL-6 levels correlate with the platelet count in MM (Nakano et al, 1998). These observations are in keeping with the finding of a positive correlation between platelet count and both MVD and weight loss in our patient series.

Other biological prognostic markers in MM include the cytokeratin marker Cyfra 21-1 (Schouwink et al, 1999), syndecan-1 (Kumar-Singh et al, 1998), bFGF (Kumar-Singh et al, 1999) and Simian virus-40 sequences (Procopio et al, 2000). We have recently presented data in operable non-small-cell lung cancer indicating that MVD contributes to a biological prognostic model which is independent of TNM stage (Cox et al, 2001). Using a similar approach, it may be possible to create a biological staging system in MM, which would avoid the current difficulties in predicting outcome associated with TNM staging. In addition to bFGF and IL-6, a number of other angiogenic factors have been studied in MM, although correlation between their expression and angiogenesis is poorly understood. These include HGF/SF (Tolnay et al, 1998), IL-8 (Antony et al, 1996) and urokinase plasminogen activator (Shetty et al, 1995). VEGF expression was correlated to MVD in MM by Ohta (Ohta et al, 1999) but not in the Kumar-Singh study (Kumar-Singh et al, 1999). However VEGF expression was not found to be a significant poor prognostic factor in either study.

Research into the mechanisms underlying angiogenesis has resulted in the discovery of a number of potential anti-angiogenic agents and endogenous angiostatic peptides, which are currently undergoing investigation in solid tumours (Bicknell and Harris, 1996; Twardowski and Gradishar, 1997; Cherrington et al, 2000; Eatock et al, 2000; O'Byrne et al, 2000; Talks and Harris, 2000). These include: synthetic matrix metalloproteinase inhibitors (e.g. Batimastat (Macaulay et al, 1999) and Marimastat (Steward, 1999)), cytokines and their modulators (e.g. interferon α-2a, IL-12 (Duda et al, 2000) and thalidomide (Calabrese and Fleischer, 2000)) and angiostatic factors (e.g. angiostatin (O'Reilly et al, 1994), endostatin (O'Reilly et al, 1997), TNP-470 (Gervaz and Fontolliet, 1998) and platelet factor-4 (Gupta et al, 1995)).

In conclusion, assessment of angiogenesis may have an important role in the prognostic evaluation of MM and contribute to currently established prognostic scoring systems. Investigation of the mechanisms of angiogenesis in MM may provide further prognostic information and help to rationalise therapy. Such markers may be useful in the selection of patients for radical surgery and chemotherapeutic treatment protocols including the use of anti-angiogenic agents.

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Cyclooxygenase-2 Expression Is a Novel Prognostic Factor in Malignant Mesothelioma[1]

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ABSTRACT

Malignant mesothelioma (MM) is a fatal tumor of in-
creasing incidence, which is resistant to current therapy.
Cyclooxygenase-2 (COX-2) plays an important role in solid
tumor growth, invasiveness, and angiogenesis, in part
through the synthesis of prostaglandins such as prosta-
glandin E_2 (PGE$_2$). In a prospective study, we evaluated
COX-2 expression in snap-frozen, surgically resected MM
tissue specimens using immunohistochemistry and semi-
quantitative Western blotting. PGE$_2$ was assessed by en-
zyme immunoassay. Thirty epithelioid, 10 biphasic, and 8
sarcomatoid tumors were evaluated. Immunohistochemistry
demonstrated strong cytoplasmic tumor cell and variable
stromal staining in all of the cases. COX-2 protein levels
were correlated with clinicopathological prognostic factors
using Kaplan-Meier and Cox proportional hazards models.
High COX-2 band densitometry values correlated with poor
survival ($P = 0.008$). In multivariate analysis, high COX-2
expression ($P = 0.0005$), nonepithelioid cell type ($P = 0.002$),
and chest pain ($P = 0.04$) were independent predictors of
poor prognosis. Furthermore, COX-2 expression contrib-
uted in multivariate analysis to both European Organization
for Research and Treatment of Cancer ($P = 0.001$) and
Cancer and Leukemia Group B ($P = 0.003$) prognostic
scoring systems. The presence of PGE$_2$ was demonstrated in
all of the samples. These results suggest that COX-2 expres-
sion is a prognostic factor in MM. COX-2 is a potential
therapeutic target in MM, and trials are required of COX-2
inhibitors alone or in combination with existing treatment
modalities.

INTRODUCTION

MM[3] is a fatal cancer of increasing incidence associated
with asbestos exposure (1). MM responds poorly to surgery,
chemotherapy, and radiotherapy (2), and has an appalling prog-
nosis. The median survival is approximately 6–12 months (3–
5). TNM staging (6) is of limited value in the majority of
patients. In contrast, evaluation of clinicopathological features
of MM has led to the formation of prognostic scoring systems.
These have been derived from multivariate analyses of patients
in chemotherapy trials conducted by the EORTC (4) and
CALGB (5). We have validated previously these systems in a
surgical series (3). Furthermore, investigation of biological
markers of prognosis has attracted interest in other solid tumors
and may provide information independent from TNM stage (7).

Cyclooxygenases catalyze the initial, rate-limiting steps of
prostaglandin synthesis from arachidonic acid (8). Compared
with its isoform COX-1, COX-2 leads preferentially to the
formation of prostaglandins such as PGE$_2$ (9). COX-2 has been
implicated in carcinogenesis through the down-regulation of
cell-mediated immunity, promotion of angiogenesis, and the
formation of carcinogenic metabolites such as malondialdehyde
(10, 11). COX-2-expressing cancer cell lines are associated with
increased proliferative and invasive potential (12). COX-2 over-
expression has been noted in many solid tumors, including
colorectal (13), breast (14), gastric (15), esophageal (16), lung
(17), and brain (18) tumors. Selective inhibition of COX-2 is a
novel therapy under investigation in both the chemoprevention
and treatment of solid tumors (19, 20).

This study evaluated the expression of COX-2 in MM by
both immunohistochemistry and Western blotting, and its prod-
uct, PGE$_2$, by enzyme immunoassay. The contribution of
COX-2 expression to clinicopathologic prognostic factors, and
the CALGB and EORTC prognostic scoring systems was as-
sessed.

PATIENTS AND METHODS

Patients. In a prospective study, patients referred to the
regional Department of Thoracic Surgery for surgical biopsy,
management of pleural effusion or empyema, or for radical

[3] The abbreviations used are: MM, malignant mesothelioma; CALGB,
Cancer and Leukemia Group B; CI, confidence interval; COX, cy-
clooxygenase; EORTC, European Organization for Research and Treat-
ment of Cancer; HR, hazard ratio; EGFR, epidermal growth factor
receptor; IMIG, International Mesothelioma Interest Group; ECL, en-
hanced chemiluminescence; TNM, Tumor-Node-Metastasis; PGE$_2$,
prostaglandin E$_2$.
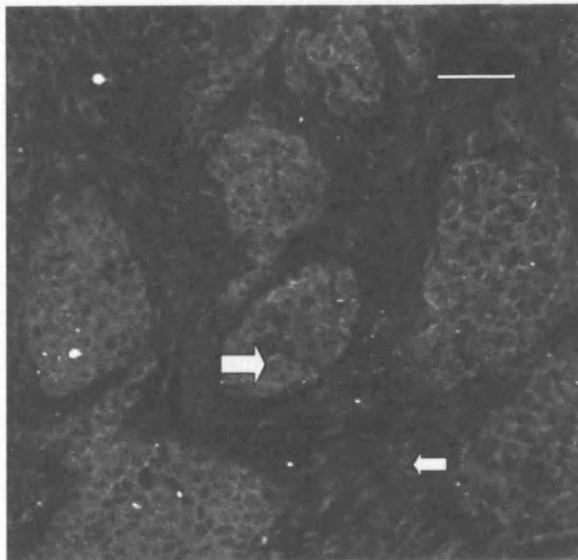
Fig. 1 COX-2 immunofluorescence. There is strong immunostaining in epithelial MM tumor islets (*large arrow*) but weaker staining in surrounding stroma (*small arrow; bar,* 40 µm).

surgery for MM were identified before their operative procedure. Case notes were reviewed and patients interviewed to retrieve relevant demographic, clinical, and pathological data. Prediagnostic variables, such as performance status and hematological indices, were taken immediately before surgery. After surgery, the detailed histopathology report was examined in each case and the pathologic IMIG stage (6) derived, where possible. Clinicopathologic prognostic factors, including CALGB (5) and EORTC (4) prognostic groups, were derived in all of the cases, as described previously (3). In regard to the CALGB prognostic system, groups 1 and 2, 3 and 4, and 5 and 6 were combined, respectively, for statistical analyses, because of the low numbers of patients in the even-numbered groups. Causes of death and patterns of recurrence were noted, and cancer-specific survival was calculated from the date of the diagnostic biopsy.

Samples. MM specimens were collected at video-assisted thoracoscopic biopsy or at thoracotomy, inspected macroscopically by a histopathologist, and representative blocks of tumor were snap-frozen and stored in liquid nitrogen. Histopathology slides were reviewed to assess the presence of suitable tumor for immunohistochemistry within each block.

Immunohistochemistry. Sections 7-µm thick were cut from snap-frozen tissue blocks with a cryostat, maintaining a chamber temperature of −27°C, before mounting on silane-treated slides and drying overnight at −20°C. Slides were fixed with acetone, washed, and permeabilized in 0.1% Triton X-100 (Sigma, Gillingham, United Kingdom). Nonspecific binding was blocked with 10% normal rabbit serum. Sections were incubated overnight at 4°C with a monoclonal COX-2 primary antibody (SC-1745; Santa Cruz Biotechnology/Autogen Bioclear, Calne, United Kingdom) at a dilution of 1:80. An FITC-labeled secondary antibody (F2016; Sigma) was used at a dilu-
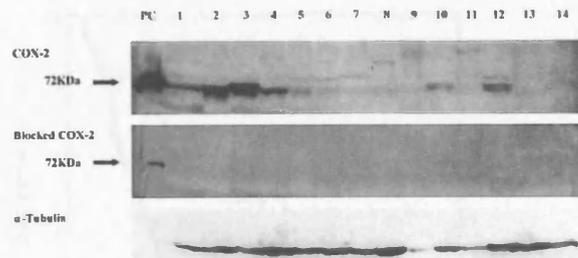
Fig. 2 Western blotting. The top gel shows the variations in COX-2 band density for the constant protein load (PC, *positive control*). In the middle gel, the effects of preabsorbing the COX-2 primary antibody with a specific COX-2 blocking peptide are seen; there is minimal immunoreactivity for the positive control, but the COX-2 bands in all of the samples are blocked. The bottom gel demonstrates the bands obtained after probing for α-tubulin.

tion of 1:50. COX-2 immunostaining was visualized with laser scanning confocal microscopy (Leica TCS4D) at low and high power. Omission of the primary antibody was used for negative controls.

Western Blotting. Snap-frozen samples were homogenized mechanically in a buffer [150 mM sodium chloride, 0.1 M Tris (pH 8), 1% Tween-20, 50 mM diethyldithiocarbamic acid, 1 mM EDTA pH 8 (Sigma)] containing protease inhibitors, before sonication and centrifugation at 4°C for 3 min. A Bradford assay (Bio-Rad, Hemel Hempstead, United Kingdom) was used to determine the protein concentration of each supernatant. Samples were loaded into a 10% SDS-PAGE, to give 150 µg protein/well. Once through the stacking gel, proteins were resolved at 150 V for 4 h. Transfer to a nitrocellulose membrane (Hypobond-ECL; Amersham Pharmacia Biotech, Amersham, United Kingdom) was performed by semi-dry electroblotting (Bio-Rad). Complete protein transfer was confirmed by staining both gel and membrane with Ponceau S (81462; Sigma). The blotted nitrocellulose membrane was blocked in 10% milk/Tris-buffered saline wash buffer [0.05 M Tris (pH 7.5), 0.15 M sodium chloride, 0.1% Tween-20 (Sigma)] overnight at 4°C. The membrane was probed with a monoclonal COX-2 primary antibody (SC-1745; Santa Cruz Biotechnology/Autogen Bioclear) for 90 min at room temperature. After washing, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (SC-2020; Santa Cruz Biotechnology) for 1 h at room temperature. The membrane was washed and developed using ECL (Amersham Pharmacia Biotech) following the manufacturer's protocol. The membrane was exposed to Hyperfilm ECL (Amersham Pharmacia Biotech) for between 2 and 20 min. Developed films were scanned and band densitometry calculated on an densitometer (Kodak Digital Science Image Station 440CF camera and ID software; PerkinElmer Life Sciences, Cambridge, United Kingdom). Background activity, the mean value of the perimeter of each template cell, was subtracted from each sample. Four samples were chosen in addition to the COX-2 standard (NP04; Oxford Biomedical Research/Biogenesis, Poole, United Kingdom) for use as internal positive controls and run on each gel. All of the gels were run under identical conditions with the same batch of
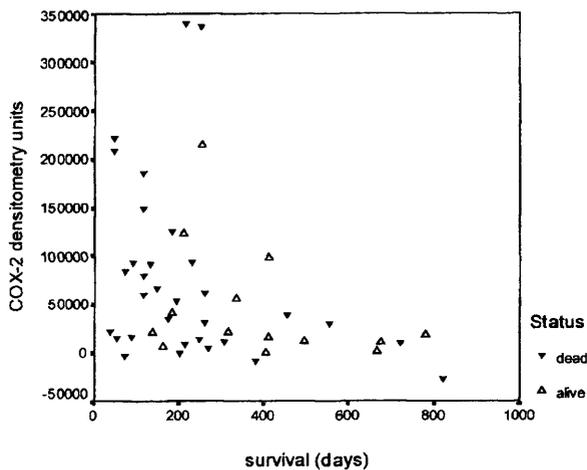
Fig. 3 Scatterplot showing the inverse relationship of COX-2 Western blot band densitometry and survival.

Fig. 4 Kaplan-Meier survival curves at a cut point of the median densitometry units ($P = 0.0057$; log rank). One patient in each group died before 30 postoperative days had passed, and these have been excluded from analysis.

reagents and densitometry results standardized between gels. COX-2 densitometry results were also normalized to membranes stripped and reprobed for α-tubulin (T-9026 primary antibody (Sigma) and P0260 secondary antibodies (Dako, Ely, United Kingdom). The semiquantitative nature of this protocol was validated by densitometric analysis of serial dilutions of the two samples with the strongest COX-2 bands. The specificity of the antibody was confirmed by preabsorbing the COX-2 primary antibody with a COX-2 blocking peptide (SC-1745P; Santa Cruz Biotechnology) overnight at 4°C.

Bicyclo-PGE$_2$ Enzyme Immunoassay. Because of its short half-life, PGE$_2$ and its physiological metabolites were measured by derivatization to their stable moiety, Bicyclo-PGE$_2$, and subsequent enzyme immunoassay, according to the manufacturer's protocol (Cayman Chemical/Alexis Biochemicals, Nottingham, United Kingdom). Briefly, homogenized tumor supernatants were derivatized to bicyclo-PGE$_2$ overnight in a 1 M sodium bicarbonate solution. Bicyclo-PGE$_2$ was then measured using an enzyme immunoassay.

Statistical Analysis. Clinicopathologic factors were assessed with Kaplan-Meier and log rank analyses. Prognostic variables on univariate analysis ($P < 0.1$) were entered into Cox proportional hazards models to examine HRs and perform multivariate analysis, as described previously (3). Patients dying within 30 days were excluded from survival analysis to avoid bias from postoperative deaths. The impact of COX-2 expression, in terms of Western blot densitometry and immunohistochemical staining, was incorporated into these models both as a continuous and a categorical variable.

RESULTS

Surgical Procedures and Outcome. Radical surgery [with intent of complete microscopic (R0) resection] was performed in 23 and palliative surgery or diagnostic surgical biopsy in 25 patients. Two patients died within 30 days: 1 from respiratory failure on day 10 and 1 from cardiac arrest after acute intrathoracic hemorrhage on day 11. Overall survival was me-

dian 8.1 (range, 0.3 to 27.0) months. Six- and 12-month survival rates were 66% and 39%, respectively. Patients who received radical surgery had a longer survival than those who had palliative or diagnostic surgery ($P = 0.02$; log rank). However, undergoing radical surgery was not an independent predictor of good outcome in subsequent multivariate Cox analyses. In univariate analyses, preoperative clinicopathologic prognostic factors significant with the log rank test were weight loss of >5% ($P = 0.007$), nonepithelioid cell type ($P = 0.002$), and hemoglobin <14 g/dl ($P = 0.02$). There was a trend toward poor survival with the presence of chest pain ($P = 0.06$) in this cohort of patients. Male gender, Eastern Cooperative Oncology Group performance status >0, WBC count >8.3 × 10^9/liter, thrombocytosis, and IMIG TNM stage were not predictors of survival. Both the CALGB ($P = 0.001$) and EORTC ($P = 0.004$) prognostic scoring systems stratified survival appropriately according to their risk groups with Kaplan-Meier analysis.
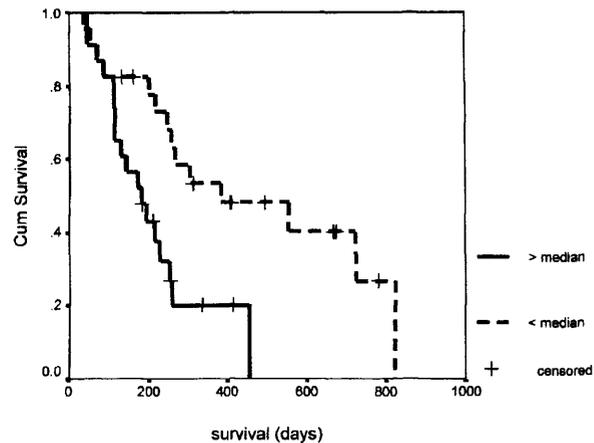
Immunohistochemistry and Western Blot Analysis. Eighteen cases were evaluated with immunohistochemistry. There was strong cytoplasmic tumor cell staining in all of the cases (Fig. 1). Surrounding stroma stained with variable immunointensity. With Western blotting, immunoreactive bands were seen, corresponding with the COX-2 standard, at M_r 72,000 and/or M_r 74,000, indicating different glycosylation states (Fig. 2). Immunoreactivity was lost when the primary antibody was blocked with a blocking peptide. The mean COX-2 band densitometry value was 66,000 (SD 83,500) units. Serial dilutional studies confirmed that the relationship between COX-2 load and band densitometry was linear within the range encountered. Correcting the results between the individual gels did not affect the rank order or categories of samples. Similarly, there was a strong correlation between the COX-2 densitometry values alone and those corrected for α-tubulin ($r = 0.92$; $P < 0.001$). There were significant correlations between weight loss >5%, and both COX-2 expression ($P = 0.004$) and the ratio to α-tubulin ($P = 0.01$). There was no correlation between either

Table 1 Multivariate Cox proportional hazards model with clinicopathologic prognostic factors

COX-2 values greater than the median were a poor prognostic factor independent of cell type. The presence of preoperative weight loss, hemoglobin <14.0 g/dl, or not undergoing radical surgery were not significant factors in the multivariate model.

		n	P	HR	95% CI
COX-2	< median	23	0.0005	1	
	> median	23		4.60	1.96–10.79
Cell type	epithelioid	30	0.0016	1	
	nonepithelioid	16		3.62	1.63–8.06
Chest pain	no	17	0.04	1	
	yes	29		2.53	1.04–6.10

Table 2 Multivariate Cox proportional hazards models

COX-2 values greater than the median were a poor prognostic factor independent of both the CALGB (a) and EORTC (b) prognostic scoring systems. The six CALGB prognostic groups were combined, because there were insufficient numbers in the even-numbered groups for analysis.

		n	P	HR	95% CI
COX-2	< median	23	0.0025	1	
	> median	23		3.77	1.60–8.91
CALGB	1, 2	14	0.001	1	
	3, 4	23		2.85	1.08–7.57
	5, 6	10		8.75	2.75–27.80
COX-2	< median	23	0.0010	1	
	> median	23		4.51	1.84–11.09
EORTC	low risk	29	0.0005	1	
	high risk	17		4.24	1.87–9.62

COX-2 or COX-2:α-tubulin ratio and IMIG TNM stage in the 32 cases in which the latter could be derived accurately.

Prognostic Impact of COX-2. In univariate analysis, high COX-2 band densitometry values correlated with poor survival (Fig. 3) when assessed as a categorical variable [greater or less than median value; $P = 0.008$; HR, 2.9 (95% CI, 1.3–6.3); Cox regression; Fig. 4] or as a continuous variable ($P = 0.003$). When normalized to the corresponding α-tubulin band, the mean COX-2:α-tubulin ratio was 0.22 (SD 0.25). A COX-2:α-tubulin ratio greater than the median value was associated with poor prognosis [$P = 0.02$; HR, 2.7 (95% CI, 1.2–5.7)].

In multivariate Cox analysis with the clinicopathologic factors (identified by univariate analysis), COX-2 expression, cell type, and the presence of chest pain at diagnosis were the only independent prognostic factors (Table 1). The presence of weight loss, low hemoglobin, and patients who did not receive radical surgery did not contribute as poor prognostic factors to the multivariate model (Table 1). In regard to the CALGB and EORTC prognostic scoring systems, COX-2 contributed independently to both (Table 2). In all of the preceding multivariate analyses, COX-2 was an independent prognostic factor when entered as a continuous variable. Furthermore, COX-2 was also significant in multivariate analyses with clinicopathologic factors or prognostic scoring systems when analyzed as the ratio to α-tubulin, both as a categorical and continuous variable.

Bicyclo-PGE₂ Enzyme/Immunoassay. Presence of Bicyclo-PGE₂ was demonstrated in all of the samples with median 1.8 (range, 0.34–5.6) pg/mg protein. No correlations with either COX-2 protein levels or survival were seen.

DISCUSSION

This study demonstrates that COX-2 is expressed in MM and can be detected using immunohistochemistry and Western blotting techniques. Furthermore, PGE₂ was also detected in all of the samples. An initial report of COX-2 expression in MM failed to demonstrate COX-2 protein in either tumor samples or cell lines by Western blotting (21). Although we found little variability in MM tumor cell immunofluorescence using immunohistochemistry, high COX-2 levels on Western blotting correlated with a worse prognosis in univariate and multivariate analyses. No correlation was seen between COX-2 Western blot densitometry values and the pathological IMIG TNM stage. This is in keeping with the lack of impact of the IMIG staging

system on prognosis in this and other patient series (22), an observation suggesting that the biological behavior of the tumor is more important than disease extent in determining patient outcome.

The prognostic significance of COX-2 tumor cell expression has been examined in other cancers. With immunohistochemistry, COX-2 overexpression has been correlated with colorectal tumor growth (23), lymph node metastasis (13), and recurrence (24), although results were not consistent between studies. COX-2 expression has been proposed as a significant poor prognostic factor in colorectal and gastric cancer but only with univariate analysis (25, 26). In non-small cell lung cancer, COX-2 immunostaining has been found to be associated with a significantly worse prognosis in stage I adenocarcinomas (27).

Clinicopathologic correlations with COX-2 protein levels, as assessed by Western blotting and semiquantitative densitometry, have been less well characterized. COX-2 overexpression, according to this method, has been described in 15 gastric tumor samples, compared with paired normal mucosa. Samples with a COX-2 densitometry ratio between tumor and normal mucosa >2 were associated with lymphatic invasion, lymph node metastasis, and TNM stage (15). Similarly, COX-2 Western blot densitometry values are greater in Barrett's esophagus and esophageal adenocarcinoma than in normal tissues (28). Our study is the first to demonstrate by Western blotting that increasing COX-2 protein levels in tumor tissue correlate with poor outcome. Furthermore, COX-2 protein levels were an independent prognostic factor in multivariate analyses and contributed to both the CALGB and EORTC prognostic scoring systems, suggesting that COX-2 is an important factor in MM.

COX-2 inhibitors have a number of beneficial effects in solid tumors. COX-2 inhibition has been shown to increase tumor cell apoptosis (29, 30), and reduce proliferation (31), invasion (12), and angiogenesis (32–34). COX-2 inhibitors are an effective means of chemoprevention in a number of carcinogenic models (35–40).

A number of previous experimental studies support a role for COX activity in the pathogenesis of MM. Asbestos causes release of PGE₂ from alveolar macrophages and inhibition of mesothelial cell-mediated cytotoxicity (41, 42). Indomethacin, an inhibitor of both COX-1 and COX-2 activity, has been demonstrated to restore the depressed lymphokine-activated

killer cell activity seen in patients with MM in an *ex vivo* model (43). Therefore, COX-2 may be implicated in the T-cell anergy seen in MM (44). The suppression of antitumor immune responses may be reversed by administration of COX-2 inhibitors (45).

We have observed previously increased expression and activation of the EGFR in pleural mesothelial cells after exposure to carcinogenic asbestos fibers (46). EGFR autophosphorylation results in activation of the transcription factor nuclear factor κB (47). A nuclear factor κB binding site is present in the promoter region of the COX-2 gene (48). In keeping with this EGFR activation has been demonstrated to be associated with up-regulation of COX-2 expression (49, 50). A number of other growth factors, which play important roles in the pathogenesis of MM, have been shown to induce COX-2 expression. These include hepatocyte growth factor/scatter factor (51, 52), transforming growth factor β (53, 54), and platelet-derived growth factor (55, 56). Collectively these data suggest an important role for COX-2 in MM carcinogenesis.

In conclusion, increasing levels of COX-2 protein, as assessed by Western blot analysis, are a poor prognostic factor in MM, which contributes independently to the CALGB and EORTC prognostic scoring systems. These data support an important role for COX-2 in pathogenesis of this malignancy. The cell signaling pathways involved in the regulation of this important immunomodulatory and tumor-promoting factor require additional investigation. The inhibition of COX-2 is a potential novel therapeutic target, alone or in combination with cytotoxic chemotherapy, for the management of MM. Furthermore, COX-2 inhibitors may have a role to play in the chemoprevention of the disease.

ACKNOWLEDGMENTS

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Appendix II

Selected Presentations

a)   Regional

Midlands Cardiothoracic Surgical Meeting
Oct 1999   Leicester
1.   Matrix metalloproteinase activity in malignant mesothelioma and benign pleura.
Edwards JG, McLaren J, Walker RA, Waller DA, O'Byrne KJ,
(poster discussion.)

Feb 2000   Birmingham
2.   Angiogenesis is an independent prognostic factor in malignant mesothelioma.
Edwards JG, Cox G, Walker RA, Waller DA, O'Byrne KJ,
(oral presentation.)

Feb 2001   Stoke-on-Trent
3.   Radical surgery for malignant mesothelioma in a specialist unit. Results of the early experience.
Martin-Ucar AE, Edwards JG, Waller DA,
(oral presentation.)

March 2002   Birmingham
4.   The influence of a multidisciplinary team approach on the surgical management of malignant mesothelioma.
Stewart D, Edwards JG, Waller DA,
(oral presentation.)

Leicestershire Research Prize Day
June 2000
5.   Angiogenesis is an independent prognostic factor in malignant mesothelioma.
Edwards JG, Cox G, Walker RA, Waller DA, O'Byrne KJ,
(oral presentation.)

June 2001
6.   Cyclooxygenase-2 expression in malignant mesothelioma: prognostic implications.
Edwards JG, Faux SP, Plummer SM, Walker RA, Waller DA, O'Byrne KJ,
(oral presentation.)

b) National

Society of Cardiothoracic Surgeons of Great Britain and Ireland Annual Meeting
1999   Nottingham
7.   Cytoreductive surgery for malignant pleural disease: is it worthwhile?
Edwards JG, Rengarajan A, Spyt TJ, Waller DA,
(poster discussion.)

2000   London
8.   Matrix metalloproteinase activity in malignant mesothelioma and benign pleura.
Edwards JG, McLaren J, Walker RA, Waller DA, O'Byrne KJ,
(oral presentation.)

2001   Nottingham
9.   Cyclooxygenase-2 is expressed and is an independent prognostic factor in malignant mesothelioma.
Edwards JG, Faux SP, Plummer SM, Walker RA, Waller DA, O'Byrne KJ,
(oral presentation.)

British Thoracic Society Winter Meeting
1999   London

10. Matrix metalloproteinase activity in malignant mesothelioma and benign pleura.
Edwards JG, McLaren J, Walker RA, Waller DA, O'Byrne KJ, *(thematic poster presentation.)*
Thorax 54(Suppl 3) A81

11. Increased Chalkley microvessel counts are related to platelet count and convey poor prognosis in malignant mesothelioma.
Edwards JG, Cox G, Walker RA, Waller DA, O'Byrne KJ,
(thematic poster presentation.)
Thorax 54(Suppl 3) A81

12. Prognostic factors for malignant mesothelioma in Leicester: validation of EORTC and CALGB scores.
Edwards JG, Leverment JN, Spyt TJ, Waller DA, O'Byrne KJ,
(thematic poster presentation.)
Thorax 54(Suppl 3) A82

2000   London
21. Radical surgery for malignant mesothelioma: the role of contrast enhanced magnetic resonance staging.
Edwards JG, Entwistle J, Jeyapalan K, Waller DA,
(thematic poster presentation.)
Thorax 55(Suppl 3) A84

2001   London
22. A prospective phase I study of radical surgery for malignant mesothelioma in a stage-selected patient population.
Martin-Ucar AE, Edwards JG, Waller DA,
(oral presentation.)
Thorax 56(Suppl III) iii8

British Association for Cancer Research
1999   Edinburgh
23. Evaluation of matrix metalloproteinase expression in malignant and benign mesothelium.
Edwards JG, McLaren J, Walker RA, Waller DA, O'Byrne KJ,
(poster presentation.)
Br J Cancer 80 (Suppl 2); 35

24. Ten years of malignant mesothelioma in Leicester: prognostic factors.
Edwards JG, JN Leverment, TJ Spyt, Waller DA, O'Byrne KJ,
(poster presentation.)
Br J Cancer 80 (Suppl 2); 106

2002   Glasgow
25. Tumour necrosis correlates with angiogenesis and is a prognostic factor in malignant mesothelioma.
Edwards JG, Swinson D, Jones JL, O'Byrne KJ,
(poster presentation.)
Br J Cancer

Imaging Oncology Science
2000   Birmingham
26. Magnetic resonance imaging of malignant mesothelioma: preliminary results.
Alaeddin F, Edwards JG, Aslam M, Entwisle J, Jeyapalan K, Waller DA,
(oral presentation.)

c) International

European Society for Thoracic Surgery
2000   London
27. Contrast enhanced magnetic resonance imaging in the selection for radical surgery in malignant mesothelioma.
Edwards JG, Alaeddin F, Entwisle J, Jeyapalan K, Waller DA,
(oral presentation.)

Istituto Nazionale Tumori, Italy
1999   Lignano Sabbiadoro
28. Cytoreductive surgery for palliation of malignant mesothelioma.
Edwards JG, Rengarajan A, Waller DA,
(oral presentation.)

European Conference of Clinical Oncology
1999   Vienna, Austria
29. Matrix metalloproteinase expression in normal, inflamed and malignant mesothelial tissues.
Edwards JG, McLaren J, Walker RA, Waller DA, O'Byrne KJ,
(poster presentation.)
Eur J Cancer 35 (Suppl 4); S107

International Mesothelioma Interest Group
1999   Grantham, UK
30. Ten years of malignant mesothelioma in Leicester: prognostic factors.
Edwards JG, Leverment JN, Spyt TJ, Waller DA, O'Byrne KJ,
(poster discussion.)
Proc 5th Meeting Int Mesothelioma Interest Group; 62

31. A "one-stop" protocol of palliative debulking surgery for malignant mesothelioma gives lasting symptom relief.
Edwards JG, Rengarajan A, Waller DA,
(oral presentation.)
Proc 5th Meeting Int Mesothelioma Interest Group; 47

32. Cyclooxygenase-2 expression in malignant mesothelioma.
Edwards JG, Faux SP, Walker RA, Waller DA, O'Byrne KJ,
(oral presentation.)
Proc 5th Meeting Int Mesothelioma Interest Group; 26

33. Evaluation of matrix metalloproteinase activity in malignant mesothelioma and benign pleura.
Edwards JG, McLaren J, Walker RA, Waller DA, O'Byrne KJ,
(poster discussion.)
Proc 5th Meeting Int Mesothelioma Interest Group; 59

34. Chalkley microvessel counts are related to prognosis in malignant mesothelioma.
Edwards JG, Cox G, Walker RA, Waller DA, O'Byrne KJ,
(poster discussion.)
Proc 5th Meeting Int Mesothelioma Interest Group; 63

European Respiratory Society Annual Congress
2002   Stockholm, Sweden
35. Predictors of survival in patients undergoing radical surgery for malignant mesothelioma.
Edwards JG, O'Byrne KJ, Waller DA,
(poster discussion.)
Eur Resp J 20 (Suppl 38); 187S

36. Tumour necrosis correlates with angiogenesis and is a poor prognostic factor in malignant mesothelioma

Edwards JG, Swinson D, Jones JL, Waller DA, O'Byrne KJ,

(poster discussion.)

Eur Resp J 20 (Suppl 38); 232S

American College of Chest Physicans Meeting
1999   Chicago, USA

37. Palliative debulking surgery for malignant pleural disease gives lasting symptom relief.

Edwards JG, Rengarajan A, Waller DA,

(poster discussion.)

Chest 116 (4 Suppl 2); 357S-358S

2000   San Francisco, USA

38. The use of contrast enhanced magnetic resonance imaging in the selection for radical surgery in malignant mesothelioma.

Edwards JG, Alaeddin F, Entwisle J, Jeyapalan K, Waller DA,

(oral presentation.)

Chest 118 (4 Suppl); 90S

2001   Philadelphia, USA

39. Decortication of trapped lung syndrome in malignant mesothelioma: predictors of successful palliation.

Martin-Ucar AE, Edwards JG, Waller DA

(oral presentation.)

40. Thrombocytosis is a stage-independent predictor of survival after radical surgery for malignant mesothelioma.

Edwards JG, Martin-Ucar AE, Waller DA,

(poster presentation.)

American Association for Cancer Research
2000   San Francisco, USA

41. Cyclooxygenase-2 and Prostaglandin E_2 in malignant mesothelioma.

Edwards JG, Faux SP, Sharma R, Shepherd P, Plummer SM, Walker RA, Waller DA, O'Byrne KJ,

(poster presentation.)

Proc AACR 41; 204

2001   New Orleans, USA

42. EGFR induced activation of NF-κB in mesothelial cells by asbestos is important in cell survival.

Faux SP, Houghton CE, Swain WA, Edwards JG, Sharma RA, Plummer SM, O'Byrne KJ,

(poster presentation.)

Proc AACR 42; 244

2002   San Diego, USA

43. EGFR activation of the PI3K/Akt pathway plays a role in human mesothelial cell survival following asbestos exposure.

Swain WA, O'Byrne KJ, Houghton CE, Edwards JG, Faux SP,

(poster presentation)

Proc AACR 43; 352

American Society of Clinical Oncology
2000   New Orleans, USA

44. Angiogenesis is an independent prognostic factor in malignant mesothelioma.

Edwards JG, Cox G, Walker RA, Waller DA, O'Byrne KJ,

(poster presentation.)

Proc ASCO 19; 550a

2001   San Francisco, USA
45. Cyclooxygenase-2 expression and angiogenesis in malignant mesothelioma.
Edwards JG, Faux SP, Plummer SM, Walker RA, Waller DA, O'Byrne KJ,
(poster presentation.)
Proc ASCO 20; 436a

2002   Orlando, USA
46. Towards a biologic prognostic model for malignant mesothelioma.
Edwards JG, Faux SP, Jones JL, Waller DA, O'Byrne KJ,
(poster presentation.)
Proc ASCO 21; 445a

International Association for the Study of Lung Cancer
2000   Tokyo, Japan
47. High microvessel density is an independent poor prognostic factor in malignant mesothelioma.
Edwards JG, Cox G, Walker RA, Waller DA, O'Byrne KJ,
(oral presentation.)
Lung Cancer 29 (Suppl 1); 192

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