CLINICOPATHOLOGICAL STUDIES OF MALIGNANT PLEURAL MESOTHELIOMA

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Malignant Mesothelioma is a primary tumour of the serosal membranes. It most commonly affects the pleura, and the majority of these cases are caused by previous exposure to amphibole asbestos. There are no effective treatments for patients with mesothelioma, and median survival from diagnosis is often less than 12 months. The UK is currently in the midst of a mesothelioma epidemic: The incidence of malignant pleural mesothelioma (MPM) has risen more than 10-fold in the last 40 years, and is predicted to continue rising for at least another decade. Despite this increase, there are many aspects of the aetiology, epidemiology and pathology of MPM that are not fully understood.

In this thesis I have summarised what is known of the causes of malignant change in the pleura, including mechanisms by which asbestos induces mesothelioma. I have also examined the evidence implicating radiation and simian virus 40 as causes of MPM.

Mesothelioma can exhibit many different patterns of differentiation, making it difficult to distinguish from other processes affecting the pleura. I have described the difficulties associated with confirming the diagnosis of MPM, detailed the different diagnostic modalities used to diagnose MPM, and performed a meta-analysis of published papers that have assessed the value of diagnostic immunohistochemistry in this area.

The relationship between patient factors and prognosis is also not clearly understood. I have analysed a cohort of 553 patients with MPM in terms of their presenting features, pathology and survival, in order to identify factors that may be of prognostic value.

Finally, recent advances in molecular biology have shed light on the mechanisms that control normal and malignant cells. The cytokine transforming growth factor-β1 (TGF-β1) critically influences the extra-cellular matrix, and is implicated in the development of tissue fibrosis, malignant invasion and metastasis. I have investigated the influence that TGF-β1 DNA polymorphisms may have on lung fibrosis, and on histological subtype and patient survival in MPM.
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I cannot begin to quantify the enormous amount of support, time and encouragement that my supervisor Professor Philip Hasleton has given me over the last five years. A thousand thanks to him, and also to his wife Sandy for her limitless hospitality during the many “thesis sessions” that were needed to finish this work.

Finally, a big thank you to all my family, friends and colleagues who together have supported, cajoled and encouraged me to undertake, finish and submit this thesis.
### List of Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>ALCL</td>
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<td>AgNOR</td>
<td>Argyrophil nuclear organising region</td>
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<td>AM</td>
<td>Alveolar macrophage</td>
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<td>ARMS-PCR</td>
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<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
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<td>cdk</td>
<td>cyclin dependent kinase</td>
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<td>ER</td>
<td>Oestrogen receptor</td>
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<td>LDR</td>
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<td>Pneumoconiosis Medical Panel</td>
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<td>SRP</td>
<td>Signal recognition particle</td>
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<td>SSP</td>
<td>Sequence specific primers</td>
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<td>tag</td>
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<td>Tumour suppressor gene</td>
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<td>Thyroid transcription factor-1</td>
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<td>UA</td>
<td>Univariate Analysis</td>
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<td>UICC</td>
<td>Union International Contre Cancer</td>
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<td>UIP</td>
<td>Usual interstitial pneumonia</td>
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<td>UK</td>
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<tr>
<td>US</td>
<td>United States of America</td>
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<td>UV</td>
<td>Ultra-violet</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>WHO</td>
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<td>WT-1</td>
<td>Wilms tumour gene product-1</td>
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CHAPTER ONE
THE EPIDEMIOLOGY AND AETIOLOGY OF MALIGNANT PLEURAL MESOTHELIOMA

INTRODUCTION
Malignant mesothelioma is a primary tumour of the serosal membranes. It is most commonly described in the pleura, but can also arise in the peritoneum, pericardium and tunica vaginalis. This thesis is primarily concerned with malignant pleural mesothelioma (MPM).

Malignant pleural mesothelioma is an uncommon tumour. In the first half of the 20th century the incidence of MPM approximated 1 - 2 cases per million per annum in the United Kingdom (UK). The recognition of the causal link with asbestos in 1960 provoked the introduction of several environmental health measures.\(^1\) It also implied that there could be an increase in the incidence of mesothelioma secondary to widespread asbestos usage. This predicted increase has already been seen in the United States (US).\(^2\) The incidence of mesothelioma has been rising in the UK for the last thirty years, and is predicted to continue rising for at least another two decades.\(^3\) The increased incidence of MPM closely parallels the level of amphibole asbestos usage in the UK (Figure 1.1 overleaf), as demonstrated by Peto and colleagues.\(^4\)

The clinical diagnosis and management of mesothelioma is challenging. It is a tumour that can pose significant diagnostic difficulty, it responds poorly to most currently available treatments, and its pathobiology is not well understood. There is increased recognition that mesothelioma is becoming a major health issue in the UK, particularly as there is little to offer patients in the way of treatment. It is therefore essential that research into the causes, biological behaviour, diagnosis and treatment of mesothelioma is made a priority.

In the first chapter of this thesis I will summarise what is already known about the aetiology of mesothelioma, from the acceptance that it existed as a separate pathological entity, through to current theories as to its cause. In subsequent chapters I will discuss why mesothelioma causes diagnostic difficulty, and explore how patient-related factors may influence this tumour's behaviour.
Figure 1.1
Predicted Mesothelioma Deaths in British Men and UK Asbestos Imports

*Bar chart indicates annual UK asbestos imports in thousands of tons (1900 - 95). Line graph demonstrates actual and predicted mesothelioma deaths as cited by J Peto et al.*

(Reproduced with permission from Professor Peto).
1.1: Historical Perspectives
Tumours involving the pleura had been reported as long ago as the eighteenth century.⁵ E. Wagner is credited as one of the first to formally publish a description of mesothelioma in 1870.⁶ In this paper he describes the gross and microscopic appearances of the lungs and pleura in 13 patients who had initially been diagnosed as having tuberculosis. He felt that they all had an alternative diagnosis. The title page from this paper is reproduced in Figure 1.2 overleaf.

Although malignant tumours involving the pleura had been recognised and reported by pathologists, the cell of origin of these tumours was disputed, and the concept of a primary mesothelial tumour was not generally accepted. Many pathologists thought that all pleural tumours were secondary tumours, invading the pleura from adjacent lung or metastasizing from elsewhere in the body.⁷ The subject remained contentious, despite increasing evidence that mesothelioma was histologically distinct from primary pulmonary tumours.⁸ By the 1950’s opinion was changing. The pioneering work of Klemperer and Rabin,⁹ Stout and Murray,⁹ Campbell,¹⁰ and McCaughey¹¹ was reinforced by the publication of a landmark paper in 1960. Wagner and colleagues described 33 cases of diffuse pleural mesothelioma arising in patients who had previously been exposed to the amphibole asbestos crocidolite whilst living or working in the North Western Cape Province, South Africa.¹ Asbestos had already been implicated in the development of lung cancer, lung fibrosis (asbestosis) and non-malignant pleural disease e.g. pleural fibrosis, effusions and plaques.¹²,¹³ A few case reports describing pleural tumours in asbestos workers had previously been published, but it was the Wagner paper that highlighted the causal association between asbestos exposure and mesothelioma. It is now accepted that mesothelioma exists as a distinct pathological entity, and that amphibole asbestos is the predominant cause of the majority of cases of pleural and peritoneal mesothelioma in adults. This realisation has driven the introduction of several public health measures regulating the asbestos industry, and has led to the banning of the more dangerous amphibole asbestos varieties in most European countries and the United States.
XXXIII. Das tuberkelähnliche Lymphadenom.
(Der sytogene oder reticulirte Tuberkel.)

Von

E. Wagner.

(Hierzuf Taf. VI und VII.)

Diejenigen Neubildungen, welche für das bloße Auge den sog. miliaren Tuberkeln gleichen, sind bekanntlich von sehr verschiedener Struktur. Sehen wir von den miliaren Abscessen und den echten miliaren Tuberkeln ab, so gehören dazu die miliaren Fibrome, Lymphome, Syphilome, Sarkome und Carcinome.

Außer den genannten, übrigens bald miliaren, d. h. ungefähr hirsekorngrossen, bald etwas grösseren Neubildungen kommt aber noch eine weitere vor, welche ich am häufigsten an der Pleura, seltener an andern serösen Häuten und in der Synovialhaut, wiederum häufiger in den Lungen, der Leber, den Nieren, der Luftwege- und Darmschleimhaut, den Hirnhäuten, der Milz und den Lymphdrüsen gefunden habe. Diese gleicht in allen wesentlichen Beziehungen dem sog. cyto- 
genem oder reticulirten oder adenoiden Gewebe und kommt wie diese bald als eine diffuse Infiltration, ähnlich dem betreffenden Gewebe in der Schleimhaut des Verdauungskanals, bald in Form von kleinen und kleinsten Knötchen, ähnlich den sog. conglobirten Drüsen oder lymphatischen Follikeln, vor. Sie ist entweder allein vorhanden oder sie findet sich gleichzeitig mit chronischer oder acuter Entzündung der betreffenden Gewebe.

Ich fand diese Neubildung zuerst an der Pleura, erst später in den meisten übrigen Organen des Körpers. Ich beschreibe dieselbe zuerst nach ihrem Verhalten in den einzelnen Organen, um am Schlusse ihre Stellung zu anderen Neubildungen, insbesondere zum Tuberkel, näher zu betrachten.

Das Lymphadenom der Pleura
kommt an allen Stellen dieser Haut vor: an der Lungen-, Costal-, Intercoastal- und Zwerchfellpleura. Es findet sich bald als

Figure 1.2
Reproduction of the Title Page of E. Wagner’s Paper Describing the Pathology of Pleural Mesothelioma in 1870.
1.2: Asbestos as a Cause of Mesothelioma

Studies of the epidemiology of MPM have supported a causal link with asbestos. The majority of series have found approximately 85% of cases are asbestos-related. Most asbestos exposure in Western Europe and the United States occurs via the subjects occupation. Rates are therefore higher in those areas based around industries utilising asbestos, such as shipbuilding and insulation work. Mesothelioma accounts for more than 10% of deaths in workers in these industries. As these occupations are markedly male-dominated, the male to female ratio in MPM is high, and the greatest increase in incidence attributed to asbestos usage has been seen in men. A notable exception is in women who were exposed to asbestos during war-work, e.g. gas-mask manufacturing, who have an incidence of mesothelioma similar to that of men.

Some geographical areas are associated with higher mesothelioma rates. These include areas where asbestos occurs naturally and is mined e.g. Russia, Quebec Province in Canada, Northern Italy, Wittenoom in Australia, Kazakhstan and Cape Province, South Africa. The main areas involved in commercial production of asbestos minerals are shown in Figure 1.3, page 6. Non-asbestiform fibrous minerals are also capable of causing mesothelioma. In Turkey, high mesothelioma rates are associated with the fibrous zeolite erionite. Erionite is a crystalline aluminosilicate whose fibres have similar dimensions to those of amphibole asbestos.

Ten to 15 percent of MPM cases have no apparent asbestos exposure. A proportion of these may have unknowingly been exposed to asbestos at work or in the home. The ability of asbestos to cause mesothelioma is not dose-dependent, and relatively minor asbestos exposure can probably induce MPM in predisposed individuals. This is classically demonstrated in women with mesothelioma whose only exposure is via their husband’s work-clothes, or from living, but not working, in the vicinity of asbestos mines or factories. There have been reports of families with more than one member afflicted by MPM. In these cases it is often difficult to exclude common environmental or para-occupational factors. Mesothelioma is rare in children. Childhood tumours are far less likely to relate to asbestos exposure, as it appears that a minimum lag phase of ten years from asbestos exposure is required for tumour development. As of yet, no other causal agents have been confirmed, although chronic pleural inflammation, simian virus 40 (SV40) and ionising radiation have been suggested as candidates. These are discussed in greater detail later in this chapter.
Figure 1.3
World Map Showing Commercially Important Asbestos Deposits
Blue markers indicate countries producing more than $100 \times 10^3$ metric tonnes (mt) of asbestos in 1998. Red markers indicate sites of important crocidolite mines.
1.3: Asbestos Mineralogy

Asbestos is the commercial collective name for a group of naturally occurring fibrous hydrated silicates that share similar chemical and physical properties. Named from the Greek for “unquenchable” (ανσβετοσ), they are particularly valued for their thermal resistance, tensile strength, flexibility and durability. Asbestos deposits are widespread, although relatively few of these are commercially exploitable. Small numbers of fibres are normally found in the environment and can contaminate water supplies.

Asbestos was known and valued from ancient times, although widespread commercial mining and manufacturing only began in earnest towards the second half of the 19th century. By the middle of the 20th century, sufficient workers had been exposed, and were surviving long enough after exposure, for the increasing incidence of MPM to be recognised.

Asbestos minerals are sub-classified into two groups, amphibole and serpentine, according to fibre morphology. Both consist of a silicate core. The type and proportion of other metals within the core structure influences the physical and chemical properties of asbestos, and may explain differing carcinogenic potential between fibres. Amphibole asbestos consists of sharp, brittle, javelin-shaped fibres, with a high length to width ratio. This group includes crocidolite (blue asbestos), amosite (brown asbestos), tremolite, actinolite and anthophyllite. By contrast serpentine asbestos, of which chrysotile (white asbestos) is the only commercial form, has long curved fibres that are less aerodynamically shaped and more friable.

The amphiboles show great chemical diversity. Although described by an idealised (standardised) formula, cation substitutions occur freely. This results in significant chemical variation between asbestos mineral types from different geographical areas. Different asbestos types can be found together in the same area, and can contaminate other mineral deposits such as talc. The basic amphibole structure is a double chain of silica tetrahedra cross-linked by bridging cations. It is these cations that can be exchanged or substituted. All the amphiboles exhibit good stability in acid conditions and have decomposition temperatures above 400°C.

In contrast, chrysotile is a sheet silicate of uniform composition. The silica tetrahedra are planar-linked with a layer of brucite (magnesium hydroxide). Differences in size and alignment between the two layers produce a structural mismatch. The resulting fibres are scroll-like with a hollow central core. The fibres are held together by hydrogen bonds, producing bundles with splayed ends that are 1-20mm long in their natural state.
Chrysotile, in contrast to the amphiboles, is stable in alkaline solution but susceptible to acid attack.

It is recognised that fibrous minerals other that asbestos can cause mesothelioma. This implies that chemical composition alone is not the only determinant of carcinogenicity. Erionite is a fibrous zeolite (aluminosilicate) that differs substantially from asbestiform minerals in terms of its structure and chemical composition. It does resemble the amphiboles in that it is durable, has a very large active surface area, and its fibres are within the range deemed critical for mesothelioma induction. Deposits of erionite are prominent in Turkey, and have resulted in endemic mesothelioma through the use of erionite-bearing rocks for house construction. Erionite is no longer commercially exploited, although non-fibrous zeolites are widely used as catalysts and adsorbents in industry. Chemical and physical properties of the asbestos minerals and erionite are summarised in Table 1.1, page 9.

The recognition of the association between asbestos and disease has led to introduction of many health measures, and a reduction in the mining and use of amphiboles, particularly crocidolite. It is generally considered that chrysotile asbestos probably does not cause mesothelioma, but can cause both asbestosis and lung cancer in a dose-dependent manner. Chrysotile now accounts for more than 99% of the total world asbestos production.
<table>
<thead>
<tr>
<th>Name</th>
<th>Fibre Type</th>
<th>Colour</th>
<th>Decomposition Temperature (°C)</th>
<th>Idealised Chemical Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinolite</td>
<td>Amphibole</td>
<td>Green</td>
<td>620-960</td>
<td>(Ca_2(Mg,Fe)<em>3(Si_8O</em>{22})(OH)_2)</td>
</tr>
<tr>
<td>Amosite</td>
<td>Amphibole</td>
<td>Pale grey / brown</td>
<td>600-800</td>
<td>((Fe,Mg)<em>7(Si_8O</em>{22})(OH)_2)</td>
</tr>
<tr>
<td>Anthophyllite</td>
<td>Amphibole</td>
<td>White / grey / brown</td>
<td>600-850</td>
<td>((Mg,Fe)<em>7(Si_8O</em>{22})(OH)_2)</td>
</tr>
<tr>
<td>Chrysotile</td>
<td>Serpentine</td>
<td>White</td>
<td>450-700</td>
<td>(Mg_3(Si_2O_5)(OH)_4)</td>
</tr>
<tr>
<td>Crocidolite</td>
<td>Amphibole</td>
<td>Blue</td>
<td>400-600</td>
<td>(Na_2Fe^{2+}_3Fe^{3+}<em>2(Si_8O</em>{22})(OH)_2)</td>
</tr>
<tr>
<td>Erionite</td>
<td>Fibrous Zeolite</td>
<td>White</td>
<td>Not known</td>
<td>((K_2Na_2Ca)MgAl_8Si_28O_{72} \cdot 28H_2O)</td>
</tr>
<tr>
<td>Tremolite</td>
<td>Amphibole</td>
<td>White / pale grey</td>
<td>950-1040</td>
<td>(Ca_2Mg_5(Si_8O_{22})(OH)_2)</td>
</tr>
</tbody>
</table>

**Table 1.1**

**Chemical and Physical Properties of Fibrous Minerals Implicated as Causing Malignant Pleural Mesothelioma**
1.4: Lung Clearance Mechanisms

Asbestos fibres occur naturally and may contaminate water supplies. There is no definitive evidence that they are harmful unless inhaled. An appreciation of the way that the body deals with inhaled fibres is essential to understanding the mechanism behind asbestos-related toxicity in the lung and pleura.

Several mechanisms exist to prevent the passage of inhaled particles into the respiratory tract. The proportion of an inhaled particle load that remains in the lung depends on the balance between initial load size and subsequent clearance. Clearance is influenced by the physical (size, shape, density) and chemical properties of a particle. The overall process can be divided into five stages: inhalation, sedimentation, deposition, translocation and dissolution.

Inhalation of particles is maximal through the nose during normal respiration. The nasal passages trap all particles over 15μm in diameter. Mouth breathing bypasses this mechanism, and is therefore far less efficient at protecting the upper airways from particles inhaled during exercise. Physical exercise also alters the dynamics of breathing. The amount and character of an inhaled particle load is therefore be influenced by the circumstances in which it is inhaled. Once in the upper airways, most particles will be deposited on the epithelial surface, particularly at bronchial airway bifurcations. Here they are trapped in mucus and removed via the mucociliary escalator and/or phagocytosed by alveolar macrophages (AM). Mucociliary clearance and macrophage function are impaired in smokers, resulting in prolonged particle contact with the bronchial epithelium. This may partly explain the synergism between smoking and asbestos in lung cancer.

The probability of a particle being deposited increases with its size. The exceptions are straight fibres with high length to width ratios, such as is characteristically seen in amphibole asbestos. Some of these fibres will align with the long axis of the airways, allowing them to travel further down the airway than less aerodynamic particles of a similar size. In the distal airways particles are distributed by sedimentation and can impact on the epithelium or penetrate into the interstitium. From here they can only be removed by translocation into the lymphatic system, or by phagocytosis and dissolution by local alveolar macrophages.

Alveolar macrophages internalise particles into phagolysosomes, in which they are subjected to the effects of lytic enzymes and an acid environment. In particular, highly reactive oxygen species (ROS) are formed. Cell death is accompanied by the release of ROS into the local environment, causing inflammation and fibrosis. Macrophages will
attempt to ameliorate the toxic effects of persisting particles by coating them with an iron-rich protein and mucopolysaccharide layer to form ferruginous or asbestos bodies, depending on the core material. There is experimental evidence that the proximity of available iron may enhance the genotoxic effects of asbestos. Usually only a small proportion of fibres are coated. The ability of AM to clear inhaled particles is finite. If mucociliary clearance mechanisms are impaired, or a large particulate load is inhaled, then not all fibres may be cleared.

Asbestos fibres, particularly amphiboles, pose particular problems for lung clearance mechanisms. The natural fibres are friable and easily fragment into smaller particles within the respirable range. Their aerodynamic shape enables them to pass deep into the airways, where they are too large to be efficiently phagocytosed by AM. This results in a relative accumulation of long fibres, the short fibres being preferentially cleared. Finally, asbestos fibres are able to resist acid attack within the AM, stimulating prolonged release of cytokines and related factors. In summary, asbestos’ physical properties facilitate passage, and its chemical properties resist removal.

1.5: Asbestos and the Mesothelium

Inadequate fibre clearance and local cytokine induction contributes to asbestos carcinogenesis and fibrogenesis in the lung. What is less clear is how and why asbestos fibres appear to preferentially target the mesothelium to produce mesothelioma. Is the carcinogenic effect a direct one, or does neoplastic change develop in response to secreted cytokines, growth factors and ROS released from sub-pleural areas of the lung? That asbestos fibres reach the pleural space is undisputed: they have been demonstrated within pleural tissue and parietal pleural plaques in humans. This may reflect passive accumulation of fibres beyond the reach of clearance mechanisms. Alternatively, it could represent active transportation (translocation) of fibre-carrying alveolar macrophages via peri-bronchovascular spaces and lymphatic channels to the pleura, in an attempt to clear fibres.

Macrophage recruitment and mesothelial cell proliferation in the pleura occur within days of asbestos inhalation in animal models, well before asbestos fibres can be demonstrated within the pleural space. It seems likely that these changes are induced in response to increasing levels of pulmonary-derived cytokines, secreted in response to asbestos in the airways and lung parenchyma. Once asbestos fibres reach the pleural space, pleural macrophages and activated mesothelial cells will attempt phagocytosis within a
matter of hours. This will further increase pleural concentrations of chemotactic and mitogenic factors, induce an inflammatory cascade and contribute to the development of a stimulatory autocrine loop.

1.6: Pleural Translocation Pathways
It was recognised that pathways between the pleural space and sub-pleural lymphatics existed long before they could be demonstrated morphologically. Water and small molecules can pass between mesothelial cells directly into lymphatics. Larger molecules, up to a size of approximately 1μm, are phagocytosed and actively transported across mesothelial cell cytoplasm. Particles greater than 1μm cannot be transported through cells, yet they can still leave the pleural space. Electron microscope studies have demonstrated the presence of other pathways. These exist on three levels; stomata, the membrana cribiformis, and lacunae.33

A stoma is a small oval or round opening found singly or in small groups on the parietal pleural surface of the anterior inferior chest wall. These permit passage of larger molecules and cells into the membrana cribiformis. The membrana is an area of loosely woven connective tissue bundles found in association with stomata. The membrana forms part of the roof of a lacuna, a dilated lymphatic bulb that directly communicates with other sub-pleural lymphatics. Changes in intra-thoracic pressure and lung movement during the respiratory cycle produce a net flow of fluid and cells from the visceral pleural surface and pleural cavity into these lymphatic channels. A proportion of inhaled asbestos fibres will be translocated via these pathways to the parietal pleura. Persistence of asbestos fibres close to stoma may cause local inflammation that eventually produces neoplastic change in the mesothelium, and might explain why mesothelioma appears to develop first in the parietal, rather than visceral, pleura (see Section 2.3, page 34).

1.7: Growth Factors and Inflammation
The prolonged production of growth factors and inflammatory cytokines has been implicated as factor in the development of malignant change. It has been suggested that chronic pleural inflammation may induce mesothelioma in the absence of asbestos fibres.34 The cytokines transforming growth factor-β1 (TGF-β1) and platelet-derived growth factor (PDGF) have both been implicated in the development and progression of MPM. Mesothelial cells secrete these cytokines in response to an inflammatory stimulus, and their production has been shown to be increased in mesothelioma.35-37 Increased local cytokine
concentrations stimulate mesothelial cells, and may influence the local environment in such a way as to both induce and promote malignant transformation. They may also contribute to tumour angiogenesis and invasion through their effects on the extracellular matrix and fibroblasts. The role played by TGF-β1 in the pathobiology of asbestos-related pulmonary fibrosis and mesothelioma is discussed in greater detail later in this thesis (Chapters Five and Six respectively).

1.8: Asbestos Fibre Dissolution

The generation of highly reactive oxygen species is a standard macrophage response to the presence of phagocytosed intracellular particulate matter. Reactive oxygen species can be formed from oxygen in solution in several different ways, which include the following:

\[
\begin{align*}
\text{Fe}^{2+} + \text{H}_2\text{O}_2 & \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^* \quad \text{(Fenton reaction)} \\
\text{H}_2\text{O}_2 + \text{O}_2^* & \rightarrow \text{OH}^- + \text{OH}^* + \text{O}_2 \quad \text{(Haber-Weiss Reaction)} \\
\text{O}_2 + \text{Fe}^{2+} & \rightarrow \text{O}_2^* + \text{Fe}^{3+}
\end{align*}
\]

Reactive oxygen species production is catalysed at available surface redox (reduction - oxidation) sites via cation exchange, particularly in the presence of reduced iron. The most toxic ROS are superoxide (O$_2^*$) and hydroxyl (OH $^*$) ions. These can directly react with individual deoxyribonucleic acid (DNA) molecules, as well as indirectly causing damage by promoting lipid peroxidation. Under normal circumstances termination of free radical activity occurs once the initiating particle is inactivated through spontaneous decay or the production of antioxidants (e.g. transferrin, glutathione, vitamin E). Experimental chromosomal damage from ROS is partly blocked by the addition of antioxidants and iron chelators such as desferrioxamine.$^{29,38}$ The durable nature of amphibole asbestos fibres, and in particular their stability in acid solution, resists these effects. This “frustrated phagocytosis” perpetuates ROS generation and DNA damage.

1.9: Carcinogenesis and the Development of Mesothelioma

Advances in molecular biology have identified some of the cellular processes that initiate and control malignant transformation. In vitro and in vivo systems have been developed to assess the carcinogenic potential of a wide range of substances. The cellular processes that control the cell cycle, DNA repair mechanisms, and the contribution of oncogenes and
tumour suppressor genes (TSG) to the development of cancer are being unravelled. Despite these advances, the exact mechanism by which MPM develops is uncertain.

In asbestos-associated cases, the interval between exposure and mesothelioma development is often in the order of 30-40 years. This suggests that a multi-step mechanism is involved. Putative carcinogenic mechanisms for asbestos have resulted from experimental work, but discrepancies between cell culture and in vivo results, and between animal and human models, exist. Different species, and different cell types from the same species, vary greatly in their susceptibility to the effects of carcinogens and promoter substances. The experimental method of fibre delivery can also influence subsequent carcinogenic potential.

A carcinogen is defined as a substance that induces neoplastic change in a cell. A complete carcinogen can induce this change in a cell after a single exposure. Carcinogenesis is a multi-step process that begins with an “initiation” phase, during which genetic material is altered. This can range from minor damage to individual DNA molecules, right through to gross chromosomal mutations. Cells have a limited ability to repair DNA damage, without which genetic mutations and cancer development are relatively common. Badly damaged cells usually “self-destruct” via apoptosis, but occasionally these mechanisms fail. If chromosomal damage affects regions coding for an oncogenes or TSG then malignant transformation can occur.

The process of initiation is usually rapid, and the induced genetic changes are irreversible. Initiated cells may remain dormant for some time, until acted upon by a promoter substance, and are characterised by their ability to undergo mitosis many times without senescence (immortality). These cells may be phenotypically different from non-transformed cells, but they are not neoplastic: They do not demonstrate the typical nuclear changes seen in cancer cells, and they retain an ordered pattern of growth.

Promoters are substances that induce transformed cells to divide and form a tumour, but are not carcinogens or mutagens in themselves. Their effects are usually reversible. Promoters are often tissue-specific, exerting their action through inflammatory mediators and changes in intercellular signalling. These mediators can modify gene expression and subsequent cell behaviour. Cell growth depends in part on the “normalising” effect of adjacent cells. This is mediated through the expression of cell adhesion molecules and local matrix protease production. Changes in communication between surrounding cells reduces stabilising influences, and predisposes to abnormal growth and behaviour. It also explains why cells in culture are more susceptible to the
effects of carcinogens and promoters, having partly lost these local influences. The fate of a transformed cell depends upon the interaction and balance of promoters and other growth-influencing factors: Positive influences promote cell division and increase the possibility of further chromosomal changes and malignant transformation. Inhibiting factors such as age, surgery, and poor nutrient supply to the affected organ can inhibit cell growth, and may retard neoplastic change. When a tissue is exposed to a carcinogen, a variable number of cells are transformed, and an unpredictable proportion will not develop further. However, if only one or two cells eventually achieve malignant change then in time a tumour can develop.

1.10: Asbestos as a Carcinogen
Asbestos fibres are complete carcinogens for bronchial epithelial cells, as well as being fibrogenic in the lung and pleura. Whether asbestos is a complete carcinogen for the mesothelial cell has been the subject of some debate. The epidemiological evidence that amphibole asbestos produces mesothelioma is compelling. It would seem logical that the carcinogenic effects of asbestos would exert their influence directly on mesothelial cells. However, the results of experimental studies to evaluate carcinogenicity in mesothelial cells have been equivocal.

Standard experimental studies of carcinogenicity are based on bacterial (Ames’ test) or animal (usually rodent) models. Bacterial tests have failed to confirm asbestos’s carcinogenicity. As bacterial cells do not ingest asbestos fibres, the potential for toxic effects is reduced compared with phagocytic mammalian cells. Extrapolation from animal studies assumes little or no interspecies variation in sensitivity to a carcinogen’s effects. This is not the case. It is recognised that the development of cancers in humans can be critically influenced by many things, including genetic, physiological and environmental factors. Differences between species are likely to be greater than within species. Within the rodent family significant differences are seen in the sensitivity to the mutagenic effects of asbestos: hamsters are very sensitive, whilst guinea pigs are relatively resistant. It is therefore necessary to exercise caution when extrapolating animal experimental results to man.

Another problem with experimental models of MPM induction relates to the method of asbestos delivery. The possible routes by which asbestos fibres reach the mesothelium in humans have been discussed in Sections 1.5 - 1.6 of this chapter. In animal models, asbestos fibre delivery is often by non-physiological means e.g. direct intra-
tracheal or intra-pleural installation. Many of the physiological processes vital to asbestos clearance may therefore be bypassed or overloaded. This markedly changes the amount and distribution of fibres within the lung and pleura compared with standard inhalation models, and makes evaluation of a threshold level difficult.\(^{30}\)

A further potential problem is that of differences in size. The length to width aspect of amphibole asbestos is critical to mesothelioma induction.\(^{25}\) Rodent airways are obviously smaller than humans, which will limit the size of fibres that can pass beyond mucociliary clearance mechanisms. Rodent macrophages are also smaller than their human equivalents, which is an important consideration when assessing asbestos carcinogenicity according to fibre size.\(^{42}\)

The lag phase seen in the development of MPM in man can also pose problems in the construction of experimental models. The long lag-phase commonly reported in asbestos-related human cases implies that the mechanism of carcinogenesis is multi-step, and requires numerous cell cycles to occur.\(^{39,40}\) Conducting experiments over many years using human cells is impractical. Most rodents have a life span of two to three years: is it safe to assume that changes in MPM occur as a proportion of the total life span, and extrapolate from animals in this regard? Some cell culture techniques utilise transformed human mesothelial cells as their model – however in order to transform these cells you inevitably alter their genetic constitution, which can affect their susceptibility to asbestos and their ability to develop mesothelioma. Indeed, a common way of transforming cells is to utilise viruses, one of which (SV40) has been implicated in causing MPM. In addition, there is evidence that particulates, including asbestos, can facilitate transfection of foreign genes into mammalian cells.\(^{43}\) This represents another potential oncogenic mechanism that may be relevant to the development of mesothelioma.

The exact mechanisms by which asbestos causes malignant change are not completely understood. Experimental evidence has suggested several ways in which this could occur, but it has not yet been ascertained which of these, if any, is the main contributor to the development of MPM in man. In initial tests of carcinogenicity, asbestos failed to consistently produce chromosomal changes, and was considered a non-genotoxic carcinogen, with some properties of a promoter.\(^{29}\) More recently, continued research using cell culture models now suggest that asbestos is a true carcinogen for the mesothelial cell. Asbestos certainly is a mutagen (capable of causing DNA damage) in both human and animal cell models. Three different mechanisms probably contribute to its genotoxicity:
1. The chemical composition of asbestos promotes cation exchange
2. The chemical and structural durability of asbestos induces and maintains ROS production, causing direct DNA damage
3. Phagocytosed asbestos fibres physically interfere with cytoplasmic functions e.g. spindle formation during mitosis

Asbestos is an insulator and as such resists the free passage of electrons at physiological temperatures. Low-grade exchange of ions from the active surface can occur within cells, until the fibre is destroyed or its surface rendered passive.

The chemical and physical structure of asbestos is of critical importance and determines surface activity. The most active part of the fibre surface is at the free end: interestingly this is the part that shows most coating when asbestos bodies are formed. Chrysotile has a hollow central core, which can act as an ion reservoir, and may explain why it appears more toxic than some amphiboles in cell culture studies. Amphiboles have solid cores, but contain many surface sites that facilitate cation exchange. Erionite also has a very large surface area, most of which is internal and communicates with the fibre surface via tiny pores. One gram of erionite has an active surface area of over 200m²; crocidolite and chrysotile have surface areas of approximately 10m²/g and 24m²/g respectively. These surface pores facilitate the passage of cations, but are too small for larger antioxidant molecules, produced to counteract the toxic effects of ROS, to reach the most active parts of the fibre. Blocking surface pores with isopentane reduces erionite cytotoxicity by 50%. Redox reactions involving the fibre surface are perpetuated by a local supply of ions, and enhanced by the presence of reduced iron (Fe²⁺). Crocidolite is also capable of absorbing organic carcinogens, such as polyaromatic hydrocarbons, onto its surface. This may contribute to the synergistic effects of asbestos and smoking in the development of lung cancer.

The length and width of an asbestos fibre, and particularly their ratio, is deemed critical for carcinogenesis. The definitive work of Stanton and colleagues demonstrated that fibres of greater than 8μm long, and less than 0.25μm wide, were the most toxic. This fibre length is comparable to the diameter of a mesothelial cell. Intracytoplasmic asbestos fibres may have a deleterious effect during mitosis. Spindle formation is vital to achieving the correct alignment and separation of sister chromatids, ensuring each daughter cell gets an equal number of chromosomes. It has been shown experimentally that asbestos fibres collect in the perinuclear region following phagocytosis. In this position they are
well placed to affect spindle formation, thereby predisposing to chromosomal non-dysjunction and karyotypic abnormalities.

1.11: Mechanisms of Asbestos-Induced Chromosomal Damage
Chromosomal changes have been demonstrated in both animal and human models of MPM. Some of these changes appear random. Others occur frequently enough to imply that they may be critical to the development of mesothelioma. These include changes in regions containing proto-oncogenes and tumour suppressor genes implicated in the development of other tumours. There have been discrepancies between different studies, some of which can be explained by differences in experimental technique and cell models.\textsuperscript{29,43} Three main methods have been used to investigate the effect of asbestos on human cells. “Cell-free” systems evaluate the direct effects of fibres on DNA without the contribution of cell-derived radicals and enzymes, and are therefore of limited value.\textsuperscript{48} In vitro cell culture experiments allow assessment of induced chromosomal changes following incubation with asbestos fibres, but cannot entirely reproduce the cellular environment found in mesothelial cells following asbestos inhalation. Few of these experiments have been performed using human mesothelial cells (HMC). The analysis of chromosomal abnormalities in established MPM cells (primary tumours or cell lines) can indicate which changes appear most important in the development of MPM.

Some of the earliest \textit{in vitro} work investigating asbestos-related chromosomal changes was by Lechner and colleagues.\textsuperscript{49} They initially studied the mutagenic effects of asbestos on HMC derived from non-malignant pleural effusions. These were cultured with UICC standard amosite, chrysotile and crocidolite fibres, and assessed for both cytotoxicity and carcinogenicity. Human mesothelial cells were ten times more sensitive to the cytotoxic effects of asbestos than bronchial epithelial cells. Chrysotile was the most toxic, then amosite and crocidolite. In carcinogenicity testing, prolonged culture with amosite produced multiple karyotypic changes, particularly losses from chromosomes 11 and 21. The \textit{in vitro} finding that chrysotile was the most genotoxic fibre conflicts with the currently held view that pure serpentine asbestos rarely, if ever, causes mesothelioma \textit{in vivo}. This may relate to the type of fibres used. At the time of this study the UICC standard crocidolite was composed of relatively short fibres (<2.5\textmu m) which are now recognised as being less pathogenic than longer fibres.\textsuperscript{50} Using crocidolite fibres within the Stanton range has confirmed that amphiboles are more genotoxic than serpentine asbestos.\textsuperscript{45} An
alternative interpretation of this data is that chrysotile can induce mesothelioma, but only if able to reach the pleura.

In 1989 Olofsson and Mark investigated the effects of incubating HMC with crocidolite, chrysotile or amosite. All fibres caused numerical and structural chromosomal abnormalities. Although precise changes were not identical to those seen in mesothelioma, there was a tendency to structural changes and losses affecting the same chromosomes. These changes were predominantly seen in chromosomes 1, 4, 6, 9, 13 and 17. The authors concluded that asbestos has a propensity to preferentially induce changes in specific chromosomes. Their failure to detect abnormalities seen more commonly in mesothelioma, e.g. monosomy 22, may indicate that such changes develop later on, after initial asbestos-induced changes have occurred.

1.12: Observed Chromosomal Changes in Malignant Pleural Mesothelioma
Several groups have investigated the types and number of karyotypic changes in human mesothelioma cells. In contrast to the previously mentioned in vitro experiments on non-malignant mesothelial cells exposed to asbestos, these experiments describe chromosomal changes that have persisted or accompanied mesothelioma development. Comparisons can then be made with non asbestos-related tumours, to see if different genetic mechanisms are implicated in tumour development. Karyotyping studies tend to use cells from two different origins. Tumour samples, either fresh or paraffin-embedded, will include many non-malignant cells (e.g. stromal cells) which may dilute or obscure changes in neoplastic cells. Mesothelioma-derived cell lines consist of cells that have continued to divide in culture. They may therefore represent a sub-population of mesothelioma cells that can continue to divide because they are genetically different from other tumour cells.

DNA flow cytometry enables evaluation of major chromosomal changes. Normal cells are diploid. Aneuploidy, in which the chromosome number is not an exact multiple of the haploid number, is a common feature of many tumours. Several investigators have assessed the DNA content of MPM cells. Most mesotheliomas remain diploid or pseudodiploid. Aneuploid tumours appear to have a poorer prognosis.

Karyotyping studies have demonstrated that most mesotheliomas have multiple chromosomal abnormalities, the majority having ten or more identifiable alterations. In a literature search I have identified 26 published studies that have evaluated chromosomal changes in MPM cells. All of these studies have conducted their research using cells from histologically-confirmed MPM, and the majority have examined more than ten
different tumour samples. Most other studies reporting changes in MPM have been single case reports, which I have not included in this analysis. Combining the results of these 26 studies provides information on a total of 611 mesothelioma samples. These include samples from 313 tumours, 234 mesothelioma cell lines and a further 64 samples in which the cell of origin was unclear.

Unfortunately, it was not always apparent how many samples had specific changes, or whether two or more listed changes were seen in the same tumour. Only a handful of different research groups have assessed chromosomal changes in MPM, and it uncertain whether the same tumour samples were used in different papers from the same centre. The Bjorkqvist 1998 study excluded analysis of gains affecting regions 1p32-pter, 16p, 19 and 22, as false positive results were found in these regions in normal control DNA.

Chromosomal abnormalities were identified in 439 samples (72% of the total number of cases). The proportion of karyotypic abnormalities was similar for all three groups: tumours 68%, cell lines 73% and others (source not stated) 55%. Changes ranged from major chromosomal losses to point deletions and translocations. Losses were more common than gains. Abnormalities were identified in 16 different chromosomes, but predominantly affected chromosomes 1, 3, 4, 6, 7, 9, 13, 14, 17 and 22. Specific chromosomal changes, and their relationship to known and putative oncogenes and tumour suppressor genes, can provide clues as to the mechanisms underlying MPM development. I have therefore analysed these papers in detail, and summarised their findings below. These results are represented graphically in Figure 1.4, page 25).

**Chromosome 1**

Ten studies have reported karyotypic changes involving chromosome (Ch)1. A total of 111 losses and 5 gains were identified. Losses predominantly affected the short arm of Ch1, and in 74 cases could be localised to the region 1p12-22. Chromosome 1 losses have been shown to be significantly associated with high lung asbestos counts (p=0.005). It is thought that a gene relating to cell senescence is located on Ch1. Interestingly, trisomy 1 is associated with the development of mesothelioma in rats.

**Chromosome 3**

Chromosome 3 changes were reported in nine studies. Both losses and gains were identified, although losses predominated. Abnormalities mainly affected 3p, and were localised to the 3p14-21 region in five studies.
Chromosome 4
Allelic loss from Chromosome 4 is a feature of many tumours. Six studies have identified changes affecting Ch4 in MPM.\textsuperscript{64, 65, 74, 78, 79} Losses were seen in a total of 56 samples. Hagemeijer and colleagues identified monosomy 4 in 13 of their cases.\textsuperscript{65} Discrete losses and deletions primarily affected the long arm (4q), although several different regions of loss were identified. Chromosome 4 losses were shown to be significantly associated with higher levels of asbestos exposure (p=0.007).\textsuperscript{64, 67}

Chromosome 5
Chromosome 5 abnormalities in mesothelioma are predominantly duplications of 5p, which were identified in five studies.\textsuperscript{64-66, 77, 79}

Chromosome 6
Chromosome 6 losses are a feature of many tumours, suggesting that it may encode for a non-specific TSG. A possible candidate is the gene SEN6, which appears to influence cell life span, and whose expression is influenced by the SV40 large T antigen (Tag).\textsuperscript{86} Chromosome 6 losses have been consistently identified in MPM, and may be a critical step in malignant transformation, as it is the only chromosomal abnormality identified in two studies.\textsuperscript{82, 87} A total of 92 samples in eight studies have been reported as showing Ch 6 losses.\textsuperscript{60, 65, 77-80, 82, 87} Losses range from discrete point deletions to the loss of the whole of the long arm. Bell and colleagues have identified 4 separate regions of loss on 6q: 6q14-21, 6q16.3-21, 6q21-23.2 and 6q25.\textsuperscript{80}

Chromosome 7
Chromosome 7 gains are common in many tumours. It is thought to contain a metproto-oncogene regulating expression of epidermal growth factor (EGF). Putative sites have been identified at 7q22 and 7q11.1-2. Gains and duplications of Ch 7 or 7q have been demonstrated in five studies, in a total of 41 samples.\textsuperscript{62, 63, 65, 67, 79} Two studies by the Helsinki group have shown a strong inverse association between the degree of polysomy 7 and survival.\textsuperscript{67}
Chromosome 9
The short arm of Ch 9 encodes for two putative TSG’s, p15 and p16, at the 9p21 site. These genes can phosphorylate the TSG Retinoblastoma-1 (Rb1), thereby inactivating it.\textsuperscript{88} Losses affecting Ch 9 are amongst the most common seen in mesothelioma, with a total of 189 abnormal samples reported in 12 studies.\textsuperscript{60, 64, 65, 67-70, 75, 77-79, 84} All changes involved losses, and included ten cases of monosomy 9 and 167 cases of 9p loss, emphasizing the probable role of p15 and p16 loss in the development of MPM. Chromosome 9 changes are also associated with high lung asbestos fibre counts.\textsuperscript{67}

Chromosome 10
Only two studies have reported abnormalities in Ch 10. Hagemeijer and colleagues reported 4 abnormalities - three deletions and one balanced translocation, with breakpoints at 10q22-24.\textsuperscript{65} In the Bjorkqvist study changes included one sample that exhibited deletions of both 10p13-pter and 10cen-q23, and two others that each showed a single alteration (-10p12-pter and +10q).\textsuperscript{79} All of these three latter cases were men with biphasic MPM.

Chromosome 11
The Wilms tumour gene product-1 (WT1) is located on the short arm of Ch11 (11p13).\textsuperscript{89} Wilms tumours develop in the kidney, an organ that, in common with the mesothelium, embryologically exhibits a mesenchymal to epithelial phenotype shift. Mesothelial cells retain the ability to express WT1, and WT1 immunohistochemistry has been used to differentiate between mesothelioma and other pleural tumours (See Section 3.8, page 120). A small number of mesothelioma samples (n=22) have chromosomal changes involving the WT1 region.\textsuperscript{64, 67} Although in vitro experiments have demonstrated a high proportion of changes involving the WT1 site when rat mesothelial cells are exposed to asbestos, the evidence to support this as a critical factor in human tumours is less conclusive.\textsuperscript{49, 90}

Chromosome 13
Chromosome 13 contains the region coding for Rb1 (13q14). Seven studies have identified changes involving deletions and or losses in Ch 13, in a total of 42 tumours.\textsuperscript{61, 65, 66, 77-79, 82} These include ten cases of monosomy 13. All focal deletions and translations involved 13q close to the Rb1 region.
Chromosome 14
Losses and deletions are also seen exclusively in Ch 14. A total of 60 cases are reported from eight studies, of which just under one third (n=16) reported monosomy 14.\textsuperscript{61, 64, 65, 67, 77-79, 82}

Chromosome 15
Both losses and gains involving Ch 15 have been identified in MPM. Five studies have reported losses involving a total of 41 samples. Two of these have also noted gains: trisomy 15 (n=3) and 15q gain (n=1).\textsuperscript{64, 65, 77-79}

Chromosome 17
Chromosome 17 changes are seen in many tumours. Most of these relate to the presence of the ubiquitous TSG p53, sited on 17p. Heritable abnormalities of p53 (Li-Fraumeni syndrome) are associated with an increased incidence of many cancers, and spontaneous or induced changes in wild-type p53 have been implicated in the development of sarcomas, lung, colon and breast cancers.\textsuperscript{91} Chromosome 17 changes have been identified in MPM, but as of yet in small numbers. A total of thirteen abnormal samples have been identified in three studies, of which five were monosomy 17.\textsuperscript{65, 82, 84} The Bjorkqvist study identified a gain of 17q in an unspecified number of samples.\textsuperscript{78} It is perhaps unexpected to find so little apparent influence from such a vital TSG in MPM. Methodological problems may be in part to blame. Major deletions or losses are relatively easy to identify. Subtle functional changes to p53 may be as relevant to carcinogenesis, yet much harder to demonstrate: many assays of p53 cannot distinguish between active and inactive forms. Over-expression of p53 has been identified in MPM, implying previous stimulus to DNA repair or apoptosis.\textsuperscript{44} Another intriguing possibility relates to p53 and the large T antigen of SV40. SV40 sequences have been identified within MPM cells’ genome, with Tag inserted close to the p53 region. It appears that Tag in some way inhibits p53, without necessarily inducing chromosomal changes that can be readily identified. Tag inhibits Rbl function, and Tag infection of normal mesothelial cells increases PDGF secretion. All of these mechanisms represent possible pathways through which SV40 influences the development of MPM. This subject is discussed in Section 1.14, page 26.
Chromosome 19
Chromosome 19 abnormalities have only been reported in two studies. The gain of 19q in a single case of epithelioid mesothelioma, which also exhibited changes in Ch 6, 9 and 13, was identified by Bjorkqvist et al. Hagemeijer and colleagues identified a mixture of gains and losses affecting seven samples.

Chromosome 20
Changes affecting Ch 20 have only been identified in a single study: trisomy 20 in one sample, and gains or losses in a further ten.

Chromosome 22
Losses affecting Ch 22 are amongst the most common seen in MPM, and are thought to play an important role in tumour development. A total of 13 studies have identified changes in Ch 22, in a total of 137 samples. All changes were losses, and a high proportion were monosomy 22 (n=43). Chromosome 22 contains regions for two important factors in cell control: the neurofibromatosis-2 (NF2) tumour suppresser gene and the viral oncogene v-sis. The latter has a DNA sequence that is almost identical to the cytokine PDGF-B chain. This is further evidence for the influence of cytokines in mesothelioma development and progression.

In conclusion, several different chromosomal changes have been demonstrated in MPM. Some of these appear to be specific effects of asbestos. Other changes are similar to those implicated in the development of other tumours. Although contributing further insight into the aetiological mechanisms required in MPM development, at present none of these appear to have much diagnostic or prognostic value. These changes are summarised overleaf in **Figure 1.4**.
Figure 1.4
Schematic Representation of the Most Frequently Observed Chromosomal Changes in Mesothelioma and Mesothelioma Cell Lines
Losses / deletions on the left in red, gains are on the right in blue. Known oncogene and tumour suppressor gene sites listed by the appropriate chromosome.
1.13: Other Causes of Malignant Pleural Mesothelioma
There have been few indications as to whether any other aetiological factors are important in the development of mesothelioma. It remains a fact, however, that a significant number of patients with MPM irrefutably have had no personal exposure to asbestos above ‘normal’ environmental levels. These tumours appear histologically and clinically identical to their asbestos-related counterparts. This begs the question whether there are other patient-related factors or carcinogens yet to be identified that can cause MPM. Two possible causal agents have been suggested: SV40 and ionising radiation. These latter will be discussed below. The potential influence of other carcinogens and genetic factors on the development of MPM have been reviewed by Heineman et al and Peterson et al.\textsuperscript{20,94}

1.14: Simian Virus 40
Simian virus 40 is a papovavirus that is endemic and non-pathogenic in rhesus and cynomolgus monkeys. There is experimental evidence that SV40 is oncogenic in rodents, and in some human cells \textit{in vitro}. The isolation of SV40-like DNA sequences from certain human tumours, including MPM, suggests that it may also be oncogenic in Man. Millions of adults and children in the 1950’s and 1960’s were inadvertently vaccinated with polio- and adenovirus vaccines contaminated with SV40, raising the possibility of a major public health scare if SV40’s oncogenic effects were to be confirmed.

Vaccination programmes against poliomyelitis were introduced in the 1950’s. These comprised the inactivated poliovirus (Salk), which was administered parenterally, and oral attenuated poliovirus (Sabin). These vaccines were initially produced in culture from monolayers of rhesus monkey kidney cells. Simian virus 40 is non-cytopathic in its normal monkey host, hence will not produce signs of viral contamination (cell vacuolation). When culture methods were changed to use green monkey kidney cells, the presence of an infecting agent was noted. Sweet and Hilleman first reported vacuolation in the kidney cell cultures in 1960.\textsuperscript{95} By 1962 it had been confirmed that live SV40 had contaminated several batches of both forms of poliovaccines, as well as some adenovirus vaccines.\textsuperscript{96} It was still uncertain as to whether this should cause concern, as SV40 was thought to be non-pathogenic in humans. By 1964 it was confirmed that SV40 was oncogenic in rodents and was capable of transforming human cells \textit{in vitro}.\textsuperscript{97,98} Despite more than three decades of research, the risk to humans from these contaminated vaccines remains unclear.
Mesothelioma is one of the human tumours in which SV40 has been implicated. Experimental studies of SV40 oncogenesis were first performed in hamsters, which are particularly sensitive to its transforming effects. Tumours developed following inoculation by several different routes, and were most frequent in young animals. Fibrosarcomas developed at the site of subcutaneous injection, and ependymomas after intra-cerebral injection. Cicala and colleagues injected SV40 directly into the pleura and peritoneum of young hamsters: mesothelioma rates were 100% and 67% respectively within 3-6 months. Mesotheliomas also developed after intracardiac injection of SV40 in 60% of young hamsters: the remaining animals developed osteosarcomas and lymphomas.

Although SV40 appears to be inactivated when administered by the oral route, it is excreted in the stools for up to five weeks after ingestion. Antibodies to SV40 are demonstrable for at least 3 years after inoculation, and are also seen in a small proportion of those who have never received contaminated vaccines. Hence, although the parenteral forms of vaccine potentially pose the greater risk to humans, the possibility of other mechanisms of infection e.g. faeco-oral spread, cannot be entirely dismissed.

The effects of SV40 on human cells in vitro have been investigated. Simian virus 40 can transform and immortalise some human cells, and this effect is enhanced by the presence of asbestos. Transformed cells are tumourigenic when injected into human volunteers. As in the rodent model, it appears that the effects of SV40 are predominantly on ependyma, choroid plexus, bone and mesothelium.

The early region of SV40 codes for two proteins that appear critical in oncogenesis and transformation. Animals that develop tumours after administration of SV40 produce antibodies to these proteins, the large T and small t antigens (Tag and tag). It is Tag that appears responsible for cellular transformation. Tag is a mutagen, and can interact with TSG products critical in normal cell cycle control, including those of p53 and Rb1. In many tumours deletion or mutation of p53 is seen as a critical step in carcinogenesis. Insertion of Tag close to the p53 region appears to functionally inhibit its effects: p53 protein is demonstrable but is inactive. This might explain why a surprisingly small number of changes are seen in the p53 region (17p) in MPM. The SV40 tag alters TSG product expression through different mechanisms. SV40 also interacts with the gene SEN6.
1.15: Simian Virus 40 DNA Sequences and Human Cancer

After SV40 was first identified there were sporadic reports of SV40-like DNA sequences isolated from tumours in humans. The technology for DNA sequencing was still fairly primitive at that time, and it was uncertain as to whether some of these results were actually due to contamination of reagents with SV40, from cross-reactions with related viruses, or to differences in experimental technique and primers. In 1992 Bergsagel and colleagues demonstrated SV40-like sequences in human ependymomas and choroid plexus tumours, using a polymerase chain reaction (PCR) technique. Carbone and colleagues subsequently demonstrated that 60% of MPM's tested were also positive for this sequence, corresponding to a highly conserved 172 base-pair region within Tag which included the Rb1 binding site. These sequences were more than 97% homologous with SV40, and demonstrably different from two other polyomaviruses, JC and BK, which had been implicated as a possible cause of cross-reactivity in these studies. This group also demonstrated the presence of mRNA (messenger ribonucleic acid) corresponding to the SV40 Tag sequence. Normal lung tissue in individuals with mesothelioma did not contain this viral sequence. Neither did tumours originating from other tissues. Interestingly, Finnish researchers were unable to demonstrate SV40 sequences in MPM. Finland has a low incidence of mesothelioma, and Finnish poliovaccine was never demonstrably contaminated with SV40.

In order to definitively investigate the relationship between SV40 and mesothelioma, an international multi-institutional study was initiated by the International Mesothelioma Interest Group (IMIG) in 1997. Twelve samples of snap-frozen MPM provided by an independent source were sent to each of four separate laboratories, and were analysed for SV40 sequences using a fixed protocol and standard primers. Nine of the twelve samples provided identical results in all four laboratories, the others were non-consistently positive. Two of the three negative samples were also negative for the presence of Tag on immunohistochemistry. It now seems certain that these sequences do exist, and may indicate a causal relationship between SV40 and mesothelioma.

Another way of investigating the relationship between SV40 and human cancer is to see whether the incidence of those specific tumours produced by SV40 in animals has increased in humans since administration of contaminated vaccines. Three separate groups have reported the results of such studies. Unfortunately, all the tumours that may relate to SV40 are rare, which makes the evaluation of changes in incidence difficult. In particular, MPM in children has only been described in a handful of cases world-wide.
The long lag phase seen in asbestos-related cases of MPM also raises concerns when SV40 is considered. If it takes many years for mesothelioma to develop irrespective of cause, then it may be a still be a few years before the potential effects of SV40 are apparent. Looking for SV40 sequences in adults who have never been exposed to contaminated vaccines is likely to add little to the debate, and may erroneously calm fears.

In 1981 the results of a 20-year follow-up study of 1073 children born between 1960 and 1962, who were potentially exposed to SV40 as neonates, were published. No cancer deaths were seen in the children in this group. Although the results of this study were considered reassuring, there were some methodological flaws that could have affected its ability to demonstrate a causal relationship. Of the 1073 children in the study, only 159 had received parenteral poliovaccine. Follow-up rates for all children were 95% in 1977, but only 87% by 1979. Less than 50% of subjects replied to a postal enquiry regarding current health status. Only one childhood cancer death would be expected in a cohort of this size. This study could therefore have missed a statistically significant increase in cancer deaths in those lost to follow-up, and was probably not large enough to evaluate absolute risk in the higher risk group given parenteral vaccine.

Geissler has evaluated changes in cancer incidence following SV40-contaminated vaccine administration in West Germany. SV40-contaminated poliovaccine was administered in the period 1959-63. He compared cancer rates in two birth cohorts (1959-61 and 1962-4), on the presumption that the earlier cohort all received contaminated poliovaccine and the later cohort did not. This presumption is probably false, and does not account for a significant number of children who were not vaccinated at all (approximately 144,000) in the earlier group. No overall difference in cancer rates was found, although small differences in the incidence of some types of brain tumour were seen.

Another study investigating cancer rates in SV40 exposed children was published in 1998. This retrospective cohort study specifically addressed whether there was a demonstrable increase in incidence of those cancers most strongly associated with SV40: ependymomas, osteosarcomas and mesotheliomas. Cancer rates were calculated from the SEER (Surveillance, Epidemiology and End Results) data (1973-93), which covered almost 10% of the population of the USA, the Connecticut Tumor Registry (1950-69) and national mortality statistics (1947-73). Subjects were divided into three groups according to date of birth and likelihood of having received contaminated poliovaccine. There was no discernible increase in any of these tumours in exposed individuals. Criticisms relating to the study methodology have been raised, particularly regarding age comparisons, as the
cancer registry data age stratification is not comparable to the age cohorts chosen in the study group. It has again been questioned whether this study was statistically powerful enough to identify a significant increase in tumours: it reported a three-fold increase in risk for developing mesothelioma, yet this failed to reach statistical significance. It has also been claimed that although gross cancer rates have not increased, the age-specific rates for ependymomas and osteosarcomas have increased by 20% since 1973.\textsuperscript{112}

In conclusion, there is evidence of an association between SV40 and some human tumours, including MPM. Whether this is a causal association is still not clear: is SV40 a pathogen or a passenger? Simian virus 40 cannot be wholly implicated in the current increase in mesothelioma incidence, as most cases are still in adults who were never exposed to contaminated vaccines. However, asbestos was only confirmed as a carcinogen in humans after it had been used commercially for decades, and thousands of people had been exposed to it. It is still uncertain as to what the latent period for SV40 oncogenesis is in humans, whether a threshold dose exists, or whether other patient-related factors can influence the likelihood of a tumour developing. Only time will tell if SV40 represents a significant risk to man, in which case the assumption that mesothelioma rates should start to fall after the current asbestos-related peak may be unfounded. In the meantime it appears that the case against SV40 is “not proven”.

1.16: Ionising Radiation and Mesothelioma
Ionising radiation is carcinogenic, causing direct DNA damage and mutations. This property is utilised in the treatment of some tumours (radiotherapy). The development of further cancers is a recognised complication of therapeutic radiation, and usually occurs more than ten years after the first cancer is treated. There have been a few case reports of MPM following radiotherapy, particularly for Hodgkin’s disease.\textsuperscript{113,114} In a cohort of 553 cases of patients with mesothelioma from the North West of England, (described in depth in Chapter 4) there were 11 patients who had a personal history of a different cancer prior to the development of their mesothelioma. Three of the 11 had received thoracic radiotherapy as part of their treatment.

Pleural mesothelioma has been experimentally produced in rodents following inhalation of plutonium, and peritoneal tumours have resulted from the intra-peritoneal implantation of plutonium oxide ($^{239}$PuO$_2$).\textsuperscript{115} It is therefore biologically plausible that some cases of MPM have resulted from previous radiotherapy treatment or other sources of radiation.
Three retrospective studies have investigated a possible relationship between MPM and radiation. Cavazza and colleagues undertook a retrospective study to evaluate the proportion of mesotheliomas that might be radiotherapy-related. This was done in two ways. Initially they searched for mesothelioma patients with a history of previous cancers treated with radiotherapy. Eight patients were identified: six following treatment for Hodgkin’s disease, and two following treatment for breast carcinoma. Secondly, a population-based study was performed, using cancer statistics provided by the SEER registry. Out of a total of 1,489,643 registered cancer patients, 142 were recorded as having developed MPM as a second cancer. Only 37 of these 142 had undergone prior radiotherapy treatment. Most of the mesotheliomas developed within ten years of their first cancer, and not all were within the original radiotherapy field. The investigators conclusion was that radiotherapy is a very uncommon cause of mesothelioma.

In the same year, Weissmann and colleagues also investigated the possible relationship between radiotherapy and MPM. They followed up an 11 year cohort (1982-93) of patients with Hodgkin’s disease. Four cases of subsequent mesothelioma were identified, two pleural and two peritoneal. The mean interval from treatment to mesothelioma development was 17 years.

One of the largest retrospective studies has been undertaken by Neugut and colleagues. They followed up two large cohorts of patients with breast cancer (n=251,750) and Hodgkin's disease (n=13,743) registered by SEER in the period 1973 -93. Approximately one quarter (n=62,453) of breast cancer patients and one half (n=6,961) of the Hodgkin’s patients had received radiotherapy as part of their treatment. Cases diagnosed within 5 years of the first cancer were excluded, as were those within missing clinical information. Six cases of MPM were identified in the breast cancer group, four of which had not received radiotherapy. There were no cases of mesothelioma in the Hodgkin's group. The estimated relative risk for the development of MPM was calculated at 1.56 (95% confidence interval 0.18 - 5.63). With such a wide confidence interval it is difficult to make a firm conclusion regarding the influence of radiotherapy. It should also be remembered that both breast cancer and lymphoma commonly metastasize to the pleura, and may resemble epithelioid mesothelioma histologically. Thoracic radiation can cause lung cancer within the radiotherapy field, and such tumours can also mimic mesothelioma clinically, radiologically and pathologically. The authors have not stated whether histological review was undertaken in the six cases of mesothelioma in the breast cancer group.
In conclusion, it is biologically plausible that a small number of cases of mesothelioma may arise from radiotherapy treatment. The number of patients treated with thoracic radiotherapy who survive for more than ten years is small. The overall contribution of radiation to the development of MPM is therefore likely to be slight.

CONCLUSIONS
The biology of MPM is complex and incompletely understood. Amphibole asbestos is accepted as the primary cause of most cases of MPM in this country, but the mechanisms by which asbestos induces malignant change in the mesothelium have not yet been entirely elucidated. Whether chrysotile causes mesothelioma remains a contentious issue.

Other causes of MPM have not been confirmed with any certainty. Familial studies and chromosomal analysis of MPM tumour specimens have suggested that genetic factors may be relevant in MPM development, but no single specific gene or chromosome abnormality has yet been implicated. Concerns remain regarding the putative relationship between MPM and SV40, but it is likely to be some time before an unequivocal conclusion as to the importance of SV40 can be made. Chronic pleural inflammation and thoracic radiation remain as potential causes of MPM, but the overall number of cases likely to arise from them is small.

A major obstacle to the analysis of MPM pathobiology has been the well-documented difficulties associated with confirmation the histological diagnosis and classification of pleural tumours. The histopathology of mesothelioma and its differentiation from other pleural tumours is the subject of the next chapter.
CHAPTER TWO
THE PATHOLOGY OF MALIGNANT PLEURAL MESOTHELIOMA

INTRODUCTION
Malignant pleural mesothelioma can exhibit a wide range of histological appearances, making it difficult to differentiate from other pleural tumours. Advances in diagnostic histopathology, particularly in those of electron microscopy and immunohistochemistry (IHC), have contributed to this field, but mesothelioma still poses a diagnostic challenge in many cases. In this chapter I will discuss the anatomy of normal pleura and the gross and microscopic pathology of MPM and its main differentials. The contribution of IHC to the diagnosis of MPM is discussed further in Chapter Three.

PART I: THE ANATOMY OF NORMAL PLEURA
2.1: Pleural and Pulmonary Embryology
All the serosal membranes (pleura, peritoneum, pericardium and tunica vaginalis) are derived from the intracoelomic mesoderm. The mesoderm is derived from cells of the epiblast (future endoderm) in the third week of development. Other mesodermal derivatives include connective tissues, muscle, heart and blood vessels, kidneys, gonads, adrenal cortex and spleen. The thorax and peritoneum develop as a single cavity, which are later separated by the development of the diaphragm. The primitive lung bud develops from the foregut and grows into the splanchnic mesenchyme within the thorax, before dividing into bronchial segments. Each segment retains an investing layer of mesenchyme which eventually forms the pleura.

2.2: Surgical Anatomy
The pleura consists of two layers, parietal and visceral, which are continuous at the pulmonary hilum. The parietal pleura covers the internal surface of the thoracic cage, extending above the clavicle into the root of the neck. Inferiorly it covers the diaphragm and reflects over the mediastinum. The visceral pleural covers the surface of the lung and extends deep into each interlobar fissure. The visceral and parietal pleura meet at the hilum as the pulmonary ligament, which forms a loose cuff allowing for lung movement during the respiratory cycle. During quiet respiration the lungs do not fully fill the thoracic cage. As a result the parietal and diaphragmatic pleurae are opposed in the most infero-posterior and infero-lateral areas, the costo-diaphragmatic recesses.
The potential space between these two layers is the pleural cavity. Within the pleural cavity is a small volume of fluid that facilitates smooth movement of the lungs. The pleural blood supply is from underlying tissues e.g. chest wall, lung and mediastinum via intercostal and bronchial arteries. The lymphatic drainage system, including stomata, has already been described in Section 1.5, page 11.

2.3: The Histology of Normal Pleura

Both parietal and visceral pleura are composed of five distinct layers. The surface consists of a single layer of mesothelial cells, the mesothelium. These cells vary in appearance according to their level of activity, ranging from flattened “endothelial-like” cells through to cuboidal. The mesothelial cells sit on a thin layer of submesothelial connective tissue, which includes the basal lamina. The third layer, the superficial elastic layer, is separated from the deep elastic layer by an intervening layer of loose connective tissue. The deep elastic layer is adherent to underlying lung, diaphragm or chest wall. These layers are found in a fixed arrangement, but the relative thickness of each varies at different points in the pleura. It is thickest at the bases, where the lung shows the greatest proportional change in expansion during the respiratory cycle. In the parietal pleura, the fifth layer fuses with the periosteum of ribs where it overlies them. Between ribs, the deep elastic layer is indistinct, and the fourth layer is expanded and loose.

The submesothelium consists primarily of fibroblasts within a stroma rich in collagen, laminin and acid mucoproteins. Stromal constituents are produced by both mesothelial and submesothelial cell populations. Submesothelial cells resemble fibroblasts in the resting state, acquiring a more epithelioid morphology when stimulated. Immunohistochemical studies have confirmed the differing origins of mesothelial and submesothelial cells, and it is now thought that whilst malignant mesothelioma arises from the mesothelium, submesothelial cells are probably the cells of origin of solitary fibrous tumours (SFT) of the pleura.

Kampmeier foci are another distinct feature of the pleura. These were first described in 1928. They consist of aggregates of lymphocytes, histiocytes, plasma cells and monocytes clustered around a central lymphatic vessel, and are covered in mesothelium in continuity with the rest of the pleural surface. They are predominantly found in the infero-posterior pleura, and appear grossly as milky white spots. These form part of the pleural translocation pathways discussed in Section 1.6, page 12.
2.4: Mesothelial Cell Ultrastructure

Mesothelial cell morphology reflects the level of cellular activity. In the normal resting state they are flattened, with bulging nuclei. The cell diameter is normally in the range of 16 - 40μm.\(^{119}\) Cell margins are well defined, except at the apical surface, where the presence of microvilli produces a “frilly,” indistinct appearance. This is particularly conspicuous when examining mesothelial cells in effusion fluid. Cytoplasm is abundant and may contain glycogen granules and acid mucin. There is a well-developed system of pinocytotic vacuoles and vesicles. Cytoplasmic organelles are unremarkable. The nucleus is small and vesicular. Nucleoli are infrequent.\(^{119,121}\) Some cellular features are particularly characteristic of mesothelial cells, and may therefore aid their differentiation from other tumours.\(^{122}\)

Microvilli

Microvilli are a striking feature of mesothelial cells. They are most prominent on the visceral pleura, particularly caudally. They reflect the mesothelial cells role in the secretion and absorption of fluid from the pleural cavity. Not only do they increase the apical surface area available for these functions, but they also entrap glycoproteins on the cell surface. The resulting glycocalyx increases lubrication between the pleural surfaces. Microvilli are usually 1-3μm long, and are often complex, with secondary and tertiary branching.\(^{119}\) Occasionally they are much longer, resembling cilia. The length to diameter ratio (LDR) of mesothelial microvilli is high, often in the order of 12–15. This feature tends to be retained despite malignant transformation. Adenocarcinoma cells may possess microvilli, but their LDR is usually under 10.\(^{123}\) As well as being shorter, adenocarcinoma microvilli are simple and club-like, with occasional glycocalyceal bodies and visible rootlets on electron microscopy.\(^{124}\) Differences in microvillus appearance can potentially aid the distinction between epithelioid mesothelioma and adenocarcinoma. However, there is a significant degree of overlap in LDR between the two cell types, which can limit its usefulness as a diagnostic test. Examples of the electron microscopy features of mesothelioma are shown at the end of this section (Figures 2.1-2.4, pages 38-41).
Dewar and colleagues have reported another feature that may help distinguish between epithelioid mesothelioma and adenocarcinoma. In their electron microscopy study of 12 mesotheliomas and 20 pulmonary adenocarcinomas (PACA) they noted that in most mesotheliomas microvilli were also located basally, and communicated with submesothelial collagen fibrils without interposition of the basement membrane. None of the PACA showed this feature. This finding has also been described by Stoebner and Brambilla.

**Cellular Junctions**

Apical tight junctions exist between mesothelial cells. They play an important role in maintaining the integrity of the mesothelial monolayer. Tight junctions also control the passage of larger molecules to the submesothelial layers, effectively prohibiting the movement of anything greater than 4 nm in diameter. Larger molecules (< 50 nm) can be transported across the cytoplasm by pinocytosis.

**Desmosomes**

Desmosomes are structures that anchor adjacent cell membranes. Microscopically they can be identified as areas of densely thickened membrane associated with filaments radiating from them into the cytoplasm. The intercellular gap is widened or unchanged, in distinction from other types of cell junction, and may be filled with dense material. Desmosomes are predominantly features of epithelial cells, but are also seen in some endothelia and the mesothelium, where they are usually well defined with prominent intermediate lines. In particular, giant (> 1 μm) desmosomes are typical of mesothelial cells.

At the molecular level intercellular adhesion and tissue integrity is accomplished via cell adhesion molecules (CAM). Five classes of CAM exist: cadherins, integrins, selectins, the immunoglobulin-like superfamily, and CD44. The type of cadherin expressed in normal cells reflects its tissue of origin. For example, mesothelium is characterised by the presence of N-cadherin, which is also expressed by cardiac and skeletal muscle, the lens and tissue of neural origin. Epithelial tissues express E-cadherin. Malignant transformation, which is often accompanied by an increase in cell motility and reduced tissue integrity is associated with reduced levels of cadherin expression. However the class of cadherin expressed is usually retained, and has been
explored as a potential diagnostic test for the distinction of MPM from adenocarcinoma. Integrin expression is more complex. These are heterodimeric molecules, and a wide range of different combinations of dimers is possible. Changes (mainly losses) in integrin expression accompany malignant change and may contribute to phenotypic and motility changes at the cellular level.

**Intermediate Filaments**

Intracytoplasmic protein filaments are found in many cells, particularly in those with motile ability. Actin (diameter 4 – 7 nm) and myosin (11-16 nm) are characteristic of mesenchymal-derived cells. Mesothelial cells are characterised by the additional presence of two types of intermediate size (6 - 12nm diameter) filaments, vimentin and cytokeratin (CK). Vimentin filaments are found in all tissues of mesenchymal origin. Mesothelial cells also have numerous low molecular weight cytokeratin-positive filaments. These are arranged in wavy bundles in the perinuclear region, and can be readily identified on electron microscopy and by immunohistochemistry. This dual expression of epithelial and mesenchymal elements reflects the mesothelium’s mesodermal origin. The type of cytokeratin expressed is partly tissue-specific and therefore also has diagnostic potential. Electron micrographs illustrating several of the characteristic features of mesothelial cells are shown in the next four pages (Figures 2.1 – 2.4).
Figure 2.1
Electron Micrograph of a Mesothelial Cell

This illustrates several characteristic features of mesothelial cells, namely hypochromatic nucleus and long complex microvilli.

(Magnification 6.3K x 2.5)
Figure 2.2
Electron Micrograph of a Mesothelial Cell (Higher Power)

This micrograph, taken at a higher power than Figure 2.1 emphasizes the long complex microvilli that are characteristic of mesothelial cells. Perinuclear cytoplasmic filaments are also evident. (Magnification 10K x 2.5)
Figure 2.3
Electron Micrograph of a Mesothelial Cell Illustrating the Presence of Desmosomes

This micrograph, again taken at a higher power than Figure 2.2 illustrates several desmosomes close to the apical surface of the cell. The characteristic blurring of the cell membrane seen on electron microscopy is caused by the presence of interdigitating cytoplasmic filaments.
(Magnification 25K x 2.5)
Figure 2.4
Electron Micrograph of a Mesothelial Cell Illustrating the Presence of Intermediate Filaments

This micrograph demonstrates swathes of intra-cytoplasmic perinuclear intermediate filaments. This pattern of distribution is characteristic of normal mesothelium and mesothelioma cells.
(Magnification 25K x 2.5)
PART II: THE HISTOPATHOLOGY OF PLEURAL DISEASE

The pleura can be affected by many different pathological processes, and the induced cellular responses may vary depending on the nature of the initial stimulus. It is important to understand the processes involved in mesothelial inflammation and healing, as these have a direct bearing on the histopathological changes seen in both benign and malignant pleural disease.

2.5: Mesothelial Injury and Healing

Opinions differ as to the exact mechanisms involved in the process of mesothelial healing. In particular, the relative contributions of mesothelial and sub-mesothelial cells to this process have been a subject of debate.

Pleural injury provokes a local inflammatory reaction that may involve both mesothelial and submesothelial cell populations. Mesothelial cells are stimulated to divide, particularly at the margins of the pleural defect. Local collagen and protease production is induced, and granulation tissue temporarily fills the defect. Macrophages initially migrate into the wound. They are subsequently replaced by a second population of cells that replace the surface mesothelium. Under normal circumstances the mesothelium heals in 8-10 days, irrespective of the size of the mesothelial defect. It is the origin of this second cell population that is disputed. One theory is that submesothelial pluripotent cells migrate to the pleural surface across the basement membrane. Support for this concept has been taken from the observation that activated submesothelial cells acquire an epithelioid appearance and express cytokeratins when stimulated. Hence they come to resemble surface mesothelial cells. An alternative hypothesis is that mesothelial cells at the margins of the defect divide and migrate into the wound, aided by the deposition of desquamated mesothelial cells from pleural fluid. Thirdly, it has been suggested that stem cells from the bone marrow migrate to the pleura and contribute to mesothelial healing.

Initial work by Raftery and colleagues appeared to support the first theory. However, their experimental model also produced submesothelial damage, which would have directly induced local submesothelial activity. Coincident mesothelial proliferation was observed but discounted as irrelevant. Accurate tracking of cell migration is difficult without using scanning electron microscopy (SEM), which was not used for this study. No submesothelial cells were observed crossing the basement membrane.
The elegant studies of Whitaker and Papadimitriou in 1985 gave credence to the second theory: that surface healing was effected by mesothelial cell proliferation and lateral migration.\textsuperscript{135} Thermal damage confined to the mesothelium was induced in the serosa of the tunica vaginalis in rats. The healing wound was then observed using a variety of techniques that included electron microscopy. Some of the experimental animals were subjected to local or whole-body irradiation, in order to assess the contribution of local and distant circulating stem cells. They demonstrated rapid induction of mesothelial activation and proliferation, but saw no evidence of cell migration from the submesothelium. Macrophages initially filled the surface wound, but were later replaced by immature mesothelial cells. These cells adopted a cuboidal morphology as they matured at the mesothelial surface. Scrotal irradiation alone prevented mesothelial healing, whilst scrotal irradiation followed by the injection of lavaged serosal cells enabled local healing to occur. Normal healing also occurred after whole-body irradiation with scrotal protection. Whitaker and Papadimitriou concluded that mesothelial healing is an entirely local process. It is probable that replacement cells are derived from mature mesothelium in response to loss of contact inhibition at the site of injury. The inability of submesothelial cells to cross the basement membrane precludes a direct role in the restoration of the surface mesothelium. However they are likely to contribute to the local environment through the production of inflammatory effectors and matrix components. Desquamated mesothelial cells in the pleural cavity may be deposited on the fibrin mesh and granulation tissue produced in response to pleural injury. This would be of particular value in covering central defects, which are furthest from the proliferating margins.\textsuperscript{137} These conclusions are important when trying to understand mesothelial changes in response to local disease processes and malignant transformation.

2.6: Cellular Changes in Response to Mesothelial Injury

Mesothelial hyperplasia (MH) and reactive pleuritis (RP) are both terms used to describe changes in the mesothelium in response to a pathological stimulus. Infection, radiation, local neoplastic disease, acute and chronic inflammatory conditions, collagen vascular disease and intra-cavity treatments (chemical pleurodesis) can all induce these changes.\textsuperscript{138} Cellular changes are both quantitative (hyperplasia) and morphological.

Mesothelial cell proliferation and submesothelial stromal activity increases pleural thickness to a variable extent. Reactive changes in the submesothelium induce collagen production, fibroblast proliferation and an overall increase in local stroma. The pattern of
proliferation can also be important; complex papillary proliferation is considered unusual in benign disease.\textsuperscript{139,140} Morphological changes in the mesothelium include a marked increase in cell size and a reduction in cell border definition. Microvillus proliferation emphasises the frilly cell margins and may produce small intercellular gaps. These “windows” can be identified in cytological preparations derived from pleural effusions and serve to distinguish between reactive mesothelial cells and metastatic carcinoma.\textsuperscript{121} Mesothelial cell nuclei become larger, hypochromatic and vesicular, but exhibit little pleomorphism. Nucleoli are prominent. An inflammatory cell infiltrate is often seen in conjunction with MH, and may reflect its underlying aetiology. For example, an eosinophilic and histiocytic infiltration can result from pneumothorax.\textsuperscript{141} An example of the histological appearance of mesothelial hyperplasia is shown in Figure 2.5 overleaf.
Figure 2.5
Mesothelial Hyperplasia in the Pleura of a 20 year-old Man with a Spontaneous Pneumothorax

The mesothelium is a monolayer in most areas (A). In the area labelled (B) the surface layer is thicker with papillary proliferation.
2.7: Atypical Mesothelial Hyperplasia

Atypical mesothelial hyperplasia (AMH) and mesothelioma-in-situ (MIS) are terms that attempt to encompass the diagnostic grey area that can exist between benign and malignant mesothelial proliferation. The histopathological differences between AMH, MIS and mesothelioma can be subtle, but have been succinctly summarised by Henderson and colleagues:

"It is the triad of extent, atypia, and invasiveness of the cytoproliferation in combination that points to the correct diagnosis."

The extent of mesothelial proliferation can be difficult to gauge. In MH the surface mesothelium may be thickened, but this is rarely extensive. In AMH surface proliferation is more pronounced. Granulation tissue, persisting chronic inflammatory changes and adjacent pleural plaque can all produce artefactual pleural thickening, and therefore need to be correctly identified. Architectural complexity is unusual in MH. Papillary proliferation is a worrying feature, particularly if groups of cells are budding away from the pleural surface, and have established fibrous cores. Mesothelial necrosis in the absence of inflammation is uncommon in benign disease, but may be seen in up to 20% of early mesotheliomas. Atypical MH can be induced by local metastatic deposits and bronchogenic carcinoma. This can cause confusion when examining fluid from a pleural effusion, as the atypical mesothelial cells may outnumber or obscure exfoliated carcinoma cells, leading to an erroneous diagnosis of MPM.

The cellular features of AMH are intermediate between those seen in MH and mesothelioma. Nuclear changes are more pronounced than those seen in association with inflammation, and may affect a greater proportion of the mesothelium. Cellular and nuclear pleomorphism is infrequent.

The third factor to consider is that of invasion. The basement membrane is poorly defined in the pleura, making invasion difficult to assess. Extension of abnormal cells to involve chest wall structures such as fat or muscle is very suggestive of malignancy. It is important to differentiate between invasion, pseudo-invasion and mesothelial sequestration. Pseudo-invasion is an artefact resulting from tangential sectioning of biopsy material. Surface mesothelium can also become sequestered deep within the pleura, secondary to inflammation and granulation tissue formation at the pleural surface. In these cases the mesothelial cells and associated capillaries usually lie parallel to the pleural
surface, and may be accompanied by an inflammatory infiltrate. Capillaries arranged perpendicular to the pleural surface is more suggestive of a neoplastic proliferation than sequestration. Sequestered cells normally elicit little in the way of a stromal reaction themselves. A final consideration when assessing invasion is the knowledge that desquamated surface mesothelial cells can pass into pleural lymphatics via stomata, which increase in size in response to pleural injury. This can resemble lymphatic invasion.¹⁴³

2.8: Mesothelioma—in—situ

The definitive diagnosis of malignancy relies on a combination of characteristic morphological changes and the demonstration of invasion. In some epithelial tumours, e.g. cervical carcinoma, there is a stage of development during which all the cellular hallmarks of malignancy are present, but exclusively confined to cells that have not crossed the basement membrane. This is defined as carcinoma-in-situ, and will usually progress to invasive disease with time.¹⁴⁴ Whether mesothelioma-in-situ (syn: minimal bulk mesothelioma) exists as a distinct entity is debatable. Henderson defines MIS as

"... the replacement of benign surface mesothelium by mesothelial cells that have cytoarchitectural features of malignancy"

...without defining any specific level of invasion.¹⁴⁰ Using the basement membrane as a point of reference is impractical for the reasons outlined in Section 2.6. Most pathologists are reluctant to diagnose MIS except in two circumstances.¹⁴⁰

1) In the presence of invasive mesothelioma elsewhere in a surgical specimen
2) In association with definite mesothelioma in the contralateral pleura

Are there any other diagnostic techniques that can accurately differentiate between benign and malignant mesothelial disease? No specific ultrastructural features have been identified, although the complexity of microvillus architecture may be important, as previously discussed. Immunohistochemistry is of limited value in this area, but the use of mesothelial cell markers may help to emphasize the pattern and extent of sequestrated or invasive surface cells within the submesothelium. This is discussed in greater detail in Chapter Three.
2.9: The Clinical Significance of AMH and MIS

The diagnosis of AMH should alert clinicians to the possibility of early mesothelioma, or a high risk of the subsequent development of this disease, particularly in patients with previous asbestos exposure. Whether progression from MH or AMH to mesothelioma is inevitable is uncertain, and there are no good biological models to explore this subject further. There have been case reports of MPM developing in patients previously diagnosed as having AMH. Although a variety of conditions can cause mesothelial hyperplasia, few reported cases of MPM have been pre-dated by pneumothorax or other causes of pleuritis. Pneumothorax may be a presenting feature of, or may be seen in association with established mesothelioma, but there is no evidence of causality. However it is difficult to discount the effects of persisting pleural inflammation. In a cohort of 553 cases of MPM identified from our hospital’s records, only a small number of patients (n=11) had a history of pneumothorax. All but five of these occurred at the time of MPM presentation, or followed pleural aspiration. The latter were therefore likely to be iatrogenic. Given the long lag period required to develop mesothelioma from other causes, a similar length of time might be required for chronic inflammation to induce malignant change. Episodes of previous pleural disease in the long-distant past may not have been recorded in the medical notes, or recalled by the patient, which would bias this observation. Given the current increase in incidence and interest in mesothelioma, further information on this subject may become available in due course. At present, however, it seems likely that many of the changes seen in MH and AMH are reversible, and that MPM only develops in those with sustained pleural irritation who are characterised by other biological factors, such as excessive cytokine production or pre-existing chromosomal changes.

A clinical dilemma is raised by the presumption that MPM is a likely consequence of persistent AMH. Very few cases of MPM are diagnosed at an early stage, and the results of all current therapies are poor. Radical surgery, which theoretically could be curative, involves extra-pleural pneumonectomy (EPP), a procedure that is associated with significant morbidity and mortality even in experienced hands. Its value in preventing the development of MPM has therefore to be weighed up against the risks of surgery. Once a patient is diagnosed with AMH, the only effective way to ascertain disease progression would be to instigate long-term radiological follow-up, with or without further pleural biopsies. By the time MPM is diagnosable by histological criteria, the chances of successful treatment are much reduced. In the meantime the patient has been exposed to both diagnostic radiation and multiple surgical procedures that can potentially cause
pleural abnormalities or other complications in their own right. Patients would also have the psychological burden of worrying whether cancer is developing over a long period of time. Restricting follow-up to those deemed as higher-risk, e.g. with asbestos exposure, might be more effective. However, the absolute risk of MPM is still small compared with other cancers, and not everyone with a clinically significant asbestos exposure is aware of it. Indeed, it is still uncertain as to what constitutes a clinically significant exposure, as the development of MPM is not dose-dependent, and a safe threshold level has not been identified. The correct management of patients diagnosed with AMH is therefore still debatable. As the diagnosis of MIS is only made in those with invasive MPM elsewhere, this poses less of a management problem.

PART III: THE DIFFERENTIAL DIAGNOSIS OF PLEURAL MALIGNANCY

Many tumours have a propensity to involve the pleura, either by direct invasion from adjacent thoracic structures, or by metastatic spread. Mesothelioma can exhibit a variety of histological appearances that resemble other malignancies. The correct identification of a pleural tumour’s origin is vital, as this may affect future management, overall prognosis and eligibility for industrial compensation. Confirmation of the diagnosis of mesothelioma requires careful consideration of clinical, radiological and histopathological factors.

2.10: Clinical Factors and Diagnostic Methods

The majority of patients with MPM present with breathlessness and/or pleural effusion. Other features can include chest wall pain and constitutional symptoms, such as weight loss. Unfortunately these symptoms and signs are not diagnostic of mesothelioma. Pleural effusion can result from a wide range of benign and malignant disease processes. Chest wall discomfort can be difficult to distinguish from musculo-skeletal pain and cardiac ischaemia. Weight loss is a non-specific symptom that although often seen in association with malignancy, gives little clue to the primary tumour type or site. Mesothelioma remains an uncommon tumour compared with cancers of the lung, breast and gastrointestinal tract. A high index of suspicion is therefore needed to distinguish correctly between MPM and other tumours. The prognostic significance of presenting symptoms in MPM is discussed further in Chapter Four.

Lung cancer accounts for approximately one third of all cases of malignant pleural effusion. Pulmonary adenocarcinoma in particular tends to arise peripherally, facilitating spread to adjacent pleura. As the lung becomes encased and constricted by tumour the
pulmonary parenchyma collapses. Under these circumstances it can be difficult to identify a small peripheral lung primary: such tumours grossly resemble mesothelioma, the “so-called” pseudomesotheliomatous carcinomas. Adenocarcinomas preferentially metastasize to lymph nodes, and this disruption of normal lymphatic pathways is an important factor in the development of pleural effusion. Other tumours that have a propensity to spread to the pleura are breast, stomach and ovarian carcinoma, and malignant melanoma. Non-Hodgkin’s lymphoma (NHL) accounted for 10% of malignant effusions in the combined series reported by Sahn. 

Pleural effusions may obscure the extent and nature of pleural disease on chest X-ray (CXR). Non-malignant processes such as chronic empyema can mimic mesothelioma radiologically and clinically. Computed tomography (CT) can demonstrate parenchymal tumours, the extent and location of pleural thickening, and the presence of pleural plaques. Although thickening of the mediastinal pleura is highly suggestive of mesothelioma, CT alone cannot distinguish accurately between mesothelioma and pseudomesotheliomatous carcinoma. Magnetic resonance imaging (MRI) is better at distinguishing between benign and malignant pleural disease. Typical examples of CXR and CT appearances of MPM are shown overleaf in Figures 2.6 and 2.7.
Chest X-Ray of a 68 Year Old Woman with Desmoplastic Mesothelioma

This CXR demonstrates the presence of a hydropneumothorax associated with desmoplastic mesothelioma of the left pleural cavity.
Figure 2.7
Computed Tomogram of the Thorax of a 68 Year Old Woman with Desmoplastic Mesothelioma

This is the CT Thorax of the same patients whose CXR is shown in Figure 2.6. This confirms the presence of a hydropneumothorax of the left pleural cavity with compression of the underlying lung.
Despite advances in diagnostic imaging, the definitive diagnosis of MPM is heavily reliant on histological examination of a pleural biopsy specimen. Percutaneous biopsy, using a side-cutting (Abrams) needle\textsuperscript{159} has the advantage of being easily performed on the ward under local anaesthesia. However the amount of tissue obtained from needle biopsies is small, and specimens are often inadequate to confirm the diagnosis of malignancy, particularly if IHC is needed. Furthermore, it is difficult to target abnormal areas of pleura accurately, even with radiological guidance. Hence sampling errors are exaggerated, which in turn has implications for the correct diagnosis and classification of mesothelioma.\textsuperscript{160, 161}

It is now recognised that it is advantageous to have larger biopsy specimens, preferably from more than one part of affected pleura. Surgical biopsies obtained at thoracotomy or thoracoscopy facilitate the correct identification of abnormal areas of pleura which in turn increases diagnostic yield. A further advantage of using a surgical approach includes the ability to perform therapeutic and palliative procedures such as pleurectomy or chemical pleurodesis at the same time. Video-assisted thoracoscopic surgery (VATS) enables the surgeon to inspect the pleural cavity through smaller incisions. However, many patients with mesothelioma or advanced lung cancer have obliterated their pleural cavity, in which case an open biopsy performed through a mini-thoracotomy is probably the better option. The risks of surgery, irrespective of method and approach, can be significant, particularly in older patients with poor lung function or co-existent cardiac disease.

The appearance of the pleura at thoracoscopy or thoracotomy depends upon the stage of the disease.\textsuperscript{162} Early malignant change may have little effect on the gross appearance of the pleura, other than producing areas of opacification or thickening, particularly in the parietal pleura. However, mild inflammatory changes are commonly seen with effusions of any cause. Parietal plaques may be present, and cause diagnostic confusion. With advanced disease the pleura is more obviously abnormal. Initially multiple pleural nodules can be seen, which may mimic deposits of metastatic carcinoma. With time these areas coalesce, producing a thick rind that encases the lung. It may then grossly resemble chronic fibrous pleurisy. The pleural space may be completely obliterated or persist as loculated areas of effusion. Bronchopneumonia secondary to pulmonary encasement is common, and symptoms and signs of mediastinal invasion e.g. superior vena cava obstruction (SVCO) may be present.
Mesothelioma is highly invasive and characteristically crosses tissue planes and tracks along incision sites to involve the chest wall. Trans-diaphragmatic spread produces hepatic and peritoneal deposits. Distant blood-borne metastases are less common, but well recognised. An example of the post mortem appearances of the lung and pleura of a patient who died of MPM are shown in Figure 2.8 overleaf.

2.11: The Histological Classification of Malignant Pleural Mesothelioma
In Chapter One I outlined the history of mesothelioma as a recognised pathological entity. Disagreements over nomenclature were resolved with the publication of the first World Health Organisation (WHO) classification of lung tumours in 1967. Three histological subtypes were described: epithelial, mixed and fibrous (spindle cell). In the most recent WHO classification (1999) the terms epithelioid, biphasic and sarcomatoid were recommended. It has since been recognised that a variety of other histological patterns can be seen within these broad groups. Lung fibrosis and pleural plaque, seen in association with mesothelioma in larger surgical biopsies and post mortem tissue specimens, may confirm asbestos as a likely aetiological factor (Figure 2.9, page 56). Asbestos bodies are rarely seen within the tumour itself, unless it is infiltrating into lung tissue showing the features of asbestosis.
Figure 2.8
Post-Mortem Appearances of the Left Lung in a Patient Who Died of Malignant Pleural Mesothelioma

The left lung is completely encased in a thick rind of tumour (A) that infiltrates along the fissure (B) and into the hilum. The lung is consolidated (C). The hilar lymph nodes (D) are enlarged and replaced with tumour, and the basal pleural cavity contains haematoma (E).
Figure 2.9
Asbestos Body and Asbestosis

Photograph of a section of lung demonstrating the changes of asbestosis and an asbestos body (A) in a patient with coincident mesothelioma.
Mesotheliomas tend to retain many of the cellular and nuclear characteristics of normal mesothelial cells. The nuclei remain centrally located, are hypochromatic and vesicular in appearance, with prominent nucleoli. Although nuclear pleomorphism and multinucleated giant cells can be seen, they are not usually a prominent feature and mitoses are infrequent. Microvilli are still identifiable at high power, and intermediate filaments are demonstrable. Hyaluronic acid (HA) is often produced in large quantities, giving the epithelioid variant of mesothelioma a shiny gelatinous appearance. Hyaluronic acid may be demonstrated in increased quantities in mesothelioma-associated pleural effusions.167

Many cancers produce mitogenic factors and proteases (matrix metalloproteinases) that facilitate invasion and metastasis.168 Some are also produced by non-neoplastic stromal cells under the direct influence of cancer cells.169,170 Mesothelioma is no exception, and the submesothelial layer can exhibit marked proliferative changes in response to both inflammatory stimuli and malignant infiltration. These induced changes may be difficult to distinguish from areas of malignant infiltration and cause confusion in the classification of mesothelioma subtype.

2.12: The Differential Diagnosis of Malignant Pleural Mesothelioma

Many different tumours can theoretically involve the pleura, thereby entering into the differential diagnosis of MPM. With the exception of metastatic carcinoma and lymphoma, many are less common than mesothelioma itself - and may therefore be unfamiliar to a pathologist who does not have a specialist interest in this field.

The experiences of the Joint US / Canadian Mesothelioma registry serves as a good illustration of the most common problems that a pathologist has to face when correctly classifying pleural tumours.171 In a review of the first two hundred referred cases, of which three-quarters were pleural, areas causing difficulty were as follows. The most common problem was that of differentiating between epithelioid mesothelioma (EM) and carcinoma involving the pleura. The next largest group included cases in which confirmation was requested from the panel. This accounted for fewer than 20% of cases. The differentiation between MH and mesothelioma, and between reactive pleuritis and desmoplastic mesothelioma (DM) both accounted for approximately 13% of cases. The problem of sarcoma vs sarcomatoid mesothelioma (SM) was seen in 9% of cases, and the remaining 13% of referrals comprised a mixture of other diagnostic problems, such as unusual variants of mesothelioma. This pattern of referrals is similar to that reported by other diagnostic panels.161 The panel graded each referred case on a four-point scale, after
examination of haematoxylin and eosin (H&E) stained sections. The results of a panel of immunohistochemical markers were available for cases in the EM vs carcinoma category. The extent of agreement within the panel for any given case was variable. A consensus opinion (>75%) was achieved in 70.5% of all cases overall. An even split was seen in less than five percent. The categories that afforded the least disagreement were those involving simple confirmation of mesothelioma, and the distinction between EM and carcinoma. Consensus was achieved in 70 - 83% of these cases. Least agreement was seen in the SM vs sarcoma (46%) and MH vs mesothelioma (59%) categories.

As reflected in the experience of the Joint US / Canadian Mesothelioma Panel, the most common diagnostic problem for a pathologist faced with a pleural tumour is the distinction between epithelioid mesothelioma and pulmonary adenocarcinoma metastatic to the pleura. Several other differentials may need to be considered, and the most likely alternatives may be suggested by clinical factors as well as cell morphology and the results of immunohistochemistry. The main differentials of each of the main subtypes of mesothelioma are listed in Figure 2.10 overleaf.
Figure 2.10
The Differential Diagnosis of Pleural Tumours
(Reproduced from Histopathology 2001, 38:471-476 with permission)
2.13: The Differential Diagnosis of Epithelioid Mesothelioma

Epithelioid mesothelioma is defined as:

"A pattern consisting of tubules, acini, papillae or sheets of atypical epithelioid mesothelial cells." (WHO 1999)\textsuperscript{166}

The cells in EM resemble those of reactive mesothelium; predominantly cuboidal cells with open vesicular nuclei and prominent nucleoli. The cytoplasm is eosinophilic. Epithelioid mesothelioma can differentiate into tubo-papillary, solid, microcystic, clear cell, small cell or deciduoid cell types, as well as showing several different glandular patterns of growth.\textsuperscript{172} A combination of different patterns of glandular differentiation in adjacent areas of the same tumour is therefore suggestive, but not diagnostic, of EM. The most common pattern is that of tubo-papillary differentiation, which can be either simple or complex. Elongated clefts and microcystic spaces resembling adenomatoid tumour or lymphangioma are occasionally seen.\textsuperscript{173} Epithelioid mesothelioma may also exhibit a predominantly acinar pattern of growth, or consist of sheets of cells with no glandular differentiation. If these cells are well-differentiated with nuclear regularity then the term polygonal EM is sometimes applied. Less well-differentiated mesotheliomas exhibit greater nuclear pleomorphism and nucleolar prominence. Mitoses and giant cell formation are more common, but the tumour cells retain an overall deceptively bland appearance. Occasionally, the cells are completely undifferentiated or anaplastic.

The stroma in EM is variable in both amount and appearance. A small amount of fibroblastic stroma is common, and may contain an inflammatory cell infiltrate. The stroma can undergo reactive changes in response to infiltrating tumour cells. In these cases it can be difficult to distinguish between a reactive stroma and areas of sarcomatoid differentiation in a biphasic mesothelioma.

Examples of some of the different subtypes of EM are shown overleaf along with an example of pulmonary adenocarcinoma metastatic to the pleura for comparison (Figures 2.11-2.14).
Figure 2.11
Epithelioid Mesothelioma – Tubo-papillary Subtype

This demonstrates a complex tubo-papillary pattern of differentiation that is frequently seen in epithelioid mesothelioma
Figure 2.12
Epithelioid Mesothelioma – Example of a Tumour with Areas of Clear Cell Differentiation (A)
Figure 2.13
Epithelioid Mesothelioma – Example of a Pleomorphic Area
Figure 2.14
Pulmonary Adenocarcinoma
This tumour has cuboidal cells arranged in a glandular pattern
The differential diagnosis of EM potentially encompasses a wide range of other tumours. Carcinoma metastatic to the pleura is the most common, either from distant sites or direct invasion from adjacent lung or mediastinum. Less common differentials that need to be considered include melanoma, lymphoma, thymoma, monophasic epithelioid synovial sarcoma, epithelioid haemangioendothelioma and haemangiopericytoma.

Carcinoma of the Lung
Well-differentiated lung cancer often retains enough cellular characteristics to enable its correct identification. For example, squamous cell carcinoma of the lung (SqCCL) may retain features of keratinisation and intercellular bridges. Well-differentiated adenocarcinomas retain a glandular pattern of differentiation by definition. The cells are columnar rather than cuboidal, and cytoplasmic mucin can be demonstrated in many cases. Nuclei are relatively hyperchromatic, although some may morphologically be very similar to mesothelioma, as shown by comparing nuclear features in Figures 2.11 and 2.14. Mitoses are more common than in EM. Metastatic adenocarcinoma deposits may retain features of the primary tumour e.g. goblet cells in colonic carcinoma. Renal cell carcinomas (RCC) are histologically and immunohistochemically similar to mesotheliomas that exhibit clear cell differentiation (Figure 2.12). Although microvillus morphology is more typical of carcinoma, in the absence of other more typical areas of EM, a renal primary needs to be excluded whenever clear cell mesothelioma is diagnosed.

Small cell carcinoma of the lung (SCCL) rarely adopts a pseudomesotheliomatous pattern of growth. The diagnosis may be suggested by clinical criteria e.g. a small centrally-placed bronchial primary with disproportionately prominent mediastinal lymphadenopathy and widespread distant metastases. A small cell variant of mesothelioma has been described, and may cause confusion if only a small biopsy specimen is available. In these cases the mesothelioma cells are uniform and have a high nuclear to cytoplasmic ratio. The nuclei retain mesothelial features, rather than exhibiting the “salt and pepper” appearance more typical of SCCL. More typical areas of mesothelioma are usually seen elsewhere in the specimen. Other features characteristically seen in SCCL e.g. nuclear moulding, rosette formation and DNA staining of vessels, are absent. Neuroendocrine marker expression is another point of distinction: Chromogranin A and synaptophysin are usually positive in SCCL but negative in mesothelioma.
Malignant Melanoma

Metastatic melanoma can involve the pleura, and may resemble mesothelioma. Conversely mesothelioma can be associated with haemosiderin-like deposits that grossly resemble melanin. Specific immunohistochemistry is usually required to confirm the diagnosis if metastatic melanoma is a possible differential.

Thymoma

Thymomas are uncommon tumours, which can invade and encase the lung in a pseudomesotheliomatous manner. They can also arise as primary pleural tumours from ectopic thymic tissue. Thymomas classically comprise a mixed population of lymphocytes and neoplastic epithelioid cells arranged in lobules that are separated by fibrosis. The epithelioid component may form nests or pseudo-rosettes, but tubo-papillary differentiation is not seen. If the lymphocyte population is prominent then it can be difficult to distinguish thymoma from Non-Hodgkin’s lymphoma. Clinical factors such as the presence of a significant anterior or middle mediastinal mass or symptoms of myasthenia gravis may suggest the true diagnosis. The demonstration of a T-cell lymphocyte population helps to confirm this diagnosis.

Lymphoma

Lymphoma, particularly Hodgkin’s disease, is a recognised cause of malignant pleural effusion. Although a pseudomesotheliomatous pattern of growth is uncommon, pleural involvement can mimic early stages of MPM. Hodgkin’s disease can involve the mediastinal pleura, a radiological sign normally suggestive of MPM. Lymphoma can also arise in the setting of chronic empyema. Another lymphoid malignancy that can cause diagnostic confusion is anaplastic large cell lymphoma (ALCL). Also known as Ki-1 (CD30) positive lymphoma, this uncommon disease constitutes less than 10% of all lymphomas. It actually encompasses a heterogeneous subset of non-Hodgkin’s lymphoma, unified by their CD30 expression. It exists in two forms, cutaneous and systemic. The systemic disease is aggressive and often involves extra-nodal sites, including the lung and mediastinum. It uncommonly presents as a localised pleural mass and malignant effusion. Anaplastic LCL is most common in the first four decades of life. There is an association between ALCL and acquired immune deficiency syndrome (AIDS), but most cases are in human immunodeficiency virus (HIV) negative patients. Lymph nodes are replaced by sheets of large, occasionally giant cells, with abundant cytoplasm
and prominent nucleoli. Predominant epithelioid differentiation has been described. Increased cell size and pleomorphism may obscure the lymphoid nature of the malignant cells; they may also be mistaken for histiocytes or the Reed-Sternberg cells of Hodgkin’s disease. Differentiating ALCL from MPM may be a problem when only a small biopsy is available. Clinical factors, such as patient age and evidence of node involvement at multiple sites are strongly suggestive of lymphoid malignancy. This tumour can be positive for epithelial membrane antigen (EMA), vimentin and CD15.

**Tumours of Vascular Origin**

I have already mentioned the fact that mesothelial cells can morphologically resemble endothelial cells. Vascular tumours therefore enter into the differential of MPM. Those most likely to cause diagnostic difficulty are haemangioendothelioma, angiosarcoma and haemangiopericytoma (HPC). A handful of cases have been described in the pleura, where they can resemble any of the subtypes of MPM, depending on their pattern of differentiation. The distinction between haemangioendothelioma and angiosarcoma depends on both histological and clinical criteria as these tumours probably represent divergent points in a spectrum of vascular tumours: haemangioendotheliomas are considered relatively low-grade, whilst the term angiosarcoma tends to be reserved for high grade vascular tumours.

Epithelioid haemangioendothelioma (EHE) was first described by Weiss and Enzinger in 1982, and is the more common variant. It characteristically arises from deep tissues in the limbs, particularly in young women. Other primary sites include liver, lungs and bone. It is uncertain whether the few reported cases of pleural EHE represent primary tumours developing from pleural blood vessels, or are the result of sub-pleural spread from a primary pulmonary tumour (previously called intravascular bronchioloalveolar tumour, IVBAT). An example of EHE is shown overleaf in Figure 2.15.

Epithelioid haemangioendothelioma usually adopts a solid pattern of growth with occasional pseudoglandular foci within a highly myxoid stroma. The cells are polygonal and bland: like EM there may be little nuclear pleomorphism and few mitoses. Intracytoplasmic vacuoles are a prominent feature, and may produce a clear cell appearance. In contrast to those in EM, the vacuoles in EHE may contain erythrocytes, which is a useful pointer to the correct diagnosis. Immunohistochemistry is of significant value in correctly identifying EHE, as is discussed in the next chapter.
Figure 2.15
Epithelioid Haemangioendothelioma Metastatic to the Pleura
Haemangiopericytoma is a tumour derived from pericytes, the contractile vascular supporting cells that control vascular calibre. It was originally described by Stout and Murray in 1942.\textsuperscript{184} Haemangiopericytomas classically arise from the deep soft tissues of the retroperitoneum, thighs, head and neck, and trunk. The latter could theoretically involve the chest wall and pleura, simulating mesothelioma. Haemangiopericytoma is characterised by the presence of a monomorphic population of closely packed polygonal, round, oval or blunted fusiform spindle cells associated with prominent vascular channels.\textsuperscript{185} The individual cells are epithelioid in appearance, but focal areas of sarcomatoid differentiation can be seen. An HPC-like pattern of differentiation has been described in other mesenchymal tumours, solitary fibrous tumours and MPM, which can cause diagnostic difficulty.\textsuperscript{186} However HPC is usually cytokeratin negative: Careful examination of the distribution of CK positivity within a pleural tumour should correctly distinguish between HPC and HPC-like differentiation within a mesothelioma.

**Synovial Sarcoma**

Synovial sarcoma (SS) is another important differential of MPM. This tumour is recognised as being capable of both epithelioid and sarcomatoid differentiation and can therefore mimic all three subtypes of mesothelioma. Indeed the term “mesothelioma of the joint” has previously been suggested for this tumour.\textsuperscript{187} Synovial sarcomas classically arise in proximity to large synovial joints, and were originally thought to arise from the synovium itself, hence their name. It is now believed that they are composite epithelial/fibroblastic malignancies, derived from arthrogenic mesenchyme, which in many ways resemble carcinosarcomas.\textsuperscript{174} Synovial sarcomas are most commonly seen in young and middle-aged adults. Although metastatic SS involving the pleura can resemble mesothelioma histologically, a history of SS in a young patient with pleural or pulmonary malignancy should be sufficient to alert a pathologist to its presence. Primary intrathoracic SS is exceedingly rare, with only a handful of cases described.\textsuperscript{188-190}

Biphasic SS and monophasic (sarcomatoid) SS are the most common patterns, but the epithelioid component may predominate, particularly in small biopsies. Several histological factors help distinguish epithelioid SS from EM.\textsuperscript{174} The epithelioid cells are columnar or cuboidal and arranged in a solid or pseudoglandular pattern. Squamous metaplasia and keratin pearls may be seen focally. Cells have large vesicular nuclei with abundant cytoplasm. Less well differentiated tumours may resemble HPC. The epithelioid component of SS is a true epithelial derivative and resembles adenocarcinoma on
ultramicroscopic examination, having short microvilli with rootlets. Biphasic SS and monophasic sarcomatoid SS are discussed later in this chapter. An example of sarcomatoid SS is shown in later in this chapter, Figure 2.19, page 80.

2.14: The Differential Diagnosis of Biphasic Mesothelioma

Biphasic mesothelioma (BM) is defined as:

"A combined epithelioid and sarcomatoid pattern with each comprising at least 10 per cent of the tumour." (WHO 1999)\(^{166}\)

Although EM is the most common pattern of differentiation, biphasic mesothelioma is considered the “classical” pattern. Other tumours that exhibit a biphasic pattern of differentiation are relatively uncommon in the pleura. The differential diagnosis includes pleomorphic carcinoma, carcinosarcoma of the lung, biphasic synovial sarcoma, sarcoma with areas of epithelioid differentiation, and pulmonary blastoma.

The diagnosis of BM requires unequivocal malignancy in both components. Differentiating between reactive stroma and sarcomatoid areas of mesothelioma can be very difficult. This can produce errors in classification between EM and BM, and probably explains discrepancies in the proportion of tumours classified in either group in some studies. Another factor that can affect the correct identification of BM relates to the way that the pleural tissue is sectioned. The characteristic nuclear and cytoplasmic appearances of mesothelioma are retained irrespective of epithelioid or sarcomatoid differentiation. Sectioning a sarcomatoid cell in a plane perpendicular to its long axis produces an appearance very similar to a cuboidal epithelioid cell.\(^{191}\) Most studies have found that BM comprises between 30-40% of pleural mesothelioma. It is well-recognised and easily understandable that the proportion of mesotheliomas designated as biphasic increases with the size of the biopsy specimen.\(^{160}\) The potential for errors of classification to bias the results of survival analyses in mesothelioma is discussed in more detail in Chapter Four.

Two other factors can potentially cause diagnostic confusion. Adenocarcinoma and other metastatic tumours can produce a fibroblastic or desmoplastic stroma, producing a biphasic appearance. The stromal cells are predominantly fibroblasts, and are cytokeratin negative. Benign bronchial or alveolar cells may also become entrapped within areas of sarcomatoid mesothelioma as it invades lung, simulating foci of epithelioid differentiation. These alveolar cells are morphologically benign and will react with the appropriate
epithelial IHC markers. The epithelioid and sarcomatoid areas in BM are commonly intermixed: adjacent epithelioid and sarcomatoid foci are not separated by fibrous strands, as is the case in biphasic synovial sarcoma.\textsuperscript{187} The presence of different patterns of subdifferentiation e.g. both solid and glandular differentiation within adjacent epithelioid areas, is a distinctive feature of mesothelioma.

Areas of sarcomatoid differentiation in BM resemble fibrosarcoma (FS) or malignant fibrous histiocytoma (MFH). The cells are elongated and spindle-shaped with moderate amounts of cytoplasm, but retain characteristic mesothelial nuclei: hypochromatic, vesicular with prominent nucleoli. Other patterns of differentiation are described (see Section 2.15, page 76). Although not easily identifiable on light microscopy, a proportion of cells appear to be transitional forms on electron microscopy: they retain some features of epithelioid cells, such as microvilli and desmosomes, yet have a gross sarcomatoid appearance.\textsuperscript{174} Desmoplasia is most commonly associated with sarcomatoid areas. Heterologous elements e.g. chondroblastic or osseous differentiation are described in mesothelioma, and may make it difficult to distinguish BM from carcinosarcoma and osteosarcoma. An example of biphasic mesothelioma is shown in Figure 2.16, page 73.

Pleomorphic Carcinoma

Pleomorphic carcinoma (PC) of the lung is an important differential of BM, not the least because they share a very similar immunohistochemical profile. It is an uncommon variant of non-small cell carcinoma of the lung that exhibits prominent sarcomatoid and/or giant (multinucleate) cell areas. Sarcomatoid carcinomas are a well-recognised phenomenon in other organs, such as the urinary tract,\textsuperscript{192} but PC accounts for only a small proportion of lung tumours. Sarcomatoid carcinoma of the lung, spindle cell carcinoma and giant cell carcinoma are all terms that have all been interchangeably used to describe this neoplasm. Fishback and colleagues, who published a series of 78 cases in 1994, predominantly use the term pleomorphic carcinoma, arguing that a combination of both spindle cells and giant cells are commonly seen in the same tumour.\textsuperscript{193} The 1981 WHO classification considered that spindle cell carcinoma was a variety of squamous cell carcinoma, whilst giant cell carcinoma was a form of large cell carcinoma.\textsuperscript{194} The 1999 WHO classification\textsuperscript{166} defines pleomorphic carcinoma as:
"A poorly differentiated...squamous cell carcinoma, adenocarcinoma or large cell carcinoma containing spindle cells and/or giant cells, or a carcinoma consisting only of spindle cells and giant cells."

The term spindle cell carcinoma (SPCC) is reserved for carcinomas that are exclusively sarcomatoid, with no other recognisable pattern of differentiation. The cells are often pleomorphic with frequent mitoses. The presence of sarcomatoid areas in PC is thought to reflect an epithelial to mesenchymal shift in appearance of carcinoma cells, possible under the influence of tumour-derived growth factors and mitogens. This is a recognised phenomenon in other cancers e.g. metaplastic carcinoma of the breast. A less likely explanation is that the local tumour environment induces malignant change in benign fibroblasts. Finally, there is evidence that radiation can induce sarcomatoid change in carcinomas. This might account for cases of PC in patients with carcinoma of the lung treated by radiotherapy. An example of PC is shown in Figure 2.17, page 74.

There are several points of distinction that help differentiate between PC and mesothelioma. The greatest difficulty is likely to be with small biopsy specimens, which may not contain areas of differentiation more typical of PC i.e. squamous. As well as being very pleomorphic, cells often exhibit prominent nuclear atypia and frequent abnormal mitoses, all of which are uncommon in mesothelioma. Vascular invasion is common, and may be associated with areas of necrosis and infarction. Even in the absence of vascular invasion, these tumours provoke prominent reactive changes, inflammation and desmoplasia. Giant cells, which are a recognised but uncommon feature in MPM, are a prominent feature in the majority of cases of PC.

Carcinosarcoma
Carcinosarcoma (CS) is an uncommon primary lung cancer that is characterised by a biphasic pattern of differentiation allied with prominent heterologous elements. It is considered by some to represent one extreme of the spectrum of sarcomatoid lung carcinomas that includes PC and SPCC. Others believe that, in common with synovial sarcoma, it is derived from a pluripotent mesenchymal precursor cell. Epithelioid elements usually exhibit squamous differentiation, and sarcomatoid areas resemble undifferentiated sarcoma. Genetic studies have confirmed the monoclonal nature of this tumour. Differentiating CS from BM is primarily based on the prominence of the heterologous areas, and the fact that the sarcomatoid areas in CS do not usually express cytokeratin.
Figure 2.16
Biphasic Mesothelioma

*This photograph demonstrates the presence of a pleomorphic epithelioid area (A) and a sarcomatoid area (B) within the same tumour section.*
Figure 2.17
Pleomorphic Carcinoma of the Lung
Biphasic Synovial Sarcoma
Synovial sarcoma is an important differential of MPM. The epithelial variant has already been described earlier in this chapter. The sarcomatoid regions of SS consist of interweaving fascicles of densely packed plump spindle cells, which are morphologically similar to those seen in fibrosarcoma. These areas are more cellular than seen in SM, with a crowded appearance. Mitoses are relatively infrequent and may be accompanied by a mast cell infiltrate. The nuclei are large and hyperchromatic with scant cytoplasm. Nuclear palisading is uncommon. Calcification is a feature in approximately 20% of tumours, and may be extensive. Band-like areas of condensed myxoid stroma separating epithelioid and sarcomatoid areas may be present. This feature is not usually seen in SM. All subtypes of SS are associated with a specific genetic abnormality: a balanced translocation between Ch 18 and the X chromosome, t(X;18)(p11.2; q11.2). This results in a genetic fusion between the SYT gene (Ch 18) and one of two genes on Ch X (SSX1 or SSX2). The gene product SYT/SSX can now be identified cytogenetically, and is therefore the gold standard for confirming a diagnosis of synovial sarcoma.

Epithelioid Sarcomas
The phenomenon of focal epithelioid differentiation within connective tissue tumours is well-recognised. In epithelioid sarcomas (particularly in leiomyosarcomas, peripheral nerve sheath tumours and chondrosarcomas) and biphasic SS, epithelioid areas show true epithelial differentiation both in terms of their IHC profile, and at the ultrastructural level. This can produce a biphasic appearance that obscures the overall sarcomatoid nature of the tumour. Epithelioid areas usually comprise only a small proportion of the tumour, a fact that should be apparent on larger specimens. Overall the histological appearances of the sarcomatoid areas may not be distinctive, and IHC may be necessary to distinguish the cell of origin.

Pleuropulmonary Blastoma
Pulmonary blastomas are biphasic tumours which can be subdivided into three separate subtypes: classic biphasic pulmonary blastoma, well-differentiated fetal type adenocarcinoma of the lung and pleuropulmonary blastoma of childhood. Classical pulmonary blastoma is biphasic, the epithelial component resembling fetal adenocarcinoma and the sarcomatoid element comprising a primitive myxoid stroma. Pleuropulmonary blastoma of childhood is a cystic or solid tumour exclusively seen in children below the
age of six. Solid forms may exhibit anaplastic sarcomatoid areas. Mesothelioma is exceedingly rare in children.

2.15: The Differential Diagnosis of Sarcomatoid Mesothelioma

Sarcomatoid mesothelioma is defined as a tumour that exhibits:

"A pure spindled pattern, resembling a fibrosarcoma or a malignant fibrous histiocytoma." (WHO 1999)²⁶⁶

Sarcomatoid mesothelioma is composed of relatively featureless spindle-shaped cells. Although small foci of epithelioid cells may be seen, by definition these should comprise less than ten per cent of the tumour overall. Transitional forms, in which the fibroblastic cells retain some features of epithelioid cells, such as microvilli, may be seen on electron microscopy. Sarcomatoid mesothelioma is frequently seen in association with desmoplasia - dense, collagenous, paucicellular stroma that may resemble chronic fibrous pleurisy and pleural plaque. Rarely this desmoplasia may account for the majority (>50%) of the sarcomatoid element within the specimen, in which case the tumour should be classified as a desmoplastic mesothelioma in its own right. Pure SM represents 15-20% of classified tumours in most series. Few published studies have reported the incidence of DM separately, but it seems to account for less than 5% of all mesotheliomas.²⁰² Sarcomatoid mesothelioma can exhibit areas of sub-differentiation that can make it difficult to distinguish from soft tissue tumours on small biopsies. A recognised subtype of SM is lymphohistiocytoid mesothelioma, which can be difficult to differentiate from lymphoma.²⁰³,²⁰⁴ Conversely, some of the tumours that have already been discussed in the differential diagnosis of EM and BM (melanoma, HE, HPC) can contain prominent areas of sarcomatoid differentiation: sampling errors can obscure the predominant pattern. The most common problems involve differentiating between other sarcomas, solitary (localised) fibrous tumours of the pleura, malignant fibrous histiocytomas, and peripheral nerve sheath tumours (PNST). An example of sarcomatoid mesothelioma is shown in Figure 2.18 overleaf.
Figure 2.18
Sarcomatoid Mesothelioma

This photograph demonstrates spindle cells arranged in a storiform pattern.
Sarcomas

Most sarcomas involving the pleural cavity are tumours that have metastasized to the lung from distant sites, and then spread into the pleura. A distant primary site may therefore be clinically apparent. Less commonly, primary sarcomas can arise from chest wall structures, filling the pleural cavity in such a way as to mimic mesothelioma. Close inspection may reveal some features that hint at the cell of origin e.g. bone formation in osteogenic osteosarcoma. Chondroblastic, osteoblastic, liposarcomatous and rhabdomyosarcomatous differentiation have all been described in MPM, but are rarely prominent.

Monophasic Sarcomatoid Synovial Sarcoma

Although monophasic sarcomatoid SS is as common as the biphasic subtype, it predominantly arises in the distal limbs and has a low metastatic potential. For these reasons it less commonly enters into the differential of SM, despite their histological similarities. The sarcomatoid areas are similar in both monophasic and biphasic variants, and the demonstration of the SYT/SSX fusion gene remains of diagnostic importance. An example of a sarcomatoid synovial sarcoma is shown in Figure 2.19, page 80.

Solitary Fibrous Tumours of the Pleura

Solitary (localised) fibrous tumours probably arise from submesothelial mesenchymal cells. They were initially considered a benign variant of MPM ("benign mesothelioma") and they were often included in early studies of the epidemiology and pathology of MPM. It is now recognised that they represent a separate entity, and are probably related to localised fibrous tumours of the orbit and aero-digestive tract. Their morphology and clinical behaviour is quite different from that of MPM.

Solitary fibrous tumours typically arise from the visceral pleura as a solid pedunculated mass, whose cut surface is fibrous and whorled. The stalk is usually well-defined and vascular. Occasional the tumour is sessile or broad-based, particularly in tumours arising from the parietal pleura. Local recurrence after resection is more common in sessile tumours. They do not adopt a diffuse, pseudomesotheliomatous pattern of spread even if malignant change occurs. Pleural effusion is uncommon in benign forms. Neoplastic transformation can occur, but is uncommon except in those greater than ten centimetres in diameter. Solitary fibrous tumours are associated with finger clubbing and hypoglycaemia, the latter resulting from the secretion of insulin-like substances. Histologically SFTs consist of spindle cells which are morphologically
benign, arranged in the "pattern-less pattern of Stout". Connective tissue is prominent and interweaves imperceptibly with more cellular areas. Individual cells are spindle shaped, with pale cytoplasm and inconspicuous nucleoli. They possess fibroblastic rather than mesothelial features on electron microscopy. In cases of malignant transformation mitoses are more common (> 4 per 10 high power fields), and areas of necrosis or haemorrhage are seen. A differential of SFT is the desmoid tumour, which has rarely been described in the thorax. These can grossly and histologically resemble an SFT, but are less likely to be pedunculated, and differ from SFT in terms of their IHC profile. Interestingly, trisomy 8 is the most common chromosomal abnormality described in both desmoid and solitary fibrous tumours.

Malignant Fibrous Histiocytomas
Malignant fibrous histiocytoma is the most common soft tissue sarcoma seen in later adult life, is more common in men and usually affects the lower limb. Although the storiform growth pattern is classically described, it may also exhibit areas of pleomorphic, myxoid, giant cell and inflammatory differentiation. Individual cells are very similar to those seen in sarcomatoid mesothelioma. It was first described in the pleura in 1983 by Yang et al since which a handful of other cases have been reported.

Malignant Peripheral Nerve Sheath Tumours
Malignant peripheral nerve sheath tumours are one of the other main differentials of sarcomatoid mesothelioma. They arise from the Schwann cells of peripheral nerves and classically appear as a monomorphic sarcomatoid cell population on light microscopy. They are morphologically similar to monophasic synovial sarcoma and mesothelioma. An uncommon variant, cellular schwannoma, is characterised by areas of glandular differentiation. A storiform pattern is uncommon.
Figure 2.19
Synovial Sarcoma

In this example a predominantly sarcomatoid pattern of differentiation is seen.
2.16: The Differential Diagnosis of Desmoplastic Mesothelioma

Desmoplastic mesothelioma is an uncommon variant of SM. Initially described by Kannerstein and Churg in 1980 it is a tumour type whose bland and relatively acellular appearance belies its highly invasive behaviour.\(^{202}\) It is defined as

\[\text{"A sarcomatoid mesothelioma with a predominance (}>50\%\) of dense collagenous stroma and haphazardly arranged slit-like spaces made up of cells with slightly atypical nuclei."} (WHO 1999)\(^{166}\)

The bulk of the tumour therefore consists of dense collagenous stroma arranged in a storiform pattern. Small nests of sarcomatoid mesothelioma cells are scattered within the stroma. Significant epithelioid foci are uncommon, and by definition should comprise < 10% of the total area examined. The stroma may show “collagen necrosis” - bland, pale, featureless areas where the definition of individual collagen fibrils is indistinct. This is uncommon in benign pleural disease. Desmoplastic areas may abut more typical sarcomatoid areas. Cleft-like spaces are also common, and may resemble the basket-weave pattern normally associated with benign pleural plaques. An example of desmoplastic mesothelioma is shown overleaf in Figure 2.20.

In contrast to the other subtypes of mesothelioma, some differentials of DM are benign rather than malignant processes. Misdiagnosis will therefore potentially have an even greater patient impact, particularly as DM has a very poor prognosis (see Chapter Four). The main problems are distinguishing between DM, pleural plaques (PP) and chronic fibrosing pleurisy. Other diagnostic differentials include SFT and desmoid tumours. Immunohistochemistry is of limited use in the diagnosis of DM, and this subject has been highlighted as a significant problem by mesothelioma diagnostic panels.\(^{171}\)

The degree of cellularity within the stromal portion of DM is an important clue to the underlying diagnosis. A very cellular infiltrate is more characteristic of reactive or inflammatory processes, such as tuberculosis pleurisy or chronic fibrosing pleuritis. At the other end of the spectrum are benign pleural plaques. These asbestos-related phenomena are comprised almost exclusively of bundles of collagen arranged in a more regular, basket-weave pattern than is seen in DM. Cell nests are infrequent. These plaques usually appear more than twenty years after asbestos exposure, and calcify with time. There is no evidence that DM arises in pre-existing PP, but this impression may be given if a plaque is invaded by coexisting mesothelioma.
Figure 2.21
Desmoplastic Mesothelioma

A storiform pattern is evident but the tumour is less cellular with areas of collagen necrosis (A).
Another important diagnostic feature is that of fat invasion. Pleural plaques are usually located on the parietal pleural surface, and do not involve underlying submesothelial structures. Reactive processes may cause pleural thickening, but the line of the original pleural surface is usually identifiable parallel to the surface. Extension of fibrous tissue to deep structures such as fat or muscle signify invasion and make DM the likely diagnosis.

The diagnosis of DM is essentially one made on H&E sections. Immunohistochemistry can not reliably distinguish between benign and malignant mesothelial cells: a subject which is addressed in more detail in the next chapter. Cytokeratin stains may help delineate the distribution of mesothelial cells within chest wall structures e.g. fat or muscle, thereby emphasising areas of invasion.

CONCLUSIONS

The physiology and pathology of the mesothelial cell is still not fully understood. The increase in incidence of the tumour is enabling further research into its cellular behaviour that may eventually identify a specific histological or immunohistochemical feature to enable its unequivocal diagnosis. Until that time pathologists will have to rely on a combination of clinical, radiological and pathological factors to guide diagnosis.

The process of assessing pleural biopsy specimens should begin with consideration of clinical factors. The age and sex of the patient may be relevant to diagnosis, and a personal history of cancer at another site is of critical importance when considering likely differentials. The extent and distribution of pleural disease and the presence of intrapulmonary masses may also be relevant, particularly if primary pulmonary carcinoma is the most likely clinical diagnosis. Evidence of prior asbestos exposure should be noted, but not given undue weight, as some degree of asbestos exposure is common in the age group in which both lung cancer and mesothelioma are found (sixth and seventh decades). A clinical suspicion of connective tissue disorders or mycobacterial infection is also of great significance.

The appearance of the pleural biopsy in H&E should enable the differentiation between benign and malignant mesothelial processes in the majority of cases. The presence of florid papillary proliferation, cellular atypia and local necrosis are all worrying features which should be considered as evidence of a highly atypical pleural reaction, and very suspicious of a malignant process. These are patients in whom a high index of suspicion of mesothelioma should be maintained. Further investigation or biopsy and close follow-up
are warranted. The diagnosis of mesothelioma –*in-situ* is difficult, and use of this term is unlikely to clarify the clinical situation in most cases. The diagnosis of desmoplastic mesothelioma should be considered in all patients with atypical pleural thickening, and a careful search for evidence of the hallmark chest wall invasion made. Correct distinction between a biphasic pattern of differentiation and prominent stroma is important, and will influence which other differentials are to be considered.

At the end of the day, it may not be possible to make a definitive diagnosis on the available tissue. Further biopsies may well be required, assuming that the patient is fit enough to tolerate surgery. Surgical colleagues should be aware of the potential difficulties inherent in this field of pathology, and encouraged to provide best possible biopsy specimens in terms of size, and also to sample at several different sites within the pleura. In many cases further investigations such as histochemistry and immunohistochemistry will be required before a diagnosis can be made with confidence. Electron microscopy can be of great help as a final diagnostic arbiter in difficult cases, but its limited availability, cost and the time taken to process sections can limit this.

The medico-legal implications of the diagnosis of mesothelioma may also influence a pathologist, albeit subliminally. The designation of mesothelioma as an industrial disease for which compensation is available has placed an additional onus onto clinicians and pathologists alike. They cannot help but be aware that, in equivocal cases, a diagnosis of mesothelioma rather than of any other pleural tumour, will secure some compensation for the patient and their family. Although this is not a subject that has been studied in any detail, I think it likely that a subtle influence does exist. However it is not the pathologist's role to apportion blame or facilitate compensation and this factor should not be allowed to over-ride the evidence of what is seen down the microscope.
CHAPTER THREE
IMMUNOHISTOCHEMISTRY AND THE DIAGNOSIS OF MALIGNANT PLEURAL MESOTHELIOMA

INTRODUCTION
Tumour diagnosis frequently requires consideration of clinical, histological, and immunohistochemical factors. This diagnostic process can be considered as a hierarchy: simple microscopy of H&E sections is the first level, followed by tissue histochemistry, then immunohistochemistry and finally electron microscopy.\(^{155}\) Although the first three of these are readily available in most pathology departments, electron microscopy is often limited to research facilities. Immunohistochemistry therefore tends to be the highest diagnostic arbiter in routine practice.

Immunohistochemistry utilises antibodies created against specific cellular epitopes. Ideally these epitope sites are exclusive to a particular cell type, and can often but by no means always, identify a tumours tissue of origin. Initial hopes that IHC could solve most diagnostic dilemmas have been tempered with the realisation that whilst useful, its value is limited in some areas.

Numerous factors influence the results of IHC staining. Differences in tissue preparation, fixation and processing can critically affect epitope sites, as can the degree of tumour differentiation.\(^{210}\) Sampling errors can be introduced by the inclusion of non-representative areas, especially when the specimen is small.\(^{161}\) The pattern of IHC staining may vary between initial biopsy and subsequent post-mortem material in the same patient, particularly if tumour progression is associated with de-differentiation and loss of epitope expression.\(^{211,212}\) Antibody specificity and sensitivity varies according to the manufacturer batch, and some require antibody retrieval techniques to ensure reproducible results.\(^{213}\) Interpretation of the pattern and intensity of staining is prone to both inter- and intra-observer variation, and may be overly influenced by clinical factors such as a history of asbestos exposure.\(^{214}\)

The ideal diagnostic antibody would be 100% sensitive (no false negatives) and 100% specific (no false positives) for any given epitope. Although IHC has made a significant contribution to the distinction between mesothelioma and other pleural tumours, the hope that a specific mesothelioma marker would be identified has not yet been realised.\(^{215-222}\)
In this chapter I will explain how IHC was used as part of the diagnostic process to identify a twenty-year cohort of patients with mesothelioma. The histological and immunohistochemical profile of this cohort is then described, and compared with the results of published studies that have addressed the problem of distinguishing mesothelioma from other benign and malignant processes affecting the pleura. Local research ethical committee approval was sought to enable us to undertake further molecular and IHC analysis of tissue blocks from patients with pleural disease, which had been stored in the histopathology department archives, or collected prospectively. This was granted on 1st April 1999 (South Manchester Local Research Ethics Committee reference SOU/99/059/CA).

**METHODOLOGY**

3.1: Identification of a Mesothelioma Cohort

A cohort of cases of mesothelioma and other pleural tumours diagnosed between 1979 and 2000 was identified from records held in the Department of Pathology at Wythenshawe Hospital. This was achieved by searching several different sources of information, which included the following:

1. A search of Wythenshawe Hospital’s computerised pathology database using the keywords mesothelioma, pleura and pleural biopsy. This covered all biopsy and post mortem (PM) cases from January 1993 to October 2000.
2. A search of the written records from 1980 - 1993 to identify all specimens described as pleural biopsies, and of PM reports for the same period for patients in whom mesothelioma was given as a cause of death.
3. All cases referred to Professor Hasleton for a second opinion or for medico-legal reasons e.g. for the Pneumoconiosis Medical Panel (PMP). Cases referred from outside the South Manchester University Hospital Trust area are allocated a laboratory reference number, but information regarding specimen type and final diagnosis are not entered into the computer. These cases will therefore not be identified using the methods mentioned in 1 and 2 above.
4. Prospective identification of any patients with pleural disease admitted under the care of the cardiothoracic surgery department at Wythenshawe Hospital.
Having identified an initial cohort of patients the pathology report(s) of each case was examined. The conclusions of pleural biopsies and subsequent PM results were compared in each patient. Where these conflicted, the PM diagnosis was the one that was accepted as these were usually based on larger tissue samples, and were more likely to have been reviewed and confirmed by a pathologist with a special interest in mesothelioma. All patients within the cohort were traced via the hospital Patient Administration System in both Wythenshawe Hospital and the Christie Hospital, and the date of last review or death noted. The post mortem findings relating to patients who had died outside the South Manchester area were obtained from their local hospitals wherever possible.

Each case was classified according to histological subtype. A significant proportion had been classified by the original pathologist, or could be classified by reading the report alone. All unclassified cases were reviewed (see below). The IHC profile for each specimen was also reviewed. All cases that had not had IHC performed, and those in whom the results of IHC were not what would be expected in mesothelioma were also reviewed. Finally, given that the prognosis of mesothelioma is so poor, it was decided to review the histology of all cases that had survived more than three years (more than 1 year in cases of desmoplastic mesothelioma).

Each sample requiring review was assessed by myself initially and then checked by Professor Hasleton. The original H&E stained 5μm slides were initially reviewed. If these were inadequate to confirm subtype then new H&E sections were taken from paraffin blocks using our standard laboratory protocol. Each specimen was classified according to the WHO 1999 guidelines.166

A total of 578 presumed cases of mesothelioma were identified after the initial search and review of histology reports. Fourteen were excluded from further study because they were referred from abroad, with little clinical information and no remaining tissue blocks. There were 89 cases which required further study in order to either confirm the diagnosis, classify the tumour or because they were long term survivors in whom the diagnosis might be questioned. Eleven further cases were excluded at this stage. Five of these were initially diagnosed as desmoplastic mesothelioma and reclassified as chronic fibrosing pleuritis on review. A further five cases were reclassified as non-small cell lung cancer (NSCLC), and one case as sarcoma (not otherwise specified, NOS).

A small number of cases (n=5) could not be classified. In two of these cases the only available blocks consisted of badly autolysed or completely undifferentiated tissue. We were confident of the diagnosis of mesothelioma on clinical grounds and the evidence
of pathology reports from other centres in each of these cases. The remaining three cases had no available blocks for re-study, but again there was strong evidence to support the diagnosis of mesothelioma. These cases were included in our cohort in order to analyse demographic data such as age and sex, but were excluded from analyses specifically based on histological subtype. I continued to collect data prospectively for the duration of my research period. Hence a further 34 cases of mesothelioma were eventually added to the cohort and are included in the description of IHC profile. In addition, a total of 33 cases of benign pleural disease, 123 cases of pulmonary adenocarcinoma, 19 cases of peritoneal mesothelioma and 18 cases of other tumours involving the pleura were identified and recorded in a separate database.

3.2: Immunohistochemical Analysis of Reviewed Cases

Eighty nine cases from the initial cohort were considered in need of histopathological review. Of these, 52 cases were re-studied using IHC to confirm the diagnosis of mesothelioma. Because of potential diagnostic difficulty, it has become common practice to employ a panel of several IHC markers when mesothelioma is suspected. These panels usually comprise a mixture of antibodies, some of which are positive for mesothelioma, and others that are positive for its main differentials, e.g. carcinoma. The IHC performed for each of our cases was specifically chosen according to the H&E appearances and the most likely differential diagnosis, rather than applying a broad panel to every case. A minimum of one positive and one negative IHC marker was accepted to confirm epithelioid and biphasic mesothelioma, although many cases had a more extensive IHC characterisation performed. Several cases were also studied with the vascular markers CD31 and CD34. Sarcomatoid and desmoplastic mesothelioma were primarily diagnosed on their light microscopy appearances. Anti-cytokeratin stains helped to emphasise cell distribution, and in several cases assisted the identification of fat or muscle invasion, an important diagnostic feature. Several of these cases were also examined with calretinin, and with CD34 to exclude solitary fibrous tumours. The IHC profile of the reviewed cases is detailed in Table 3.1 below. Another three cases were reclassified as NSCLC rather than mesothelioma after review of their IHC results (cases 36, 42 and 50). A summary of the diagnostic antibodies used, and our antigen retrieval methods can be found in Appendix One, page 265.
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Abbreviations:


Table 3.1

Summary of Immunohistochemistry Results for Reviewed Patients
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Abbreviations:

P = Positive staining result, - denotes negative staining result.

Table 3.1 (Continued)
Summary of Immunohistochemistry Results for Reviewed Patients
3.3: Analysis of Published Studies That Have Investigated the Value of IHC in the Diagnosis of Malignant Pleural Mesothelioma

The ability of IHC to distinguish between mesothelioma and other benign and malignant processes involving the pleura has been the subject of numerous publications over the last three decades. Although the accumulated number of cases of MPM and other tumours studied is impressive, it is still unclear which antibodies are the most reproducible and reliable for everyday practice. To clarify this subject I have performed a meta-analysis of papers devoted to the value of IHC in the diagnosis of mesothelioma and its differentials. Papers were identified initially by performing a literature search using the medical electronic libraries Medline and Pubmed, and the keywords Mesothelioma, diagnosis and immunohistochemistry. Individual papers were then studied to determine which diagnostic criteria had been used, whether mesotheliomas had been classified according to histological subtype, and whether monoclonal or polyclonal antibodies had been used in each study. Positive staining that was focal in nature (involving <30% of tumour cells) was not counted as a positive result for the purposes of this analysis. Wherever possible I extracted the individual results for each case of EM, BM and SM separately, and have compared them with the IHC results of the tumours that are their main differential in normal practice. For example I have compared the results for EM and epithelioid areas within BM with that of pulmonary adenocarcinoma, and those for SM, DM and sarcomatoid areas within BM with other sarcomas and benign pleural disease. This assumes that the epithelioid and sarcomatoid areas within BM behave in an analogous way to pure epithelioid or sarcomatoid tumours respectively. To date there is little evidence that this is not the case. I have excluded cases of non-pulmonary adenocarcinoma from this study wherever possible. This is because it is most difficult to clinically distinguish between MPM and PACA as lung encasement and pleural thickening potentially obscures the presence of a lung mass. Carcinoma metastatic from non-pulmonary sites may well be confirmed by consideration of past medical history, clinical examination and ancillary investigations e.g. radiology. A different panel of IHC antibodies might well be chosen if non-pulmonary metastatic carcinoma was a likely differential (see Section 3.10, page 122).

The results from a total of 86 papers were incorporated in the study of the diagnostic sensitivity and specificity of IHC markers used to distinguish between mesothelioma with epithelioid areas and PACA. A further 21 papers were used to calculate
the incidence of IHC staining in mesothelioma. Twenty nine papers were analysed for the study of sarcomatoid mesothelioma and its differentials, some of which were also used for the EM vs PACA analysis.

3.4: Immunohistochemistry and Effusion Cytology
Epithelioid mesothelioma and metastatic pleural carcinoma can both produce pleural effusions. Effusion cytology is frequently utilised when trying to differentiate between benign and malignant effusions, but has relatively poor diagnostic value for mesothelioma. Two cell populations may be identified within malignant effusions; tumour cells and reactive (benign) mesothelial cells. The characteristic features of reactive and malignant mesothelial cells have been discussed already in Chapter Two (Sections 2.4-2.8, pages 35-48). Immunohistochemistry may be of value in helping to distinguish between epithelial and mesothelial cells, in the same way that it serves to distinguish between PACA and EM in tissue sections. However it remains difficult to confidently distinguish between reactive and malignant mesothelial cells even when IHC is employed. Furthermore, even advanced pleural malignancy may fail to shed significant numbers of malignant cells into associated effusions. Although cytological specimens are often examined, most patients will require a more definitive test, such as a needle, thoracoscopic or open pleural biopsy. For these reasons, I have not investigated the value of IHC in effusion cytology in detail.
RESULTS

3.5: Immunohistochemical Profile of the Mesothelioma Cohort

The final IHC cohort consisted of 587 cases of MPM of which 576 were examined with at least one diagnostic antibody (98% of cohort). Five cases could not be classified into one of the four main variants of mesothelioma and are excluded from the rest of this analysis.

Epithelioid Mesothelioma

Immunohistochemistry was used most frequently to differentiate between EM and pulmonary adenocarcinoma. A total of 297 cases of EM were included in our IHC cohort. The most commonly used IHC markers were low molecular weight cytokeratins (LMWCK), carcinoembryonic antigen (CEA) and Ber-EP4. The LMWCK vary in their specificity for MPM: CAM 5.2 and AE1/AE3 decorate epithelial and mesothelial cells, whereas CK5/6 is thought to discriminate between them. Novel antibodies such as calretinin and thyroid transcription factor-1 (TTF-1) were used in more recent cases and those in the review group. The IHC profiles for cases of EM are summarised in Table 3.2 overleaf.

Biphasic Mesothelioma.

A total of 167 cases of biphasic mesothelioma was included in the IHC cohort. As with EM the most commonly used antibodies were CEA, Ber-EP4 and LMWCK. One difficulty assessing the IHC of BM is that it is not always clear whether IHC results refer to both epithelioid and sarcomatoid portions of the tumour. The IHC results are summarised in Table 3.3, page 95.

Sarcomatoid and Desmoplastic Mesothelioma

A total of 80 cases of SM and 27 cases of DM were in the cohort. Markers used to identify carcinoma are of less value in this group. Low molecular weight cytokeratins have a greater diagnostic value in SM as they may help to distinguish between SM and sarcomas with epithelioid differentiation. The diagnosis of desmoplastic mesothelioma can often be made on H&E sections. The main differentials are benign mesothelial processes, which will have a similar IHC profile to DM. The distribution of tumour cells can be emphasized by the use of IHC, and help to assess invasion, which is a critical diagnostic feature. The vascular marker CD34 was also used in a few cases to exclude solitary fibrous tumours of the pleura. A summary of the IHC results for SM is listed below in Table 3.4, page 96.
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<th>IHC Result</th>
<th>DPAS</th>
<th>CEA</th>
<th>Ber-EP4</th>
<th>Leu-M1</th>
<th>CAM 5.2</th>
<th>AE1/AE3</th>
<th>CK5/6</th>
<th>Calretinin</th>
<th>TTF-1</th>
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<td>87</td>
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Footnote: * Figures in brackets are percentage of cases with that IHC staining result. See text for IHC antibody names in full.

Table 3.2
Summary of the Immunohistochemical Profile for Cases of Epithelioid Mesothelioma
<table>
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<th>CAM 5.2</th>
<th>AE1/3</th>
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Footnote: * Figures in brackets are percentage of cases with that IHC staining result. See text for IHC antibody names in full.

Table 3.3
Summary of the Immunohistochemical Profile of Cases of Biphasic Mesothelioma.
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<th>AE1/3 (In DM)</th>
<th>CK5/6 (In SM)</th>
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Footnote: *A small number of cases were excluded because of equivocal IHC staining. See text for IHC abbreviations in full.

Table 3.4

Summary of the Immunohistochemical Profile for Cases of Sarcomatoid and Desmoplastic Mesothelioma.
3.6: The Statistical Evaluation of Diagnostic Tests and Immunohistochemistry

Many of the antibodies utilised to diagnose mesothelioma are selected for their ability to differentiate between mesothelial and epithelial cells, and as such can be divided into two broad groups. Those that identify cells of epithelial origin include mucins, carcinoembryonic antigen, the glycoprotein markers Ber-EP4 and B72.3, Leu-M1 and thyroid transcription factor-1 among others. These are often referred to as “carcinoma markers.” This is not strictly accurate as they may also be positive in cells of non-epithelial origin such as epithelial sarcomas, but this serves as a useful generic term to distinguish them from those antibodies that are usually positive in cells of mesothelial origin (“mesothelioma markers”). Many of these antibodies do not distinguish between benign and malignant proliferations of a given cell type, in which case basic principles of tumour diagnosis, such as cellular and nuclear morphology, the presence of invasion, and pattern of spread are employed. Commonly used carcinoma and mesothelioma markers are in Table 3.5.

<table>
<thead>
<tr>
<th>Carcinoma Markers</th>
<th>Mesothelioma Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial Mucins (DPAS)</td>
<td>CK 5/6</td>
</tr>
<tr>
<td>Carcinoembryonic Antigen</td>
<td>Calretinin</td>
</tr>
<tr>
<td>Ber-EP4</td>
<td>HBME-1</td>
</tr>
<tr>
<td>B72.3</td>
<td>Thrombomodulin</td>
</tr>
<tr>
<td>Leu-M1</td>
<td>N-cadherin</td>
</tr>
<tr>
<td>MOC-31</td>
<td>Wilms’ Tumour Product-1</td>
</tr>
<tr>
<td>E-cadherin</td>
<td></td>
</tr>
<tr>
<td>Thyroid Transcription Factor-1</td>
<td></td>
</tr>
<tr>
<td>Lewis’ (BG8)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.5

Immunohistochemical Markers Frequently Used in Diagnostic Panels to Distinguish Between Mesothelioma and Pulmonary Adenocarcinoma
There are other IHC antibodies used to confirm the diagnosis of mesothelioma that cannot be exclusively classified into one or other of these groups. For example LMWCK and some glycoprotein antigens (e.g. EMA) are present in both PACA and mesothelioma, but the pattern of distribution of the IHC marker may have discriminatory value. In addition the vascular markers CD31 and CD34 may help to distinguish between EM and epithelioid haemangioendothelioma, and between DM and solitary fibrous tumours.

In trying to evaluate the ability of an antibody to correctly identify a particular tissue or tumour type, results are best expressed in terms of diagnostic sensitivity and specificity. Sensitivity is defined as the ability of a diagnostic test to correctly identify positive cases, implying a low false negative rate. Specificity is defined as the ability of a test to correctly identify a negative result i.e. it has a low false positive rate. Sensitivity and specificity are expressed as percentages, and convention dictates that results are expressed with reference to the positive test result. They are calculated using the equation shown overleaf in Table 3.6. Not all published studies of the IHC of mesothelioma have compared staining results with other tumours. These results cannot therefore be expressed in terms of sensitivity and specificity, merely in terms of the incidence of positive and negative staining for that antibody. I have therefore expressed the cumulative results of my analysis in two ways. Those studies that have only reported IHC staining in mesothelioma have been combined to calculate an overall incidence of positive staining for each antibody. Those that have compared mesothelioma with pulmonary adenocarcinoma have been combined to calculate overall sensitivity and specificity for each antibody. Ordonez has recently published a study of 19 different diagnostic antibodies, and included a meta-analysis similar to mine as part of his discussion. There are a few points of distinction between our studies. Firstly he has used different criteria for IHC evaluation, accepting any level of positive staining as a positive result. I excluded cases with <30% positive staining in my analysis, as I felt that most pathologists were unlikely to accept minimal focal staining as a meaningful diagnostic result. Secondly, he has included studies performed on cytology samples. I have excluded these from my study. Finally, it does not appear that all cases of peritoneal mesothelioma, non-pulmonary adenocarcinoma and sarcomatoid mesotheliomas have been excluded from his analysis.
<table>
<thead>
<tr>
<th>Test Result</th>
<th>Disease Present (Number of cases)</th>
<th>Disease absent (Number of cases)</th>
<th>Total number of cases tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>a</td>
<td>b</td>
<td>a+b</td>
</tr>
<tr>
<td>Negative</td>
<td>c</td>
<td>d</td>
<td>c+d</td>
</tr>
</tbody>
</table>

Sensitivity = \( \frac{a}{a+c} \)  
Specificity = \( \frac{d}{b+d} \)  
Positive Predictive value = \( \frac{a}{a+b} \)

Table 3.6

Calculation of the Sensitivity and Specificity of a Diagnostic Test$^{226}$

The results of my analysis of IHC diagnostic sensitivity and specificity are summarised in Tables 3.7-3.8, pages 100-101 and Figures 3.1 -3.2, pages 102-103. They are discussed in detail in the next part of this chapter.
<table>
<thead>
<tr>
<th>IHC Marker</th>
<th>Number of Studies Analysed</th>
<th>Number of PACA Cases Included</th>
<th>Number of EM Cases Included</th>
<th>Sensitivity of Antibody for PACA</th>
<th>Specificity of Antibody for PACA</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cases CEA</td>
<td>51</td>
<td>1524</td>
<td>1818</td>
<td>83</td>
<td>95</td>
</tr>
<tr>
<td>Monoclonal CEA</td>
<td>24</td>
<td>949</td>
<td>1007</td>
<td>81</td>
<td>97</td>
</tr>
<tr>
<td>Ber-EP4</td>
<td>17</td>
<td>702</td>
<td>899</td>
<td>80</td>
<td>90</td>
</tr>
<tr>
<td>B72.3</td>
<td>16</td>
<td>769</td>
<td>700</td>
<td>80</td>
<td>93</td>
</tr>
<tr>
<td>Leu-M1</td>
<td>26</td>
<td>1473</td>
<td>1204</td>
<td>72</td>
<td>93</td>
</tr>
<tr>
<td>E-Cadherin</td>
<td>7</td>
<td>183</td>
<td>218</td>
<td>86</td>
<td>82</td>
</tr>
<tr>
<td>MOC-31</td>
<td>7</td>
<td>213</td>
<td>276</td>
<td>93</td>
<td>93</td>
</tr>
<tr>
<td>TTF-1</td>
<td>5</td>
<td>366</td>
<td>240</td>
<td>72</td>
<td>100</td>
</tr>
<tr>
<td>Lewis^BG8</td>
<td>4</td>
<td>231</td>
<td>197</td>
<td>93</td>
<td>93</td>
</tr>
</tbody>
</table>

Table 3.7
Summary of the Results of Published Studies That Have Evaluated the Sensitivity and Specificity of Immunohistochemical Markers in Distinguishing Between Adenocarcinoma and Mesothelioma: Part I – "Carcinoma Markers".
<table>
<thead>
<tr>
<th>IHC Marker</th>
<th>Number of Studies Analysed</th>
<th>Number of PACA Cases Included</th>
<th>Number of EM Cases Included</th>
<th>Sensitivity of Antibody for EM (%)</th>
<th>Specificity of Antibody for EM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK 5/6</td>
<td>8</td>
<td>284</td>
<td>402</td>
<td>83</td>
<td>85</td>
</tr>
<tr>
<td>Vimentin</td>
<td>17</td>
<td>815</td>
<td>773</td>
<td>62</td>
<td>75</td>
</tr>
<tr>
<td>Calretinin</td>
<td>17</td>
<td>912</td>
<td>885</td>
<td>82</td>
<td>85</td>
</tr>
<tr>
<td>HBME-1</td>
<td>14</td>
<td>676</td>
<td>769</td>
<td>85</td>
<td>43</td>
</tr>
<tr>
<td>Thrombomodulin</td>
<td>17</td>
<td>964</td>
<td>831</td>
<td>61</td>
<td>80</td>
</tr>
<tr>
<td>N-Cadherin</td>
<td>5</td>
<td>121</td>
<td>151</td>
<td>78</td>
<td>84</td>
</tr>
<tr>
<td>WT1</td>
<td>6</td>
<td>213</td>
<td>264</td>
<td>77</td>
<td>96</td>
</tr>
</tbody>
</table>

Table 3.8
Summary of the Results of Published Studies That Have Evaluated the Sensitivity and Specificity of Immunohistochemical Markers in Distinguishing Between Adenocarcinoma and Mesothelioma: Part II – “Mesothelioma Markers”.


Figure 3.1. Histogram Depicting Sensitivity and Specificity of IHC Antibodies for Distinguishing Between Pulmonary Adenocarcinoma and Mesothelioma with Epithelioid Differentiation (Carcinoma Markers)
Figure 3.2.
Histogram Depicting the Specificity and Sensitivity of IHC Antibodies for Distinguishing Between Pulmonary Adenocarcinoma and Mesothelioma with Epithelioid Differentiation (Mesothelioma Markers)
DISCUSSION
The propensity of mesothelioma to demonstrate several different patterns of differentiation within a single tumour can test the diagnostic acumen of the most expert of pathologists, particularly if only a small biopsy is available. Immunohistochemistry is of value in two ways. Firstly, it can help to distinguish between cells of different origin. Secondly, it can also emphasise the distribution of tumour cells within a biopsy. This can assist the identification of important features such as invasion, and in the case of biphasic mesothelioma specifically, distinguish between areas of sarcomatoid tumour and reactive stroma.

There are other factors that need to be considered when attempting to critically analyse the contribution of IHC to mesothelioma diagnosis. The discriminative value of a diagnostic test can only be definitively assessed if you are entirely confident of the diagnosis in each test group. This is one of the biggest problems when evaluating IHC in mesothelioma. Because we do not yet have a gold standard test for identifying mesothelioma, there is always the possibility that results could be skewed by the inclusion of even a small number of other tumours. Studies often state that the diagnosis of mesothelioma was made using standard diagnostic criteria, without saying what those were. We know that diagnosing mesothelioma can be very difficult, and that most diagnostic adjuncts, including electron microscopy, are not 100% accurate. Reliance on clinical factors such as a history of asbestos exposure can also influence a pathologist’s willingness to diagnose mesothelioma. As asbestos also predisposes to the development of carcinoma of the lung, this actually has limited discriminatory value. Many studies of mesothelioma have included small numbers of cases. The inclusion of even one or two carcinomas in a group of mesotheliomas can give a false impression of the value of a diagnostic antibody. Indeed, this could explain the small numbers of CEA and BER-EP4 positive mesotheliomas identified in some studies.

It is also important to appreciate the effect that IHC staining techniques can have when comparing results from different institutions. Any individual laboratory may have slightly different ways of processing tissue blocks and performing IHC that may influence the sensitivity or specificity of an antibody. Staining methodologies can vary between manufacturers, particularly in terms of optimum antibody concentration. Antigen retrieval techniques, such as microwave or pressure cooker heat treatments may also have been used. Poorly preserved or autolysed tissue may also lose characteristic epitope sites, and therefore fail to stain with an antibody that would normally be positive. Conversely,
necrotic tissue may have an inflammatory infiltrate that produces a positive reaction with some IHC markers such as Leu-M1. Pooling of antibody complexes at the periphery of a tissue section can be misinterpreted as a positive reaction.

Another potential area of difficulty relates to interpretation of patterns of distribution and intensity of staining. This is potentially prone to both inter- and intra-observer variation. Assessment of staining intensity and distribution may be performed by a single, or more than one pathologist, and with or without blinded checking. Alternatively, computerised systems may be used. The criteria for evaluation of staining may differ between pathologists; some count any positive staining even in 1% of tumour cells as a positive result, others have a higher cut-off level. In this meta-analysis I excluded cases with positive staining that was classified as focal or <30% of the total tumour, which is the cut-off level employed in our laboratory. The distribution of staining is also important. For example, the antibody TTF-1 is almost exclusively positive in thyroid carcinoma and some carcinomas of the lung. A positive result is denoted by nuclear staining alone: cytoplasmic staining however strong is considered a negative result. Cytoplasmic staining could erroneously be interpreted as a positive result by those unfamiliar with this antibody. These are technical factors of which an experienced histopathologist should be fully aware. However, many studies do not provide enough methodological detail in their published results to ascertain whether any of these factors could be relevant.

Publication bias is another factor that needs to be considered. When a new diagnostic technique is first reported, it often represents the fruits of a long period of research by an enthusiastic and committed group, who may have spent a long time ensuring best possible results. Subsequent groups will not have the advantage of familiarity with the test and allied reagents, and may not be able to reproduce similar results. It is less than ideal to combine the results of several different studies when trying to evaluate a diagnostic test. By including a large number of cases, and by excluding those studies in which the methodology, case selection or diagnostic criteria are a concern, some of these reservations can be ameliorated. It is possible to obtain a good feel for the overall usefulness of those antibodies in common use, and select the most appropriate to help in a specific diagnostic dilemma. However it is essential that the limitations of this sort of study are not forgotten when trying to extrapolate results to one's own practice.
3.7: IHC in the Distinction Between EM and PACA—Value of Carcinoma Markers

Mucins

Histochemistry was one of the first diagnostic tests used to distinguish between PACA and mesothelioma. Both epithelial and mesothelial cells can produce intracellular mucins that are demonstrated by relatively simple and cheap means. Mucins are polyanionic compounds composed of a protein core associated with variable chains of glycosaminoglycans (GAG). The predominant type of GAG varies between different tissues of origin. The mesothelium predominantly produces hyaluronic acid which has excellent elastic and lubricant properties. There are several different mucins stains that are routinely used for diagnostic histochemistry. The most commonly utilised are mucicarmine, periodic acid-Schiff (PAS) and alcian blue. Several studies have confirmed that between 60 and 70% of pulmonary adenocarcinomas are mucin positive. Mesotheliomas can exhibit positive mucin staining, but this is much less common when combined diastase and PAS (DPAS), rather than mucicarmine or alcian blue are employed. Positive mucin reactions in mesothelioma are frequently due to HA, and as such are usually abolished by hyaluronidase pre-treatment. Staining for HA itself is often unhelpful, as it is leached from tissues when water-based fixatives e.g. formalin are used. Mesotheliomas also frequently contain large amounts of glycogen, which can stain positively with DPAS. The incidence of mucin-positive mesotheliomas in series that have used hyaluronidase pre-treatment is in the order of 5% or less. Basement membrane contains GAG that may cross-react with DPAS; in some microscopic sections these areas may appear intracytoplasmic. Both Henderson and Hammar have cautioned against misinterpretation of such areas.

Mucin stains continue to have a diagnostic role despite the introduction of IHC. Diffuse, strongly positive DPAS in an epithelioid pleural tumour is highly suggestive of adenocarcinoma, and should therefore direct the pathologist to selecting an IHC panel that will both confirm this and help identify tissue of origin. Weak and/or focal DPAS staining is of less diagnostic value.

In our cohort DPAS was frequently employed as part of the diagnostic panel. A total of 134 epithelioid and 71 biphasic tumours was studied. One biphasic tumour exhibited positive staining, one epithelioid tumour was focally positive, and a further case of both BM and EM were focally positive. Overall, this corresponds to a >99% negative staining rate.
Carcinoembryonic Antigen

Carcinoembryonic antigen is an oncofetal glycoprotein of approximately 200kD that was first identified by Gold and Freeman in 1965.\(^\text{234}\) There are several CEA epitope sites which differ according to their carbohydrate moiety. Carcinoembryonic antigen can be reproducibly demonstrated in adenocarcinoma arising from different tissues, but is particularly associated with those from the colon and lung. Mesothelial cells characteristically do not possess CEA epitopes. The CEA antibody was therefore recognised early on as a potential discriminator between these two tumours and has since been extensively investigated.\(^\text{235}\) Carcinoembryonic antigen has repeatedly shown itself to be one of the most useful diagnostic antibodies for distinguishing between EM and PACA.

Initial studies with CEA used a polyclonal antibody. This also reacted with CEA-related sites (non-specific cross-reacting antigen, NCA). These NCA sites can be demonstrated in mesothelioma cells, resulting in false positive CEA staining. Studies evaluating polyclonal CEA in mesothelioma have reported positive results in up to 45% of cases.\(^\text{236-248}\) Pre-absorption with spleen powder or NCA can reduce the false positive rate, but the value of polyclonal CEA is still limited. The introduction of monoclonal (Mo) CEA antibodies has improved its diagnostic specificity, with far fewer mesotheliomas reported as CEA-positive (0-8%). Interestingly, CEA-positive mesotheliomas often also give positive reactions for mucin. Both Robb\(^\text{249}\) and Hammar\(^\text{299}\) have demonstrated that CEA reactivity in mesotheliomas is abolished or diminished by pre-digestion with hyaluronidase. It therefore appears likely that other cross-reacting antigens exist and may influence results with both polyclonal and monoclonal antibodies.

I identified a total of 58 studies that had evaluated CEA expression in mesothelioma.\(^\text{122, 124, 213, 217, 227, 228, 230, 232, 233, 235-248, 250-284}\) Carcinoembryonic antigen positivity in pulmonary adenocarcinoma had been used for comparison in all but seven of these studies.\(^\text{122, 232, 239, 264, 266, 276, 281}\) The results of CEA staining for 1524 cases of PACA and 2077 cases of epithelioid (or biphasic) mesothelioma were reported. The incidence of positive CEA staining was 83% in PACA and 4.4% in EM. When only those studies that had compared PACA with EM were considered, the sensitivity and specificity of CEA for PACA was 83% and 95% respectively. In the 24 studies that had utilised MoCEA sensitivity was reduced to 81% but specificity improved to 97%.\(^\text{124, 213, 228, 233, 238, 240, 250, 253-255, 257, 258, 260, 262, 263, 265, 267, 268, 275, 277, 279, 280, 282, 284}\)

Carcinoembryonic antigen was a frequently employed diagnostic antibody in our own cohort. A total of 253 cases of EM (85% of all EM cases studied by IHC) and 148 of
BM (89% of all EM cases studied by IHC) were examined with monoclonal CEA (Dako). The incidence of diffuse positive CEA staining was zero (0%), with focal positivity seen in less than 3% of cases of EM (n=6). In BM there was one tumour with diffuse positive staining (0.7%) and six tumours with focal staining (4%). It should be born in mind however, that supposed cases of mesothelioma that were subsequently shown to be strongly positive for more than one “carcinoma marker” were normally excluded from our cohort in the early stages. By doing so, we will have biased our IHC results against the inclusion of “CEA positive mesotheliomas”, if such tumours do indeed exist.

Glycoproteins Ber-EP4 and B72.3

Ber-EP4 is a formalin-resistant antibody raised against a 34 - 49kD glycoprotein that is expressed on most epithelial cells, except those with squamous differentiation. It has no cross-reactivity with cytokeratins. Early studies suggested that it had a high sensitivity and specificity for adenocarcinoma whilst positive reactions were reported in less than 1% of mesotheliomas by others.

I identified 17 studies that compared Ber-EP4 staining in PACA and EM. In these studies the proportion of mesotheliomas exhibiting positive reactions with Ber-EP4 ranged from 0-20%. Three of the studies with the lowest proportion of mesothelioma cases positive for Ber-EP4 (range 0 - 5.3%) also had the lowest percentage of positive reactions in adenocarcinoma (range 50 - 67%). These results may therefore reflect differences in staining technique and evaluation rather than Ber-EP4 expression per se. A total of 702 cases of PACA and 899 cases of mesothelioma were examined. The overall sensitivity and specificity of Ber-EP4 for distinguishing between PACA and EM was 80% and 90% respectively.

Monoclonal Ber-EP4 (Dako) was the third most commonly employed IHC marker used to study our mesothelioma cohort. It was used in 172 cases of EM and 94 cases of BM. Only four cases of EM were positive for Ber-EP4 (2%) with a further 22 cases (13%) exhibiting minimal and /or focal staining. The results for BM were similar with one case positive (1%) and a further six cases focally positive (7%).

B72.3 is a monoclonal antibody raised in mice against a tumour-associated glycoprotein complex expressed in breast carcinoma cell lines. As with Ber-EP4, initial studies suggested that B72.3 could be an important tool for differentiating between PACA and EM. Further investigation led to a dampening of enthusiasm, with some authors reporting positive reactions in up to 47% of EM. However an analysis of 16 studies that
include 769 cases of PACA and 700 cases of EM show it to have an 80% sensitivity and 93% specificity for adenocarcinoma, figures that compare favourably with more established IHC antibodies. B72.3 was not routinely employed by our laboratory during the study period therefore I am unable to provide comparative results for our own cohort.

Leu-M1
Leu-M1 (CD15) is a monomyelocytic marker that has also been shown to have diagnostic merit in the distinction between adenocarcinoma and mesothelioma. Focal staining for Leu-M1 has been reported in up to 80% of pulmonary adenocarcinomas. I have identified five studies that have evaluated Leu-M1 staining in mesothelioma and a further 26 studies that have evaluated Leu-M1 expression in mesothelioma in comparison with adenocarcinoma. The latter studies include a total of 1473 adenocarcinomas and 1204 mesotheliomas. Overall sensitivity for the diagnosis of adenocarcinoma was only 72%, but specificity was better at 93%. The overall incidence of positive Leu-M1 staining in mesothelioma was 6%, with 16 of the 28 studies reporting no positive staining in any cases of mesothelioma. Conversely four studies reported Leu-M1 in mesothelioma in more than 10% of cases (range 12.8-28%).

Leu-M1 was studied in 123 cases of EM and 47 cases of BM within our cohort. The vast majority of cases were Leu-M1 negative, with positive reactions in only four cases of EM (3%) and three cases of BM (6%). A further 14 cases of EM and six cases of BM demonstrated minimal focal staining.

MOC-31
MOC-31 is a monoclonal antibody that reacts with a 38 kD epithelium-associated transmembranous glycoprotein (epithelial glycoprotein-2) raised from a small cell lung cancer cell line. The epitope is similar to that targeted by the monoclonal antibody to EMA and has been investigated in a small number of studies (n=7) as a means of distinguishing PACA (n=213) from mesothelioma (n=276) in tissue block preparations. These studies have confirmed that MOC-31 has a high sensitivity and specificity for PACA (93% for both). There is, however, a single study of MOC-31 in mesothelioma and metastatic pleural carcinoma that reports a positive staining incidence of MOC-31 in mesothelioma of 17%. This data was not incorporated into our meta-analysis as the
primary source of the metastatic carcinomas was not reported. MOC-31 positivity in
cytological preparations has also been studied by three groups, who report slightly less
impressive results, with a sensitivity of 88% and a specificity of 93% overall. 223, 292, 293

**E-cadherin**

The cadherins are a group of heterodimeric calcium-dependent, membrane-associated
glycoproteins within the family of cell adhesion molecules. 294 They are responsible for
cell-to-cell adhesion at the molecular level, communicating with the cytoskeleton via a
group of regulatory proteins, the catenins. 295 Reduced levels of cadherin expression are
associated with increased cell motility and tissue invasion. 130 The type of cadherin
expressed by a cell reflects its embryological origin. E-cadherin is typically expressed in
epithelia. In contrast, N-cadherin is expressed by cells originating from mesodermal and
neural crest tissue, such as mesothelium. During morphogenesis homophilic attraction
between cadherin molecules facilitates the clustering of cells of the same origin within a
tissue or organ. The class of cadherin expressed by a cell persists despite malignant change
although the level of expression may change. Reduced E-cadherin expression is associated
with increased invasiveness and a poorer prognosis in many epithelial malignancies. 131
Such changes are often a result of reduced expression or altered phosphorylation of the
cadherin-catenin complex.

It has therefore been recognised that cadherin expression could have diagnostic
value. 131, 132 To date I have identified seven studies that have evaluated E-cadherin as a
discriminator between mesothelioma and PACA based on a total of 183 adenocarcinomas
and 218 mesotheliomas. 131, 132, 222, 227, 267, 281, 296 E-cadherin appears to have reasonable
diagnostic value with an overall sensitivity and specificity for adenocarcinoma of 86% and
82% respectively. E-cadherin was not routinely used in our department during the study
period.

**Thyroid Transcription Factor-1**

Thyroid transcription factor is a relatively new addition to the diagnostic armamentarium.
It is a member of a homeodomain transcription factor family that is selectively expressed
in thyroid and lung epithelium. 297, 298 The gene for TTF-1 is highly conserved: in the
thyroid it regulates thyroglobulin and thyroperoxidase gene transcription, and in the lung it
plays an important role in controlling surfactant production from type II alveolar cells. 299
Thyroid transcription factor-1 expression is retained despite malignant transformation.
Hence it has been recognised as potentially able to distinguish between primary and secondary lung adenocarcinoma, a problem that had previously relied on a combination of clinical factors, cell morphology and CK subset expression. The potential for TTF-1 to contribute to the distinction between EM and PACA has also been recognised. These five papers investigated the differential expression of TTF-1 in a total of 366 pulmonary adenocarcinomas and 240 mesotheliomas. None of the mesotheliomas were positive for TTF-1, whilst 85 carcinomas were negative (28%). Sensitivity and specificity of TTF-1 for identifying PACA are therefore 72% and 100% respectively.

The TTF-1 (Dako) antibody was used as a diagnostic adjunct in our own mesothelioma IHC cohort in only a small number of cases (EM=10, BM=2, SM=1). All cases studied were negative in epithelioid areas. The ability of TTF-1 to distinguish between MPM and pleomorphic carcinoma is discussed in more detail later in this chapter (Section 3.15, page 138).

Blood Group Antigens (Lewis')
Several blood group antigens have been examined as potential discriminators between PACA and EM. These include Lewis', ABO blood group-related antigen and Helix pomotia agglutinin. Lewis' is the only one of these that has consistently proven its worth.

Initially Noguchi and colleagues studied the NCC-St-433 antibody. This gave disappointing results: although the majority of adenocarcinomas (95%) stained positively, so did almost half of the mesotheliomas (44%). In the same year Jordon and colleagues performed a similar study using the BG8 antibody, which is raised from the SK-LU-3 lung cancer line. They found that 100% of PACA and 23% of EM were positive for this antibody. More recently two larger studies using the BG8 antibody have been undertaken. In 1997 Riera et al published the results of the largest study of this antibody, which included 123 cases of PACA and 57 cases of EM. Sensitivity and specificity were 93% and 92% respectively. In 2000, Ordóñez reported more impressive results in a smaller study in which only one case of mesothelioma demonstrated more than 1% positive staining with BG8 (sensitivity 98% and specificity 98%). He has since published a second study that confirms the moderate sensitivity but high specificity of BG8 for PACA. Overall the combined sensitivity and specificity of the BG8 antibody are both 93%. These four studies include a total of 231 cases of PACA and 197 cases of mesothelioma.
Intermediate Filaments and Cytokeratins

The contribution of intermediate filaments to the cytoskeleton of mesothelial cells has been discussed in Section 2.4, page 35. Intermediate filaments are sub-classified into six groups (I-VI). Groups I and II comprise the cytokeratins. There are 20 different cytokeratins which can be classified according to their molecular weight and by their biochemical properties. Intermediate filament Group I comprises acidic cytokeratins (CK9-20) and Group II the basic cytokeratins (CK1-8). Cytokeratins share a similar structure, comprising a 310 amino acid central rod domain, with heterogeneous head and tail areas (30-50% sequence homology). The type of CK expressed by a cell is influenced by embryological origin and differentiation, and is usually retained despite malignant transformation. The cytokeratins frequently exist in pairs, with one acidic and one basic CK within each pair. Low molecular weight cytokeratins are preferentially expressed in glandular tissue, with heavier CK seen in tissues with transitional or squamous differentiation. Studying the distribution of specific CK types within a tumour cell can therefore help to identify its tissue of origin, a fact that has been widely exploited in diagnostic histopathology.

Two of the more commonly used CK antibodies are AE1/AE3 and CAM 5.2. AE1/AE3 is a mix of two antibodies that between them identify most CK found in human epithelium (CK 1-6, 8, 10, 15, 16, 19) but have little cross-reactivity with members of the other intermediate filament groups. CAM5.2 is a monoclonal antibody raised in human colonic carcinoma cell line that preferentially recognises the CK8/18 pair. Numerous studies have confirmed that the majority of mesotheliomas (87%, n= 963) and adenocarcinomas (90%, n= 768) express LMWCK. The use of broad spectrum LMWCK is therefore limited when distinguishing between EM and PACA, as both would be expected to produce a positive result with a similar pattern of distribution. However, the demonstration of LMWCK positivity in an epithelioid tumour that is negative for other common carcinoma markers (CEA, Ber-EP4 and Leu-M1) supports a diagnosis of EM. Cytokeratins are also valuable in distinguishing between sarcomatoid mesothelioma and its differentials. The latter is discussed in more detail later in this chapter (Section 3.12, page 131).

In our own cohort both CAM5.2 and AE1/AE3 were frequently employed. CAM5.2 was studied in 204 cases of EM and 115 cases of BM. Only three cases of EM were negative for CAM5.2, and one further case showed only focal staining, corresponding to a positive staining rate of 98%. The results for BM were similar with one focally
positive and two negative cases (97% positive staining rate). The AE1/AE3 antibody was tested in 115 cases of EM, of which two were negative (98% positive staining rate). All 66 cases of BM tested were positive.

3.8: IHC in the Distinction Between EM and PACA—Value of Mesothelioma Markers

The other antibodies that have been extensively investigated are “mesothelioma markers.” These are antibodies that preferentially decorate cells of mesothelial origin. The most commonly used markers were listed earlier in Table 3.5, page 97. There are significant advantages to including mesothelium-specific antibodies in a mesothelioma diagnostic panel. Firstly, the antibody can usually be used to evaluate all three histological subtypes of mesothelioma. Secondly, assuming that a negative “carcinoma marker” result confirms the diagnosis of mesothelioma does not take account of the possibility of false negative results. A negative result could just as easily reflect poor staining technique or inadequate antigen retrieval, although the appropriate use of positive and negative controls should reduce misinterpretation of results. Hence it is always reassuring to have a confirmatory result based on positive staining to limit such bias.

Cytokeratin 5/6.

Cytokeratin expression in mesothelioma has been briefly mentioned earlier in this chapter. Because both mesothelial cells and carcinomas express cytokeratin, the use of broad spectrum LMWCK is of limited diagnostic value in distinguishing between these two groups. However expression of specific CK subtypes is now possible and does have a diagnostic role. Mesothelial cells express both LMWCK (pairs CK5/14, 8/18, 7 and 19) and vimentin (intermediate filament group III member). Pulmonary ACA expresses CK7, 8/18 and 19. It can therefore be seen that the presence of CK5 and/or CK14 is an important discriminator between PACA and MPM.

The diagnostic potential of CK5 was recognised by Blobel and colleagues in 1985. At that time there were no commercially available antibodies that could identify CK5 in formalin-fixed paraffin-embedded tissue blocks. This is no longer the case, and six groups have studied CK5/6 expression in pleural tumours. The first two of these studies used the commercial antibody D5/16B4 (Boehringer Mannheim, UK). Their combined number of cases totalled 57 adenocarcinomas and 63 epithelioid mesotheliomas. Clover and colleagues reported strong CK5/6 positivity in all 23 cases of epithelioid and biphasic mesothelioma. Ten sarcomatoid mesotheliomas were also
studied; only one demonstrated positive staining. Weak CK5/6 positive staining was demonstrated in five of 27 PACA (19%), although this was only focal in one case, and weak in the other four.

The following year Ordóñez and colleagues published the results of their own study of CK5/6 in mesothelioma and several different types of lung tumours. They reported CK5/6 negativity in all 30 PACA, and positive results in all 40 EM. Two of the cases of EM had less than 25% positive cells, and in a quarter of cases less than 50% stained positively. Some CK5/6 positivity was seen in examples of squamous and undifferentiated lung cancers that were also included in their study. The sensitivity and specificity of CK5/6 for mesothelioma was 86% and 91% respectively when their results were combined. Ordóñez has recently published a further study evaluating CK5/6 using a different selection of mesothelioma cases with similar results. Another four studies evaluating CK5/6 have been published. Kayser and colleagues evaluated the antibody keratin 5/6 (Zymed, Berlin) as part of a larger investigation of comparative IHC staining in 111 cases of mesothelioma (epithelioid and biphasic) and 82 cases of lung carcinoma metastatic to the pleura. They found the sensitivity of keratin 5/6 to be 76% and the specificity only 60%. The majority of the sarcomatoid areas in biphasic mesotheliomas stained positively with keratin 5/6 (n=6, 86%). Cury and colleagues studied CK5/6 in 61 cases of EM in comparison with ACA of various origins, including 19 arising from the lung. They found that CK5/6 was expressed in 92% of EM and only one case of the PACA (5%). Carella et al also using the D5/16B4 (Boehringer Mannheim, UK) antibody report a positive staining incidence of 87% in their study of 46 mesotheliomas. Abutaily and colleagues have recently published their study of a variety of novel antibodies including CK5/6 (Dako, Ely, UK) in 35 pulmonary adenocarcinomas and 41 mesotheliomas, of which 35 of the latter had areas of epithelioid differentiation (EM=11, BM=23). The majority of their cases of EM (90%) stained positively for CK5/6, but only 14 of the 23 biphasic tumours were positive. The authors did not stipulate their cut-off levels for staining, or comment whether positivity was required in both epithelioid and sarcomatoid areas in these cases. Only two cases of PACA were positive for CK 5/6, and only one sarcomatoid mesothelioma stained positively. Their results suggest a sensitivity and specificity for mesotheliomas with epithelioid differentiation of 71% and 94% with the Dako antibody. Finally, Chu and colleagues have recently published the results of a comprehensive study of CK5/6 (Chemicon, Temecula, CA) expression in a large number of epithelial tumours of various tissues of origin (n=509), which includes a small number
of mesotheliomas (n=29) and PACA (n=21).$^3$ Although most tumours showed far less prominent CK5/6 positivity than the mesotheliomas, focal staining was seen in one case of PACA, in 25% of ovarian carcinomas, 40% of ductal breast carcinomas and 38% of pancreatic carcinomas. All of these tumours can potentially enter into the differential diagnosis of mesothelioma, particularly in the peritoneum, which may limit the value of CK5/6 in such cases. Furthermore, all eight thymomas studied were CK5/6 positive.

The combined results of these eight studies include a total of 284 cases of PACA, and 402 mesotheliomas. The overall sensitivity and specificity of CK5/6 for mesothelioma were 83% and 85% respectively.

The incidence of positive CK5/6 staining (diffuse and focal) in our own cohort of mesotheliomas using the D5/16 antibody was as follows. Of the 87 cases of EM studied, 73 were positive, with a further 4 cases of focal positive staining. For BM, 40 of the 74 tumours were positive, with focal positivity seen in a further nine. The overall rate of diffuse positive staining was therefore 84% and 54% respectively. Our results support the findings of those studies mentioned earlier that CK5/6 is of only reasonable diagnostic value when comparing EM with PACA.

Vimentin
The other intermediate filament that has been studied in mesothelioma is vimentin. Vimentin is a group III intermediate filament that primarily identifies cells of mesodermal origin. It is therefore expressed in both benign and malignant connective tissue, as well as in mesothelial cells and mesothelioma.$^{308,314}$ The co-expression of LMWCK and vimentin within a cell is highly suggestive of a mesothelial origin, particularly if the filaments are prominent and in a perinuclear distribution. Vimentin can be demonstrated in PACA, both within tumour-associated stroma and the tumour cells themselves.$^{315}$ However vimentin expression is usually distributed diffusely within the cytoplasm, rather than in a perinuclear pattern, and staining is often much weaker than that seen in mesothelioma.$^{167,250,252,314}$

Vimentin expression in mesothelioma and its differentials has been studied by several groups, both in isolation, and in terms of co-expression with LMWCK. I have identified 20 studies that have addressed this topic, of which 17 have also examined vimentin expression in adenocarcinoma.$^{213,227,228,233,236,238,240,250-252,258,260,266,271,275,277}$ A total of 773 mesotheliomas and 815 adenocarcinomas was studied, and overall sensitivity and specificity of vimentin for mesothelioma was 62% and 75% respectively. Overall the incidence of vimentin expression was 59% in mesotheliomas and
22% in PACA. Vimentin was not used on a regular basis during the study period, being studied in only 50 patients in our cohort. Vimentin was studied in 21 cases of EM, of which 8 were negative and one focally positive. This corresponds to a positive staining rate of only 57%.

**Calretinin**

Calretinin is a 29 kDa calcium-binding molecule that is a member of the EF-hand protein group. This group is characterised by the presence of an amino acid sequence that forms a helix-loop-helix configuration in the calcium-binding part. Calretinin contains six of these stretches. It is related to the other diagnostic antibodies S100 and calbindin, and is thought to be involved in the calcium-dependent intracellular signalling mechanisms that control the cell cycle. Although characteristically expressed in central and peripheral nervous system tissue, it can also be demonstrated in mesothelium, as shown by Gotzos in his study of the role of calcium-binding proteins in cancer of the colon. The first specific study of calretinin as a marker for mesothelioma was undertaken by Doglioni and colleagues in 1996. They compared calretinin positivity in 44 mesotheliomas and 294 carcinomas using two different antibodies: the 7696 antiserum produced by Swant (Bellinzona, Switzerland) and the AB149 antiserum from Chemicon (Temecula, CA, USA). Thirty five of the carcinomas were of pulmonary origin, as were five cytological specimens. Calretinin was positive in all cases of EM and BM, and over 65% of sarcomatoid mesotheliomas were also positive. Nine of the 40 adenocarcinoma samples showed some calretinin positivity, although this was weak in all cases. In a similar study, Ordóñez evaluated calretinin immunostaining using a different polyclonal calretinin antibody (Zymed, San Francisco, CA). He found calretinin positivity in all 38 cases of EM studied. He also studied calretinin expression in a total of 155 adenocarcinomas, of which 38 were of pulmonary origin. Only three of the PACA exhibited positive calretinin staining. Hence calretinin was promoted as a major advance in the identification of mesothelioma.

To date a total of 17 studies have assessed the value of calretinin in distinguishing between mesothelioma and pulmonary adenocarcinoma. These comprise a combined total of 912 cases of PACA and 885 cases of EM. Overall calretinin sensitivity (82%) and specificity (85 %) are less than might be expected, given the encouraging results achieved by Ordóñez. Two of these studies in particular have negatively influenced the overall result, compared with the other studies. Riera and
colleagues\textsuperscript{213} studied calretinin expression in 57 cases of mesothelioma, whilst Roberts and colleagues studied 94 cases.\textsuperscript{262} They reported calretinin positivity in 42\% and 48\% of cases respectively. Both of these studies used the Chemicon calretinin polyclonal antibody. Three other groups that used this antibody found it to have excellent sensitivity (100\%, 90\% and 87\% respectively) at the expense of low specificity (50\%, 60\% and 90\% respectively).\textsuperscript{221, 280, 283} The more recent studies by Abutaily \textit{et al}, Cury \textit{et al},\textsuperscript{312} and Miettinen \textit{et al}\textsuperscript{320} using the Zymed polyclonal antibody also found calretinin to be highly sensitive (92\%, 90\% and 96\% respectively) and specific (95\%, 94\% and 93\% respectively) for mesothelioma.

It has also been suggested that the pattern of calretinin distribution within a tumour is important, (Professor Hasleton, personal communication), with nuclear staining being of more diagnostic value than cytoplasmic staining. The majority of those groups that have studied calretinin in mesothelioma have found it to be primarily expressed within the cytoplasm, although four groups have also reported nuclear staining.\textsuperscript{221, 237, 262, 319} Roberts and colleagues, in a separate report did comment that nuclear staining was less evident in post-mortem samples compared with pre-mortem samples from the same patients.\textsuperscript{211} As many studies use post-mortem specimens of mesothelioma this may be of some relevance. No groups have specifically reported differences in pattern of staining between EM and PACA so at present this hypothesis remains unproven. The value of calretinin in confirming the diagnosis of sarcomatoid mesothelioma is discussed later (Section 3.12, page 131).

In our own cohort a total of 60 cases of EM and 77 cases of BM were examined with calretinin, using the Swant polyclonal antibody. Positive staining was seen in 87\% of EM, with a further 7\% showing focal staining. Only 54\% of biphasic tumours showed diffuse staining, with focal staining see in a further 12\%. Our conclusion was that calretinin had a similar diagnostic value to CK5/6 in distinguishing between EM and PACA, but was less useful in tumours with areas of sarcomatoid differentiation.

The disappointing sensitivity and specificity results for calretinin may have a biological explanation above and beyond that of IHC technique. In Gotzos' original paper calretinin was described as only being expressed in activated (cuboidal) mesothelial cells with an epithelioid appearance. Quiescent endothelial-like cells were negative, as were areas of sarcomatoid differentiation.\textsuperscript{317} Other studies have confirmed that between 10 - 30\% of mesothelial cells are consistently negative for calretinin. It has therefore been suggested that the expression of calretinin may be cyclical, and is most common during the
G1 phase of the cell cycle. The precise role that calretinin plays in mesothelial cell regulation, and the factors that control its expression are still poorly understood. As of yet there appear to be no specific antigen retrieval methods that can reliably unmask the calretinin epitope in all mesotheliomas.

HBME-1
ME-1 and HBME-1 are monoclonal antibodies raised from the mesothelioma cell line SPC111. The exact nature of the target antigen is unknown but is predominantly located on microvilli. ME-1 was the first of these antibodies to be introduced, and was shown to react with both normal mesothelial and epithelioid mesothelioma cells in frozen section specimens by O'Hara and colleagues in 1990. They compared ME-1 staining in 20 epithelioid mesotheliomas and 28 PACA. All cases of EM were positive for ME-1, with only one of twenty adenocarcinomas also yielding a positive result. The latter was positive in areas exhibiting squamous differentiation. The second part of their study compared a further 20 cases of predominantly peritoneal EM with 60 extra-pulmonary adenocarcinomas. Again all EM cases were positive, as were many of the adenocarcinomas. In particular 80% of ovarian carcinomas were also ME-1 positive. An antibody suitable for use on paraffin-embedded tissue (HBME-1) was subsequently produced by Battifora and colleagues. An early study by Bateman and colleagues compared HBME-1 staining in 17 mesotheliomas and 14 adenocarcinomas. They reported 100% HBME-1 positivity in the mesotheliomas, but 10 of the adenocarcinomas were also positive. Three of the adenocarcinomas were of pulmonary origin, and their results were not stratified according to tissue of origin. Hence this study has not been included in our meta-analysis. Since then a total of 14 other studies have reported their experiences using HBME-1 in paraffin-embedded tissue to distinguish between EM and PACA.

The majority of EM and epithelioid areas of BM stained strongly for HBME-1, in a predominantly membranous distribution in these studies. The incidence of positive staining ranged from 72-100%. Very few sarcomatoid mesotheliomas demonstrated any positive staining. A smaller number of mesotheliomas exhibited a degree of cytoplasmic staining, although this tended to be diffuse and weak. The results in adenocarcinomas were more variable. Whereas Ordóñez, Dahlstrom, Attanoos and Comin limited their studies to using PACA, other studies used carcinomas of varying origin. This makes it difficult to effectively evaluate HBME-1 as a discriminator. Positive staining with
HBME-1 in PACA ranged from 100%\textsuperscript{282} down to 42%.\textsuperscript{324} In most cases this was cytoplasmic and weak.

Dahlstrom and colleagues performed the most thorough evaluation of HBME-1 in mesothelioma.\textsuperscript{324} They compared results for similar numbers of mesothelioma (n=26) and PACA (n=27) using the Dako HBME-1 antibody at four different dilutions with antigen retrieval technique (Dako Corporation, Carpinteria, CA, USA). They also utilised gold immunostaining techniques with electron microscopy to better characterise the nature of the HBME-1 positive staining in both groups.

The overall sensitivity and specificity of HBME-1 for mesothelioma compared with PACA were 85% and 43% respectively when the results of these 14 studies are combined. These are based on a total of 769 mesotheliomas (EM / BM) and 676 PACA. HBME-1 was not routinely used as part of the diagnostic panel for mesothelioma in our department during the period under study.

**Thrombomodulin (CD141)**

Thrombomodulin (TM) is a glycoprotein of 75 kD that is expressed by endothelium, mesothelium, synovium and placental syncytiotrophoblasts.\textsuperscript{253} It has anticoagulant activity through its effects on protein C and other elements of the coagulation cascade, and can bind thrombin. It was first described in 1982\textsuperscript{325} and its value in the recognition of vascular tumours was soon recognised.\textsuperscript{326} Collins and colleagues\textsuperscript{253,277} were the first to investigate the expression of TM in mesothelioma. They found it to have excellent sensitivity (100%) and specificity (92%) for EM compared with adenocarcinoma. Thrombomodulin has subsequently been evaluated in a further 15 studies, but with less impressive results.\textsuperscript{213,217,219,227,228,237,238,262,280-283,312,319,320,322} Combining the results of these 17 studies indicates that the sensitivity and specificity of thrombomodulin for mesothelioma is poor, at 61% and 80% respectively. This is based on a total of 964 cases of PACA and 831 cases of EM.

The incidence of TM expression in ACA appears highly variable, ranging between 6%\textsuperscript{213,281} and 87%\textsuperscript{280} which limits its usefulness as a diagnostic antibody. Why such varied results have been reported remains uncertain but there are some possible explanations.

Firstly, thrombomodulin expression may be heterogenous within a tumour section, and is probably influenced by local cytokine expression.\textsuperscript{212} Secondly, although normal bronchial epithelium does not usually express TM, it has been demonstrated in areas of squamous metaplasia.\textsuperscript{217} It is therefore possible that some of the TM positive tumours in these series were actually poorly differentiated squamous or adeno-squamous tumours.
N-Cadherin
The diagnostic value of cell adhesion molecules, and in particular the cadherins has been previously mentioned. Mesothelium is of mesodermal origin and therefore expresses N-cadherin. An initial study by Peralta-Soler and colleagues using frozen section material, confirmed that N-cadherin showed excellent diagnostic sensitivity and specificity (both 100%) when used to distinguish between EM and PACA. The recent introduction of an antibody suitable for use with paraffin-embedded tissue has enabled further study of N-cadherin. Han and colleagues also found N-cadherin to have high sensitivity and specificity for mesothelioma (100% and 94% respectively). Thirkettle and colleagues reported slightly less impressive results for N-cadherin, at 90% sensitivity and 83% specificity for EM in a study that was relatively small, with 29 mesotheliomas but only 6 PACA studied. More recently Abutaily and colleagues reported N-cadherin sensitivity and specificity as 74%. Ordonez has reported more disappointing results, with a sensitivity of 63% and a specificity of 82%. The combined result of these five studies implies an overall sensitivity of 78% and specificity of 84%, which is less impressive than the early studies suggested. Whether better results will be seen when greater numbers of cases are studied remains to be seen.

WT1
The role that oncogenes and tumour gene products such as p53 and WT1 play in the pathogenesis of mesothelioma has already been discussed in Chapter 1 (Section 1.12, p19). As well as being implicated in the pathogenesis of mesothelioma the Wilms tumour gene product has also been investigated as a potential diagnostic marker for mesothelioma. WT1 is expressed in fetal spleen, mesothelium and mesonephric ridge derivatives such as the kidney. In the adult WT1 continues to be expressed by mesothelium, spleen, the glomerular cells of the kidney, testicular Sertoli cells, uterine decidual cells, granulosa cells of the ovary and myoepithelial cells in the breast. WT1 positivity can also be seen in stromal cells and blood vessels. The value of WT1 as a diagnostic marker for mesothelioma in tissue samples has been investigated by eight groups. Initial results using frozen section material were encouraging. Amin and colleagues were able to demonstrate WT1 nuclear positivity in 20 out of 21 samples of mesothelioma. These results were supported by Kumar-Singh et al who found all 50 samples of mesothelioma studied were positive for WT1 when a mixture of anti-WT1 monoclonal antibodies were used. A subsequent study by Oates and Edwards was less
encouraging. In a comparative study that primarily examined differences in IHC staining between biopsy and post-mortem tissue samples, 18 of 42 (43%) cases of mesothelioma demonstrated WT1 positivity. In particular they found that none of the post-mortem samples stained with WT1 and that biopsy sample results were highly dependent on fixation technique. This study utilised the polyclonal Santa Cruz WT1 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA). More recently Foster and colleagues reported WT1 expression in 42 of 50 cases of EM, and Ordóñez described WT1 expression in 36 of 50 cases of EM, both groups also using the Santa Cruz polyclonal antibody. A recent small study of cell blocks by Hecht et al reported that WT1 was 100% sensitive and specific for mesothelioma. This study utilised the 6F-H2 clone (Dako, Carpinteria, CA), one of the antibodies also used by Kumar-Singh et al.

Overall WT1 has a sensitivity of 77% and specificity of 96% in distinguishing between EM (n=264) and PACA (n=213) in tissue sections. Potential problems relating to WT1 expression when renal and ovarian tumours form part of the differential diagnosis are discussed later in Section 3.10, page 122.

3.9: Miscellaneous Immunohistochemical Markers

Several other antibodies have been investigated for their potential to differentiate between EM and PACA. Epithelial membrane antigen and human milk fat globulin (HMFG) are related members of a family of high molecular weight trans-membranous glycoproteins. Epithelial membrane antigen is positive in both EM and PACA: It is the distribution of EMA that differs between them. In mesothelial cells EMA is located predominantly on the cell surface, in association with microvilli. By contrast EMA is located within the cytoplasm in adenocarcinoma. Human milk fat globulins, and HMFG-2 in particular, share a similar pattern of distribution to EMA. Both EMA and HMFG stain PAS-positive material, implying a common antigenic moiety. Almost 50% of anaplastic (CD30+) large cell lymphomas are positive for EMA, and EMA positivity has also been described in the epithelioid areas of synovial sarcomas and epithelioid sarcomas. Although EMA and HMFG-2 have been evaluated as discriminators between EM and PACA, it is difficult to make direct comparisons between papers because of differences in methodology and assessment of positive staining. I have therefore not discussed them in any more detail in this thesis.

Other antibodies that have been assessed include the HA receptor CD44S, 222, 227, 262, 282, 312, 333-335 IOB-3, 336 OV632, 292, 323, 337 SM3, 217 hepatocyte growth factor/scatter factor, 296
antibodies to the anti-tyrosine kinase receptor met and erbB-2, mesothelin (K1), 44-3A6, and AMAD-1 and -2. None of these have been shown to have reproducible diagnostic value above and beyond the antibodies already discussed, and have been studied in small numbers only.

3.10: IHC in the Distinction Between EM, PACA and Other Epithelioid Pleural Tumours

Carcinomas of pulmonary and non-pulmonary origin commonly metastasize to the pleura. Indeed the incidence of non-mesothelioma related malignant pleural effusions is much higher than those caused by mesothelioma. Lung cancer and lymphoma account for the majority of cases. Common extra-pulmonary sources include breast and ovary in women, prostate in men, and colon and kidney in both sexes. The presence of diffuse DPAS expression and the judicious use of appropriate carcinoma and hormone markers will often resolve any diagnostic difficulty if the patient is known to have a personal history of carcinoma at another site. More problematic is the presence of a malignant pleural effusion without an obvious extra-thoracic primary site. In these cases the combination of clinical i.e. patient examination and radiological investigations may be of value. A summary of the usual IHC profile of EM and its common differentials can be found at the end of this section (Table 3.9, page 126).

Carcinoma of the Breast and Ovary

Carcinoma of the breast and ovarian carcinoma are both considerably more common than mesothelioma in women. Both can metastasize to the pleura, thereby entering into the differential diagnosis of EM. Trans-diaphragmatic spread of mesothelioma into the peritoneum is well-recognised in advanced cases, and two of the major differentials of peritoneal mesothelioma in women are also ovarian carcinoma and metastatic breast cancer. Hence differentiating between these three tumours on clinical and radiological grounds can be difficult.

Immunohistochemistry still has a valuable role in this area, but the IHC profile of breast and ovarian carcinoma can differ considerably from that of pulmonary ACA. For example the proportion of breast and ovarian carcinomas expressing CEA is much smaller than that described in PACA, hence the inclusion of monoclonal CEA in a diagnostic panel is of limited value. By contrast Ber-EP4 is highly expressed in ovarian carcinoma (> 90%) and MOC-31 remains a highly sensitive and specific discriminator (both >
Although several studies have addressed the subject of IHC distinction between ovarian carcinoma and peritoneal mesothelioma, few have evaluated mesothelioma marker expression, specifically CK5/6, calretinin and WT1, in metastatic breast or ovarian cancer.

CK5/6 expression has been described in a small but significant proportion of ovarian serous carcinomas: 22% (Attanoos et al.), 24% (Ordoñez), and 33% (Ordoñez). Its expression in breast cancer is less common: 9% (Cury et al.) and 5% (Ordoñez).

Calretinin appears to be a better discriminator. In his initial paper describing calretinin distribution Doglioni found calretinin expression in 11% of ductal breast cancers, most of which was in a minority of cells. Only one case of ovarian serous carcinoma exhibited weak positivity (7% cases), however calretinin positivity was seen in a small number of granulosa cell, Sertoli cell and Leydig cell ovarian tumours. This last observation has been confirmed by other groups, who have described 100% calretinin positivity in ovarian sex-cord-stromal tumours. Ordoñez reported calretinin positivity in 9% of ovarian carcinomas whilst none of the cases of ovarian carcinoma (n=23) studied by Attanoos et al were calretinin positive.

WT1 is of less value when either breast or ovarian carcinoma is in the differential diagnosis. Ovarian serous carcinomas express WT1 in over 85% of cases. Breast carcinoma has been reported as expressing WT1 in more than 70% of cases. It would therefore seem prudent to recommend that WT1 positivity be viewed with caution in tumours from women unless combined with other relevant IHC markers e.g. oestrogen (ER) and progesterone (PR) receptors, and a clinical exclusion of primary tumours of the breast or ovary.

Renal Cell Carcinoma

Renal cell carcinoma can be difficult to confidently differentiate from EM as they share a similar IHC profile (CEA/Ber-EP4 negative, CK positive, vimentin positive). Renal cell carcinoma may express many different patterns of differentiation, which include clear cell areas. However clear cell variants of EM have been reported and clear cell change has also been described in chondro- and osteosarcomas. Renal cell carcinomas may also contain areas of sarcomatoid differentiation resembling SM.

The ability of IHC to distinguish between mesothelioma and RCC has not been investigated by many groups. In 1995 Attanoos and colleagues studied the ability of Ber-
EP4, Leu-M1, thrombomodulin and Tamm-Horsfall protein to distinguish between mesothelioma and RCC. They concluded that Leu-M1 was the best discriminator with a sensitivity and specificity of 70% and 95% for RCC respectively. In his original description of calretinin immuno-localisation, Doglioni reported that all four RCC studied were calretinin negative. More recently Osborn and colleagues studied the IHC profile of 37 cases of mesothelioma with epithelioid differentiation and 40 cases of RCC with five antibodies commonly used in mesothelioma panels (CEA, Ber-EP4, CK5/6, thrombomodulin and calretinin). They concluded that calretinin was the most useful antibody, staining 97% of mesotheliomas and only 10% of RCC cases. CK5/6 positivity was seen in 78% of mesotheliomas and only 5% of RCC. None of the other antibodies had significant discriminatory value. The value of CK5/6 has been independently confirmed by Chu and Weiss, who found no positive CK5/6 staining in a series of 19 RCC, and Ordoñez who reported negative staining in 10 cases of RCC.

Although WT1 expression has been extensively investigated in paediatric renal tumours, expression in RCC has been infrequently studied. Overall WT1 positivity has been described in six out of a total of sixteen cases. It would seem prudent therefore to avoid the use of WT1 if RCC is a differential and use calretinin and CK5/6 instead. It is also important that a renal origin is excluded by appropriate radiology e.g. renal ultrasound or abdominal CT whenever RCC enters into the differential diagnosis.

Metastatic Melanoma

In common with renal cell carcinoma and mesothelioma, malignant melanoma is capable of many different patterns of differentiation. Although malignant melanoma rarely metastasizes to the pleura, it may cause diagnostic difficulty because the primary tumour may not be clinically evident or may have occurred many years previously. Furthermore metastatic melanoma deposits may have a different IHC profile from the original tumour. Malignant melanoma is usually CK negative and vimentin positive. Anomalous CK expression, predominantly CK8, has been described in some malignant melanomas, particularly in metastatic rather than primary tumours. The use of specific melanoma markers should be encouraged in cases where melanoma is a potential differential, as positivity for less specific markers such as S-100 has been described in mesotheliomas. A further point of distinction is that melanosomes should be identifiable on electron microscopy.
Thymoma and Lymphoma

Thymoma and several types of lymphoma can produce a clinical and radiological picture that can be difficult to differentiate from mesothelioma. Although thymoma has a distinctive light microscopy appearance with lobulation secondary to fibrous septal formation, this may be obscured in small biopsies. Rarely, adenocarcinoma can arise in the thymus. The epithelial areas of thymoma are usually positive for epithelial CK’s and Ber-EP4. Chu et al found that all eight thymomas in their series of epithelial tumours stained positively with CK5/6, a finding that needs to be considered if thymoma is in the differential of a pleural tumour.

Anaplastic large cell lymphoma can also enter the differential of pleural tumours, as discussed earlier in Section 2.13, page 60. This tumour is reactive with CD30 (Ki-1), and can also be positive for EMA, vimentin and Leu-M1. Interestingly, there has been a published case report of a mesothelioma that demonstrated strong CD30 positivity.

Vascular tumours

Vascular tumours (haemangioendothelioma, angiosarcoma and haemangiopericytoma) rarely metastasize to the pleura. They are most commonly seen in younger patients, particularly females, and therefore do not fall into the usual demographic profile associated with mesothelioma (older males with asbestos exposure). In common with MPM, these tumours can stain for both cytokeratins and vimentin. However the pattern and intensity of staining is different: EHE and angiosarcoma both exhibit very strongly vimentin positivity, whilst cytokeratin expression is often weak, focal, and restricted to those areas with an epithelioid appearance. Cytokeratin positivity in HPC is uncommon. All of these tumours usually stain with at least one vascular marker. The most commonly used are CD31, CD34 and von Willebrand factor. Of these, CD31 is reported as being the most specific and sensitive endothelial marker. It has the added advantage, unlike CD34, of not reacting with epithelioid sarcomas or solitary fibrous tumours of the pleura. It is therefore recommended that the vascular marker CD31 be used as part of the diagnostic antibody panel for EM whenever a vascular tumour is part of the differential.
<table>
<thead>
<tr>
<th>Tumour</th>
<th>CEA</th>
<th>Ber-EP4</th>
<th>CK5/6</th>
<th>Cal</th>
<th>EMA</th>
<th>WT1</th>
<th>Vim</th>
<th>CD34</th>
<th>Other Markers</th>
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<td>+ (C)</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>BG8 + TTF-1 + MOC-31 +</td>
</tr>
<tr>
<td>Breast CA</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>DPAS+ ER/PR + or - MOC-31 +</td>
</tr>
<tr>
<td>RCC</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Leu-M1 +</td>
</tr>
<tr>
<td>Ovarian CA</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>MOC-31 + HBME-1 +/-</td>
</tr>
<tr>
<td>Thymoma</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>CD99 +</td>
</tr>
<tr>
<td>Metastatic Melanoma</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>HBM-45+ CK8 +/- E &amp; N-cad +</td>
</tr>
<tr>
<td>Vascular Tumours</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>CD31 + WVF +</td>
</tr>
</tbody>
</table>

Footnote: + = normally positive, - = normally negative, +/- = Positivity described in 0-50% cases in some series. * = Sex cord ovarian tumours only. M = membranous staining, C= cytoplasmic staining, N = Nuclear staining. See text for tumour type and IHC antibody abbreviations.

**Table 3.9**

Summary of the Usual Immunohistochemical Profile of Epithelioid Mesothelioma and its Differentials
3.11: Algorithm for Differentiating Between EM and PACA

The introduction of novel antibodies that can aid in the distinction between EM and PACA has tended to expand the diagnostic panel employed by many pathologists. Although this may improve diagnostic accuracy, it has the disadvantages of being expensive and time-consuming. Studies that have assessed the contribution of IHC to the diagnosis of mesothelioma have reported conflicting results. Moch and colleagues found that only 25% of suspected cases of mesothelioma were confidently diagnosed using clinical factors, H&E and DPAS stains.\textsuperscript{360} The additional use of a panel of IHC antibodies increased the proportion confidently diagnosed to almost 80%. The most powerful discriminatory antibodies in their study were Ber-EP4, HEA-125, Anti-BGR antigens and CEA. Conversely, Betta and colleagues considered that 63% to 81% of prospective mesotheliomas could be diagnosed without the use of IHC.\textsuperscript{361} Using IHC improved the diagnostic rate to between 67% and 92%.

Problems may also occur if results for different antibodies conflict. The ideal panel would consist of a small number of complementary antibodies, some with high sensitivity, others with high specificity. This panel would at best identify all cases of mesothelioma, or at least act as a useful screening panel to guide further analyses. Several groups have attempted to extrapolate from their own IHC results to produce a small but robust antibody panel and/or associated algorithm to address this subject.\textsuperscript{228,267,281,254}

Brown \textit{et al} looked at the diagnostic value of combined results for epithelial markers.\textsuperscript{228} They found that a positive result for both CEA and B72.3 was 100% specific and 88% sensitive for ACA, whilst a negative result for both markers was 99% specific and 97% sensitive for EM.

Dejmek and colleagues performed a logistic regression analysis to evaluate the diagnostic value of several antibodies.\textsuperscript{254} Their analysis suggested that the most useful pattern of IHC expression was the co-existence of:

1) Vimentin positivity in epithelioid areas \textit{and}

2) CAM5.2 positivity in sarcomatoid areas.
Using both of these criteria and the presence of any of the following three factors identified 77% of all mesotheliomas:

1) The co-expression of vimentin and CAM5.2
2) EMA membrane positivity
3) Negative staining for Leu-M1, Ber-EP4 or B72.3

Monoclonal CEA was the most valuable single antibody studied, as it was 100% specific for PACA. Including CEA staining results did not improve the diagnostic accuracy of their algorithm, but they suggested using this as an initial screening tool, and reserving their algorithm for CEA-negative tumours only.

Leers and colleagues advocate the use of Calretinin and E-cadherin as the most effective combination of IHC markers. They reported that a positive result for E-cadherin and a negative result for calretinin was 100% specific and 91% sensitive for pulmonary adenocarcinoma. Conversely, the combination E-cadherin negative, calretinin positive was 100% specific and 80% sensitive for mesothelioma.

Comin and colleagues suggested a four-antibody panel to diagnose mesothelioma:

Extrapolating from my analysis, I would suggest a different algorithm. Having identified the presence of an epithelioid pleural tumour on H&E sections, a DPAS stain should be performed. A diffusely positive DPAS is highly suggestive of adenocarcinoma and should direct further investigations towards identifying tissue of origin. These would include the use of specific cytokeratin, hormone or tumour markers depending on the mostly likely diagnosis according to patient sex, age and clinical factors. It should be remembered that CEA is usually negative in secondary carcinomas of the breast or ovary. A negative DPAS result would guide you towards a “mesothelioma panel”:

This would consist of two antibodies with the best sensitivity and specificity for PACA and EM respectively. Monoclonal CEA (97% specific, 81% sensitive) has repeatedly shown itself to be of great value in a large number of cases, and should therefore be recommended
as the antibody of choice. This should be combined with another antibody with better sensitivity such as MOC-31 or Lewis\textsuperscript{3}. Thyroid transcription factor-1 could be considered as an alternative, but is of less value when non-pulmonary carcinoma is a differential. The panel should also contain two antibodies that specifically identify mesothelioma. The best two antibodies for this appear to be N-cadherin and WT-1. At present calretinin seems less reliable, although it has been investigated in far greater numbers than N-cadherin and WT-1. It will be necessary to adapt this panel if ovarian or renal tumours are differentials. The presence of histological or clinical factors atypical for mesothelioma or adenocarcinoma should alert you to the possibility of other rarer differentials. This algorithm is shown in Figure 3.3 overleaf.
Algorithm for the Use of Immunohistochemistry in Distinguishing Between Epithelioid Pleural Tumours
3.12: IHC in the Distinction Between Mesothelioma with Sarcomatoid Areas and its Differentials

Far less attention has been paid to the role of IHC in characterising pleural tumours that exhibit areas of sarcomatoid differentiation. This is partly due to the fact that biphasic and sarcomatoid mesotheliomas are less common that their epithelioid counterpart, and therefore numerically at least, constitute less of a diagnostic problem. A biphasic pleural tumour has a small number of differentials, which includes mesothelioma, carcinosarcoma, biphasic synovial sarcoma and pleomorphic carcinoma of the lung. Pure sarcomatoid tumours involving the pleura are also uncommon. The main differentials of SM include primary and secondary sarcomas, vascular tumours, spindle cell carcinoma, malignant melanoma and solitary fibrous tumours (Figure 2.10, page 59). An important differential of desmoplastic mesothelioma is benign pleural fibrosis.

I therefore performed a second analysis, this time investigating the value of IHC in mesotheliomas with areas of sarcomatoid differentiation. The methodology was comparable to that described earlier for the EM vs PACA analysis (Section 3.3, page 91). Several of the papers that investigated IHC in mesothelioma and PACA included small numbers of biphasic and sarcomatoid tumours. I extracted individual results for these tumours wherever possible. There are also a small number of papers that have specifically investigated the value of IHC in distinguishing SM from other pleural tumours. In total I have identified 29 studies that reported specific results for BM and SM. Many of these were not comparing the IHC profile of mesothelioma with that of other tumours. These results cannot therefore be used to accurately calculate the diagnostic sensitivity and specificity, as there is no comparator. However, it can act as a crude surrogate marker for IHC expression in SM, for comparison with other pleural tumours, and the results of IHC expression in our own IHC cohort. The results of this analysis are summarised in Table 3.10 overleaf and compared with the IHC profile our own cohort in Figure 3.4, page 133.
<table>
<thead>
<tr>
<th>IHC Marker</th>
<th>Number of Studies Analysed</th>
<th>Number of SM Cases</th>
<th>Range of Positive Staining (%)*</th>
<th>Incidence of Positive Staining (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAM 5.2</td>
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<td>106</td>
<td>90 - 94</td>
<td>93</td>
</tr>
<tr>
<td>AE1/AE3</td>
<td>4</td>
<td>72</td>
<td>77 - 100</td>
<td>89</td>
</tr>
<tr>
<td>CK5/6</td>
<td>7</td>
<td>94</td>
<td>0 - 86</td>
<td>22</td>
</tr>
<tr>
<td>Vimentin</td>
<td>11</td>
<td>207</td>
<td>55 - 100</td>
<td>81</td>
</tr>
<tr>
<td>Calretinin</td>
<td>13</td>
<td>141</td>
<td>0 - 100</td>
<td>46</td>
</tr>
<tr>
<td>HBME-1</td>
<td>9</td>
<td>81</td>
<td>0 - 86</td>
<td>25</td>
</tr>
<tr>
<td>Thrombomodulin</td>
<td>7</td>
<td>92</td>
<td>16 - 71</td>
<td>37</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>3</td>
<td>22</td>
<td>86 - 100</td>
<td>95</td>
</tr>
<tr>
<td>WT1</td>
<td>5</td>
<td>43</td>
<td>0 - 83</td>
<td>37</td>
</tr>
</tbody>
</table>

*Range refers to results for each individual study, incidence is mean percentage of total cases showing positive staining for that antibody.

Table 3.10
Summary of Results of Published Studies Evaluating Immunohistochemical Staining in Mesotheliomas With Areas of Sarcomatoid Differentiation
Figure 3.4
Summary of the Results of Published Studies Evaluating IHC Staining in Mesotheliomas with Areas of Sarcomatoid Differentiation
3.13: Cytokeratin and Vimentin Expression in Pleural Tumours with Biphasic or Sarcomatoid Differentiation

Cytokeratin expression is a useful diagnostic feature when trying to correctly identify sarcomatoid pleural tumours. Cytokeratin expression in sarcomas can be either of two types. Some sarcomas are characterised by areas of true epithelial differentiation, that is to say, their epithelioid areas express epithelial markers. The classical examples of this are epithelioid sarcomas and synovial sarcomas. The other pattern of cytokeratin expression involves the production of anomalous cytokeratins. In these cases CK is expressed in areas that are not epithelioid in appearance. These are usually non-specific LMWCK such as CK8/18, and are seen in dot-like aggregations in a perinuclear distribution, usually in a small proportion of the tumour overall. Anomalous CK expression has been described in smooth muscle tumours, melanomas and vascular tumours. It may be enhanced by the use of heat-induced epitope retrieval techniques.

The patterns and types of broad-spectrum low molecular weight cytokeratin expression in BM and SM have been reported in eleven papers. Four of these studied CAM 5.2 expression, with a combined total of 106 tumours. The range of positive staining was similar in each individual group (90-94%). Overall CAM 5.2 was expressed in 99 tumours, corresponding to an overall frequency of 93%. Four other studies used the AE1/AE3 antibody in a total of 72 tumours, with positivity reported in 64 cases (89%). Lucas and colleagues studied the expression of a pan-cytokeratin antibody cocktail in sarcomatoid mesotheliomas and other sarcomatoid tumours. Their antibody cocktail contained CAM 5.2, AE1/AE3 and CK-904. The majority of sarcomatoid areas in BM (n=9, 90%) and cases of SM (n=7, 70%) demonstrated positive staining, albeit less strong than in epithelioid mesothelioma. A further two studies have reported CK expression using a pan-cytokeratin cocktail of antibodies, which included AE1/3, CAM5.2 and CK7. The incidence of Pan-CK expression was 83%.

In my IHC cohort both CAM5.2 and AE1/AE3 were studied. CAM 5.2 was examined in 61 cases of SM and 16 desmoplastic mesotheliomas. The majority of SM (n=55, 90%) were positive for CAM5.2, with a further five tumours (8%) exhibiting focal positivity. The antibody AE1/AE3 was employed in a smaller number of cases but with similar results. A total of 30 out of 34 cases of SM were positive (88%) with two other cases focally positive (6%). All cases of DM were positive for this antibody (n=5).
The intermediate filament that has most extensively been studied in SM is vimentin. There have been eleven published studies, which have included a total of 207 tumours.\textsuperscript{203, 210, 233, 236, 239, 240, 250, 262, 266, 280, 291, 309} Vimentin positivity was reported to range from 55% to 100%, with an average of 81% overall. Vimentin was studied in only a small number of cases in our cohort. Five of six cases of SM (83%) were diffusely positive, with focal positivity seen in the other case examined. All three cases of DM examined were positive.

3.14: Mesothelioma Marker Expression in Pleural Tumours with Biphasic or Sarcomatoid Differentiation

CK5/6

The expression of the CK5/6 antibody in sarcomatoid mesothelioma has been evaluated in seven studies that include a total of 94 tumours.\textsuperscript{201, 225, 233, 281, 313, 362, 364} CK5/6 positivity ranged from 0 to 86% for individual studies. The highest incidence was reported by Kayser et al with six of seven tumours CK5/6 positive. Interestingly, they also reported a relatively high incidence of CK5/6 positivity in the cases of PACA (40%) and EM (77%) within their study, which used the Zymed antibody.\textsuperscript{233} In contrast, four other groups have reported CK5/6 incidence of less than 20% in SM, despite an incidence of 70% or greater in epithelioid mesotheliomas assessed within the same study.\textsuperscript{201, 225, 281, 364} Indeed Lucas et al specifically comment on the marked and abrupt change in CK5/6 staining in adjacent epithelioid and sarcomatoid areas with the BM cases they evaluated.\textsuperscript{364} The overall incidence of CK5/6 positive staining is 22% when the results of all of these studies are combined. It would therefore appear that although of low sensitivity, a positive result for CK5/6 in a sarcomatoid tumour is supportive of a diagnosis of mesothelioma.

In our own cohort only a small number of SM (n=7) and DM (n=5) were studied with CK5/6 but with similar results. Four sarcomatoid (57%) and three desmoplastic tumours (60%) were CK5/6 positive, with focally positivity seen in one further case of SM.
Calretinin

Thirteen groups have studied calretinin expression in mesotheliomas showing sarcomatoid differentiation. They include a combined total of 141 mesotheliomas. Five of these have reported high levels of calretinin expression in SM. Chenard-Neu et al, Doglioni et al, Leers et al, and Meittenen et al, all reported 100% calretinin positivity, albeit in small numbers of cases, whilst Oates and Edwards report an incidence of 86%. Conversely, four other studies report calretinin positivity in less than a third of tumours. The mean incidence of calretinin positivity when all these studies were combined was 46%.

Meittenen et al, Attanoos et al, and Lucas et al have each independently evaluated calretinin expression in mesothelioma and other sarcomatoid pleural tumours. In the first of these studies calretinin expression was assessed in 103 synovial sarcomas, 30 mesotheliomas and a small number of other sarcomas. Calretinin positivity was seen in all mesotheliomas. Sarcomatoid areas within biphasic and sarcomatoid synovial sarcomas demonstrated focal calretinin positivity in a little over half of all cases (55%), with much less positivity in any epithelioid areas (14%). Two peripheral nerve sheath tumours demonstrated focal calretinin positivity but all other sarcomas were negative.

Attanoos and colleagues reported conflicting results. In their comparative study of 31 sarcomatoid mesotheliomas and a similar number of other spindle cell tumours, calretinin positivity was demonstrable in less than half of the mesotheliomas (n=12, 39%). However none of the other spindle cell tumours were positive for calretinin, suggesting low sensitivity but 100% specificity for mesothelioma. In a recently published paper Lucas and colleagues have also evaluated calretinin expression in sarcomatoid mesothelioma and its differentials. They studied IHC expression in ten cases each of EM, BM, SM, sarcomatoid carcinoma of the lung and 24 cases of other non-pleural sarcomas. WT1 and thrombomodulin were studied in addition to calretinin and cytokeratins, which have been discussed already earlier in this chapter. Nuclear and cytoplasmic calretinin positivity was seen in all cases of EM, and the majority of epithelioid areas in BM (n=9, 90%). The sarcomatoid areas in BM (n=6, 60%) and SM (n=7, 70%) were also calretinin positive in the majority of cases. However four cases of sarcoma (17%) and six of the sarcomatoid carcinomas (60%) also demonstrated calretinin positivity. In the sarcomas calretinin positivity was <5% in three tumours. Only two of the six sarcomatoid carcinomas exhibited strong and diffuse calretinin positivity. Interestingly, almost a third of the
sarcomatoid carcinomas were reported as showing extensive pleural and intra-pulmonary spread and four of the ten cases were also thrombomodulin positive. It could therefore be questioned as to how confident the authors were in the diagnosis of sarcomatoid carcinoma for the cases included in this study. The overall incidence of positive calretinin staining when all of these studies are considered was 46%. In our cohort calretinin was studied in a small number of sarcomatoid (n=4) and desmoplastic (n=6) tumours. We did not separately evaluate calretinin positivity in epithelioid and sarcomatoid areas within BM. Only one of the SM cases was positive for calretinin. Four of the DM tumours were positive for calretinin, with a fifth case showing focal staining only.

It therefore seems that calretinin is a useful adjunct to an IHC panel in epithelioid tumours, but that a positive result in biphasic or sarcomatoid tumours is not necessarily diagnostic of MPM. Calretinin positivity is uncommon in other sarcomatoid pleural tumours, and biphasic SS should always be considered as a differential diagnosis.

HBME-1, Thrombomodulin, N-Cadherin and Wilms Tumour Product-1

Nine groups have studied HBME-1 expression in sarcomatoid mesothelioma. Thrombomodulin expression in SM has been studied by seven groups. Thrombomodulin positivity was noted in 34 out of a total of 92 cases of SM (37%). Although studied by only three groups, and in a very small number of cases of SM (n=22), N-cadherin expression was reported in all but one tumour (95% expression). None of these studies simultaneously evaluated N-cadherin expression in other sarcomatoid pleural tumours so it is difficult to predict its diagnostic value. More recently Laskin and colleagues have studied cadherin expression in a series of sarcomatoid tumours with epithelioid features that included cases of mesothelioma and synovial sarcoma. They reported 100% expression of E-cadherin in the epithelioid areas of biphasic SS, with > 50% E-cadherin expression in cases of melanoma and monophasic sarcomatoid SS. By contrast only 20% of mesotheliomas were positive. When N-cadherin was considered, high levels of expression were seen in biphasic SS (86%), malignant melanoma (56%) and mesothelioma (70%). It would therefore seem likely that although N-cadherin is of great diagnostic value in differentiating mesothelioma from other epithelioid tumours, it may be less useful when synovial sarcoma is a differential. The appropriate use
of an epithelial marker such as Ber-EP4, or demonstration of the SYT/SSX gene product would help this distinction.

Wilms tumour product-1 has also been evaluated in only a small number of studies (n=5) and tumours (n=43).\textsuperscript{201, 221, 319, 329, 364} Overall incidence of WT-1 expression was 37%. Only one study reported no WT-1 positivity in any case of SM.\textsuperscript{201} WT-1 is therefore of limited value in the distinction of SM from other sarcomatoid tumours.

3.15: IHC Profile of Other Biphasic and Sarcomatoid Pleural Tumours

The differentiation between BM and other biphasic tumours may in part be possible by the consideration of clinical factors. Many sarcomas have an obvious extra-thoracic primary site and are most common in a much younger group of patients. Pleomorphic carcinoma and carcinosarcoma characteristically produce discrete intrapulmonary masses, at least in their early stages, rather than the diffuse pattern of growth seen in mesothelioma. Immunohistochemistry is still a useful diagnostic adjunct. The usual pattern of IHC expression in BM, SM and their differentials are discussed in this section and summarised in Table 3.12, page 141.

Pleomorphic Carcinoma

Pleomorphic carcinoma of the lung is characterised by the presence of spindle cells and/or giant cells. These areas may comprise only a small area of the tumour overall, but if seen in a limited biopsy specimen may effectively obscure the overall pattern of differentiation. Pleomorphic or spindle areas within an adenocarcinoma or poorly differentiated squamous carcinoma may therefore resemble biphasic mesothelioma. Immunohistochemistry is of limited value in distinguishing PC from MPM. Both epithelial and sarcomatoid elements of PC express cytokeratins.\textsuperscript{198} Vimentin can also be expressed by both elements. Pleomorphic areas within adenocarcinoma may express CEA or Ber-EP4.

As a part of my analysis of the ability of IHC to distinguish between MPM and other tumours, I undertook a small study to evaluate CK5/6, calretinin and TTF-1 in mesothelioma and pleomorphic carcinoma. Sixteen mesotheliomas that had previously been identified as showing areas of pleomorphism were chosen from our cohort. The cases of PC were identified from Professor Hasleton’s own records, and re-examined to confirm that they fulfilled the WHO diagnostic criteria.\textsuperscript{166} Each tumour was stained for each of the
three IHC antibodies using our standard laboratory protocol and with appropriate positive and negative controls (see Appendix One, page 265). Individual diagnostic sensitivity and specificity were calculated for each antibody. A small number of the PC cases initially gave equivocal IHC results: unfortunately we did not possess enough tissue to repeat the IHC for every case. Hence each antibody was studied in a different number of pleomorphic carcinomas. The results are shown in Table 3.11 below.

Only calretinin differentiated between these two tumours with any reliability, with a sensitivity of 81% and a specificity of 94% for mesothelioma. CK5/6 was positive in almost half the cases of PC, and three quarters of mesotheliomas. None of the cases of PC were positive for TTF-1, a disappointing result given its high specificity for other pulmonary tumours.

<table>
<thead>
<tr>
<th></th>
<th>Incidence of Positive CK 5/6 Staining</th>
<th>Incidence of Positive Calretinin Staining (%)</th>
<th>Incidence of Positive TTF-1 Staining (%)</th>
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</thead>
<tbody>
<tr>
<td>Mesothelioma</td>
<td>75%</td>
<td>81%</td>
<td>0%</td>
</tr>
<tr>
<td>(n=16)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pleomorphic Carcinoma</td>
<td>41%</td>
<td>6%</td>
<td>0%</td>
</tr>
<tr>
<td>(Total n=17)</td>
<td>(Total n=16)</td>
<td>(Total n=23)</td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>75%</td>
<td>81%</td>
<td>0%</td>
</tr>
<tr>
<td>Specificity</td>
<td>59%</td>
<td>94%</td>
<td>0%</td>
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</tbody>
</table>

Table 3.11
Diagnostic Ability of CK5/6, Calretinin and TTF-1 to Distinguish Between Pleomorphic Mesothelioma and Pleomorphic Carcinoma
Carcinosarcoma

Carcinosarcomas are biphasic lung cancers composed of epithelioid areas of squamous differentiation, and a sarcomatoid component that resembles fibrosarcoma with prominent heterologous elements. The sarcomatoid elements are true mesenchymal derivatives and as such do not express cytokeratins. Desmin and actin may be seen in association with the heterologous elements.

Biphasic and Monophasic Sarcomatoid Synovial Sarcoma

Synovial sarcoma is one of the few soft tissue tumours capable of true epithelial differentiation. Hence the epithelial elements in biphasic SS are positive for epithelial markers such as CEA, Ber-EP4 and E-cadherin. Epithelioid areas will also show electron microscopy features consistent with an epithelial origin, namely short microvilli with glycocalyx bodies. Calretinin has been studied in only a small number of cases of SS but appears to be infrequently expressed. The other IHC markers that are of diagnostic value in confirming the diagnosis of SS are CD99 (p30/32) and bcl-2 which are commonly positive in SS but negative in SM. CD34 is usually negative in SS, which serves to distinguish it from SFT of the pleura and epithelioid sarcomas.

Epithelioid sarcoma

Although epithelioid sarcomas (ES) can demonstrate true epithelial differentiation (CK positivity), sarcomatoid areas are usually CK negative. There may be desmin or actin positivity, which is less commonly seen in MPM. Epithelioid areas of ES rarely express E-cadherin but more than 50% express CD34.

Solitary fibrous tumours

Solitary fibrous tumours are another important differential of both sarcomatoid and desmoplastic mesothelioma. Solitary fibrous tumours are typically cytokeratin negative, reflecting their probable origin from submesothelial rather than mesothelial cells. The most valuable IHC marker for confirming the diagnosis of SFT is the vascular marker CD34. Although positive in vascular tumours, gastrointestinal stromal tumours and some sarcomas, CD34 does not label mesothelioma and as such the combined use of this and cytokeratin should confirm the diagnosis.
<table>
<thead>
<tr>
<th>Tumour</th>
<th>CEA</th>
<th>Ber-EP4</th>
<th>CK</th>
<th>Cal</th>
<th>EMA</th>
<th>WT1</th>
<th>Vim</th>
<th>CD34</th>
<th>Other Markers</th>
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<tr>
<td>BM</td>
<td>E -</td>
<td>E -</td>
<td>E +</td>
<td>E +</td>
<td>E + (M)</td>
<td>E +</td>
<td>E +</td>
<td>E -</td>
<td>E N-cad +</td>
</tr>
<tr>
<td></td>
<td>S -</td>
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<td>S +/</td>
<td>S +</td>
<td>S +/</td>
<td>S -</td>
<td>S +</td>
<td>S -</td>
<td>S N-cad +</td>
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<td>E -</td>
<td>E &amp; N-cad +</td>
</tr>
<tr>
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<td>S +/</td>
<td>S -</td>
<td>S -</td>
<td>S -</td>
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<td>S -</td>
<td>S -</td>
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</tr>
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<td>Desmin +</td>
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<td>S +</td>
<td>S -</td>
<td>Actin +</td>
</tr>
<tr>
<td>ES</td>
<td>E +</td>
<td>E +</td>
<td>E +</td>
<td>E</td>
<td>E +</td>
<td>E -</td>
<td>E +</td>
<td>E +</td>
<td>E-cad -</td>
</tr>
<tr>
<td></td>
<td>S -</td>
<td>S -</td>
<td>S -</td>
<td>S</td>
<td>S -</td>
<td>S -</td>
<td>S -</td>
<td>S -</td>
<td></td>
</tr>
<tr>
<td>SM</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>CK5/6 +/-</td>
</tr>
<tr>
<td>SFT</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Desmin Focal</td>
</tr>
</tbody>
</table>

Footnote: + = normally positive, - = normally negative, +/- has been described as positive in 0-50% cases in some series. E = epithelioid areas of biphasic tumours. S = sarcomatoid areas of biphasic tumours. M = membranous staining, C = cytoplasmic staining, N = Nuclear staining. See text for tumour type and IHC antibody abbreviations.

Table 3.12
Summary of the Usual Immunohistochemical Profile of Biphasic and Sarcomatoid Mesothelioma and their Common Differentials
3.16: The Role of Immunohistochemistry in Differentiating Between Benign and Malignant Mesothelial Proliferations

The final diagnostic consideration when studying pleural pathology is the differentiation between benign and malignant mesothelial proliferations. In some ways this is one of the most important distinctions to have to make, as the repercussions of an incorrect diagnosis can be far more devastating for a patient. I have already discussed the pathophysiology and histopathology of pleural inflammation and emphasized how histological changes can be difficult to differentiate from those of malignancy. This can be particularly challenging in the presence of infection (empyema) or when dealing with desmoplastic tumours, in which a bland appearance belies their aggressive invasive behaviour and poorer prognosis.

Immunohistochemistry is of limited value in this area as both benign and malignant mesothelial proliferations have similar IHC profiles. Staining with LMWCK can emphasize the distribution of mesothelial cells within stroma and highlight areas of invasion with are critical in confirming the diagnosis of malignancy.\(^{140}\) Similarly the use of macrophage markers (CD68) may help to better define cellular areas associated with an inflammatory infiltrate.\(^{370}\)

Several IHC antibodies have been evaluated for their ability to distinguish between benign and malignant mesothelial proliferations. The most extensively investigated include p53 and EMA. Other potential diagnostic markers include p-170, desmin, bcl-2, and the gene products \textit{c-fos} and \textit{c-myc}.\(^{140}\) Another diagnostic technique that has been studied is the quantitative distribution of silver-labelled nucleolar organiser regions (AgNOR).\(^{140}\) A summary of the results for these various diagnostic methods is shown in Table 3.13, page 146 and depicted graphically in Figure 3.5, page 147.

**p53**

Changes in the tumour suppressor gene p53 have been implicated in the origin in many human cancers including malignant mesothelioma. The normal ("wild-type") form of p53 has a very short half-life and is therefore difficult to demonstrate immunohistochemically, unless its degradation mechanisms have been altered, or subjected to high levels of cellular stress. Mutated forms of p53 are resistant to normal degradation pathways, and therefore accumulate in the nucleus in quantities that are proportional to the cells proliferation rate. The immunohistochemical demonstration of p53 is therefore suggestive, but not diagnostic
of malignant proliferation. Anti-p53 antibodies are sensitive to differences in fixation techniques, and are most reliably demonstrated in small tissue sections or cytological preparations.

p53 has been investigated as a marker for malignancy in mesothelioma by 13 groups, of which ten have also assessed its incidence in benign mesothelial proliferations. In 1992 three different groups reported the results of their analyses of p53 in benign and malignant mesothelial tissue. Kafiri and colleagues studied the CM-1 antibody in 20 cases each of reactive pleuritis and malignant mesothelioma. None of the cases of reactive pleura, and 14 of the 20 (70%) mesotheliomas were positive for p53. Ramael and colleagues studied three different antibodies (CM-1, DO7 and pAb240) directed against p53 in 40 cases of RP and 36 mesotheliomas. Twenty-five per cent of the mesotheliomas were positive for p53 with both CM-1 and DO7. All of the reactive pleura were negative for p53, and no cases of either type were positive with pAb240. Mayall and colleagues studied p53 in 47 cases of mesothelioma of different histological subtypes and 20 cases of reactive pleura using both CM-1 and DO7. The incidence of p53 staining was 45% in mesothelioma, whilst no cases of reactive pleura were positive.

Since then a further seven groups have evaluated p53 in both benign and malignant mesothelial tissue. Three other groups have reported the incidence of p53 staining in mesothelioma without comparison to other disease processes.

Combining their results suggests that the incidence of p53 staining in mesothelioma is 57% (range 25-100%). The sensitivity of p53 is 58% and the specificity is 90% when used to distinguish between mesothelioma (n=416) and benign mesothelial proliferations (n=193).

Epithelial Membrane Antigen

Epithelial membrane antigen has been discussed regarding its ability to distinguish between PACA and EM earlier in this chapter. It has also been evaluated as a discriminator between benign and malignant mesothelial processes by five different groups. Although EMA can be demonstrated on the microvilli of normal mesothelial cells, the intensity of staining is much less than that seen in mesothelioma. The incidence of positive staining for EMA in reactive pleuritis was less than 20% in four of these studies.
but over 50% in the fifth. Overall EMA has a sensitivity of 74% and a specificity of 88% when compared in RP (n=170) and mesothelioma (n=353).

**Desmin**

Desmin is an intracellular intermediate filament that can be characteristically demonstrated in smooth and skeletal muscle. It has also been described in non-myogenous tumours, including primitive neuro-ectodermal tumour, Wilms tumour and mesothelioma. Three groups have specifically evaluated desmin in terms of its ability to distinguish between mesothelioma and reactive pleuritis. These studies included a total of 114 mesotheliomas and 60 cases of RP. The sensitivity and specificity of desmin for mesothelioma were equal at 83%.

**Bcl-2 and p-170**

Bcl-2 is a protein that has anti-apoptotic activity, and its over-expression in carcinomas is associated with a poorer prognosis. Three groups have studied the expression of bcl-2 in mesothelioma (n=108) and reactive pleuritis (n=93). Bcl-2 positivity was seen in 5% of mesotheliomas and no cases of RP.

p-170 is the glycoprotein product of the mdr 1 gene. It has been studied in mesothelioma and RP by three groups and found to have a sensitivity of only 45%, although specificity is better at 97%.

**AgNOR and MCM2**

Nucleolar organizer regions are argyrophil acidic proteins associated with areas within chromosomes 13, 14, 15, 21 and 22 that code for ribosomal RNA. An increase in the number of NOR’s may therefore reflect an increase in transcriptional or proliferative activity. The use of silver-labelling facilitates the recognition and counting of NOR’s within the nucleus. An increase in AgNOR numbers has been shown to be of value in distinguishing between benign and malignant lymphoid and breast tissue, and has also been studied in mesothelial tissue.

Aires and colleagues also studied AgNOR in cases of normal pleura, reactive pleuritis and mesothelioma. They found an increase in AgNOR incidence in the
malignant tumours (mean 5.5/ nucleus) compared with normal (mean 1.0) and inflammatory pleura (mean 1.8). There was no overlap in the range of values for all three tissues and the difference in mean AgNOR numbers reached statistical significance. In a later study Soosay and colleagues studied AgNOR frequency in nine samples of reactive mesothelium and seven mesotheliomas. The mean number of AgNOR’s per nucleus was 1.9 (range 1.2 – 4.2) in reactive pleura and 3.7 (range 1.6 – 5.2) in MPM respectively. Eight cases of PACA were also studied, with a mean number of AgNOR’s of 4.7 (range 2.4 – 8.1). The degree of overlap between the values for benign and malignant tissue reduced its discriminatory value overall.

Henderson and colleagues have also investigated the diagnostic value of AgNOR labelling, but using a different analysis methodology. They assessed the proportion of the nucleus that was occupied by AgNOR-positive material and expressed this as an area (μm²). They reported a diagnostic sensitivity of 95% for distinguishing between reactive and malignant mesothelium when AgNOR labelling and the use of EMA were combined.

Another marker of cell proliferation that has been assessed in this area is MCM2. The demonstration of increased levels of MCM2 is strongly associated with dysplasia and neoplasia. Unfortunately, as with AgNOR, there is significant overlap between benign and malignant tissue.
<table>
<thead>
<tr>
<th>IHC Marker</th>
<th>Number of Studies Analysed</th>
<th>Number of Mesothelioma Cases</th>
<th>Number of Reactive Pleuritis Cases</th>
<th>Sensitivity of Antibody for MPM (%)</th>
<th>Specificity of Antibody for MPM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>10</td>
<td>416</td>
<td>193</td>
<td>58</td>
<td>90</td>
</tr>
<tr>
<td>EMA</td>
<td>5</td>
<td>353</td>
<td>170</td>
<td>74</td>
<td>88</td>
</tr>
<tr>
<td>Desmin</td>
<td>3</td>
<td>114</td>
<td>60</td>
<td>83</td>
<td>83</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>3</td>
<td>108</td>
<td>93</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>p-170</td>
<td>3</td>
<td>154</td>
<td>61</td>
<td>45</td>
<td>97</td>
</tr>
<tr>
<td>AgNOR</td>
<td>2</td>
<td>32</td>
<td>19</td>
<td>6.4</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Table 3.13
Summary of the Results of Published Studies That Have Evaluated the Diagnostic Value of Immunohistochemical Markers in Distinguishing Between Benign and Malignant Mesothelial Proliferations
Figure 3.5
Histogram Depicting Sensitivity and Specificity of IHC Antibodies for Distinguishing Between Benign and Malignant Mesothelial Proliferations
CONCLUSIONS

It has long been realised that the distinction between mesothelioma and other tumours involving the pleura poses a diagnostic challenge. The acceptance of the mesothelium as the tissue of origin for malignant mesothelioma paved the way for consideration of specific histochemistry, and subsequently immunohistochemistry, as an adjunct to the diagnostic process. Indeed this subject has produced dozens of papers over the last twenty years. Unfortunately, although much has been learned from these studies we are still left without any IHC antibody that is reproducibly 100% sensitive and specific for mesothelioma compared with other pleural tumours. It is therefore essential that IHC is used to complement rather than override both clinical and histopathological factors.

The tendency to use an ever-expanding panel of diagnostic antibodies is not one which should be encouraged. This is time-consuming and expensive, and the greater the number of antibodies, the greater the difficulty in interpretation if conflicting results are found. There is a wealth of evidence, in large numbers of tumours, for the diagnostic efficacy of established IHC antibodies such as monoclonal CEA. Newer diagnostic antibodies such as TTF-1, the cadherins and WT1 show promise, but have so far only been studied in small numbers of tumours. It is often the case that the first few published studies of new diagnostic antibodies report high levels of sensitivity and specificity. As other groups begin to use these antibodies the results are often not reproducible, or less impressive. This is likely to reflect the time and effort taken to perform initial studies by enthusiasts, which is less sustainable in everyday working practice, and in laboratories where basic IHC staining techniques may be subtly different.

The use of a small but robust IHC panel, chosen after consideration of the most likely differentials of any given pleural tumour, is to be encouraged. Initial screening with DPAS or an antibody that is highly specific for the most likely differential is logical, and may limit the need for other antibodies. Individual laboratories should use antibodies that they are most familiar with, and have consistently reliable and reproducible results, in preference to newer antibodies. It should never be forgotten that the H&E sections may be the most valuable diagnostic tool, and that IHC should be used to complement and confirm the preferred diagnosis, rather than over-riding clinical and histological evidence.
CHAPTER FOUR
ANALYSIS OF PROGNOSTIC FACTORS AND SURVIVAL IN MALIGNANT PLEURAL MESOTHELIOMA

INTRODUCTION
Several aspects of the aetiology and epidemiology of MPM are now well-defined: the causative link with amphibole asbestos, the long interval from exposure to presentation, and the overall poor prognosis with little response to most anti-cancer treatments. What is less certain is whether, and to what degree, the progression of mesothelioma is affected by patient-related factors. Although several reports examining patient factors and outcome in pleural mesothelioma have been published, the results of many studies have been inconclusive or contradictory. This is because mesothelioma is a relatively rare tumour, and small patient numbers limit the statistical power of many series. In particular the effects of age, sex, histology and asbestos exposure on survival remain uncertain.

The aim of this part of the thesis was to investigate relationships between epidemiology, pathology and other factors in patients with MPM, and to assess their influence on survival. This study was accomplished by means of a retrospective analysis of a cohort of mesothelioma patients from the North West Region of England.

METHODOLOGY
4.1: Identification of a Cohort of Patients with Mesothelioma
In Chapter Three (Section 3.1, page 86) I described how a cohort of patients with malignant pleural mesothelioma in the North West Region of England was identified. The same cohort of patients was utilised for this analysis of prognostic factors and survival.

4.2: Clinical Details and Follow-up
All available information relating to diagnosis, clinical course, occupation and outcome for every patient was extracted from the identifying source and recorded on an eight by five inch index card. Basic demographic details were cross-checked with the hospital Patient Administration System and dates of further hospital admissions or death noted. A second search of current and archival (1989 - 1993) pathology computer databases was carried out for all those patients listed as having Wythenshawe Hospital or Christie Hospital records, to check for any other histology reports not identified by the initial search. A search of the Christie hospital database was also conducted. Wythenshawe and Christie Hospital
medical case records were examined where available, and the pathology and medical record departments in other hospitals within the region contacted to complete the data collection. Patients admitted for investigation of suspected pleural malignancy to our surgical unit were identified, and their medical and occupational history prospectively recorded wherever possible.

4.3: Database Construction

The information collected on the index cards was collated and transferred on to a computer-generated spreadsheet (Microsoft® Excel 5.0). Twenty four different fields were created. Similar separate data sets were created for cases of peritoneal mesothelioma, pleural adenocarcinoma, reactive pleura and other non-mesotheliomatous pleural tumours. The database comprising only of cases of MPM was converted into a Statistical Package for Social Scientists (SPSS®) file to facilitate analysis. Data fields were as follows:

- Patient source e.g. in-patient, medico-legal referral
- Name
- Sex
- Hospital number
- Date of birth
- Date of first symptoms
- Date of histological diagnosis (or clinical diagnosis if no biopsy performed)
- Date of death or last follow-up
- Status (i.e. alive or dead at end of follow-up)
- Age at diagnosis
- Age at death
- Survival (defined as time from histological diagnosis to death in months)
- Cause of death
- Diagnostic and therapeutic interventions e.g. surgery, radiotherapy, chemotherapy
- Biopsy and PM reference numbers.
- Histological sub-type
- Presence of other pulmonary conditions or neoplasia
- Occupation (classified by to job most likely to have caused asbestos exposure)
- Type of asbestos exposure (e.g. occupational, environmental)
- Duration of asbestos exposure (years)
- Maximum and minimum interval from asbestos exposure to diagnosis (years)
4.4: Confirmation of the Diagnosis of Mesothelioma
The difficulties inherent in confirming the diagnosis of mesothelioma have already been discussed. Given that even expert panels disagree on a proportion of cases, it was thought likely that some of our cases might have been misdiagnosed. Review of the pathology of our cohort was undertaken, as detailed in Section 3.1, page 86.

RESULTS (PART I):
DESCRIPTION OF COHORT DEMOGRAPHICS AND OUTCOME
4.5: Excluded Cases and Cohort Demographics
A total of 567 patients was initially included on the database. Ten cases were referrals from abroad. These and four other cases were excluded because of a lack of clinical information. This left 553 patients for the main survival analysis. Almost half the cohort had been identified via Wythenshawe Hospital pathology records (47%, n=260). The remaining cases were fairly evenly distributed between medico-legal and Pneumoconiosis Medical Panel referrals (25.3%, n=140 and 28.4%, n=157) respectively.

   It was difficult to obtain a complete data set for all patients, particularly as many had never been in-patients at Wythenshawe Hospital or had missing medical case-notes. A minimum data set comprising age, sex, date of diagnosis, survival, histological subtype and occupational history was available in 381 cases (70% of total). The date of first symptoms and occupational history were the most difficult details to confirm in cases that were not under the Coroner’s jurisdiction.

4.6: Age and Sex Distribution
The cohort comprised 482 men (87.2%) and 71 women (12.8%). Age at diagnosis was known in 442 cases (80% of cohort). Missing data reflected either that the exact date of diagnosis could not be confirmed, or that the patient had been diagnosed at death. These results are summarised in Table 4.1 and Figure 4.1.
All Patients (n = 442) | Males (n=389) | Females (n=53)
--- | --- | ---
Mean age at diagnosis (years) | 62.4 | 62.3* | 63.4*
Range (years) | 37 - 86 | 37 - 86 | 43 - 82

*Unpaired T-test, p = 0.478

Table 4.1
Age and Sex Distribution in a Cohort of Patients with Mesothelioma

Figure 4.1
Age at Diagnosis in a Cohort of Patients with Mesothelioma Stratified by Gender
4.7: Symptoms and Signs at Diagnosis

The most common presenting symptoms in MPM are breathlessness (dyspnoea) and chest pain. These may be associated with constitutional symptoms, particularly weight loss and malaise. Frequent presenting signs include clinical and radiological evidence of pleural thickening, pleural effusion, and cachexia. Less commonly there may be evidence of local or distant metastatic spread.

Contemporaneous information regarding presenting symptoms was available for only 219 patients, representing 40% of the cohort. Information relating to clinical and radiological signs at presentation was better documented: This was available for 363 patients (66%). I made some assumptions when analysing this data. Patients were categorised according to whether pain, dyspnoea or constitutional symptoms were present, either singly or in combination. Patients who presented with less well-recognised symptoms, such as pneumothorax or the effects of distant metastases, were placed in an “atypical presentation” group, even if they also had symptoms of dyspnoea or pain. Occasionally, the medical records gave conflicting information on patient presentation. In these cases greatest weight was given to contemporaneous records, rather than reports written at a later date. In the absence of medical case notes, information from the histology request card written at the time of diagnostic biopsy was used as a surrogate marker for clinical data. It is accepted that this is potentially less reliable than using medical case notes, particularly as considerable delay may have occurred between the time of initial symptoms and biopsy. It may be less likely to quantify the relative severity of each symptom, and a significant number of forms had little or no clinical information recorded on them. Symptoms were not regarded as being mutually exclusive, and the absence of the reporting of a symptom was not taken to mean that this symptom was absent. If a patient was described as having "pleurisy" then it was assumed that they had both pain and dyspnoea.

Spontaneous pneumothorax is an unusual finding in mesothelioma: most studies report few patients presenting in this way. Spontaneous pneumothorax was categorised as an “atypical presentation”, despite the fact that it usually presents with symptoms of dyspnoea and pleuritic chest pain. We found that some patients only developed a pneumothorax after pleural aspiration or pleural biopsy. These pneumothoraces were assumed to be iatrogenic rather than spontaneous and not placed in the atypical presentation group.
A small number of our patients had mass lesions on CXR with or without evidence of pleural involvement or distal pulmonary collapse. Many of these were assumed to have primary bronchopulmonary tumours until histological material was made available. In many cases this was after death.

Dyspnoea was the most common presenting symptom in our cohort, being the sole symptom in 78 patients, and in combination with pain in a further 33. Pain was present in 92 patients. It appeared to be the sole symptom in 51 patients. Weight loss as the only symptom was reported by seven patients, whilst four patients reported pain, dyspnoea and constitutional symptoms. Thirty-six patients were placed in the atypical presentation category. The frequencies of each symptom are represented in Figure 4.2, and a sub-analysis and summary of the atypical presentation group is shown in Table 4.2 overleaf.

Abbreviations: SOB = dyspnoea, Const = constitutional symptoms.
### Table 4.2
Atypical Presenting Symptoms in Patients with Mesothelioma

<table>
<thead>
<tr>
<th>Presenting Symptom</th>
<th>Number of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pneumothorax</td>
<td>8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Metastatic Disease</td>
<td>7</td>
</tr>
<tr>
<td>Incidental / Radiological&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>Empyema&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>Other Respiratory Symptoms&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>Chest Wall Disease</td>
<td>3</td>
</tr>
<tr>
<td>Mediastinal Disease&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>“Acute Abdomen”&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>Anaemia</td>
<td>2</td>
</tr>
<tr>
<td>Diabetic Complications</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>36</strong></td>
</tr>
</tbody>
</table>

**Notes:**

- a: A further 3 patients developed pneumothorax during presenting illness.
- b: Pleural disease noted on chest X-ray taken for unrelated reasons.
- c: Temperature, malaise and signs of pleural thickening following chest infection.
- d: Cough (n=2), haemoptysis (n=1) in absence of any other respiratory symptoms.
- e: Recurrent laryngeal nerve palsy (n=1), superior vena caval obstruction (n=1).
- f: Presented to general surgeons with abdominal and chest signs.
The most common clinical signs noted at presentation reflected the pattern described in other series (see Table 4.18, page 204). Clinical and radiological evidence of pleural effusion alone was noted in 275 patients. In many cases radiological evidence of significant pleural or pulmonary disease was noted after the effusion was drained. A further 23 had an effusion and other signs, such as pleural thickening or a mass lesion on CXR. Thirty-six cases had radiological evidence of pleural thickening without effusion. Eight patients had clinical evidence of chest wall disease e.g. palpable chest wall metastatic deposits at the time of presentation. Four patients presented with signs of mediastinal invasion: two had pericardial effusions, neither of which was thought to be due to primary pericardial mesothelioma. The other two patients with mediastinal invasion presented with hoarseness (left recurrent laryngeal nerve palsy) and facial swelling (superior vena cava obstruction).

4.8: Diagnostic and Therapeutic Interventions

Once a patient has presented with a pleural effusion or other evidence of a possible intra-thoracic malignancy, there are several ways in which they may be investigated. Which tests are chosen will depend upon the clinical history, CXR appearances, patient fitness and the experience and expertise of the investigating clinician. For example, aspiration of a pleural effusion with cytological examination and/or closed needle biopsy are techniques that are readily available in most hospitals, but they have low diagnostic sensitivity for mesothelioma. Patients admitted to the regional cardiothoracic unit may be more likely to undergo surgical biopsy (open or thoracoscopic). Frail patients deemed unfit for intervention may only be diagnosed at death.

Ascertaining the exact number of diagnostic or therapeutic interventions was therefore difficult. Many patients had previously undergone one or more pleural aspirations or needle pleural biopsies at their local hospitals, but these were often incompletely documented in the Wythenshawe notes. Only patients with non-diagnostic biopsies and those who were felt amenable to surgical intervention were referred for a surgical opinion to our unit: we therefore had more comprehensive data on patients in this category.

Surgical treatments have come and gone from fashion over the years, and it was not always clear whether pleurectomy or decortication was performed with curative or palliative intent. Radical surgery for mesothelioma (extra-pleural pneumonectomy, EPP) was considered optimal treatment for mesothelioma in those fit enough to withstand major surgery, before disappointing results and high morbidity and mortality rates led to a decline
in the number of these operations being performed.\textsuperscript{391,392} More recently, improved results after radical surgery in selected patients, when combined with post-operative radiotherapy and chemotherapy has led to renewed interest in EPP, and a new trial of radical surgery is currently being proposed in the UK.\textsuperscript{151} Chemotherapy and radiotherapy regimes have similarly changed with time. A small number of our patients were entered into chemotherapy trials, including phase I studies, run from the Christie Hospital.

Overall a total of 577 interventions were identified. These were performed on 391 patients (71\% of the cohort). They are listed below in Table 4.3.

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Number of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pleural aspiration</td>
<td>49</td>
</tr>
<tr>
<td>Abrams Needle biopsy</td>
<td>89</td>
</tr>
<tr>
<td>Diagnostic Surgical biopsy</td>
<td>245</td>
</tr>
<tr>
<td>Therapeutic Surgical Procedure^*</td>
<td>62</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>64</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>43</td>
</tr>
<tr>
<td>Other^#</td>
<td>25</td>
</tr>
</tbody>
</table>

\^* Includes all therapeutic thoracic surgical procedures from pleurodesis to EPP.
\^# Includes chest wall and lymph node biopsies, excision of metastases, stents etc.

Table 4.3
Diagnostic and Therapeutic Interventions in Patients with Mesothelioma
4.9: Histopathological Classification of Tumours

All but six of the cohort had their tumours sub-classified into one of the three variants of mesothelioma defined in the 1999 WHO guidelines. Two specimens were autolysed and blocks and slides were missing in the other four cases. The distribution of subtypes was similar to that found in other reported series (see Section 4.27, page 208). There was a slight excess of epithelioid tumours in women, but this difference did not reach statistical significance (63.8% vs 49.2%, p=0.15). The effect of histology on survival is examined further in the second part of this chapter (Section 4.19 page 185).

<table>
<thead>
<tr>
<th>Histological Subtype</th>
<th>All Patients</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Epithelioid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesothelioma</td>
<td>279</td>
<td>235*</td>
<td>44*</td>
</tr>
<tr>
<td></td>
<td>(51.0%)</td>
<td>(49.2%)</td>
<td>(63.8%)</td>
</tr>
<tr>
<td><strong>Biphasic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesothelioma</td>
<td>164</td>
<td>149</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>(30.0%)</td>
<td>(31.2%)</td>
<td>(21.7%)</td>
</tr>
<tr>
<td><strong>Sarcomatoid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesothelioma</td>
<td>77</td>
<td>69</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>(14.1%)</td>
<td>(14.4%)</td>
<td>(11.6%)</td>
</tr>
<tr>
<td><strong>Desmoplastic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesothelioma</td>
<td>27</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>(4.9%)</td>
<td>(5.2%)</td>
<td>(2.9%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>547</td>
<td>478</td>
<td>69</td>
</tr>
</tbody>
</table>

*Difference between groups: Pearson $\chi^2 = 5.3$, p= 0.15
Figures in brackets represent the proportion of that subtype as a percentage of all classified tumours in that group

Table 4.4

Distribution of Tumour Subtypes in a Cohort of Patients with Mesothelioma
4.10: Occupation and Asbestos Exposure

Occupational details were available in 460 patients (83.2% of cohort). Many had details of previous occupations documented as part of their claims for industrial compensation. If they had worked in several different occupations then they were classified according to the one that had apparently provided the greatest asbestos exposure, where known. If no contemporary occupational history was available then they were classified by the occupation listed in their notes. I accept that this may not always be an accurate marker for asbestos exposure, particularly in those who had retired, or whose exposure to asbestos may not have been occupational. The cohort was then sub-classified according to the type of asbestos exposure, using the classification proposed by Zeilhuis. The duration of exposure, and minimum and maximum lag period (time from first and last asbestos exposure to diagnosis of mesothelioma respectively) were also calculated for the main occupation causing asbestos exposure. The categories were defined as follows:

**Group 1 - definite asbestos exposure (n=343, 62.0 % of cohort)**

All patients had well-documented histories of working with asbestos. Patients whose occupation was uncertain but who had histological evidence of asbestos exposure (asbestosis) were included in this group. Patients with asbestos fibre counts significantly above the level seen in a non-exposed population i.e. above 1 x 10^5 fibres/g on lung digestion were also included in this group.

**Group 2 - probable asbestos exposure (n = 44, 8.0 % of cohort)**

This included patients without a comprehensive occupational history, but whose listed occupation was one associated with an increased risk of mesothelioma e.g. the insulation industry.

**Group 3 - para-occupational exposure (n = 24, 4.3 % of cohort)**

It is recognised that mesothelioma can arise in those only exposed to asbestos via spouses or other family members, through the carriage of asbestos on working clothes. This group only included patients who had no other recognised exposure to asbestos and had been accepted as having para-occupational exposure by the Pneumoconiosis Medical Panel.
Group 4 - environmental exposure (n = 11, 2.0%)
It is also recognised that many families were exposed to significant asbestos loads from living close to asbestos factories prior to the introduction of public health regulations. The distinction between para-occupational and environmental exposure is somewhat arbitrary as many of these people will also have family members who work in the local industries. This group predominantly contained plaintiffs and their families involved in the legal case Margereson v JW Roberts Ltd, Hancock v same.396

Group 5 - No asbestos exposure (n= 36, 6.5 %)
This group only included those with good occupational histories that had excluded any likely asbestos exposure. If this information was unavailable then they were classified as unknown.

Group 6 - occupational history unknown (n = 95, 17.2 %)
This group contained patients without occupational histories in whom there was no objective evidence of prior asbestos exposure.

A similar proportion of men and women had a history of any asbestos exposure irrespective of amount, with 93% of the males (n = 379) and 86% of the females (n=43) falling into groups one to four (Fishers Exact Test, p = 0.1). However the majority of women had non-occupational exposure (Groups 3 and 4, n=24, 60%). Information on the presence of pleural plaques at post-mortem examination was available in 176 patients (31.8% of cohort). One hundred and thirty two patients had plaques and 44 did not. Asbestos bodies were noted in 125 of the 281 patients in whom this was commented on in their pathology report. Detailed information regarding the duration and timing of asbestos exposure was available in almost half of the cohort. The mean duration of exposure was 15 years (range 0.5 - 49 years, n = 197). The mean interval from first asbestos exposure to the date of diagnosis was 38.9 years (range 0 - 76 years, n=219). These figures agree well with those reported in other series.232, 397, 398
4.11: Pulmonary Asbestos Fibre Counts

Pulmonary asbestos fibre burden had been measured in 273 patients, using a modification of the lung digestion technique described by Ashcroft and Heppleston. Asbestos counts performed at other centres were not included, as their experimental method was uncertain. A small number of cases had single values only (site not specified, n = 7) or SEM counts (n = 11), which were performed by The Environmental Lung Disease Research Group, Llandough Hospital NHS Trust, Penarth.

The asbestos fibre burden results from our laboratory were as follows:

Upper lobe counts: mean = 54.1 x 10^4 fibres/g  range = 0 - 43.3 x 10^6 fibres/g
Lower lobe counts: mean = 43.0 x 10^4 fibres/g  range = 0 - 36.4 x 10^6 fibres/g.

Asbestos lung fibre burdens and the incidence of asbestosis have fallen since the introduction of occupational health measures in 1969. It was our impression that asbestos fibre burdens in our cohort reflected this change. To test this hypothesis all cases with known asbestos counts were divided into five-year cohorts by the year of measurement (i.e. death) and the groups compared. Although there is a trend towards falling counts more recently, there was no statistical difference between groups, either for upper lobe or lower lobe counts. Re-examining the groups as 10 year cohorts also failed to demonstrate a statistically significant difference (data not shown).

The results for upper lobe counts are shown in Table 4.5 overleaf and represented graphically in Figure 4.3, page 163.
### Table 4.5

**Upper Lobe Asbestos Fibre Burden by Year of Death in Patients with Mesothelioma**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>$3.2 \times 10^5$</td>
<td>$10 \times 10^5$</td>
<td>$4.2 \times 10^5$</td>
<td>$1.1 \times 10^5$</td>
</tr>
<tr>
<td><strong>Median</strong></td>
<td>$1.4 \times 10^5$</td>
<td>$0.7 \times 10^5$</td>
<td>$0.5 \times 10^5$</td>
<td>$0.3 \times 10^5$</td>
</tr>
<tr>
<td><strong>Range ( x $10^5$)</strong></td>
<td>$1.0 - 11.4$</td>
<td>$0 - 433$</td>
<td>$0 - 180$</td>
<td>$0 - 17$</td>
</tr>
</tbody>
</table>

Non-parametric ANOVA, $p = 0.28$. 
Figure 4.3
Changes in Upper Lobe Asbestos Fibre Counts over Time in Patients with Mesothelioma

*A logarithmic transformation of the value of (upper lobe fibre counts +1) was performed to enable all values including zero to be represented on the y axis. Initial asbestos fibre count values measured as fibres/g dry lung using a lung digestion technique.\(^\text{386}\)
4.12: Pulmonary Fibrosis and the Presence of Other Tumours

Asbestos exposure predisposes to pulmonary fibrosis and the development of lung cancer. A proportion of the cohort (10%, n = 56) had histological evidence of asbestosis on examination of the lungs. In addition five patients had a diagnosis of non-asbestos related lung fibrosis and four had a coexisting empyema. Eleven patients had coincidentally been diagnosed as having other malignant tumours. This figure does not include patients whose mesothelioma was initially misdiagnosed as a pulmonary neoplasm. The other tumours included the following:

- Contralateral pulmonary adenocarcinoma (n=1)
- Ipsilateral small cell carcinoma of the lung (n=1)
- Colonic adenocarcinoma (n=3)
- Malignant melanoma of the skin (n=1)
- Renal adenocarcinoma (n=1)
- Breast adenocarcinoma (n=1)
- Phaeochromocytoma (n=1)
- Hodgkin’s disease, diagnosed 22 years previously (n=1)
- Non-Hodgkin’s lymphoma involving the palatine tonsil (n=1)

Both of the lymphoma patients had previously undergone thoracic irradiation as part of their treatment. All of these tumours were histologically and immunohistochemically different from the pleural tumours.

4.13: Outcome and Survival

By the end of the follow-up period 446 patients had died (80.7% of cohort) and 76 were still known to be alive (13.7%). Thirty-one patients had been lost to follow-up. I had defined survival as the time elapsed from the date of histological diagnosis until follow-up; The exact date of diagnosis or death was unknown in 53 cases. A further ten patients were diagnosed with MPM after death. Patients in whom pleural malignancy had been diagnosed pre-mortem but not correctly identified as mesothelioma were still included in the outcome analysis as if their tumour had been correctly identified at that time. This left 461 patients for the survival analysis.

Actuarial survival was calculated by the Kaplan - Meier method with censoring of cases alive at the end of follow-up. Differences in survival between groups were
quantified using the log-rank test, with the 5% level taken as significant. A summary of the results and the cohort survival curve are shown below (Figure 4.4). Further survival analyses are described in more detail in Results (Part II), page 167.

Median survival 8.0 months
Range <1 day* - 11.6 years.
30-day death rate 6.3 % (n = 29).
1-year survival rate 64.7 %
2-year survival rate 13.0 %
Long term survivors (> 5 years) n = 3
(* One patient died within 24 hours of diagnostic biopsy).

Figure 4.4
Survival in a Cohort of Patients with Malignant Pleural Mesothelioma
The cause of death recorded after PM examination (or special examination of the lungs by the PMP) was known in 406 cases. The most frequent cause of death was mesothelioma and/or bronchopneumonia, confirming that mesothelioma is directly responsible for the death of most patients that it affects. A small number of patients died of other causes.

<table>
<thead>
<tr>
<th>Cause of Death</th>
<th>Number of Patients</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesothelioma ± Bronchopneumonia</td>
<td>355</td>
<td>79.6</td>
</tr>
<tr>
<td>Other Pulmonary Causes</td>
<td>21</td>
<td>4.7</td>
</tr>
<tr>
<td>Cardiovascular Disease</td>
<td>18</td>
<td>4.1</td>
</tr>
<tr>
<td>Gastrointestinal Disease</td>
<td>9</td>
<td>2.0</td>
</tr>
<tr>
<td>Other Causes*</td>
<td>3</td>
<td>0.7</td>
</tr>
<tr>
<td>Not known</td>
<td>40</td>
<td>8.9</td>
</tr>
<tr>
<td>Total</td>
<td>446</td>
<td>100</td>
</tr>
</tbody>
</table>

*Two patients who died of diabetic complications and one from anaemia.

Table 4.6
Causes of Death in a Cohort of Patients with Mesothelioma
RESULTS (PART II): SURVIVAL ANALYSIS AND PROGNOSTIC FACTORS

Having defined the cohort in terms of demographic, histopathological and occupational factors, further analyses were performed to assess the influence of these factors on patient outcome. Previous studies have varied in their ability to identify prognostic factors, and the strength of these associations. However, it seems that age, tumour stage at presentation, performance status and histological subtype are the factors most likely to influence patient outcome. Other possible prognostic factors include gender, diagnostic delay, patient symptoms and asbestos exposure.

It can be difficult to fully evaluate prognostic factors and treatment modes in retrospective studies. Few reported studies of mesothelioma have accrued large patient numbers. Those that have, have relied on information from many different sources, and collected cases of a long period of time. The selection of patients for various treatments will be biased by clinical factors such as age and fitness, and whether any specific trials of therapy are ongoing at that time. Studies may also be biased in other ways. For example, it can become more difficult to ensure that all cases of mesothelioma have been correctly identified if many different pathologists and their laboratories are utilised. My study is similarly limited by such factors: There was insufficient clinical information for tumour stage, patient performance status and treatment to be accurately evaluated.

Each of the following factors was analysed for its influence on survival in our cohort:

- Age at diagnosis
- Gender
- Mode of presentation
- Diagnostic delay
- Histological subtype
- Asbestos exposure
4.14: Analysis of the Influence of Age at Diagnosis on Outcome

Information on the age at diagnosis and survival was available for 415 patients. Patients were subdivided into four groups by age, which corresponded approximately to the initial cohort age quartiles.

- Group 1: patients < 55 years old (n = 94)
- Group 2: patients aged 55 - 62 (n = 142)
- Group 3: patients aged 63 - 75 (n = 128)
- Group 4: patients > 75 years old (n = 51)

Actuarial survival curves were calculated for each group (Kaplan-Meier) and the difference between groups calculated using the Log-rank test. The Chi-square value was 28.34 which is highly statistically significant (p < 0.00005). The survival curve is shown in Figure 4.5.

![Survival in Patients with Mesothelioma Stratified by Age at Diagnosis](image.png)
Median survival for each age group was as follows:

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Median Survival (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 55</td>
<td>10.6</td>
</tr>
<tr>
<td>55 - 62</td>
<td>9.5</td>
</tr>
<tr>
<td>63 - 75</td>
<td>7.1</td>
</tr>
<tr>
<td>&gt; 75</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Table 4.7

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Median Survival (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 55</td>
<td>10.6</td>
</tr>
<tr>
<td>55 - 62</td>
<td>9.5</td>
</tr>
<tr>
<td>63 - 75</td>
<td>7.1</td>
</tr>
<tr>
<td>&gt; 75</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Median Survival in Patients with Mesothelioma Stratified by Age at Diagnosis

A Cox regression analysis was performed to confirm the results of the univariate analysis, and to assess the degree of influence of age on survival. Age was confirmed as being highly significant. Using the group with the best prognosis (age < 55 years) as the reference group, the relative risks of dying were then calculated for the other age groups. There was a linear increase in risk with age throughout groups, as shown overleaf in Table 4.8.

This result is not surprising given that life expectancy normally decreases with age. However it is important to remember this when assessing the results of trials of treatment in mesothelioma. If age is not corrected for in a treatment group, then this can seriously bias any results. This subject is discussed in more detail later in this chapter.
### Table 4.8
Influence of Age at Diagnosis on Relative Risk of Dying in Patients with Mesothelioma

<table>
<thead>
<tr>
<th>Age Cohort</th>
<th>Relative Risk of Dying</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1: Age &lt; 55 years</td>
<td>1.0</td>
</tr>
<tr>
<td>Group 2: Age 55 - 62 years</td>
<td>2.2</td>
</tr>
<tr>
<td>Group 3: Age 63 - 75 years</td>
<td>3.0</td>
</tr>
<tr>
<td>Group 4: Age &gt; 75 years</td>
<td>4.9</td>
</tr>
</tbody>
</table>

4.15: Analysis of the Influence of Gender on Survival

Because the majority of mesotheliomas are related to occupational asbestos exposure, most studies have a significant excess of men. Analysis of gender differences on survival can therefore be influenced by unequal group size. In our cohort there was a small survival advantage for women that just reached statistical significance ($\chi^2 = 4.2$, $p=0.04$). The details of this analysis are shown in Table 4.9 and Figure 4.6 overleaf.
Table 4.9
Median Survival in Patients with Mesothelioma Stratified by Gender

<table>
<thead>
<tr>
<th></th>
<th>Males (N = 407)</th>
<th>Females (n = 54)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median survival (months)*</td>
<td>7.9</td>
<td>11.3</td>
</tr>
<tr>
<td>Range (months)</td>
<td>0 - 124</td>
<td>0.3 - 139</td>
</tr>
</tbody>
</table>

* $\chi^2 = 4.2, p=0.04.$

Figure 4.6
Survival in Patients with Mesothelioma Stratified by Gender
This difference in survival could be a true biological effect. Alternatively it could be influenced by another confounding variable such as age or histology. Age at diagnosis had already been analysed by sex, with women being slightly but not significantly older than men in our cohort (p=0.48). This would tend to reduce rather than increase survival in women, in view of the strong association between age and outcome. Another possible influence is the tumour histology. It has been reported that the epithelioid subtype of mesothelioma is associated with a better prognosis (see Section 4.27, page 208). There was an excess of epithelioid tumours in women but this did not reach statistical significance ($\chi^2=5.3$, p= 0.15). Re-analysing survival in men and women when stratified for histology failed to demonstrate a continued survival advantage for women ($\chi^2=2.7$, p= 0.09). Survival curves for each tumour subtype stratified by gender are shown below (Figures 4.7 - 4.9, continued over page).

![Figure 4.7](image)

**Figure 4.7**

*Survival in Patients with EM Stratified by Gender*
Figure 4.8: Survival in Patients with BM Stratified by Gender

Figure 4.9: Survival in Patients with SM Stratified by Gender
Closer examination of these survival curves emphasizes the small number of women in this analysis, and how easily mean and median survival can be influenced by one or two outlying points. For example two of the three long term (>5 year) survivors in the EM group were women. In the BM group the small number of women surviving more than 500 days (n=3) markedly skews the survival curves, and appears to show a survival advantage for women with this tumour subtype. In fact there were also 20 men with BM who survived more than 500 days (16.4% of that group). Survival in months by histological subtype in both sexes is detailed below in Table 4.10.

<table>
<thead>
<tr>
<th></th>
<th>EM</th>
<th>BM</th>
<th>SM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Median Survival in Males</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Number of Patients)</td>
<td>10.8</td>
<td>7.6</td>
<td>3.9</td>
</tr>
<tr>
<td>(n=200)</td>
<td>(n=122)</td>
<td>(n=83)</td>
<td></td>
</tr>
<tr>
<td><strong>Median Survival In Females</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Number of Patients)</td>
<td>11.8</td>
<td>21.8</td>
<td>2.2</td>
</tr>
<tr>
<td>(n=36)</td>
<td>(n=9)</td>
<td>(n=8)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.10**

Median Survival in Patients with Mesothelioma Stratified by Gender and Tumour Subtype

4.16: Presenting Symptoms as Prognostic Factors

Pain and dyspnoea have been shown to have prognostic significance in a small number of studies. In order to test this hypothesis in our own cohort, patients were stratified according to the presence or absence of these symptoms, and actuarial survival was compared between groups.
Initially, the cohort was re-categorised according to whether chest wall pain was a presenting feature, irrespective of the presence of any other symptoms. The group with pain (n = 87) was compared with the rest of those in whom symptoms were known (n=95). Median survival in those with pain was 8.1 months, and 7.8 months in those without. The difference in survival between groups was not statistically significant ($\chi^2=0.78, p = 0.38$). The actuarial survival curves are shown in Figure 4.10 below.

**Figure 4.10**
Survival in Patients with Mesothelioma Stratified by the Presence or Absence of Chest Pain
The other symptom that may have prognostic significance is dyspnoea. The cohort was re-stratified according to whether dyspnoea was noted at presentation. Median survival in the breathless group was 7.8 months (n=114), and 8.9 months in those without dyspnoea (n=89). This difference also failed to achieve statistical significance (Pearson $\chi^2 = 1.1$, $p=0.29$). The survival curves are shown in Figure 4.11 below.

**Figure 4.11**

*Survival in Patients with Mesothelioma Stratified by the Presence or Absence of Dyspnoea at Diagnosis*
4.17: Analysis of the Relationship Between Symptoms and Tumour Subtype

It has been suggested that histological subtype may influence symptoms and signs in mesothelioma. This assumes that epithelioid mesothelioma behaves like carcinoma, with early lymph node spread and the production of pleural effusion. In contrast, sarcomatoid tumours metastasize via the blood stream to distant sites and are more likely to invade local structures e.g. chest wall and mediastinum. If this is the case then it is possible that presenting symptoms reflect tumour histology, and may therefore have both diagnostic and prognostic value.

The association between symptoms and histology in our cohort was examined in two ways. Firstly, the proportion of patients who presented with pain was calculated for each histological type. Pain was seen in 38% of those with EM, 43% of BM and 51% of those with SM (Figure 4.12 below). Although suggesting that pain is more commonly a feature of tumours with sarcomatoid differentiation, this difference failed to reach statistical significance (Pearson $\chi^2 = 2.3$, $p = 0.3$).

![Figure 4.12](image-url)  
**Figure 4.12**  
Incidence of Chest Pain in Tumour Subtypes
A similar analysis was performed for dyspnoea. The proportion of patients in each histological sub-group with dyspnoea was similar, at approximately 55% ($\chi^2 = 0.04$, $p=0.9$). This result is represented in Figure 4.13 below.

![Incidence of Dyspnoea in Tumour Subtypes](image)

**Figure 4.13**

**Incidence of Dyspnoea in Tumour Subtypes**

Those patients with clinically apparent distant metastases at presentation ($n=7$) were analysed by histological subtype. Four of them had SM, one had BM and two had EM. This is markedly different from the distribution of histological subtypes overall, as SM is the least common type. Despite the small number of cases involved, this difference achieved statistical significance (Fishers exact test $\chi^2 = 6.6$, $p = 0.04$) implying that distant metastases may occur earlier in SM than in other subtypes of mesothelioma.
4.18: Influence of Diagnostic Delay on Survival

The interplay between symptoms, histology and survival may have another aspect: does tumour type influence the speed of diagnosis? Pleural effusions cause significant dyspnoea due to compression of the underlying lung. The signs of an effusion are readily seen on CXR, and cytological examination of aspirated fluid may confirm the presence of malignancy. Sarcomatoid tumours are less commonly associated with effusion, and may require pleural biopsy to confirm the diagnosis. This could result in diagnostic delay, and in part explain the difference in survival between tumour types. Unfortunately, the time of first symptoms, and the subsequent interval between that and diagnosis was known in only 141 patients (25.5% of cohort). The mean time from symptoms to diagnosis was 96 days. The cohort was therefore divided into two groups, depending on whether the delay from presentation to diagnosis was greater or less than 100 days (approximate mean). Actuarial survival was compared between groups and was non-significant ($\chi^2 = 1.38$ p = 0.24). The survival curves are shown in Figure 4.14.

![Figure 4.14](image-url)

**Figure 4.14**

Survival in Patients with Mesothelioma Stratified by Diagnostic Delay
4.19: Influence of Tumour Subtype on Survival

At the time that this project was conceived, it was still uncertain whether tumour subtype had a significant effect on survival. In the last couple of years it has become increasingly apparent that epithelioid mesothelioma is associated with a better prognosis than sarcomatoid mesothelioma. Biphasic tumours appear to have an intermediate prognosis in most studies, although one group concluded that biphasic tumours have the worst prognosis.\textsuperscript{406} The incidence of desmoplastic mesothelioma as a separate subtype has been reported in only one study, and survival analysis was not performed because of the small number of cases in this group (n=4).\textsuperscript{407}

Actuarial survival was calculated for our cohort, stratifying for histological type. The difference between groups was highly significant ($\chi^2 = 116.7, p < 0.00005$, Log-rank test) confirming the strong influence of histology on survival. Epithelioid tumours survived the longest, and biphasic tumours had the next best prognosis. Sarcomatoid tumours fared the worst. Subdividing sarcomatoid tumours further demonstrated that desmoplastic tumours did indeed do badly, with only 4% of patients surviving more than twelve months. Median survival and the corresponding survival curves for each subtype are detailed in Table 4.11 below and Figure 4.15 overleaf.

<table>
<thead>
<tr>
<th>Tumour Subtype</th>
<th>EM (n = 236)</th>
<th>BM (n = 131)</th>
<th>SM (n = 66)</th>
<th>DM (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median survival (months)</td>
<td>11.8</td>
<td>7.6</td>
<td>4.5</td>
<td>2.9</td>
</tr>
<tr>
<td>Range (months)</td>
<td>0.3 - 139</td>
<td>0.4 - 28.1</td>
<td>0 - 24.8</td>
<td>1 - 15.8</td>
</tr>
</tbody>
</table>

$\chi^2 = 116.7, p < 0.00005$ Log-rank test

Table 4.11

Survival in Patients with Mesothelioma Stratified by Tumour Subtype
Figure 4.15
Survival in Patients with Mesothelioma Stratified by Tumour Subtype

As age at diagnosis also has a highly significant effect on survival, this needs to be taken into account when interpreting these results. Further analyses were therefore performed to explore the relative contributions of these two factors on survival. Initially the mean age at diagnosis for each histological subtype was calculated (Table 4.12).

<table>
<thead>
<tr>
<th></th>
<th>EM (n=237)</th>
<th>BM (n=117)</th>
<th>SM (n=64)</th>
<th>DM (n=21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age at diagnosis* (years)</td>
<td>62.2</td>
<td>60.7</td>
<td>64.9</td>
<td>67.8</td>
</tr>
</tbody>
</table>

* ANOVA F=4.5, p = 0.004.

Table 4.12
Mean Age at Diagnosis Stratified by Tumour Subtype
This demonstrated an interesting pattern, with epithelioid tumours presenting in younger patients and desmoplastic in the oldest. This difference was highly significant (p=0.004) and further analysis (Duncan post hoc test) confirmed that the SM and DM patients were significantly older than EM and BM patients at the time of diagnosis. This result is represented graphically in Figure 4.16.

![Histogram of Tumor Subtypes by Age at Diagnosis](image)

**Figure 4.16**

*Distribution of Tumor Subtypes by Age at Diagnosis*

Actuarial survival was then re-examined. Firstly survival according to histology was recalculated and stratified for age at diagnosis. The reverse was then examined: survival by age at diagnosis and stratified by histology. In both cases the differences between groups remained significant (p < 0.00005). This implies that both histology and age affect survival. This supposition was tested using a Cox regression study, which confirmed the independent effects of age and histology on survival. The relative risks of dying were then
calculated for each histological subtype, using EM as the baseline. An increase in risk was seen through the groups: the ratios are shown in Table 4.13.

<table>
<thead>
<tr>
<th>Histological Subtype</th>
<th>Relative Risk of dying</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelioid Mesothelioma</td>
<td>1.0</td>
</tr>
<tr>
<td>Biphasic Mesothelioma</td>
<td>2.1</td>
</tr>
<tr>
<td>Sarcomatoid Mesothelioma</td>
<td>4.9</td>
</tr>
<tr>
<td>Desmoplastic Mesothelioma</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Table 4.13

Effect of Tumour Subtype on Risk of Dying in Patients with Mesothelioma

4.20: Influence of Asbestos Exposure on Survival

The final prognostic factor examined was asbestos exposure. Possible mechanisms by which asbestos exposure might influence survival include reduced lung function, which could exacerbate the effects of mesothelioma, or an influence on the histological subtype. Initially the cohort was divided into two groups according to asbestos exposure. Group A (n=412) included all patients with documented asbestos exposure (groups 1-4 as defined in Section 4.10, page 159). Group B (n=34) contained those patients with no known asbestos exposure. Comparison between these two groups failed to demonstrate a survival advantage for non-exposed patients ($\chi^2 = 0.53$, p = 0.47 Log-rank test) as shown in Figure.
4.17. Although this result is non-significant it is possible that a true difference was concealed by the marked inequality of group size.

![Graph showing survival times for patients with and without asbestos exposure.](image)

**Figure 4.17**

**Survival in Patients with Mesothelioma Stratified by Asbestos Exposure**

It has been suggested by some authors that asbestos exposure is associated with biphasic tumours. If tumour type was influenced by asbestos exposure then this would need to be controlled for when investigating the relationship between asbestos exposure and survival. The proportional distribution of histological subtypes within each group was compared: there was an excess of sarcomatoid and desmoplastic tumours in asbestos-exposed patients (Table 4.14 below). The Pearson Chi-square statistic was 11.4, \( p = 0.01 \). This result should be interpreted with caution as the actual and expected counts in the DM non-asbestos exposed group were both less than five, which could artificially increase significance. The analysis was repeated combining the SM and DM groups. The difference between groups remained significant (\( \chi^2 = 8.9, \ p = 0.01 \)). Further analysis was not possible given the small numbers of patients in these categories.
<table>
<thead>
<tr>
<th></th>
<th>EM</th>
<th>BM</th>
<th>SM</th>
<th>DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asbestos Exposure</td>
<td>197</td>
<td>134</td>
<td>59</td>
<td>22</td>
</tr>
<tr>
<td>(n = 412)</td>
<td>(47.8%)</td>
<td>(32.5%)</td>
<td>(14.3%)</td>
<td>(5.3%)</td>
</tr>
<tr>
<td>No Asbestos Exposure</td>
<td>25</td>
<td>4</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>(n = 34)</td>
<td>(73.5%)</td>
<td>(11.8%)</td>
<td>(14.7%)</td>
<td>(0%)</td>
</tr>
</tbody>
</table>

Table 4.14
Distribution of Tumour Subtypes in Patients with Mesothelioma Stratified by Asbestos Exposure

(Figures in brackets represent the proportional distribution of tumour subtypes in each exposure group as a percentage of all tumours seen in that group)

Finally, the relationship between quantitative asbestos exposure and survival was also explored by comparing survival in those with high and low lung fibre counts, and correlating survival with duration of asbestos exposure. Neither of these analyses demonstrated a statistically significant relationship between exposure and survival (data not shown).
DISCUSSION

4.21: The Increasing Incidence of Malignant Pleural Mesothelioma

Despite the recent increase in incidence, malignant pleural mesothelioma is still a relatively uncommon tumour. For example, a total of 1,097 cases of mesothelioma at all sites was registered in England and Wales in 1992, an incidence of 3.7 and 0.8 per 100,000 of the population for males and females respectively. This compares with over 37,000 new cases of lung cancer registered in the same year. Reported cases of mesothelioma for the North Western Region follow a similar pattern (3.5 and 0.7 per 100,000 of the population respectively). The relatively small numbers of pleural mesothelioma cases and the difficulty of diagnosis have hindered attempts to accurately investigate factors important in the diagnosis, prognosis and treatment of this tumour.

The incidence of pleural mesothelioma has also been steadily rising in Europe and the United States for several years. This increase correlates closely with previous asbestos usage, particularly amphiboles (Figure 1.1, page 2). In the United States the maximum use of asbestos in industry and the armed forces coincided with World War II. The subsequent increase in mesothelioma incidence was seen approximately thirty years later, and is now approaching its predicted peak. Conversely, asbestos usage was widespread in the UK until the introduction of the 1969 Asbestos Regulations. The proven relationship between increasing mesothelioma incidence and time from first asbestos exposure (incidence $\propto$ interval) suggests that for Europe and the UK the worst is yet to come.

Peto and colleagues have investigated this increase in mesothelioma incidence in detail. In 1982 they highlighted the relationship between interval from exposure and subsequent risk of mesothelioma as quoted above. In 1995 they published a study of mesothelioma mortality as reported by the UK Health and Safety Executive (HSE) Mesothelioma Register from 1968 - 1991. Deaths from pleural and peritoneal mesothelioma increased in both sexes during this period of time. The mortality rate in men rose from 8.4 per million to 44 per million, a five-fold increase. The increase in women was less marked but still significant (from 2.3 to 6.4 per million). These annual rates are illustrated in Figure 4.18 below.
A statistical model based on the year of birth and age-specific mesothelioma rates was devised. Using the Peto model the increase in mesothelioma mortality was predicted to continue for the next 25 years, reflecting a maximum mesothelioma risk to men who were working at the time of greatest UK asbestos use (1960 -1980). Although occupational health measures had been introduced to protect workers in high-risk occupations e.g. the insulation industry, a great number of men were working with asbestos in less well-regulated occupations, such as the building trade. As the development of mesothelioma is not dose-dependent, it is likely that a proportion of these men (and women) may have been exposed to biologically significant levels of asbestos. The number of individuals exposed to asbestos in this way is likely to be far greater than those working in the asbestos trade. The final size of this increase is therefore difficult to predict. For those born before 1958 the peak was predicted to plateau at approximately 2,700 deaths per year in 2020. If the risk to those born after this time has been reduced by occupational health measures then mesothelioma incidence should then start to fall. Eventually the rate should reach that of the current “sporadic” rate i.e. the rate seen in subjects in whom asbestos is not an aetiological factor. However if the risk to men born after 1958 has not been completely
abolished then the incidence is likely to rise even further, to perhaps >3,000 cases a year in
the UK. The Peto model has also been applied to mesothelioma rates in other European
countries, and predicts similar increases.\textsuperscript{410, 411}

Recently the HSE has revised its estimates on predicted mesothelioma mortality.\textsuperscript{412}
The incidence of mesothelioma has continued to rise, and provisional results for 2001
indicate over 1,800 cases registered. A new statistical model, again based on previous
amphibole asbestos usage, has been created. This fits the observed rise in mesothelioma
better than the previous model. It estimates a slightly smaller peak incidence, occurring

A pattern of increasing rates can be demonstrated in our own cohort. All of our
cases were stratified by year of death into five-year cohorts. In the period 1980 - 1985
there were 14 mesothelioma deaths (annual rate 2.8 per year). During the next 5 years
(1986 - 1990) there were 130 deaths (26 per year) and during the period 1990 - 1995 the
annual death rate was 30.6. For the last 4 years of our study period the annual
mesothelioma mortality was 63.7 per year. This is a very crude estimate of true
mesothelioma mortality. The number of cases referred to Wythenshawe Hospital for
investigation or treatment will be biased in many ways. These include the preference of the
referring clinician, the need for a tissue diagnosis or surgical intervention, and the
perceived expertise of the specialists based there. Many of our cases were referred by the
PMP or local Coroners for expert opinion, or were medico-legal cases. The presence of a
pathologist with an interest in mesothelioma is likely to have influenced the number of
referred cases and is likely to account for some of this increase in incidence, particularly as
the proportional increase through each cohort is greater than the national rate.
Nevertheless, it reinforces the impression that Peto’s estimates are accurate, and that the
incidence of mesothelioma is set to continuing rising for another 10-20 years.

4.22: Causes of Bias in Studies of Prognostic Factors in Mesothelioma
Despite the prediction of an incipient mesothelioma “epidemic”, the previous low
incidence has hindered attempts to define prognostic factors and best treatment regimes. It
is over 40 years since Wagner and colleagues first highlighted the causative link between
asbestos and pleural mesothelioma, yet there are still many unanswered questions relating
to aetiology, pathogenesis, best diagnostic methods and the predictive value of patient
variables which relate to prognosis.\textsuperscript{1} Detailed analysis of results from the largest series and
trials indicates that the most important biological factors that may influence outcome in
mesothelioma appear to be age, gender, histology, diagnostic delay and asbestos exposure. However, there still remains some uncertainty as to how strong and consistent the effects of these factors are, and whether they can accurately predict response to treatment, or subsequent patient outcome. The majority of published series concerning mesothelioma include less than two hundred patients, which can limit the power of the study to identify statistically significant differences between patient outcomes. Furthermore, there are several ways in which bias can be introduced into studies, further eroding confidence in their results.

There have been four very large published studies that have examined prognostic factors in mesothelioma. In 1982 Hillerdal published an overview that included the majority of cases of mesothelioma reported in the literature to that date, a total of 4,710 patients. Even a study of this size was limited by a lack of recorded clinical data. For example, of the 4181 cases of pleural mesothelioma identified, patient gender was not stated in over 1,300 and less than 300 cases had enough information to assess the influence of histology on survival. Similarly, another large series of mesothelioma cases reported by the SEER Program of the National Cancer Institute included 1,475 patients, of whom only 290 had histological subtype recorded. Cancer Registry data, this time from Germany, has been reported by Neumann and colleagues. A total of almost 4,000 cases accrued from 1987–99 were identified: of these less than half were analysed in terms of prognostic factors, and survival was known in 404 cases, which included non-pleural tumours. More recently Magnani and colleagues have published an extensive retrospective study of patient outcome based on Mesothelioma Registry data from the Piedmont region in northern Italy. Over a nine year period a total of 590 cases of pleural mesothelioma were registered. Although clinical details and outcome were extensively recorded for all patients, histological subtype was not known in almost a quarter of cases (n=140, 24%).

This last point is important. In the previous two chapters I have discussed the problems of diagnosis in mesothelioma in great detail. The inclusion of even a small number of non-mesotheliomas may be enough to confound analysis of prognostic factors in a small study. Another potential source of bias results from the inclusion of non-pleural mesotheliomas within a cohort: peritoneal mesothelioma has its own problems relating to differential diagnosis, and may behave in a very different way from its pleural counterpart in terms of prognosis. Mesothelioma of the pericardium and the tunica vaginalis are both extremely rare. A total of 278 mesotheliomas of non-pleural origin were included in the SEER study, and several other studies have not distinguished between pleural and non-
pleural mesothelioma in some of their analyses. Discrepancies in nomenclature are another potential source of error. Solitary fibrous tumours of the pleura have previously been given many names including benign mesothelioma. This can cause confusion if cases are identified only by the term mesothelioma without histology review. Solitary fibrous tumours may be benign or of variable malignant potential, and their inclusion in a study of malignant mesothelioma could markedly skew survival analyses. There are studies describing long-term mesothelioma survivors whose clinical history is more suggestive of a diagnosis of SFT than diffuse malignant mesothelioma, when critically reviewed.

Differences in cancer registration and coding can also influence survival analyses. Malignant mesothelioma will account for the majority, but not all, cases coded as pleural cancer (ICD 163): in the UK mesothelioma represents approximately 85% of cases so classified. In other countries this proportion falls to nearer to 70%. Selecting cases by a more general code may potentially introduce a significant number of other tumours, again introducing bias.

Lead time bias is another potential source of error. Although exact numbers are difficult to define, it is clear that diagnostic difficulty may be experienced in a significant proportion of patients with mesothelioma: this certainly was the case in our cohort. Many required more than one biopsy to confirm their diagnosis, which could introduce delay in subsequent registration and treatment. Some studies of prognostic factors and treatment regimes calculate survival from the time of first symptoms, or from recruitment. Given the short median survival in some subtypes of mesothelioma, a delay of even a few weeks may be of significance.

The low incidence of mesothelioma has also had an effect on prospective trials of new treatment modalities. Mesothelioma responds poorly, if at all, to the majority of anticancer treatments and few patients are suitable for radical surgical resection. Trials of treatment tend to include highly selected cases, e.g. young patients with good performance status, "textbook" histology and early stage disease. A large proportion of our cohort would not fall into this category. Although performance status was not routinely recorded in most of our cases, it was apparent that many presented late in their disease course: twenty-nine patients died within a month of the diagnosis of mesothelioma being confirmed. Although each of these factors may be present in only a small number of cases, the cumulative effects of all these factors together may be far more influential.
Some of these biases are likely to have affected the survival analysis in this study. As a retrospective study it is impossible to standardise or compensate for referral patterns, diagnostic algorithms and treatment regimes. However, every attempt to confirm important factors such as histological subtype has been made: this will hopefully reduce the influence of some forms of bias as much as is possible.

4.23: Analysis of the Influence of Age at Diagnosis on Survival

The incidence of pleural mesothelioma increases with age. It is described in all ages including childhood although this is rare. In England and Wales the incidence in adults peaks in the seventh decade in both sexes. This reflects both the general increase in malignant disease with age, and the long lag period in asbestos-related cases. The pattern of age distribution in men and women within our cohort mirrors national figures, however the peak is slightly earlier. The subgroup that contained patients diagnosed between the ages of 56 and 60 years was the largest (n= 83, 18.8% of cohort).

Despite the fact that age at diagnosis is one of the most easily obtainable patient factors, the influence of age on outcome is still debated. The risk of death from any cause increases with age, therefore you would expect some link between age at diagnosis and survival. However the magnitude of that risk is uncertain, and makes analysis of trials of treatment difficult if age has not been corrected for. Several papers have investigated the influence of age on survival. Unfortunately these do not always report non-significant results or detail the way in which cases were stratified for age. This makes comparison between studies difficult. I have identified 20 published studies that have investigated the relationship between age at diagnosis and outcome, that contain reasonable numbers of patients and give enough details of age stratification to allow comparisons. Their results are summarised in Table 4.15 below.
<table>
<thead>
<tr>
<th>Study</th>
<th>Number of Cases in Analysis</th>
<th>Cohort Age (years)</th>
<th>Age Strata (years)</th>
<th>Survival (months)</th>
<th>p Value (Univariate analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mártensson</td>
<td>32</td>
<td>67 (Median)</td>
<td>&lt; 65</td>
<td>16.0</td>
<td>Not Significant</td>
</tr>
<tr>
<td>1984</td>
<td></td>
<td></td>
<td>≥ 65</td>
<td>9.0</td>
<td></td>
</tr>
<tr>
<td>Spirtas</td>
<td>1,475</td>
<td>Stated</td>
<td>&lt; 50</td>
<td>Not Stated</td>
<td>0.0001 Cox</td>
</tr>
<tr>
<td>1987</td>
<td></td>
<td></td>
<td>50 - 69</td>
<td>Not Stated</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>≥ 70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alberts</td>
<td>262</td>
<td>55 (Median)</td>
<td>&lt; 40</td>
<td>Not Stated</td>
<td>0.2 Mantel-Cox</td>
</tr>
<tr>
<td>1988</td>
<td></td>
<td></td>
<td>40 - 65</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt; 65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antman</td>
<td>180</td>
<td>Stated</td>
<td>&lt; 20</td>
<td>7.0</td>
<td>0.001 Log-rank</td>
</tr>
<tr>
<td>1988</td>
<td></td>
<td></td>
<td>20 - 40</td>
<td>26.0</td>
<td></td>
</tr>
<tr>
<td></td>
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<td>40 - 60</td>
<td>15.0</td>
<td></td>
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<td></td>
<td></td>
<td>&gt; 60</td>
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<tr>
<td>Calavrezos</td>
<td>132</td>
<td>Stated</td>
<td>&lt; 40</td>
<td>16.0</td>
<td>0.0005 Log-rank</td>
</tr>
<tr>
<td>1988</td>
<td></td>
<td></td>
<td>&lt; 50</td>
<td>15.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt; 60</td>
<td>11.0</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>&lt; 70</td>
<td>7.0</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>≥ 70</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>Chailleux</td>
<td>167</td>
<td>62 (Mean)</td>
<td>≤ 60</td>
<td>17.0*</td>
<td>0.02 Log-rank</td>
</tr>
<tr>
<td>1988</td>
<td></td>
<td></td>
<td>&gt; 60</td>
<td>12.0*</td>
<td></td>
</tr>
</tbody>
</table>

Notes: # - study includes non-pleural cases, n - time from symptoms not diagnosis, * - statistically significant effect (p<0.05) confirmed after multivariate analysis

Table 4.15

Summary of Published Series That Have Investigated the Influence of Age on Survival in Malignant Mesothelioma
<table>
<thead>
<tr>
<th>Study</th>
<th>Number of Cases Analysed</th>
<th>Cohort Age (years)</th>
<th>Age Strata (years)</th>
<th>Survival (months)</th>
<th>p Value (Univariate analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ruffie 1989</td>
<td>328</td>
<td>59 (Mean)</td>
<td>&lt;50</td>
<td>9.3</td>
<td>0.07* Log-rank</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50-65</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Walz 1990</td>
<td>64</td>
<td>Not Stated</td>
<td>&lt;50</td>
<td>21.0a</td>
<td>0.02 Mantel-Cox</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50-60</td>
<td>13.0a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;60</td>
<td>9.0a</td>
<td></td>
</tr>
<tr>
<td>Tammilehto 1992</td>
<td>98# (Mean)</td>
<td>58</td>
<td>≤65</td>
<td>11.0</td>
<td>0.04 Log-rank</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boutin 1993</td>
<td>188</td>
<td>63 (Mean)</td>
<td>&lt;65</td>
<td>10.0</td>
<td>0.6 Log-rank</td>
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<td></td>
<td></td>
<td>≥65</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>Fusco 1993</td>
<td>113</td>
<td>66 (Median)</td>
<td>&lt;60</td>
<td>12.0</td>
<td>0.8 Cox</td>
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<td></td>
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<td>61-70</td>
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<td></td>
<td></td>
<td>&gt;70</td>
<td>11.0</td>
<td></td>
</tr>
<tr>
<td>Manzini 1993</td>
<td>80</td>
<td>69 (Median)</td>
<td>&lt;65</td>
<td>19.0</td>
<td>0.07 Mantel-Cox</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>65-74</td>
<td>11.0</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;74</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>Van Gelder 1994</td>
<td>167 Not Stated</td>
<td>65-74</td>
<td>&lt;65</td>
<td>11.8</td>
<td>0.004* Log-rank</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;74</td>
<td>8.0</td>
<td></td>
</tr>
</tbody>
</table>

Notes: # - study includes non-pleural cases, a - time from symptoms not diagnosis, b - time from entry into trial
* - statistically significant effect (p<0.05) confirmed after multivariate analysis

Table 4.15 (Continued)

Summary of Published Series That Have Investigated the Influence of Age on Survival in Malignant Mesothelioma
<table>
<thead>
<tr>
<th>Study</th>
<th>Number of Cases in Analysis</th>
<th>Cohort Age (years)</th>
<th>Age Strata (years)</th>
<th>Survival (months)</th>
<th>p Value (Univariate analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curran</td>
<td>204</td>
<td>Not Stated</td>
<td>$\leq 55$</td>
<td>8.8$^b$</td>
<td>0.95 Log-rank</td>
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<td>$&gt; 55$</td>
<td>8.0$^b$</td>
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<tr>
<td>Herndon</td>
<td>337$^a$</td>
<td>61.3 Mean</td>
<td>$&lt; 75$</td>
<td>Not Stated</td>
<td>0.001* Log-rank</td>
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<tr>
<td>1998</td>
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<td>Stated</td>
<td></td>
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<tr>
<td>Edwards</td>
<td>121</td>
<td>64 (Median)</td>
<td>Not Stated</td>
<td>Not Stated</td>
<td>0.015 Cox</td>
</tr>
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<td></td>
<td>Stated</td>
<td>Stated</td>
<td></td>
</tr>
<tr>
<td>Metintas</td>
<td>100</td>
<td>57 (Mean)</td>
<td>$&lt; 75$</td>
<td>8.5</td>
<td>0.02$^a$ Log-rank</td>
</tr>
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<td></td>
<td>$\geq 75$</td>
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<td>Ceresoli</td>
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<td>10.5</td>
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<td></td>
<td></td>
<td>$&gt; 50$</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>Neumann</td>
<td>404$^d$</td>
<td>60.4 (Mean)</td>
<td>$\leq 60$</td>
<td>15.5$^a$</td>
<td>p $&gt; 0.03$ Log-rank</td>
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<tr>
<td>2001</td>
<td></td>
<td></td>
<td>$&gt; 60$</td>
<td>12.1$^a$</td>
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</tr>
<tr>
<td>Magnani</td>
<td>590</td>
<td>Not Stated</td>
<td>$\leq 55$</td>
<td>10.2</td>
<td>$&lt;0.0001^*$ Log-rank</td>
</tr>
<tr>
<td>2002</td>
<td></td>
<td></td>
<td>56–65</td>
<td>9.1</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$\geq 76$</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>415</td>
<td>62.2 (Mean)</td>
<td>$\leq 55$</td>
<td>10.6</td>
<td>$&lt;0.00005^*$ Log-rank</td>
</tr>
<tr>
<td>Study</td>
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<td>9.6</td>
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<td></td>
<td>$&gt; 75$</td>
<td>4.9</td>
<td></td>
</tr>
</tbody>
</table>

Notes: # - study includes non-pleural cases, a - time from symptoms not diagnosis, b - time from entry into trial
^a-statistically significant effect (p<0.05) confirmed after multivariate analysis

Table 4.15 (Cont): Summary of Published Series That Have Investigated the Influence of Age on Survival in Malignant Mesothelioma
Careful scrutiny of these papers reveals some unusual findings. Eleven of these studies found a statistically significant (p<0.05) relationship between age and survival with p values ranging from 0.04 to 0.0001 after univariate analysis. A further two studies narrowly failed to reach significance (p=0.07). The remaining six studies demonstrated no relationship. After multivariate analysis, age was confirmed as a significant prognostic factor in seven studies.

Interestingly, it does not appear that study size alone was the main determinant of whether the study was able to detect any effect of age on outcome. The SEER study, which includes over 1400 cases of mesothelioma, and the study by Magnani et al reported the strongest association (p=0.0001). The next largest study by Herndon et al (n=337, p=0.001) confirms this finding. The study by Ruffie et al (n=328) only confirmed the significance of age after multivariate analysis. Large studies by Alberts et al (n=262), Curran et al (n=204), Boutin et al (n=188) and Neumann et al (n=404) found age to be non-significant. Conversely, smaller studies such as those by Antman et al (n=180), Chailleux et al (n=167), Van Gelder et al (n=167) Tammilehto et al (n=98) Walz et al (n=64) and Metintas et al (n=100) found age to be significant after univariate analysis. In three of these, age remained significant after multivariate analysis.

One possible explanation for the differences seen in these studies is that they have stratified their cases in different ways. Choosing a lower discriminatory age e.g. 50 or 60 will allow younger, better prognosis patients to dilute the effect of a smaller number of older, poorer prognosis cases. Furthermore there is quite a variation between studies in terms of the mean and median age of the cohort. Four of these studies, including SEER, do not state a mean or median age for their cases, and the others vary as to whether age is described by reference to the cohort mean or median. There is over a 10-year difference in median patient age between some papers e.g. Antman et al and Alberts et al (median age 55 years) and Manzini et al (median age 69 years). Those studies which have evaluated different treatment modalities often have inclusion criteria that discriminate against older patients, either by defining an upper age limit or requiring a minimum predicted survival. A second possible source of confounding factors is the inclusion of non-pleural mesotheliomas and childhood cases within series. Five of the studies have included all mesotheliomas irrespective of site. In the SEER and Antman studies, the proportion of non-pleural tumours approaches 20% of the total. Inclusion of peritoneal mesothelioma cases can introduce an excess of younger females with tumours
that may be difficult to distinguish from ovarian and other intra-abdominal tumours. In men, peritoneal mesothelioma is strongly associated with high pulmonary asbestos fibre burden: impaired lung function could potentially reduce survival.\textsuperscript{398}

The influence of age at diagnosis on survival was highly significant in our cohort (\(p < 0.00005\)). Age remained significant in both sexes when analysed separately (\(p=0.0001\) and \(p=0.017\) respectively), despite the relatively small group of women in the analysis (\(n=54\)). There were three patients who survived more than five years: all were under the age of 50 when diagnosed, and two were women. The relationship between age and survival in our cohort was confirmed by regression analysis, the relative risk of dying increasing for each age group. This increase in risk is approximately linear through our first three age cohorts (i.e. patients <75 years old) but then increases exponentially for the oldest patients (Table 4.8, page 170). Two other studies have quantified the influence of age in this way\textsuperscript{404,406}. The results are shown in Table 4.16.

<table>
<thead>
<tr>
<th>Study</th>
<th>Age Stratification (years)</th>
<th>Relative Risk of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Van Gelder</td>
<td>&lt; 65</td>
<td>1.0</td>
</tr>
<tr>
<td>1994</td>
<td>65 - 74</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>&gt;74</td>
<td>2.0</td>
</tr>
<tr>
<td>Magnani</td>
<td>≤ 56</td>
<td>1.0</td>
</tr>
<tr>
<td>2002</td>
<td>56 - 65</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>66 - 75</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>≥76</td>
<td>1.7</td>
</tr>
<tr>
<td>Current</td>
<td>≤ 55</td>
<td>1.0</td>
</tr>
<tr>
<td>Study</td>
<td>56 - 62</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>63 - 75</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>&gt; 75</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Table 4.16
Summary of Studies That Have Estimated the Relative Risk of Death
Attributable to Age in Patients with Mesothelioma
It seems logical that younger patients will tend to be fitter and have less co-morbidity, thereby increasing survival. However few of our cohort died of other causes, and the majority had evidence of progressing mesothelioma at death. What is less clear is whether age can influence tumour behaviour and survival by any other mechanism - does mesothelioma in young patients behave in a different way from that in older patients? It is possible that younger patients differ from older in other important ways, such as their immune response to their tumour, and it is this that influences survival. Potential confounding factors include stage at presentation, performance status, histological subtype and asbestos exposure. Unfortunately stage at presentation, and performance status were not reliably recorded in our cohort, and therefore cannot be commented on. One hundred and nine patients were aged 55 or less in our cohort, and survival was known in 94 of these. There was no significant difference in the proportion of patients under 55 years when sub-analysed by gender or asbestos exposure, compared with older patients. However, the histological subtype of tumour did appear to be influenced by age.

Initial analysis of the age at presentation and histological subtype confirmed the clinical impression that sarcomatoid and desmoplastic tumours tend to present in older patients (Table 4.12, page 181). Analysis of tumour distribution by age cohort confirmed an excess of sarcomatoid and desmoplastic tumours in patients over the age of 63 years (p<0.03), Figure 4.16, page 182. Why the phenotype of a tumour should be influenced by age is unclear, but an excess of SM in older patients has been reported in other studies. There are no studies that have suggested a link between tumour type and other age-linked biological factors, such as sex hormones. Although two of the three longest survivors were pre-menopausal women, differences in survival between sexes were eliminated when tumour type was corrected for.

To summarise, our study confirms the finding that age is an important prognostic factor in patients with pleural mesothelioma. The relative risk of dying is doubled in those over the age of 55 years compared with those below 55, and continues to rise with increasing age. Although some of this effect is likely to be a reflection of general health it seems that other factors such as histological type may also interact with age to influence outcome. The exact mechanisms for this remain obscure at present. It is vital however, that future studies of outcome carefully stratify subjects by age if this influence is to be accounted for.
4.24: Analysis of the Influence of Gender on Survival

The limitations incurred in studies of pleural mesothelioma due to small study numbers are even more pronounced when prognostic factors are investigated in women. Because the majority of pleural mesotheliomas result from occupational asbestos exposure, most studies of pleural mesothelioma in adults have a significant excess of men, usually of the order of 4:1 or more. A greater proportion of women may have usually had contact with asbestos through para-occupational or environmental exposure, rather than through their work. The small numbers of women thus affected, along with the difficulties identifying relevant para-occupational asbestos exposure has hindered systematic study of mesothelioma in this group. Diagnostic bias can also be a significant problem in women. The main differential of EM is adenocarcinoma involving the pleura: Pulmonary adenocarcinoma is proportionally more common in women than men, and metastatic breast cancer is a common cause of malignant pleural effusion in women.

I have identified only two papers that have exclusively studied pleural mesothelioma in women. In 1978 Vianna and Polan studied the occupational histories of 52 women with mesothelioma, and those of their relatives. They demonstrated an excess of first degree male relatives working with asbestos in their study population, compared with controls. In 1993 Dawson et al published a study of 177 women with mesothelioma, of which 125 involved the pleura. They analysed histological subtype, the presence of lung fibrosis and lung fibre burden and concluded that the pathology of mesothelioma in women appears to be the same as in men. Gender has been analysed as a potential prognostic factor in many series, but no unequivocal conclusion can be drawn as to whether true differences in outcome exist, and whether differences are the result of confounding factors such as age, histology or asbestos exposure.

I have identified 19 published series that have examined the link between gender and survival. In seven of these a statistically significant survival advantage for women compared with men was confirmed (p<0.05). A significant influence was confirmed by multivariate analysis in only three studies. The two studies that contained the largest number of women (SEER, n=352 and Magnani et al, n=208) failed to confirm this finding (p=0.6 and p=0.2 respectively). In the SEER study only 63% of the women had pleural tumours, whereas the study by Magnani et al was unusual due to its high proportion of women (35% of the cohort), many of whom had themselves worked in the asbestos industry or had well-documented para-occupational asbestos exposure. The study by Metintas et al consisted
of patients from Turkey with predominantly environmental exposure to asbestos and erionite. There were almost equal numbers of men and women in this study, and no difference in survival was shown. A summary of the results of all of these studies, except that by Chailleux et al which does not report actual survival figures by gender, can be found in Table 4.17 overleaf.

There is, however, a suggestion that real differences do exist in terms of outcome between men and women. Women are disproportionately represented in the long-term survivor groups in three studies, but this fails to impact on the survival analysis overall because of their small numbers. Of the two cases described by Ruffie et al the first described a young woman who underwent five resections of a pleural-based tumour over a period of 14 years. This may well be a solitary fibrous tumour. The second case was a young woman who was diagnosed as having a desmoplastic mesothelioma that subsequently "underwent complete regression". In neither case was histological review carried out to confirm the diagnosis of malignant mesothelioma. Interestingly, in our initial cohort five cases initially diagnosed as DM were reclassified as reactive pleuritis after review. The study by Fusco et al fails to demonstrate a difference in survival between sexes, but states a 20% 3-year survival for women compared with an 8.6% 3-year survival rate in men. Of the six women included in the study by Mårtensson, one died at 57 months from diagnosis, and two further women survived more than five years. By contrast only one of 26 men in their study survived more than 5 years. Similarly, our cohort contained one woman who is alive and well five years post-diagnosis, and another one who developed chest wall metastatic disease almost 11 years after treatment. Both of these women had epithelioid tumours and were below the age of 50 at diagnosis.

The initial actuarial survival comparison between males and females in our cohort demonstrated a survival advantage for women (P=0.04). This was despite being slightly older at diagnosis than men (difference not significant, p=0.48). Women demonstrated the same survival pattern as men when stratified for age, although the statistical significance was less marked, probably because of smaller group size. Our two long-term female survivors may account for this survival advantage, particularly as the Log-rank test is most sensitive to differences in late events and long-term survival.
<table>
<thead>
<tr>
<th>Study</th>
<th>Male : Female</th>
<th>Number of Women In Study</th>
<th>Median Survival (months)</th>
<th>p Value (Univariate analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mårtensson 1984</td>
<td>4 : 1</td>
<td>6</td>
<td>10.0 36.5</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Adams 1986</td>
<td>3.4 : 1</td>
<td>21</td>
<td>8.4 18.0</td>
<td>0.03 Cox</td>
</tr>
<tr>
<td>Spiritas 1988</td>
<td>3.2 : 1</td>
<td>352#</td>
<td>Not Stated</td>
<td>0.6 Log-rank</td>
</tr>
<tr>
<td>Alberts 1988</td>
<td>2.6 : 1</td>
<td>72</td>
<td>8.6 12.6</td>
<td>0.05 Mantel-Cox</td>
</tr>
<tr>
<td>Antman 1988</td>
<td>3.2 : 1</td>
<td>43#</td>
<td>15.0 14.0</td>
<td>0.01 Log-rank</td>
</tr>
<tr>
<td>Calavrezos 1988</td>
<td>2.1 : 1</td>
<td>42</td>
<td>8.0 11</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Ruffie 1989</td>
<td>3.7 : 1</td>
<td>68</td>
<td>9.2 9.0</td>
<td>0.03 Log-rank</td>
</tr>
<tr>
<td>Tammilehto 1992</td>
<td>3.7 : 1</td>
<td>21#</td>
<td>8.0 13.0</td>
<td>0.02* Log-rank</td>
</tr>
<tr>
<td>Boutin 1993</td>
<td>5.7 : 1</td>
<td>28</td>
<td>8.0 9.0</td>
<td>0.9 Log-rank</td>
</tr>
<tr>
<td>Fusco 1993</td>
<td>15 : 1</td>
<td>7</td>
<td>10.0 10.0</td>
<td>Not Significant</td>
</tr>
</tbody>
</table>

Notes: # - study includes non-pleural cases, a - time from symptoms not diagnosis, * - statistically significant effect (p<0.05) confirmed after multivariate analysis

Table 4.17
Summary of Studies That Have Investigated the Influence of Gender on Survival in Mesothelioma
<table>
<thead>
<tr>
<th>Study</th>
<th>Male : Female</th>
<th>Number of Women In Study</th>
<th>Median Survival (months)</th>
<th>p Value (Univariate analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manzini 1993</td>
<td>10 : 1</td>
<td>7</td>
<td>M: 14.0, F: 10.0</td>
<td>0.4 Mantel-Cox</td>
</tr>
<tr>
<td>Van Gelder 1994</td>
<td>11.8 : 1</td>
<td>13</td>
<td>M: 8.9, F: 4.6</td>
<td>0.3 Log-rank</td>
</tr>
<tr>
<td>Curran 1998</td>
<td>8 : 1</td>
<td>23</td>
<td>M: 8.1, F: 11.7</td>
<td>0.06* Cox</td>
</tr>
<tr>
<td>Edwards 2000</td>
<td>10.5 : 1</td>
<td>12</td>
<td>M: Not Stated</td>
<td>0.013* Cox</td>
</tr>
<tr>
<td>Metintas 2001</td>
<td>1 : 1</td>
<td>51</td>
<td>M: 6.7, F: 11.5</td>
<td>0.09 Log-rank</td>
</tr>
<tr>
<td>Ceresoli 2001</td>
<td>3.6 : 1</td>
<td>26</td>
<td>M: 11.0, F: 10.0</td>
<td>0.14 Log-rank</td>
</tr>
<tr>
<td>Magnani 2002</td>
<td>1.8 : 1</td>
<td>208</td>
<td>M: Not Stated</td>
<td>0.2 Log-rank</td>
</tr>
<tr>
<td>Current Study</td>
<td>7.5 : 1</td>
<td>54</td>
<td>M: 7.9, F: 11.3</td>
<td>0.04 Log-rank</td>
</tr>
</tbody>
</table>

Notes: # - study includes non-pleural cases, a – time from symptoms not diagnosis
* - statistically significant effect (p<0.05) confirmed after multivariate analysis

Table 4.17 (Continued)
Summary of Studies That Have Investigated the Influence of Gender on Survival in Mesothelioma
It is also possible that differences in tumour subtype alone could account for the difference in survival between the sexes, given the small but non-significant ($p=0.15$) excess of epithelioid tumours in women (EM comprised 50% of all tumours in men and 64% of tumours in women). Recalculating actuarial survival in men and women stratified by tumour subtype abolished the women’s survival advantage ($p=0.09$). There are two possible reasons for this. Firstly, a significant difference between sexes will be harder to demonstrate statistically as the groups become smaller. Secondly, the skewing effect of the two women who survived more than five years from diagnosis will be diluted, as the longest-surviving male also had an epithelioid tumour. Regression analysis confirmed that gender did not exert a significant effect on survival.

To summarise, the influence of gender on survival is small when other factors are corrected for. There appear to be a small number of young women with predominantly well-differentiated epithelioid tumours who have much better life expectancy compared with other patients with mesothelioma. As of yet we have been unable to demonstrate any epidemiological factors or pathological features to explain this phenomenon, the biological explanation for which remains obscure.

4.25: Analysis of the Influence of Patient Symptoms on Survival

There are plausible reasons why patient symptoms and the mode of clinical presentation may relate to survival. The development of a significant pleural effusion usually produces dyspnoea, and can be readily diagnosed by CXR and pleural aspiration. Conversely, chest pain may present more of a diagnostic challenge, and may be difficult to differentiate from pain of cardiac, oesophageal and musculo-skeletal origin. This will have implications regarding ease and speed of diagnosis, as discussed earlier in this chapter. Considering the strong influence of histology on survival, the demonstration of an association between tumour subtype and symptoms might explain why some studies have shown the mode of presentation to be of prognostic significance.\(^{162,389,415,416,421,425,432}\)

The difficulties associated with data collection and verification in retrospective studies are well recognised. These are greatest in categories that are highly subjective, such as dyspnoea. Symptoms are rarely recorded by reproducible objective means, e.g. dyspnoea grades or visual analogue pain scores, outside the setting of prospective clinical trials. This might explain why half of those studies that have demonstrated an association between symptoms and survival have predominantly included patients enrolled in trials...
evaluating different therapies, as they often require strict recording of clinical factors at presentation.415,421

Most published studies of MPM are produced by tertiary referral centres or using cancer registry data. It is possible that symptoms have changed or are less well remembered by the time patients are referred. Given the fact that diagnostic delay can be significant in these patients, the listed symptoms may differ significantly from records taken at the time of initial presentation to peripheral hospitals. A comparison of our records with those of the referring hospital was beyond the scope of this project. These sources of error should be borne in mind when evaluating published results.

Although several studies have reported the incidence of specific presenting symptoms and signs, few have analysed them in terms of survival.162,389,397,413,415,416,420,421,424-427,432 Even fewer have investigated any association between symptoms and tumour subtype.389,405 Overall, it appears that chest pain as a presenting symptom may be associated with a poorer prognosis. This has been demonstrated in five studies.415,416,421,425,432 Two of these, and one other study, have demonstrated that dyspnoea at presentation is also a poor prognostic factor.389,415,421 Ruffie et al, Herndon et al and Boutin et al also demonstrated that weight loss was associated with a poorer prognosis.162,389,415 These studies are summarised in Table 4.18 below. Whether these findings are a simply a reflection of tumour stage at presentation, or reflect some other aspect of tumour behaviour or subtype is uncertain.

I was unable to demonstrate a relationship between symptoms and prognosis in my cohort. This may relate to the relatively small proportion of patients that were included in the analysis. Alternatively, inaccuracies in symptom recording could have masked a potential relationship. The question of whether tumour subtype and symptoms are related is less clear-cut. Although failing to achieve statistical significance, there was a trend towards pain being more common in sarcomatoid tumours. Given the strong association between survival and tumour subtype, this factor alone could explain the results of some studies, if the link between pain and SM was confirmed. The presence of distant disease at presentation was also significantly more common in this group, and might account for some of their reduced survival. However, this analysis is based wholly on clinically apparent metastases, and involves small numbers of patients. Sub-clinical dissemination may well have been present but undiagnosed or unreported in other tumour types, and could significantly influence the strength of this association if included in the analysis.
<table>
<thead>
<tr>
<th>Study</th>
<th>No of Patients Analysed</th>
<th>Incidence of Pain (%)</th>
<th>Incidence of SOB (%)</th>
<th>Incidence of Weight Loss (%)</th>
<th>Survival Analysis (Log-rank)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alberts 1988</td>
<td>262</td>
<td>58</td>
<td>74</td>
<td>3.1</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Antman 1988</td>
<td>180*</td>
<td>48</td>
<td>60</td>
<td>15</td>
<td>Pain: p&lt;0.001*</td>
</tr>
<tr>
<td>Calavrezos 1988</td>
<td>132</td>
<td>72</td>
<td>88</td>
<td>-</td>
<td>Pain p = 0.0008*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SOB p = 0.004</td>
<td></td>
</tr>
<tr>
<td>Hulks 1989</td>
<td>68</td>
<td>47</td>
<td>67</td>
<td>23</td>
<td>Pain vs SOB p &lt; 0.01</td>
</tr>
<tr>
<td>Ruffe 1989</td>
<td>332</td>
<td>33*</td>
<td>28*</td>
<td>-</td>
<td>Pain = Not Sig</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SOB p = 0.07*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Weight p &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Tammilehto 1992</td>
<td>98*</td>
<td>47</td>
<td>46</td>
<td>-</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Boutin 1993</td>
<td>168</td>
<td>-</td>
<td>-</td>
<td>50</td>
<td>0.001* (Log-rank)</td>
</tr>
<tr>
<td>Manzini 1993</td>
<td>80</td>
<td>51</td>
<td>65</td>
<td>29</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Fusco 1993</td>
<td>113</td>
<td>55</td>
<td>65</td>
<td>14</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Yates 1997</td>
<td>272*</td>
<td>38</td>
<td>33</td>
<td>-</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Herndon 1998</td>
<td>257*</td>
<td>60</td>
<td>70</td>
<td>41</td>
<td>Pain p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SOB p = 0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Weight p = 0.004</td>
<td></td>
</tr>
</tbody>
</table>

Notes: # = non-pleural cases included, SOB = shortness of breath
* confirmed as significant (p <0.05) after multivariate analysis

Table 4.18
Summary of Studies That Have Analysed the Influence of Symptoms on Survival in Mesothelioma
Table 4.18 (Continued)

Summary of Studies That Have Analysed the Influence of Symptoms on Survival in Mesothelioma

4.26: Analysis of the Influence of Diagnostic Delay on Survival

The time elapsing from first symptoms to the date of diagnosis can vary widely between patients. It has been suggested that diagnostic delay appears to be of prognostic significance: the greater the delay, the longer the survival. Two studies have reported a statistically significant relationship between delay and outcome after univariate analysis. Three have confirmed the influence of delay after multivariate analysis. These results are summarised in Table 4.19 at the end of this section.

There are two potential ways in which diagnostic delay might reflect tumour biology and behaviour, and thereby influence survival. Firstly, tumours with a better prognosis e.g. EM, may grow more slowly. If this is the case then patients might take longer to develop symptoms severe enough to warrant seeking medical advice, and therefore be diagnosed later. An alternative explanation may also help explain the influence of histology on survival. Epithelioid mesotheliomas most frequently present as pleural effusions, which can be readily diagnosed on CXR. Aspiration cytology is of limited specificity for unequivocally diagnosing mesothelioma, but the demonstration of atypical or malignant cells within the effusion should ensure further diagnostic investigations. By contrast, sarcomatoid mesothelioma more frequently presents with chest wall pain. This can be more difficult to diagnose, and the CXR changes associated with
pleural thickening are not always diagnostic of malignancy. Hence a lag-phase bias may be introduced, patients with SM being diagnosed later in their disease course, thereby artificially reducing survival when calculated from time of diagnosis rather than presentation. Difficulties associated with the confirmation the histological diagnosis of mesothelioma, particularly desmoplastic mesothelioma, will introduce further delay. Hence it is important to note how reports define delay. Is it a measure of the time from symptoms to presentation and diagnosis? Alternatively is it a measure of the duration of the diagnostic process itself, defining the time from presentation to diagnosis?

To test this hypothesis we sub-classified our cohort according to diagnostic delay. We defined this as the time from first symptoms to histological confirmation of the diagnosis of mesothelioma. Group One included patients with a delay of less than 100 days. Group Two those with a delay of 100 or more days. As mentioned in Section 4.18, 100 days approximated to the mean delay (96 days). A total of 141 patients was included in the analysis. Delay did not significantly affect survival (p=0.24, Figure 4.14, page 179).

The relationship between diagnostic delay and histological subtype was also analysed. The mean time from symptoms to diagnosis approximated to 100 days for the three main histological subtypes (EM=102 days, BM = 91 days, SM = 94 days). This difference was not statistically significant (p = 0.9). Similarly, looking at the proportions of cases diagnosed more or less than 100 days from symptoms within each histology type failed to demonstrate any difference ($\chi^2 =0.9$, p = 0.6). Overall we could find no real evidence of a relationship between delay and survival in our cohort. Our one caveat is that this information was only available in a small proportion of our cohort, which makes it less likely that we would demonstrate a difference even if it did exist.
<table>
<thead>
<tr>
<th>Study</th>
<th>No of Subjects in Study</th>
<th>Diagnostic Delay</th>
<th>Median Survival (months)</th>
<th>P Value (Univariate analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alberts 1988</td>
<td>262</td>
<td>Delay &lt; 6 months</td>
<td>12.1</td>
<td><strong>0.008</strong>* Mantel-Cox</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Delay &gt; 6 months</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>Antman 1988</td>
<td>180*</td>
<td>Delay &lt; 6 months</td>
<td>13.0</td>
<td><strong>0.2</strong>* Log-rank</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Delay &gt; 6 months</td>
<td>16.0</td>
<td></td>
</tr>
<tr>
<td>Calavrezos 1988</td>
<td>132</td>
<td>Delay &lt; 6 months</td>
<td>8.0</td>
<td>Not Sig Log-rank</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Delay &gt; 6 months</td>
<td>11.0</td>
<td></td>
</tr>
<tr>
<td>Chailleux 1988</td>
<td>167</td>
<td>Delay &lt; 2 months</td>
<td>8.0*</td>
<td>&lt; 0.001 Log-rank</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Delay &gt; 2 months</td>
<td>16.0*</td>
<td></td>
</tr>
<tr>
<td>Tammilehto 1992</td>
<td>98*</td>
<td>Delay &lt; 6 months</td>
<td>8.0</td>
<td><strong>0.07</strong>* Log-rank</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Delay &gt; 6 months</td>
<td>14.5</td>
<td></td>
</tr>
<tr>
<td>Boutin 1993</td>
<td>188</td>
<td>Delay &lt; 4 months</td>
<td>7.3</td>
<td>0.07 Log-rank</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Delay &gt; 4 months</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>Manzini 1993</td>
<td>80</td>
<td>Delay &lt; 31 days</td>
<td>14.0</td>
<td>0.9 Mantel-Cox</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Delay 31-36 days</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Delay &gt; 36 days</td>
<td>15.0</td>
<td></td>
</tr>
<tr>
<td>Curran 1998</td>
<td>204</td>
<td>Delay ≤50 days</td>
<td>8.8 (mean)</td>
<td>0.23 Cox</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Delay &gt; 50 days</td>
<td>7.6 (mean)</td>
<td></td>
</tr>
<tr>
<td>Current Study</td>
<td>141</td>
<td>Delay &lt; 100 days</td>
<td>7.4</td>
<td>0.3 Log-rank</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Delay &gt; 100 days</td>
<td>7.6</td>
<td></td>
</tr>
</tbody>
</table>

Notes: # - Study included non-pleural cases, a – time from symptoms not diagnosis, * confirmed as significant \((p < 0.05)\) after multivariate analysis

Table 4.19: Summary of Studies That Have Investigated the Relationship Between Diagnostic Delay and Survival
4.27: Analysis of the Influence of Tumour Subtype on Survival

Tumour subtype was the other highly significant factor influencing survival in our cohort (p<0.00005). Survival was longest in patients with epithelioid mesothelioma and slightly less in biphasic tumours. The sarcomatoid group fared worse still, with desmoplastic tumours associated with the poorest prognosis. Four patients with sarcomatoid tumours survived more than a year (6.1% of all SM patients) and only one with desmoplastic mesothelioma (4% of all DM patients). This contrasts with epithelioid and biphasic subtypes, which had one-year survival rates of 54.7% and 37.4% respectively. This pattern was seen in both men and women when analysed separately, and remained significant when age at diagnosis was corrected for. Regression analysis confirmed histology as an independent prognostic factor. The relative risks of dying from BM, SM and DM were 2.1, 4.9 and 7.4 respectively, compared with EM (Table 4.13 page 183).

Analyses of the influence of histological subtype on survival have been reported by several researchers, with conflicting results. Some of these studies have only stratified into epithelioid and non-epithelioid tumours. Others do not state median survival times or relative risk values for individual tumour subtypes. This makes comparisons between studies difficult. There are 22 published series that have analysed the influence of histology in a methodical way, with reasonable patient numbers and tumour classification according to the WHO guidelines.

Fifteen of these studies have demonstrated a statistically significant relationship between histology and outcome, but not all agree on the relative effects of each subtype. Eleven have confirmed the effect of histology after multivariate analysis. Although most agree that epithelioid tumours are associated with the best prognosis, two studies have reported that biphasic have either the best or worst prognosis overall. A summary of their reported findings are detailed in Table 4.20 overleaf.

Unfortunately the SEER study, which potentially contains enough patients to categorically confirm or refute a prognostic influence, does not require histological classification or pathology review. Only one quarter of all cases were sub-classified, and none had histology reviewed once included on the registry database. A large number of non-pleural mesotheliomas were also included in this analysis (n=278). Similarly, in the German mesothelioma registry data reported by Neumann et al only 10% of all cases were sub-typed, and the analysis also included peritoneal cases.
<table>
<thead>
<tr>
<th>Study</th>
<th>No of Cases in Survival Analysis</th>
<th>Median Survival All Cases (months)</th>
<th>Survival For EM (months)</th>
<th>Survival For BM (months)</th>
<th>Survival For SM (months)</th>
<th>p Value (Univariate analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hillerdal</td>
<td>153</td>
<td>10.0</td>
<td>11.0</td>
<td>10.0</td>
<td>5.0</td>
<td>Not stated</td>
</tr>
<tr>
<td>Mårtensson</td>
<td>32</td>
<td>11.0</td>
<td>11.5</td>
<td>17.0</td>
<td>3.5</td>
<td>&lt;0.05 Rank sum</td>
</tr>
<tr>
<td>Adams</td>
<td>92</td>
<td>8.0</td>
<td>13.2</td>
<td>8.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.6</td>
<td>0.0001 Log-rank</td>
</tr>
<tr>
<td>Antman</td>
<td>180&lt;sup&gt;#&lt;/sup&gt;</td>
<td>15.0</td>
<td>17.0</td>
<td>13.0</td>
<td>7.0</td>
<td>0.04* Log-rank</td>
</tr>
<tr>
<td>Calavrezos</td>
<td>132</td>
<td>9.0</td>
<td>11.0</td>
<td>9.0</td>
<td>5.0</td>
<td>0.0002* Log-rank</td>
</tr>
<tr>
<td>Ruffie</td>
<td>286</td>
<td>9.0</td>
<td>9.9</td>
<td>9.2</td>
<td>5.2</td>
<td>0.009 Log-rank</td>
</tr>
<tr>
<td>Walz</td>
<td>43</td>
<td>13.0</td>
<td>23.0</td>
<td>13.0</td>
<td>6.0</td>
<td>Not Significant</td>
</tr>
<tr>
<td>Tammilehto</td>
<td>98&lt;sup&gt;#&lt;/sup&gt;</td>
<td>9.0</td>
<td>14.0</td>
<td>9.0</td>
<td>2.5</td>
<td>0.001* Log-rank</td>
</tr>
</tbody>
</table>

Notes: # - Study includes non-pleural mesotheliomas, a - from time of symptoms
* Statistically significant difference (p <0.05) confirmed on multivariate analysis

Table 4.20

Summary of Studies That Have Investigated the Effect of Histology on Survival in Mesothelioma
<table>
<thead>
<tr>
<th>Study</th>
<th>No of Cases in Survival Analysis</th>
<th>Median Survival All Cases (months)</th>
<th>Survival For EM (months)</th>
<th>Survival For BM (months)</th>
<th>Survival For SM (months)</th>
<th>p Value (Univariate analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boutin 1993</td>
<td>188</td>
<td>Not Stated</td>
<td>11.2</td>
<td>7.0</td>
<td>5.3</td>
<td>0.01* Log-rank</td>
</tr>
<tr>
<td>Fusco 1993</td>
<td>113</td>
<td>10.0</td>
<td>12.0</td>
<td>7.0</td>
<td>4.0</td>
<td>0.001* Mantel-Cox</td>
</tr>
<tr>
<td>Manzini 1993</td>
<td>73</td>
<td>13.0</td>
<td>13.0</td>
<td>15.0</td>
<td>8.0</td>
<td>0.001* Mantel-Cox</td>
</tr>
<tr>
<td>Van Gelder 1994</td>
<td>83</td>
<td>8.0</td>
<td>8.3</td>
<td>6.2</td>
<td>6.8</td>
<td>0.04* Log-rank</td>
</tr>
<tr>
<td>Johanssen 1996</td>
<td>85</td>
<td>Not Stated</td>
<td>17.6^</td>
<td>13.3^</td>
<td>12.8^</td>
<td>0.01 Mann-Whitney</td>
</tr>
<tr>
<td>Yates 1997</td>
<td>272^</td>
<td>14.0^</td>
<td>12.5^</td>
<td>11.0^</td>
<td>9.4^</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Curran 1998</td>
<td>184</td>
<td>12.6</td>
<td>8.4</td>
<td>9.1</td>
<td>5.0</td>
<td>0.002* Log-rank</td>
</tr>
<tr>
<td>Gore 1998</td>
<td>69^</td>
<td>9.9</td>
<td>11.2</td>
<td>9.4</td>
<td>7.5</td>
<td>0.08</td>
</tr>
<tr>
<td>Edwards 2000</td>
<td>121</td>
<td>5.9</td>
<td>Not Stated</td>
<td>Not Stated</td>
<td>Not Stated</td>
<td>&lt;0.0001* Cox</td>
</tr>
</tbody>
</table>

Notes: ^ -Study includes non-pleural mesotheliomas, a- from time of symptoms
* Statistically significant difference (p <0.05) confirmed on multivariate analysis

Table 4.20 (Continued)
Summary of Studies That Have Investigated the Effect of Histology on Survival in Mesothelioma
<table>
<thead>
<tr>
<th>Study</th>
<th>No of Cases in Survival Analysis</th>
<th>Median Survival All Cases (months)</th>
<th>Survival For EM (months)</th>
<th>Survival For BM (months)</th>
<th>Survival For SM (months)</th>
<th>p Value (Univariate analysis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Merritt 2001</td>
<td>101</td>
<td>7.0</td>
<td>7.9</td>
<td>8.7</td>
<td>2.7</td>
<td>0.0016* Log-rank</td>
</tr>
<tr>
<td>Metintas 2001</td>
<td>77</td>
<td>8.0</td>
<td>9.0</td>
<td>6.7</td>
<td>7.0</td>
<td>0.16 Log-rank</td>
</tr>
<tr>
<td>Ceresoli 2001</td>
<td>121</td>
<td>10.5</td>
<td>11</td>
<td>8.5</td>
<td>6</td>
<td>0.048 Log-rank</td>
</tr>
<tr>
<td>Neumann 2001</td>
<td>404&quot; (mean)</td>
<td>Not Stated</td>
<td>Not Stated</td>
<td>Not Stated</td>
<td>Not Stated</td>
<td>&lt;0.001 Log-rank</td>
</tr>
<tr>
<td>Magnani 2002</td>
<td>450</td>
<td>8.5</td>
<td>Not Stated</td>
<td>Not Stated</td>
<td>Not Stated</td>
<td>&lt;0.00001* Log-rank</td>
</tr>
<tr>
<td>Current Study</td>
<td>458</td>
<td>8.0</td>
<td>11.8</td>
<td>7.6</td>
<td>3.9</td>
<td>&lt;0.00005* Log-rank</td>
</tr>
</tbody>
</table>

Notes: # - Study includes non-pleural mesotheliomas, a- from time of symptoms
* Statistically significant difference (p < 0.05) confirmed on multivariate analysis

Table 4.20 (Continued)

Summary of Studies That Have Investigated the Effect of Histology on Survival in Mesothelioma
The studies in Table 4.20 confirm a median survival for all patients ranging from eight to fifteen months. Four studies included non-pleural mesotheliomas within their series. The relative proportion of each tumour subtype also varied between studies. Five studies have reported that over 60% of cases were classified as EM. Most of these reports were from units that utilised thoracoscopy in the diagnosis of mesothelioma: this high proportion of epithelioid tumours may reflect their choice of patients with demonstrable pleural effusions. Biphasic mesothelioma was the most common subtype reported by Neumann et al. The incidence of SM is between 10 – 20% in most series, although it is over 40% in the SEER study. The incidence of DM has been reported in only one study, at 4%. Our cohort comprised 51% EM, 30% BM, 14.1% SM and 4.9% DM, which reflects the pattern seen in most of the larger studies that have sub-classified their mesotheliomas.

Two factors need to be considered when considering the distribution of subtypes. Firstly there is good evidence that the proportion of tumours classified as biphasic directly relates to the number of sections examined. Sampling errors of this type will tend to be more common in small biopsy sections i.e. needle and thoracoscopic biopsies, and less so in open biopsies and post-mortem specimens. In our cohort, post-mortem tissue was available for more than three-quarters of patients who had died (77.6%, n = 346). If there was a difference between biopsy and PM material then the subtype of the larger specimen was used. Secondly, distinguishing between a true biphasic tumour and an epithelioid mesothelioma with a prominent stromal reaction can be difficult, particularly without the contribution of immunohistochemistry to aid diagnosis. Chailleux and colleagues re-categorised cases of epithelioid mesotheliomas with prominent stroma in their cohort as biphasic tumours, and re-analysed them. This still failed to demonstrate a statistically significant difference between tumour types in terms of survival. Biases of these types may be enough to explain the differing results reported in the literature.

Only six of the studies listed in Table 4.20 have attempted to quantify the influence of histology. The relative risk for each tumour histological subtype calculated after multivariate analysis are shown in Table 4.21 overleaf.
<table>
<thead>
<tr>
<th>Study</th>
<th>Relative Risk for EM</th>
<th>Relative Risk for BM</th>
<th>Relative Risk for SM</th>
<th>Relative Risk for Non-EM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusco 1993</td>
<td>1.0</td>
<td>1.7</td>
<td>3.2</td>
<td>Not Stated</td>
</tr>
<tr>
<td>Manzini 1993</td>
<td>1.0</td>
<td>1.5</td>
<td>4.5</td>
<td>Not Stated</td>
</tr>
<tr>
<td>Van Gelder 1994</td>
<td>1.1</td>
<td>1.7</td>
<td>0.9</td>
<td>Not Stated</td>
</tr>
<tr>
<td>Curran 1998</td>
<td>1.0</td>
<td>1.0</td>
<td>2.7</td>
<td>Not Stated</td>
</tr>
<tr>
<td>Edwards 2000</td>
<td>1.0</td>
<td>Not Stated</td>
<td>Not Stated</td>
<td>2.3</td>
</tr>
<tr>
<td>Magnani 2002</td>
<td>1.0</td>
<td>0.7</td>
<td>1.4</td>
<td>Not Stated</td>
</tr>
<tr>
<td>Current Study</td>
<td>1.0</td>
<td>2.1</td>
<td>4.9</td>
<td>Not Stated</td>
</tr>
</tbody>
</table>

Table 4.21
Summary of Studies That Have Reported the Relative Risk of Death Attributable to Tumour Subtype in Patients with Mesothelioma

In summary, the histological sub-type of pleural mesothelioma was the most influential prognostic factor in our series. This yet again emphasizes the critical importance of accurate histopathological evaluation of pleural tumours prior to registration or the commencement of treatment. There appears to be a complex interplay between tumour subtype and other patient factors such as gender and patient age, which we have not yet been able to fully unravel. For example why should older patients be more prone to sarcomatoid tumours? Does this relate to asbestos exposure or other as yet unidentified patient factors? What is clear is that the randomisation of patients into therapeutic trials
without controlling for tumour subtype is likely to introduce substantial bias into any subsequent survival analysis.

4.28: Analysis of the Influence of Asbestos Exposure on Survival

Most studies that have investigated the incidence of mesothelioma in a non-selected population have found that approximately 85% of patients have a history of definite or possible asbestos exposure. This was the case in our cohort. What is less clear is whether asbestos exposure can affect survival, either through an influence on tumour subtype or behaviour, or via other mechanisms.

What are the possible mechanisms by which asbestos exposure acts as a prognostic factor in pleural mesothelioma? Other than inducing mesothelioma, asbestos exposure also causes lung fibrosis (asbestosis). The development of fibrosis seems to be dose-dependent. Asbestos also increases an individual’s risk of developing lung cancer, and is synergistic with cigarette smoking in this respect. This introduces two confounding factors. People with asbestosis have reduced lung function. This could affect their ability to tolerate the pulmonary effects of mesothelioma, and further reduce survival. Conversely, given the long lag period from exposure to mesothelioma development, it is possible that a significant number of people exposed to asbestos will die from other causes long before mesothelioma ever has the chance to develop or become clinically apparent, particularly if they are smokers. Hence, it could be inferred that those who develop mesothelioma are self-selected from a group with less asbestos exposure than average for their occupation! It is also important to remember that it requires a high index of suspicion to diagnose mesothelioma. Several patients within our cohort were misdiagnosed as lung cancer in life. Unless a post-mortem examination is performed, the true pleural nature of their disease may not be apparent. How many other cases of mesothelioma are never diagnosed? This may well have a bearing on our ability to identify and quantify prognostic factors if those individuals who are studied represent a pre-selected group.

It can be difficult to evaluate the amount of asbestos exposure an individual has had, particularly when the mode of exposure is thought to be environmental or para-occupational. Total asbestos exposure is difficult to quantify, as it depends on a complex balance between the number of fibres inhaled and the efficiency of an individual's bronchopulmonary clearance mechanisms. There can be great variation in the amount of exposure to individuals in similar occupations. Given the long lag phase in mesothelioma, diagnosis is usually decades after exposure, which can reduce the accuracy of an
occupational history, particularly if taken from a spouse. The risk of mesothelioma also relates to the type of asbestos. More dangerous forms, e.g. crocidolite may contaminate less dangerous forms, greatly increasing the theoretical risk of mesothelioma from what might otherwise have been considered a clinically insignificant exposure.

Finally, it has been suggested that asbestos exposure may influence the type of tumour that develops. Given the strong influence of histological subtype on survival, this could be the mechanism by which asbestos influences survival. It could also explain the difference in tumour histology distribution between the sexes, as women generally have less asbestos exposure than men.

In 1980 Law and colleagues suggested that asbestos exposure had both a prognostic and a causative effect in mesothelioma. In 1983 they published a paper in which they analysed the relationship between asbestos and survival, using both occupational history and mineralogical analysis to assess asbestos exposure. They found that survival was significantly longer in patients without asbestos exposure (p = 0.01). Since then, few other groups have studied this subject further, and only one author has reported a statistically significant influence of asbestos exposure on survival. In this study of 332 patients, 44% had a definite or probable history of asbestos exposure, although complete occupational details were only available in 60% of their cohort. They found no difference between exposed and non-exposed groups on univariate analysis (p=0.6). However after multivariate analysis, asbestos exposure was found to be significant (p=0.02), with non-exposed subjects enjoying an improved survival. This and other relevant studies are listed in Table 4.22 below.
<table>
<thead>
<tr>
<th>Study</th>
<th>No of Subjects in Study</th>
<th>Proportion of Cohort Exposed to Asbestos</th>
<th>Median Survival in Exposed Subjects (months)</th>
<th>Median Survival in Non-exposed Subjects (months)</th>
<th>p Value (UA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Law 1983</td>
<td>106</td>
<td>78%</td>
<td>10.0</td>
<td>19.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Antman 1988</td>
<td>180&lt;sup&gt;a&lt;/sup&gt;</td>
<td>68%</td>
<td>27.0</td>
<td>34.0</td>
<td>0.24</td>
</tr>
<tr>
<td>Chailleux 1988</td>
<td>139</td>
<td>88%</td>
<td>Not stated</td>
<td>Not Stated</td>
<td>NS</td>
</tr>
<tr>
<td>Calavrezos 1988</td>
<td>132</td>
<td>78%</td>
<td>8.0</td>
<td>9.0</td>
<td>NS</td>
</tr>
<tr>
<td>Ruffie 1989</td>
<td>332</td>
<td>44%</td>
<td>9.3</td>
<td>8.6</td>
<td>0.6&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tammilehto 1992</td>
<td>98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60%</td>
<td>8.5</td>
<td>10.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Boutin 1993</td>
<td>118</td>
<td>76%</td>
<td>8.3</td>
<td>10.5</td>
<td>0.15</td>
</tr>
<tr>
<td>Fusco 1993</td>
<td>113</td>
<td>37%</td>
<td>12.0</td>
<td>9.0</td>
<td>NS</td>
</tr>
<tr>
<td>Van Gelder 1994</td>
<td>82</td>
<td>83%</td>
<td>10.1</td>
<td>6.6</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Notes: a-cases with predominantly environmental exposure, # includes non-pleural cases. UA-univariate analysis, NS – not statistically significant
* effect of asbestos confirmed as significant (p <0.05) after multivariate analysis

Table 4.22
Summary of Studies That Have Investigated the Influence of Asbestos Exposure on Survival in Mesothelioma
### Table 4.21 (Continued)

**Summary of Studies That Have Investigated the Influence of Asbestos Exposure on Survival in Mesothelioma**

In our cohort there was no statistically significant difference between groups after univariate analysis (p = 0.74, **Figure 4.17, page 184**). This was a crude comparison, looking at those with any asbestos exposure compared with those with none. Obviously there may be great differences between patients within each of these groups that may confound results. However re-examining survival with further stratification into three groups (Group A = definite + probable exposure, Group B = para-occupational + environmental exposure, Group C = no exposure) also failed to detect a difference (p = 0.7). Finally I attempted to examine whether the amount of asbestos exposure influenced survival. Two analyses were performed: in the first subjects were stratified according to the maximum length of asbestos exposure recorded. Strata were as follows; no exposure, exposure < 5 years, 5-10 years exposure, 10 - 20 years exposure. There was no difference between groups (p = 0.8). Secondly subjects were stratified according to pulmonary asbestos fibre burden. Groups consisted of those with zero counts, counts greater than zero.

<table>
<thead>
<tr>
<th>Study</th>
<th>No of Subjects in Study</th>
<th>Proportion of Cohort Exposed to Asbestos</th>
<th>Median Survival in Exposed Subjects (months)</th>
<th>Median Survival in Non-exposed Subjects (months)</th>
<th>p Value (UA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edwards 2000</td>
<td>107</td>
<td>81%</td>
<td>Not Stated</td>
<td>Not Stated</td>
<td>0.7</td>
</tr>
<tr>
<td>Metintas 2001</td>
<td>100*</td>
<td>81%</td>
<td>8.5</td>
<td>6.7</td>
<td>0.9</td>
</tr>
<tr>
<td>Current Study</td>
<td>389</td>
<td>73%</td>
<td>9.0</td>
<td>8.8</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Notes: a-cases with predominantly environmental exposure, # includes non-pleural cases. UA - univariate analysis, NS – not statistically significant

* effect of asbestos confirmed as significant (p <0.05) after multivariate analysis
but less than 100,000 and those with counts greater than 100,000. Again no difference was demonstrable (p = 0.8). It therefore seems that asbestos exposure per se does not influence survival.

It does however appear that asbestos exposure may influence the histological type of tumour that develops. We found a significant excess of tumours with sarcomatoid differentiation in those with a history of asbestos exposure (p=0.01), Table 4.14, page 185. If this were the case it might also help to explain the excess of pure epithelioid tumours in women, who tend to have much less asbestos exposure compared with men. Another confounding factor is age. Patients with sarcomatoid and desmoplastic tumours were also older than those with other subtypes. Given the strong effect of age on survival, it is difficult to tease out the relationship between these three factors.

What is less easy to explain is why we cannot demonstrate an influence of asbestos exposure on survival, given the strong association between histology and survival. The group without asbestos exposure consisted of only 34 patients, and occupational history was unknown in 95 - this may have had enough of an effect to mask an influence on survival. In conclusion, the influence of asbestos exposure on survival appears negligible when considered separately from its causative role. Although there is evidence from our cohort that asbestos exposure can influence the type of tumour that develops, this effect is not translated into a difference in survival, and has not been validated by any other studies. The difficulty inherent in accurately assessing the amount and significance of asbestos exposure, particularly in those without occupational exposure remains a major problem when analysing asbestos as a prognostic factor in mesothelioma.
CONCLUSIONS

The increasing incidence of mesothelioma is forcing us to further examine factors that cause this tumour, and those that may influence treatment and survival. The effects of therapy have been disappointing, but there is renewed interest in the role of surgery as treatment, and new chemotherapy regimes are being evaluated in the setting of clinical trials. It is vital that those factors known to influence survival, such as age and histological subtype, are controlled for when patients are randomised, and the studies analysed. Significant bias can otherwise be introduced. Clinical trials will tend to exclude older patients with poor performance status. Most surgeons will not consider undertaking radical surgery on patients with sarcomatoid mesothelioma, as the improved survival for EM is becoming more widely accepted. The overall result of selection bias is that clinical trials are likely to contain an excess of patients whose prognosis was better than average to begin with. Extrapolating the results of these trials to all patients with mesothelioma may not be appropriate. For example, although the median survival for all patients in our cohort was eight months, for the best prognosis group (EM, age < 55) years this rose to 16 months. Survival has potentially been doubled through selection bias alone.

Age at the time of diagnosis will be known for all patients. The significant influence of histological subtype on prognosis emphasizes the vital need to correctly classify mesothelioma wherever possible. The possible relationship between age, histology and asbestos exposure is intriguing, but its significance is difficult to gauge at present. As more patients with mesothelioma are enrolled into therapeutic trials, it is possible that new aspects of its pathobiology are revealed, enabling better understanding of other biological influences such as the role of cytokines. These may be potential therapeutic targets in the future.
CHAPTER FIVE
TRANSFORMING GROWTH FACTOR–BETA1 POLYMORPHISMS IN PULMONARY FIBROSIS

INTRODUCTION

Transforming growth factor–beta (TGF-β) is a cytokine that regulates many physiological processes characterised by tissue remodelling and matrix deposition. It therefore plays a central role in normal embryogenesis and development, wound healing and both acute and chronic inflammation.

The gene encoding for transforming growth factor-β is highly conserved across animal species, including man. Minor differences within the TGF-β DNA sequence (DNA polymorphisms) have been identified. Some of these polymorphisms are located within areas of the DNA sequence that control the transcription and secretion of the active cytokine, and as such can significantly influence the amount and duration of active TGF-β1 production. The DNA polymorphisms involving codons 10 and 25 of the signal sequence have been shown to influence the presence and severity of pulmonary fibrosis, and to predict the development of pulmonary fibrosis following lung transplantation.

Pulmonary fibrosis has several aetiologies, but all are characterised by distortion of normal lung architecture and the deposition of excess extra-cellular matrix. Pulmonary fibrosis may be seen as part of a systemic chronic inflammatory condition. Others result from exposure to inhaled fibrogens e.g. asbestos. In a proportion of cases no underlying cause can be identified: under these circumstances the clinical term idiopathic pulmonary fibrosis is often employed.

It is both possible, and biologically plausible, that individuals who are genetically predisposed to produce excess TGF-β1 may be at increased risk of developing pulmonary fibrosis. They may respond to fibrogens at a lower dose, or in an exaggerated manner. This project was designed to investigate the distribution of the codon 25 polymorphism in patients with fibrotic lung disease and different levels of asbestos exposure. Paraffin-embedded formalin-fixed lung tissue was used as the source of DNA, and an amplification refractory mutation system – polymerase chain reaction (ARMS-PCR) technique utilising sequence-specific primers (SSP) used to identify the TGF-β1 polymorphism genotype for each case. I personally undertook the preparation of tissue blocks, DNA extraction and
PCR amplification and analysis at the Tissue Typing Laboratory, The Royal London Hospital, under the supervision of Dr Paul Sinnott.

MATERIALS AND METHODS

5.1: Patient Selection

A cohort of 40 patients who had died with histologically-confirmed severe pulmonary fibrosis was identified from the pathology records held at Wythenshawe Hospital. All cases had undergone a special examination of the lungs after death because of a possible occupational cause of their pulmonary disease, and lung asbestos fibre burden had been calculated using the lung digestion technique of Ashcroft and Heppleston.\(^{386}\)

The patients were subdivided into two further groups according to the accepted aetiology of their pulmonary disease. Group 1 (patient numbers 1-20) consisted of patients with clinical and radiological evidence of pulmonary fibrosis and a history of asbestos exposure, however the degree of fibrosis was greater than would be expected for their lung asbestos fibre counts.\(^{444}\) Group 2 (patients 21-40) consisted of patients with histologically-confirmed asbestosis. These patients either had asbestos fibre counts of greater than \(1 \times 10^5\) fibres/g in at least one pulmonary lobe, asbestos bodies visible on lung section or had been accepted as cases of asbestosis by the Pneumoconiosis Medical Panel by virtue of prolonged or heavy occupational asbestos exposure. Only those asbestos fibre estimates performed in our laboratory were considered, although in some cases higher values had been obtained elsewhere, or been confirmed by electron microscopy studies. A summary of patient characteristics, fibre counts and cause of death are shown overleaf in Table 5.1.
<table>
<thead>
<tr>
<th>Case No</th>
<th>Age at Death</th>
<th>Smoker</th>
<th>Known Asbestos Exposure</th>
<th>Pulmonary Asbestos Fibre Count (UL) ((x \times 10^6 /\text{g}))</th>
<th>Pulmonary Asbestos Fibre Count (LL) ((x \times 10^6 /\text{g}))</th>
<th>Cause of Death</th>
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</tr>
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</tr>
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</tr>
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<td>Gastric Ulcer</td>
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<td>PF</td>
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</tr>
<tr>
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<td>Y</td>
<td>0.19</td>
<td>0.15</td>
<td>PF</td>
</tr>
</tbody>
</table>

*denotes female patients. Y=yes, N=no, U = unknown. BPA= bronchopneumonia, PF= Pulmonary fibrosis, NSCLC = Non-small cell lung cancer, PE= Pulmonary embolus, CAD= cardiac disease

An asbestos fibre count of 20,000 fibres /g is the upper limit of normal for our laboratory.

Table 5.1: Summary of Patient Characteristics and Causes of Death
5.2: De-paraffinisation of Tissue Blocks

Genotyping using PCR and sequence specific primers is most commonly performed on peripheral blood leucocyte DNA. For the purposes of this study we had access to samples of formalin-fixed paraffin-embedded lung tissue. Although genotyping from paraffin-embedded samples has been described, DNA fragmentation and degradation secondary to the fixation process can critically affect the quality of extracted DNA. However, quantitative evaluation of similar material by Bateman et al. had suggested that our tissue blocks could be an adequate source of DNA.

The blocks used for this study corresponded to tissue from the inferior aspect of an upper lobe. Pulmonary fibrosis preferentially affects the lower zones. It was therefore anticipated that upper lobe sections would contain relatively more normal lung tissue than those from the lower lobe. The first part of the method involved the removal of paraffin wax from each patient sample. A portion of tissue approximately 0.5 cm$^3$ was cut from the lung block and the covering layer of paraffin wax scraped away. This tissue was placed in a 1.8 ml Eppendorf tube and the paraffin wax was removed by the addition of 1 ml xylene (BDH) and intermittent shaking over a period of ten minutes. Excess xylene was removed from the tube with a bulb pipette taking care not to disrupt the tissue. Residual xylene was removed by the addition of 1 ml of absolute alcohol (BDH) and left for ten minutes with periodic shaking. The excess ethanol (with xylene in solution) was removed with a micropipette, and the residual ethanol evaporated by placing the open Eppendorf tube in a water bath at 55$^\circ$C for five minutes.

5.3: DNA Extraction from Tissue Samples

Having removed the paraffin wax, DNA and RNA was then extracted from the tissue samples. Each specimen was incubated overnight at 55$^\circ$C after the addition of 500$\mu$l of stock lysis solution (100 mM TRIS/HCL, 4 mM EDTA, 0.145% Nonidet P-40, 0.45% Tween 20) and 20$\mu$l of 10 ng/ml stock proteinase K solution (all Sigma). This mixture lysed the cell membrane releasing cytoplasmic and nuclear material into solution.

Following incubation each sample was spun down in a microfuge at 13,000 rpm for five minutes. The supernatant was transferred to a second 1.8 ml Eppendorf tube and 100$\mu$l of 6 M NaCl (BDH) added to denature the proteinaceous contents. After vigorous mixing the samples were spun down (five minutes at 13,000 rpm) and the resultant supernatant transferred to a new Eppendorf tube. Absolute ethanol (1 ml) was added to the decanted
supernatant and mixed well before being placed in a freezer (-70°C) for a minimum of one hour, to promote precipitation of the DNA.

After freezing the samples were spun down for five minutes (13,000 rpm) and the supernatant removed and discarded. The solid residue which contained the precipitated DNA was re-suspended in 1ml of 70% ethanol solution (Hayman) and the pellet broken up with the tip of a glass pipette. Use of 70% rather than absolute alcohol dissolves any residual NaCl. The sample was pelleted and the ethanol-rich supernatant removed. Residual ethanol was evaporated by placing each tube in a water bath at 55°C for five minutes. The solid residue was re-suspended in 100µl distilled water, producing a suspension of ethanol-free salt-free DNA in solution.

5.4: Verification of DNA Quality
A small amount of each sample was removed using a glass capillary tube. The solution was assessed for DNA concentration and the RNA:DNA ratio using spectrophotometry (Pharmacia GeneQuant RNA / DNA Calculator). Ideally the RNA:DNA ratio should be ≤ 1.8 and the DNA concentration in the order of 0.5 - 1.5 µg/µl. All samples yielded DNA and RNA. The ratios (mean 1.4, range 1.0 - 1.8) and concentrations (mean 0.8µg/µl, range 0.4 - 1.4 µg/µl) were within the desired range. These results are detailed in Table 5.2, page 230.

5.5: Amplification of the Polymorphic DNA Sequence
A single base substitution at codon 25 of the TGF-β1 signal sequence has been shown to influence the amount of active TGF-β1 produced in response to a fibrogenic stimulus. Substitution of a guanine (G) nucleotide for a cytosine (C) encodes for arginine (CGG) rather than proline (CCG) at this position. Individuals who are homozygous for arginine at codon 25 produce significantly more active TGF-β1 ("TGF-β1 high-producers") than arginine/proline heterozygotes. The proline/proline genotype is very uncommon (<1% in most population studies).

The sequence-specific primers used for this study, were kindly donated by Professor Hutchinson’s group in Manchester, and were identical to the primers used in their own study. These primers specifically target the codon 25 area and differ only by the presence of a guanine (G-primer) or cytosine (C-primer) in this position. The
nucleotide sequence of each primer is shown below, the polymorphic region indicated in red.

Generic primer (antisense)  5' - GGCTCCGGTTCTGCACTC - 3'
Primer G (sense)  5' - GTGCTGACGCCTGGCCG - 3'
Primer C (sense)  5' - GTGCTGACGCCTGGCCC - 3'

(Product size 233 base pairs)

The optimum concentration of target DNA for PCR is in the order of 50 - 150ng/µl. Each patient sample was therefore diluted with distilled water to achieve a DNA concentration within this range in a total volume of 5µl.

One µl of each DNA sample was placed in a standard 10µl PCR tube, along with 1µl of either of the two TGF-β1 primers (which contained equal amounts of the generic and specific primers) and 3µl of standard PCR solution (comprising 60µl PCR mix [AB Technologies] and 1.2µl of the DNA polymerase Taq).

The reagents were made up to a volume of 10 µl by the addition of 5µl of distilled water. The tubes were sealed and DNA amplification commenced using the GeneAmp® PCR System 9700. The following cycling sequence was used, 59°C being the primer-specific annealing temperature#

\[
\begin{align*}
95^\circ C & \quad 1 \text{ minute} \\
95^\circ C & \quad 15 \text{ seconds} \\
65^\circ C & \quad 50 \text{ seconds} \\
72^\circ C & \quad 40 \text{ seconds} \\
95^\circ C & \quad 20 \text{ seconds} \\
59^\circ C & \quad 50 \text{ seconds} \\
72^\circ C & \quad 50 \text{ seconds}
\end{align*}
\]

(10 cycles)  \hspace{1cm}  (20 cycles)
5.6: Verification of Patient Genotype Using Gel Electrophoresis

The post-amplification DNA samples were transferred to wells in a 2% agarose (FMC Bioproducts) gel containing ethidium bromide (0.5%) within a TBE buffer bath (Sigma). Electrophoresis was performed at 250 V and 250 mA for 20 minutes. The amplified DNA was visualised using an Ultra-Violet (UV) light source and then photographed (Alpha Innotech Transilluminator TM-26).

Each patient sample was examined with both sets of primers to ascertain genotype. The primer product size was 233 base pairs, which would be expected to travel approximately two-thirds of the way along the gel under the conditions we used. An internal control was used in selected known samples to confirm that the PCR was working. The control amplified human growth hormone (hGH) and the product size was 400bp.

**Figure 5.1** below is a graphical representation of the results of electrophoresis. For each patient sample (1,2,3) amplified DNA solution with either the G-primer pair or C-primer pair are run on adjacent strips. The black bars represent the wells the solution is initially placed in and the white bars the position of the product after the run. In the schematic shown below, sample 1 contains the G-primer amplicon only (genotype GG), sample 2 has both G and C-primer amplicons (genotype GC) and sample 3 is CC.

![Figure 5.1](image)

**Figure 5.1**

Schematic Representation of PCR Product Electrophoresis
RESULTS
Initially only the first eight patient samples were examined, in order to assess the efficacy of our method. The post-amplification results for these samples are shown in Figure 5.2.

Only sample 8 gave a positive result (genotype GG). Sample 6 demonstrated a weakly fluorescent band at the expected position for the G-primer allele product. The majority of the fluorescence accumulated around the distal end of the electrophoresis track and probably represented aggregates of small DNA fragments and amplified primer - “DNA dimers”. In view of these disappointing results the process was repeated using a higher concentration of DNA (mean 224, range 194 - 246 ng/L) for samples 1-7. Sample 8 was replaced by normal genomic human DNA to act as a control for the PCR and the hGH internal control was also included. The results for the second run are shown in Figure 5.3.
Sample 6 was confirmed a genotype GG, but none of the other samples demonstrated the presence of either the TGF-β₁ amplicon or the hGH internal control, except for the control DNA (well 8). This confirmed that the primers and PCR were working. The remaining 32 samples were then examined using a DNA concentration in the higher range (150 -250ng). A primer band was demonstrable in 22 of the 40 samples, all samples being homozygous for the G polymorphism (Figure 5.4).
In studies that have assessed TGF-β1 polymorphisms, approximately 20% of the population are genotype GC. We had failed to identify any samples that were heterozygous within our study population. This result could be interpreted in one of two ways. Either all the samples were truly G homozygous or alternatively the C primer might not be working and some of these cases were in reality heterozygous. To ascertain which of these was the most likely, the C-specific primer was evaluated on samples of genomic DNA from patients whose TGF-β1 genotype was already known. These controls had been genotyped by the group who had initially developed the TGF-β1 primers. The control DNA samples were examined using our primers and their genotype confirmed. We therefore felt that the apparent excess of the GG genotype was not an artefact resulting from a problem with the C-primer.

The remaining 18 samples that had previously failed to amplify were re-examined (samples 1-5, 7, 11, 22-24, 31-34 and 37-40) using our original PCR protocol and the higher DNA concentration. In addition, one of the control DNA samples (GC genotype) and a random selection of 5 samples (8, 16, 20, 30, and 36) previously designated as genotype GG were re-amplified to check the reproducibility of our results. Samples 4, 5 and 39 still failed to produce a positive result, all the remaining patient samples were GG homozygous and the GC control genotype was confirmed. The three inconclusive samples (4, 5 and 39) were re-amplified along with 2 control DNA samples (both GC heterozygous). Samples 4 and 5 were negative for both primers and 39 was genotype GG.

It is possible that the difficulties experienced in amplifying the target DNA in some of our samples related to the presence of residual reagents in the final PCR solution. To overcome this problem the DNA extraction method was repeated for the remaining two samples that had so far failed to produce a result (samples 4 and 5). Two separate portions of lung tissue were taken from each block and processed according to the original methodology. For each patient sample two solutions of different DNA concentrations (approximately 100 ng/μl or 200 ng/μl) were created in the hope that dilution of the DNA would not significantly reduce the efficacy of the PCR but might diminish the effect of any inhibitors. Each of these solutions and one control DNA sample (GC) underwent amplification as previously detailed. Two of the four samples derived from lung block 4 and one of the samples from lung block 5 demonstrated a band corresponding to the genotype GG. A summary of our results is shown below in Table 5.2.
<table>
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<th>Sample No</th>
<th>RNA:DNA</th>
<th>DNA Concentration (μg/μl)</th>
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<td>0.5</td>
<td>172</td>
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</tr>
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<td>0.8</td>
<td>196</td>
<td>GG</td>
</tr>
<tr>
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<td>1.5</td>
<td>0.7</td>
<td>227</td>
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</tr>
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</tr>
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<td>1.3</td>
<td>245</td>
<td>GG</td>
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<td>1.5</td>
<td>0.9</td>
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<td>GG</td>
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<td>0.6</td>
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<td>GG</td>
</tr>
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<td>23</td>
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<td>0.9</td>
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<td>GG</td>
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<tr>
<td>Control DNA</td>
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<td>0.4</td>
<td>203</td>
<td>GG</td>
</tr>
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</table>

Table 5.2
Summary of Patient Sample RNA and DNA Characteristics and Codon 25 Genotype
5.7: Statistical Analysis

The distribution of the codon 25 TGF-β₁ DNA polymorphisms in controls with normal lung function in patients from the North West Region have previously been assessed by Awad *et al.* In their study 80% of controls were TGF-β₁ high producers (GG homozygous), 19% were heterozygous (GC) and only 1% were genotype CC. This distribution is similar to those seen in other studies that have assessed the codon 25 frequency (see Table 5.4, page 239). The patients from our study were compared with this normal population for the presence of the G allele using the Chi-square test. The Chi-square value was 7.3 which corresponded to a value of *p*<0.009 (1 df). These results are shown below in Table 5.3.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Lung Fibrosis (No of Patients)</th>
<th>No Lung Fibrosis (No of Controls)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>40</td>
<td>87</td>
</tr>
<tr>
<td>GC or CC</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>107</td>
</tr>
</tbody>
</table>

\( \chi^2 = 7.3, \ p < 0.009 \)

Table 5.3

Comparison of the Frequency of the GG Genotype in Patients with Lung Fibrosis Compared with a Normal Population
DISCUSSION
The lungs have an important role in orchestrating the responses to inhaled and circulating toxins and pathogens. To this end they are rich in inflammatory effector cells, which are activated when the integrity of the alveolar-capillary membrane is threatened. Although usually a protective mechanism, sustained or excessive response to such stimuli can be detrimental.

Exposure to an inhaled pathogenic substance initially provokes an acute inflammatory response. The presence of a foreign substance initiates endothelial cell activation and neutrophil recruitment into the lung. Local production of chemotactic substances such as tumour necrosis factor-α (TNF-α), interleukin -1 (IL-1) and -8 (early response cytokines) promotes neutrophil migration into the pulmonary interstitium and alveolae. The expression of cell adhesion molecules is altered and matrix deposition and new capillary growth initiated. These mechanisms normally persist until clearance or dissolution of the initial stimulus occurs. The final result of this process can either be resolution, scarring or chronic inflammation and fibrosis. Pulmonary fibrosis is the end result of an excess, imbalance or abnormal persistence of pro-fibrogenic mediators. Because so many different cells, cytokines and other inflammatory mediators are involved in this process, it can be difficult to predict the effect that changes in any one component may have. The specific effects of any given cytokine can also depend on the local environment. For example, TGF-β1 can be an activator or suppressor of fibroblast proliferation, depending on the predominant influence of other local inflammatory mediators.

5.8: The Classification and Pathology of Interstitial Lung Disease
Pulmonary fibrosis is one of a group of diverse diffuse lung conditions that are classified within the blanket term interstitial lung disease (interstitial pneumonias). The classification of interstitial lung diseases has undergone many changes in the last few decades as a greater understanding of the underlying pathogenic mechanisms, and the diagnostic and prognostic significance of different histological patterns have been recognised.

Pulmonary fibrosis is a progressive condition characterised by persistent chronic inflammatory reaction, alveolar architecture disruption and excess fibrotic matrix deposition within the lung interstitium. The end result is distortion and destruction of the normal alveolar-capillary diffusion barrier, with consequent hypoxaemia and respiratory failure. Patients with severe pulmonary fibrosis are also at increased risk of developing
carcinoma of the lung, above and beyond any risk they may have secondary to smoking. Indeed eight of the patients in our cohort developed lung cancer that contributed to their death. Pulmonary fibrosis can exist as part of a systemic inflammatory disease such as scleroderma, sarcoidosis or rheumatoid arthritis, or as part of an organ-specific fibrotic condition. It can result from exposure to fibrogenic substances which have been inhaled (asbestos fibres, silicates) or administered systemically (bleomycin). A proportion of cases have no recognisable cause or association (idiopathic pulmonary fibrosis, IPF).

Pulmonary fibrosis is difficult to treat and has a poor prognosis. The mainstays of management are corticosteroids and immunosuppressants, with lung transplantation a possibility in a small number of cases. Not all cases of pulmonary fibrosis respond to treatment: in particular asbestos-related pulmonary fibrosis (asbestosis) responds poorly. It is therefore important to correctly identify the cause of pulmonary fibrosis if appropriate treatment is to be initiated. As with mesothelioma, confirmation of an occupational cause also has medico-legal implications.

Idiopathic pulmonary fibrosis and asbestosis both typically exhibit a usual interstitial pneumonia (UIP) pattern histologically. This is characterised by gross patchy lung involvement, preferentially affecting the sub-pleural, para-septal and peri-bronchial areas with relative central sparing. In advanced cases honeycombing of the lung may be seen. At the microscopic level there is accumulation of alveolar macrophages, lymphoid aggregates and smooth muscle proliferation.

Idiopathic pulmonary fibrosis is a diagnosis of exclusion: there should be no clinical or histological evidence of other causes, such as a history of occupational exposure to inhaled fibrogenes. It can be difficult to accurately assess the relative contribution of occupational exposures, smoking and other disease processes in those with end-stage pulmonary fibrosis.

5.9: Clinical Features and Pathology of Asbestosis
Asbestosis is defined as interstitial pulmonary fibrosis secondary to the inhalation of asbestos fibres. Asbestosis was recognised as resulting from inhalation of asbestos fibres at the beginning of the twentieth century. It was more common in the unregulated conditions prevalent at that time, but is now decreasing in incidence. As with mesothelioma, the development of asbestosis is associated with a long latency period from initial exposure. Unlike mesothelioma, the development of asbestosis is directly related to asbestos fibre dose, and usually requires prolonged and / or heavy exposure. It has been
suggested that exposures in the region of 25-100 fibres/ml/year are usually required, with a latency period of 10-20 years.\textsuperscript{454} Lower levels of exposure associated with the introduction of public health regulations has also had the effect of increasing latency period; it effectively takes longer to amass the critical level of inhaled fibres to induce fibrosis.

The clinical diagnosis of asbestos is usually made on the basis of appropriate symptoms (dyspnoea and dry cough), inspiratory basal crepitations, and an occupational history of significant asbestos exposure. Pulmonary function testing confirms a restrictive defect with reduced diffusion coefficient. Radiological evidence includes the presence of bilateral reticulo-nodular shadowing primarily affecting the lower zones and peripheral regions of the lung.\textsuperscript{454} There may also be evidence of other asbestos-related pathologies, such as pleural fibrosis or plaques.

It can be difficult to distinguish between UIP and asbestosis histopathologically. Both conditions are characterised by the presence of a persistent inflammatory reaction and prolonged generation of pro-inflammatory and pro-fibrotic cellular mediators. In advanced cases honeycombing may be seen in both diseases. The presence of coated asbestos fibres (asbestos bodies) within the lung interstitium is one of the criteria for a diagnosis of asbestosis.\textsuperscript{444} The quantitative measurement of pulmonary asbestos fibre burden is often performed using the using the light microscopy method.\textsuperscript{386} Unfortunately this method is prone to inaccuracy, and it is recognised that absolute values can be difficult to reproduce between laboratories. This can make it difficult to confidently confirm asbestos as cause of fibrosis, even with an appropriate occupational history. Electron microscopy evaluation of asbestos fibre counts is also not without methodological problems.

This is a difficult area of pulmonary pathology, and one that can have significant effect on patients in terms of their eligibility for industrial compensation. It was interest in this group of patients that prompted the choice of our two study groups: we wondered if a difference in TGF-β\textsubscript{1} producer status could account for the presence of severe fibrosis in the presence of lesser asbestos fibre counts.
5.10: Asbestos Fibrogenicity, Pulmonary Clearance Mechanisms and Burden Estimation

I have already discussed the biological properties of asbestos and related minerals in Chapter One (Section 1.3, page 7). The bio-persistence of asbestos, coupled with its high iron content and large surface area, predisposes to prolonged generation of reactive oxygen species. Although the earlier discussions focussed on the ability of asbestos to induce malignant change in mesothelial cells, it is also recognised as being a profound fibrogen in the lungs and pleura. The local release of bioactive molecules incites an inflammatory response that includes cytokine release. These substances are toxic to alveolar macrophages, and result in cell death and release of more inflammatory mediators that further contribute to a sustained inflammatory response.

Limited exposure to inhaled asbestos does not appear to cause fibrosis, with all inflammatory changes resolving within a matter of days to weeks. Prolonged or repeated exposures saturate pulmonary asbestos fibre clearance mechanisms and result in chronic deposition of bioactive fibres within the pulmonary interstitium. Cigarette smoking reduces the ability of the lung to clear fibres, particularly long thin amphibole fibres, and this may account for the synergistic effect of asbestos and smoking on pulmonary fibrosis. As with mesothelioma, it appears to be fibres with high length to width ratios that pose the greatest risk.

The positive association between the amount of asbestos exposure, retained pulmonary fibre burden and the subsequent severity of pulmonary fibrosis is well established in humans. However even in the presence of a clear dose-dependent relationship, there is still some variability in the eventual severity of fibrosis in individuals, even when confounding factors such as smoking are accounted for. It is therefore likely that inter-individual differences in asbestos handling exist. What is not clear is whether these differences primarily relate to fibre clearance, or to the cellular response to retained asbestos such as cytokine production.
5.11: The Biological Effects of Transforming Growth Factor-β1

Transforming growth factor-β is a pleotropic polypeptide cytokine that is intimately involved in both normal and pathological conditions characterised by tissue remodelling and matrix deposition. As such it plays a critical role in normal embryogenesis and development, growth, and wound healing. It is also implicated in the development and maintenance of the chronic inflammatory reaction. Transforming growth factor-β was named for its ability to induce cellular changes in vitro that resemble the effects of viral transformation, and it has been implicated in the process of carcinogenesis in some tumours.\textsuperscript{457}

Transforming growth factor-β was first characterised in 1985.\textsuperscript{458} The gene encoding TGF-β\textsubscript{1} was localised to Ch19q13 by Fujii \textit{et al}\textsuperscript{459} and the DNA sequence reported by Derynck \textit{et al}.\textsuperscript{460} Five isoforms of TGF-β have been identified. Of the three TGF-β isoforms seen in Man (TGF-β\textsubscript{1,3}) TGF-β\textsubscript{1} is the most biologically important, although all isoforms have broadly similar actions. The biological effects of TGF-β\textsubscript{1} are complex: it influences many different cell types and is itself influenced by the local environment via autocrine and paracrine feedback loops. The net overall effect therefore depends on a complex interaction of numerous factors, and even slight local changes can induce markedly different responses. Biological effects of TGF-β\textsubscript{1} include the following:

1. Increased expression and transcription of genes involved in the synthesis and secretion of extra-cellular matrix components, particularly collagen and matrix proteins e.g. fibronectin, elastin and decorin.\textsuperscript{461}
2. Increased transcription of matrix protein receptors including those involved in cell-cell adhesion.
3. Decreased collagen degradation (↓ collagenase production).\textsuperscript{461}
4. Increased protease inhibitor production (plasminogen activator inhibitor-1).\textsuperscript{440}
5. Stimulation and transformation of local fibroblasts.
7. Mitogenic effects on pulmonary fibroblasts and mesothelial cells.\textsuperscript{462}

Transforming growth factor-β\textsubscript{1} can be an agonist or antagonist for cell proliferation and inflammation depending on the local environment. It is capable of auto-induction and exhibits synergism with other cytokines, particularly TNF-α, IL-1 and platelet-derived
growth factor. These three cytokines are critically important in the initiation and early phases of cellular and humoral responses to injury.\textsuperscript{461} Transforming growth factor-\(\beta_1\) is essential for growth, tissue homeostasis and angiogenesis: knockout mice deficient for TGF-\(\beta_1\) die at two weeks of age, once maternal levels of have waned, and show evidence of major vascular anomalies.\textsuperscript{463}

Under normal circumstances acute injury results in a transient increase in TGF-\(\beta_1\) transcription and secretion. Repeated or persistent stimulation results in sustained TGF-\(\beta_1\) production and loss of normal termination mechanisms. Overall control is determined by local auto-induction and repeated stimulation of other cells e.g. alveolar macrophages and fibroblasts.\textsuperscript{452} The biopersistence of asbestos fibres within the pulmonary interstitium sustains the inflammatory reaction until tissue fibrosis and destruction occur.

5.12: Experimental Evidence for the Role of Transforming Growth Factor-\(\beta_1\) in Fibrotic Lung Disease

The biological properties of TGF-\(\beta_1\) make it a likely mediator in the pathogenesis of fibrotic diseases.\textsuperscript{461} Fibroblast proliferation, disordered collagen metabolism and inflammatory cell infiltration are all recognised sequelae of TGF-\(\beta_1\) activity. Experimental evidence confirming this role exists in both \textit{in vitro} and \textit{in vivo} settings.

Early studies confirmed that immunohistochemical TGF-\(\beta_1\) expression was localised to areas of pulmonary fibrosis, and much greater than that seen in normal lungs.\textsuperscript{464,465} More recently Croker and colleagues evaluated the expression of TGF-\(\beta\) isoforms in normal and fibrotic lung using anti- TGF-\(\beta_1\) antibodies in an \textit{in situ} hybridisation study.\textsuperscript{466} They confirmed that mRNA transcripts of TGF-\(\beta_1\) were widely distributed within normal lung tissue, within alveolar macrophages, bronchiolar epithelium and endothelia. In fibrotic lung there was increased expression of TGF-\(\beta_1\), and the TGF-\(\beta_3\) isoform was also present. In addition, metaplastic type II alveolar cells expressed TGF-\(\beta_1\) transcripts, a finding that was not seen in normal lung. Changes in type II cell morphology is thought to be an important factor in the development of pulmonary fibrosis.

Transforming growth factor-\(\beta_1\) can induce epithelial to mesenchymal shift in both normal and neoplastic cells, and may be responsible for some of the changes in type II cells. Khalil and colleagues have studied the differential production and localisation of latent and active TGF-\(\beta_1\) in patients with pulmonary fibrosis in comparison with patients with non-fibrotic pathology.\textsuperscript{467} They evaluated TGF-\(\beta_1\) production both from the lungs of patients
with IPF, and also from alveolar macrophages obtained from broncho-alveolar lavage fluid. They demonstrated that patients with IPF produce similar amounts of TGF-β₁ to controls, but that in IPF patients the proportion of active rather than latent-form TGF-β₁ produced was much greater. Excess active TGF-β₁ was also produced from the alveolar macrophages in culture compared with controls.

There have also been studies that have confirmed the importance of TGF-β₁ in the development of a fibrotic reaction to asbestos in the lung. Perdue and colleagues have demonstrated up-regulation of TGF-β₁ in a murine model in which animals were exposed to inhaled chrysotile fibres. The areas of increased activity corresponded well to the areas in which the asbestos fibres were deposited.⁴⁶⁸

The net result of these studies appears to confirm that TGF-β₁ plays an integral role in the development and progression of fibrotic lung disease. What is less clear is how the normal feedback mechanisms that regulate TGF-β₁ are modified, and whether other genetic or environmental factors are important. One possibility is that patients with IPF may be constitutionally predisposed to produce an excess of pro-fibrotic cytokines such as TGF-β₁.

In the last few years much has been learnt regarding the factors that control TGF-β₁ production and in vivo activation of the latent form. In particular it has been recognised that minor changes in the gene encoding for TGF-β₁ (DNA polymorphisms) can critically affect TGF-β₁ production, and may partly explain an individuals’ propensity to the development of fibrotic disease.

### 5.13: Transforming Growth Factor-Beta₁ Polymorphisms

The gene sequence that encodes for TGF-β₁ consists of 6 introns and 7 exons. The first major translation site commences at the +841 position relative to the 5’ terminal. A total of seven different polymorphisms affecting the TGF-β₁ gene have now been identified.⁴³⁸ The positions of and substitutions in these polymorphic regions are as shown overleaf in Table 5.4.
<table>
<thead>
<tr>
<th>Polymorphism Position (Relative to 5')</th>
<th>Nucleotide Substitution</th>
<th>Effect on Active TGF-β₁ Production</th>
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<tr>
<td>-988</td>
<td>C → A</td>
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</tr>
<tr>
<td>-800</td>
<td>G → A</td>
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<td>C → T</td>
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<td>Increase</td>
</tr>
<tr>
<td>(Codon 263)</td>
<td>C → T</td>
<td>Unknown</td>
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</tbody>
</table>

Table 5.4

Known Polymorphic Sites in the Transforming Growth Factor-β₁ Gene

In common with other polypeptide cytokines, TGF-β₁ is initially produced in an inactive precursor form. The active portion comprises of two 112 amino-acid chains linked by a disulphide bond to produce a 25kDa dimer. It is predominantly produced in platelets, stored within their α–granules in a latent form (pre-pro-TGF-β₁) and activated by cleavage of the pre-protein. It can also be activated in vitro by acidification. Many other cells are capable of producing TGF-β₁ de novo in response to local inflammatory mediators. These include alveolar macrophages, pulmonary epithelial cells and endothelia.

The first stage of the series of steps that enables secretion of soluble proteins and polypeptides involves translocation of the precursor molecule across the endoplasmic reticulum. This requires correct identification of the polypeptide by a signal recognition particle (SRP) and its appropriate docking protein. The process of translocation is initiated by the emergence of a specific DNA segment, the signal sequence, from the ribosome. The
signal sequence characteristically comprises 8-12 amino-acids arranged in an α-helix with a hydrophobic core. The signal sequence controls the formation of the “targeting complex” (ribosome, signal sequence chain and SRP). The subsequent interaction of the targeting complex and docking protein releases the precursor protein from the mRNA, catalyzing transfer of the protein into the cytosol.

The amino acid profile of the signal sequence has a critical effect on this process, and therefore can directly affect the rate of translocation and eventual secretion of the polypeptide. The +915 position (codon 25) polymorphism involves an amino acid substitution within this important signal sequence. Arginine is a large polar amino acid, whereas proline is small and neutral. It is therefore plausible that this substitution could affect the signal sequence characteristics in terms of size, spatial arrangement or size of hydrophobic core, thereby altering its affinity for the SRP.

5.14: Biological Significance of the Codon 25 Polymorphism in Pulmonary Disease
Several groups have determined the distribution of TGF-β1 DNA polymorphisms in normal populations in comparison with those affected by fibrotic lung disease.438, 440-442, 446, 449, 469

Cambien and colleagues were one of the first groups to identify TGF-β1 polymorphisms and investigate their relationship to disease.438 There was experimental evidence that TGF-β1 was important in the control of endothelial function and vascular remodelling. They therefore investigated whether TGF-β1 production was associated with the development of ischaemic heart disease (IHD). A group of 1192 men, aged 25-64, from four different geographical areas were stratified according to the presence or absence of IHD. TGF-β1 genotyping was performed using allele-specific oligonucleotide primers on blood. Seven polymorphic areas were identified. There were significant differences in the distribution of the codon 25 polymorphism between the two study groups, with fewer patients with the TGF-β1 GG genotype in the group without IHD (p < 0.05). There were also notable geographical differences in the relative distribution of genotypes, with an excess on the GG genotype in patients in Belfast and Toulouse. Part of the criteria for selection was that the subjects and their parents were natives of their city, which might explain such a difference.

Since this initial work other groups have studied the distribution of TGF-β1 polymorphisms in disease, particularly in the lung. Combined research undertaken by Professor Hutchinson’s group, the Department of Cardiothoracic Transplantation and the
Cystic Fibrosis Unit in Manchester has produced much valuable information regarding the influence of TGF-β₁ polymorphisms in fibrotic lung disease. In one of their first papers they confirmed the existence of TGF-β₁ DNA polymorphisms and developed the ARMS-PCR method that we adapted for our own study. Their subsequent studies investigated the distribution and biological significance of these polymorphisms in patients with severe fibrotic lung disease of varying aetiologies, including those who underwent pulmonary transplantation.

In 1998 Awad and colleagues first reported an association between the codon 25 polymorphism and the presence and progression of fibrotic lung disease of different aetiologies. There were two parts to their study. The first part confirmed the presence of the codon 10 and 25 polymorphisms using the primers developed by Perrey et al. Secondly they demonstrated a significant excess (p< 0.02) of the genotypes associated with increased TGF-β₁ production in patients with end-stage lung disease of fibrotic aetiology compared to those with non-fibrotic pathology. The TGF-β₁ high producers were also more likely to develop pulmonary fibrosis following lung transplantation. This clinical observation was confirmed by an in vitro study of activated and latent TGF-β₁ secretion from blood leucocytes in response to the addition of two mitogens. Those patients with the GG codon 25 genotype produced significantly more TGF-β₁ in response to the mitogenic stimulus than those who were genotype GC (p< 0.02). The association between TGF-β₁ activity and the codon 10 polymorphism in patients with fibrotic pathology was even stronger (p< 0.005).

Subsequent studies suggested a possible mechanism for this observation in transplanted patients. Transforming growth factor-β₁ has the greatest immunomodulatory effects of all known cytokines. The lung is rich in lymphoid tissue and immune effector cells. A significant degree of immunosuppression is therefore required following pulmonary transplantation if rejection is to be avoided or minimised. They found that lung transplant recipients had increased levels of circulating active TGF-β₁ compared with normal controls, and that there was a direct correlation between TGF-β₁ levels and the use and dose of the immunosuppressant cyclosporine A.

There is one study that has failed to demonstrate any relationship between TGF-β₁ polymorphisms and fibrotic lung disease. Xaubet and colleagues evaluated the incidence of the codon 10 and 25 polymorphisms in a group of patients with IPF (n=128) compared with normal controls (n=140) in a Spanish Caucasian population. They found no excess of
the high-producer phenotype in those with lung fibrosis, although the incidence of the
codon 25 GG genotype was slightly higher, at 92.2%, in the control group than in other
control populations described in the literature.\textsuperscript{438,440} They did note, however, that the
codon 10 high-producer genotype was associated with a more rapid decline in pulmonary
function. Less than half of the patients in the IPF group had histological confirmation of
their underlying pathology, which may have influenced their results.

5.15: Codon 25 Polymorphism Frequency in Idiopathic and Asbestos-related
Pulmonary Fibrosis

The discovery that all the patients in our study group were TGF-β\textsubscript{1} high-producers
supports our hypothesis that TGF-β\textsubscript{1} is an important mediator of chronic inflammation and
fibrosis in the lung. Although the numbers included were small, the excess of the high-
producer genotype was sufficient to achieve statistical significance at the five percent
level.

An initial concern was that this apparent excess was a reflection of problems with
our DNA amplification technique. Failure of amplification of the C-primer could account
for the excess of apparent GG genotype cases. The inclusion and correct identification of
control DNA from a known GC heterozygote in the majority of PCR runs made us more
confident that the C-primer was amplifying correctly. Random repeat genotyping of a
proportion of samples confirmed the reproducibility of our results. The need to repeat
DNA extraction and PCR reactions for several samples was disappointing, but not
surprising given the likely poor quality of DNA obtained from formalin-fixed paraffin-
embedded tissue. Indeed, some of the samples used were from patients who had died more
than 15 years prior to the start of this study.

Are there any other factors that could influence or confound our results? It has been
shown that there are small geographical differences in the population frequencies of TGF-
β\textsubscript{1} polymorphisms.\textsuperscript{438} All the subjects in our group were known or suspected to have an
industrial cause for their lung fibrosis. Although we used control data from patients who
lived in the same region as our study group, it is possible that people who work in
asbestos-related industries share similar backgrounds or origins that might be reflected in a
different TGF-β\textsubscript{1} genotype distribution, compared to our control group. Large population
studies, corrected for confounding factors, would be necessary to investigate this
possibility.
Although our results imply an important role for TGF-\(\beta_1\) in pulmonary fibrosis, it is highly likely that TGF-\(\beta_1\) is only one of many factors that contribute to the development and progression of IPF. The TGF-\(\beta_1\) high producer genotype is the most common of the three possible polymorphisms identified at codon 25, being present in approximately 80% of normal controls. Using an individual's TGF-\(\beta_1\) polymorphism genotype as a means to identify susceptibility to inhaled fibrogens and risk of pulmonary fibrosis is limited if it will place almost all of those tested in the high risk category. It is interesting to conjecture whether the converse is true; that those with the GC genotype have an innate protection against fibrogens and the development of fibrotic lung disease.

CONCLUSIONS

In this study we have demonstrated an excess of individuals who are genetically predisposed to produce higher levels of the pro-fibrogenic cytokine TGF-\(\beta_1\) within a group of patients with fibrotic lung disease and a history of asbestos exposure. This finding might suggest a mechanism to explain inter-individual differences in the development of pulmonary fibrosis, particularly after exposure to an inhaled fibrogen.

It is almost certain that there are other biological factors that are important in patients who develop pulmonary fibrosis. It is possible that it is the possession of a number of “high-risk” cytokine genotypes in one individual that predisposes them to fibrotic disease. The identification of other cytokine polymorphisms and confirmation of their biological effects may shed light on the pathophysiological mechanisms involved in this disease process and suggest new diagnostic and therapeutic avenues.
CHAPTER SIX
TRANSFORMING GROWTH FACTOR–BETA_1 POLYMORPHISMS IN MALIGNANT PLEURAL MESOTHELIOMA

INTRODUCTION
In the previous chapter I reported an excess of the high TGF-β_1 producer genotype in a group of patients with pulmonary fibrosis. As well as being an important cytokine in the chronic inflammatory reaction, TGF-β_1 has also been implicated as a key effector in tumour development and progression. In particular TGF-β_1 promotes changes in the local extracellular matrix that increases cell invasiveness and angiogenesis.

Malignant pleural mesothelioma is a tumour characterised by extreme local invasiveness. It commonly spreads around and ultimately encases the lung, and frequently spreads along local tissue planes and the path of surgical incisions. In Chapter Four I discussed how the prognosis of mesothelioma is influenced by the histological subtype of tumour: the worst prognosis is associated with the desmoplastic variant of mesothelioma. In desmoplastic mesothelioma the bland microscopic appearance of the tumour, which predominately consists of collagenous matrix, belies its highly aggressive nature. Given the relationship between TGF-β_1 and matrix formation, I therefore wondered whether differences in TGF-β_1 production between individuals could influence the histological subtype of mesothelioma. To investigate this hypothesis further, I evaluated the incidence of the TGF-β_1 high-producer polymorphism in patients with different subtypes of mesothelioma and benign pleural fibrosis.

MATERIALS AND METHODS
The evaluation of the TGF-β_1 genotype of patients with benign and malignant pleural disease used a similar methodology to that already described in Chapter Five. Parts of this methodology are repeated here for ease of reference.

6.1: Patient Selection
A total of 88 patient samples were selected for this study. These were divided into four groups: desmoplastic mesothelioma (n=24), epithelioid mesothelioma (n=24), sarcomatoid mesothelioma (n=24) and benign pleural fibrosis (n=16). In contrast to the previous chapter, samples of pleural tissue or tumour blocks were used rather than lung tissue.
sections. This was because not all cases, and particularly those with benign disease, had archival lung tissue available for examination. All mesothelioma patients were members of the cohort that had been reviewed in terms of pathology and immunohistochemistry to confirm their diagnosis and tumour subtype. The cases of benign pleural fibrosis were identified through the pathology department electronic database. In none of these cases was mesothelioma or other pleural malignancy suspected clinically or pathologically.

6.2: De-paraffinisation of Tissue Blocks
A portion of tissue approximately 0.5cm³ was cut from the tissue block and the covering layer of paraffin wax scraped away. This tissue was placed in a 1.8 ml Eppendorf tube and the paraffin wax was removed by the addition of 1ml xylene (BDH) and intermittent shaking over a period of ten minutes. Excess xylene was removed from the tube with a bulb pipette taking care not to disrupt the tissue. Residual xylene was removed by the addition of 1ml of absolute alcohol (BDH) and left for ten minutes with periodic shaking. The excess ethanol (with xylene in solution) was removed with a micropipette, and the residual ethanol evaporated by placing the open Eppendorf tube in a water bath at 55°C for five minutes.

6.3: DNA Extraction from Tissue Samples
Having removed the paraffin wax, DNA and RNA was extracted from the tissue samples. Each specimen was incubated overnight at 55°C after the addition of 500μl of stock lysis solution (as previously detailed, Section 5.3, page 223).

Following incubation each sample was spun down in a microfuge at 13,000 rpm for five minutes. The supernatant was transferred to a second 1.8 ml Eppendorf tube and 100μl of 6M NaCl (BDH) added to denature the proteinaceous contents. After vigorous mixing the samples were spun down (five minutes at 13,000 rpm) and the resultant supernatant transferred to a new Eppendorf tube. Absolute ethanol (1 ml) was added to the decanted supernatant and mixed well before being placed in a freezer (-70°C) for a minimum of one hour, to promote precipitation of the DNA. After freezing the samples were spun down for five minutes (13,000 rpm) and the supernatant removed and discarded. The solid residue was re-suspended in 1ml of 70% ethanol solution (Hayman) and the pellet broken up with the tip of a glass pipette. The sample was pelleted and the ethanol-rich supernatant removed. Residual ethanol was evaporated by placing each tube in a water bath at 55°C for
five minutes. The solid residue was re-suspended in 100µl distilled water, producing a suspension of ethanol-free salt-free DNA in solution.

6.4: Verification of DNA Quality
A small amount of the sample was removed using a glass capillary tube. The solution was assessed for DNA concentration and the RNA: DNA ratio using spectrophotometry as previously detailed.

6.5: Amplification of the Polymorphic DNA Sequence
The primers donated by Professor Hutchinson and colleagues were used to identify the patients TGF-β1 genotype. DNA samples from each patient were diluted to achieve a concentration within the optimum range (50 - 150ng/µl). One µl of each DNA sample was placed in a standard 10µl PCR tube, along with 1µl of either of the two TGF-β1 primers (which contained equal amounts of the generic and specific primers) and 3µl of standard PCR solution (comprising 60µl PCR mix [AB Technologies] and 1.2µl of the DNA polymerase Taq). Given the difficulties experienced in amplifying the hGH control, this was omitted, and instead a sample of normal blood of known genotype (GC) was amplified with each sample batch as a control for both the primers and the PCR.

The reagents were made up to a volume of 10 µl by the addition of 5µl of distilled water. The tubes were sealed and DNA amplification commenced using the GeneAmp® PCR System 9700. The same cycling sequence was used, 59°C being the primer-specific annealing temperature.

6.6: Verification of Patient Genotype Using Gel Electrophoresis.
The post-amplification DNA samples were transferred to wells in a 2% agarose (FMC Bioproducts) gel containing ethidium bromide (0.5%) within a TBE buffer bath (Sigma). Electrophoresis was performed at 250 V and 250 mA for 20 minutes. The amplified DNA was visualised using a UV light source and photographed (Alpha Innotech Transilluminator TM-26).
RESULTS
A summary of patient groups, DNA concentration, DNA:RNA ratio and genotype results are shown in Tables 6.1 and 6.2 at the end of this section, pages 249-251. Photographs of the amplified DNA results are shown in Figures 6.1 – 6.3 below. X denotes the sample of control blood. Unfortunately I was unable to confirm the TGF-β1 genotype in a total of 12 mesothelioma samples despite repeating the PCR reaction. Due to time constraints I was unable to prepare and test replacement samples from other patients.

Figure 6.1
Results of TGF-β1 Genotyping for Samples 1 - 45
Figure 6.2
Results of TGF-β1 Genotyping for Samples 46 – 72

Figure 6.3
Results of TGF-β1 Genotyping for Samples 73 – 88 and Repeat Runs for Samples that Previously Failed to Amplify
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D=Desmoplastic mesothelioma, E=Epithelioid mesothelioma.

Table 6.1
Sample RNA and DNA Characteristics and Codon 25 Genotype in Patients with Malignant Pleural Mesothelioma
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E= epithelioid mesothelioma, S= sarcomatoid mesothelioma.

**Table 6.1 (Continued)**

Sample RNA and DNA Characteristics and Codon 25 Genotype in Patients with Malignant Pleural Mesothelioma
Table 6.2
Sample RNA and DNA Characteristics and Codon 25 Genotype in Patients with Fibrosing Pleurisy

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FP = Fibrosing pleurisy

6.7: Statistical Analysis
The frequency of the three possible TGF-β1 genotypes in each group are summarised overleaf (Table 6.3). The distribution of genotypes was similar in the three different subtypes of mesothelioma, and reflected that seen in a general population. There was a relative excess of the high producer genotype in the pleural fibrosis group (GG = 94%) but this failed to reach statistical significance in comparison with the normal population (Fishers exact test p > 0.5).
<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Genotype GG</th>
<th>Genotype GC</th>
<th>Genotype CC</th>
<th>Genotype Not Ascertained</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desmoplastic Mesothelioma</td>
<td>16</td>
<td>3</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>(n=24)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelioid Mesothelioma</td>
<td>16</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>(n=24)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sarcomatoid Mesothelioma</td>
<td>13</td>
<td>3</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>(n=24)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pleural Fibrosis</td>
<td>15</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(n=16)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6.3
Distribution of Transforming Growth Factor -β₁ Polymorphisms in Patients with Benign and Malignant Pleural Disease

6.8: Influence of TGF-β₁ Genotype on Survival
Transforming growth factor-β₁ can behave as either a tumour promoter or suppressor, depending on its local environment and the influence of other signalling pathways (see Section 6.9, page 257). We therefore wondered whether TGF-β₁ could influence tumour progression, and subsequent patient survival. The mesothelioma cohort was therefore stratified for both histological subtype and TGF-β₁ genotype. Actuarial survival was compared between genotypes for each tumour subtype and the difference between groups
calculated (log rank test). Although there was the suggestion of an improved survival in patients with the genotype CC in the EM and DM groups this failed to reach statistical significance \((p>0.5)\). The mean and median survival, and actuarial survival curves are shown in Table 6.4 below and Figures 6.4 – 6.6, pages 254-256 respectively.

<table>
<thead>
<tr>
<th>TGF-β1 Genotype</th>
<th>Survival For Epithelioid Mesothelioma (days)</th>
<th>Survival For Sarcomatoid Mesothelioma (days)</th>
<th>Survival For Desmoplastic Mesothelioma (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>Mean 588</td>
<td>Mean 121</td>
<td>Mean 136</td>
</tr>
<tr>
<td></td>
<td>Median 329</td>
<td>Median 94</td>
<td>Median 88</td>
</tr>
<tr>
<td></td>
<td>((n=15))</td>
<td>((n=12))</td>
<td>((n=15))</td>
</tr>
<tr>
<td>GC</td>
<td>Mean 585</td>
<td>Mean 76</td>
<td>Mean 98</td>
</tr>
<tr>
<td></td>
<td>Median 380</td>
<td>Median 82</td>
<td>Median 79</td>
</tr>
<tr>
<td></td>
<td>((n=4))</td>
<td>((n=3))</td>
<td>((n=2))</td>
</tr>
<tr>
<td>CC</td>
<td>Mean 837</td>
<td>Mean 118</td>
<td>Mean 148</td>
</tr>
<tr>
<td></td>
<td>Median 837</td>
<td>Median 91</td>
<td>Median 148</td>
</tr>
<tr>
<td></td>
<td>((n=1))</td>
<td>((n=3))</td>
<td>((n=1))</td>
</tr>
<tr>
<td>Unknown (Cohort Controls)</td>
<td>Mean 542</td>
<td>Mean 175</td>
<td>Mean 73</td>
</tr>
<tr>
<td></td>
<td>Median 359</td>
<td>Median 150</td>
<td>Median 66</td>
</tr>
<tr>
<td></td>
<td>((n=216))</td>
<td>((n=48))</td>
<td>((n=7))</td>
</tr>
</tbody>
</table>

Table 6.4
Actuarial Survival in Patients with Mesothelioma Stratified by Histological Subtype and TGF-β1 Genotype
Figure 6.4

Actuarial Survival Curves Stratified by TGF-β1 Genotype in Patients with Epithelioid Mesothelioma
Figure 6.5

Actuarial Survival Curves Stratified by TGF-β1 Genotype in Patients with Sarcomatoid Mesothelioma
Figure 6.6
Actuarial Survival Curves Stratified by TGF-β1 Genotype in Patients with Desmoplastic Mesothelioma
DISCUSSION

Malignant transformation is a complex process that frequently involves changes in those signalling pathways that regulate normal cellular behaviour. These changes may directly involve DNA coding for known oncogenes or tumour suppressor genes. They may also affect other downstream members of the signalling pathways, such as receptor molecules, and alter the influence of the local environment on cell proliferation ("autocrine responsiveness").

There are striking similarities between the processes of wound healing and carcinogenesis. Both are characterised by cell proliferation, increased cytokine activity, angiogenesis and changes in the local extracellular matrix. The main difference is that wound healing is usually a highly regulated and finite process, whilst in carcinogenesis, cell proliferation, angiogenesis and growth factor production are relatively unregulated. It is therefore not surprising that many of the cytokines involved in wound healing and inflammation are also implicated in carcinogenesis.

Malignant transformation is associated with a deregulation of the normal mechanisms that control cell proliferation, differentiation and programmed cell death (apoptosis). Transformed cells are characterised by a higher proliferation index, de-differentiation and reduced intercellular adhesion. These changes are consequent on both genetic alterations e.g. activation of oncogenes, and alterations in the local environment. The local production of growth factors and cytokines by the tumour itself can have an important effect on subsequent tumour development and progression, as can changes in the constituents of the extracellular matrix (ECM). An increase in stroma is often seen in association with tumours. Although the constituent cells within this stroma have not undergone malignant transformation themselves, they contribute to tumour invasion, angiogenesis and metastasis through their own production of cytokines, proteases and pro-angiogenic factors.

6.9: Transforming Growth Factor-β1; Oncogene or Tumour Suppressor Gene?

The identification of TGF-β1 through its ability to induce cellular transformation suggested that it may play an important role in carcinogenesis. The subsequent recognition that TGF-β1 is secreted by most normal and malignant cell types, and has strong anti-proliferative effects on some cell populations, seemed to negate this role. It is now clear that TGF-β1 has both tumour-promoter and tumour suppressor activity.
Almost all cells will produce TGF-β₁ in culture, but this secretion is repressed by the addition of ECM or active TGF-β₁, via negative feedback loops. Transforming growth factor-β₁ is stored within the ECM in its latent form, thereby guarding against inappropriate activation, but providing a large pool of readily-available TGF-β₁ should it be needed. The activation of latent TGF-β₁ can be achieved via several different mechanisms and is facilitated by an acid environment. These mechanisms include the action of proteases e.g. plasmin and matrix metalloproteinases, and changes in local integrin expression. Disruption of the ECM through injury or tumour invasion can therefore both release and activate latent TGF-β₁. An increase in the concentration of active TGF-β₁ will influence local signalling pathways and may further enhance tumour progression.

Under normal circumstances TGF-β₁ has a growth inhibitory effect on epithelial cells. High levels of expressed TGF-β₁ induce growth arrest in the late G1 stage of the cell cycle, and prevent dephosphorylation of the Rb1 gene product.⁴⁶³ Despite this anti-proliferation effect, TGF-β₁ expression is often up-regulated in carcinomas, a seemingly contradictory finding. It therefore seems likely that tumour cells must derive some other advantage by expressing rather than inactivating TGF-β₁. It is now clear that in carcinomas the inhibitory effects of TGF-β₁ are often negated by downstream changes in TGF-β₁ signalling pathways. Mutations in the genes controlling receptor molecule or cyclin dependent kinase (cdk) expression are now recognised as common in many cancers, and may explain how the growth-inhibitory effects of TGF-β₁ are avoided. At the same time other parts of the TGF-β₁ signalling pathway are preserved as their effects, e.g. matrix remodelling, immune suppression, are seen as favouring tumour progression.⁴⁶³,⁴⁷⁰

In contrast to its effects on epithelial cells, TGF-β₁ is mitogenic for cells of mesenchymal origin. Fibroblast migration and proliferation are induced by TGF-β₁ directly, and also through its effects on the local concentration of other cytokines e.g. EGF, IL-1 and PDGF-B. Interleukin-1 and PDGF-B have both been implicated in the development of mesothelioma as discussed in Section 1.7, page 12.

Transforming growth factor-β₁ may also influence tumour progression through its effects on the immune system. The development of immune tolerance towards cancer cells is recognised as a mechanism that facilitates tumour progression. Transforming growth factor-β₁ possesses immunosuppressant activity against T-cells, and may therefore enable cancer cells to escape T-cell mediated immune surveillance.⁴⁷¹ Reversal of this immune-
suppressant effect has been shown to induce tumour regression in murine models of mesothelioma.\textsuperscript{472}

The final effect that TGF-$\beta_1$ may have on tumour development and progression is through angiogenesis. The development of new blood vessels is a critical part of tumour development as tumours often have a high metabolic requirement that may outstrip the capabilities of the local blood supply. An increased local vascular bed will also facilitate tumour metastasis. Transforming growth factor-$\beta_1$ plays an important role in normal vascular development: mice deficient for TGF-$\beta_1$ in experimental models die from gross vascular malformations. In both acute and chronic inflammation, TGF-$\beta_1$-induced angiogenesis contributes to the production of granulation tissue, as discussed in the previous chapter. Transforming growth factor-$\beta_1$ plays an equally important role in tumour angiogenesis, and exerts its pro-angiogenic effects through several different mechanisms. Firstly, TGF-$\beta_1$ directly stimulates the production and secretion of vascular endothelial growth factor (VEGF), which is a potent inducer of new vessel formation and endothelial cell migration.\textsuperscript{463} Secondly, TGF-$\beta_1$ directly induces capillary formation, and promotes vascular invasion through its effects on local protease production and the ECM.\textsuperscript{473} Finally TGF-$\beta_1$ is a potent chemo-attractant for monocytes, which are capable of producing angiogenic cytokines in their own right. The importance of these effects is attested to by the observation that the addition of anti-TGF-$\beta_1$ antibodies reduces angiogenesis in several carcinoma models.\textsuperscript{463}

So to summarise, TGF-$\beta_1$ has both tumour suppressor and tumour-promoter abilities. How tumours are able to disassociate the opposing growth inhibitory and mitogenic effects of TGF-$\beta_1$ is still unclear, but may depend on alterations in signalling thresholds, or through effects on downstream signalling molecules that have not yet been fully characterised.\textsuperscript{470} What is clear is that TGF-$\beta_1$ has been implicated as having an important role in the development and progression of many tumours. Although there has been relatively little research on the importance of TGF-$\beta_1$ in mesothelioma, evidence is emerging to suggest that TGF-$\beta_1$ may play a significant role in the pathobiology of this tumour.
6.10: Transforming Growth Factor-β1 in Malignant Pleural Mesothelioma

There have been few published papers that have investigated the relationship between TGF-β1 and malignant mesothelioma. Immunohistochemical studies indicate that the majority of mesotheliomas express TGF-β, and that the β1 isoform primarily locates to the ECM (tumour stroma). Transforming growth factor-β1 is secreted by both normal and malignant mesothelial cells, and malignant transformation of mesothelial cells is accompanied by changes in the production of, and responsiveness to, cytokines that include TGF-β1 and PDGF. Malignant mesothelioma cells are also capable of activating latent TGF-β1 within the ECM. Increased levels of TGF-β1 have been demonstrated in pleural effusions associated with mesothelioma, when compared with other types of malignant effusion.

Transforming growth factor-β1 is not the only cytokine implicated in the development and progression of mesothelioma. Several other cytokines and related growth factors have been investigated for their influence on mesothelioma, including the interleukins, insulin-like growth factor (IGF), basic fibroblast growth factor (bFGF) and PDGF. Platelet derived growth factor-β is exclusively produced by mesothelioma cells, rather than mesothelial cells, and is therefore thought to be intimately involved in the development of mesothelioma. It is also mitogenic for human mesothelial cells and fibroblasts, and as such may be a more significant contributor to mesothelial oncogenic transformation than TGF-β1. However, as TGF-β1 is a potent inducer of PDGF-β and many of the other cytokines and interleukins implicated in mesothelioma, it is difficult to separate the direct and indirect effects of TGF-β1, and therefore identify which cytokine is the prime effector in this tumour. Similarly it is difficult to dissect the inter-relationship between TGF-β1 and the interleukins. Interleukins are key effectors of the inflammatory response and their production is regulated by TGF-β1. The production of IL-4, -8 and -12 are increased in malignant mesothelioma, and all three interleukins have been investigated as potential targets for gene therapy in the treatment of this tumour.

The third potential way in which TGF-β1 might contribute to mesothelioma development is via its effects on angiogenesis. Mesothelioma is an extremely vascular tumour, and its high microvascular density has been shown to relate to prognosis. Mesothelioma expresses increased levels of several angiogenic cytokines, including VEGF and fibroblast growth factors-1 and -2. Vascular endothelial growth factor
is a powerful mitogen for endothelial cells, whilst bFGF is also mitogenic for mesothelial cells in its own right.483

6.11: The Pathobiology of Desmoplastic Mesothelioma
The pathobiology of MPM is still poorly understood. Epithelioid mesothelioma tends to metastasize via lymphatics, in a similar way to carcinomas, whilst sarcomatoid mesothelioma favours haematogenous spread. However, other differences in the pathobiology of the different mesothelioma subtypes have not been studied in any great detail. Although it would seem logical that such a locally invasive tumour is likely to produce cytokines and growth factors that facilitate invasion and metastasis, there have been few studies that have specifically examined this aspect of its behaviour.

6.12: Transforming Growth Factor-β1 Polymorphisms in Malignant Pleural Mesothelioma
The aim of this part of my thesis was to see if there was an association between the high TGF-β1 producer genotype, and the histological subtype of mesothelioma that the patient developed. In particular, was the incidence of the codon 25 TGF-β1 high producer polymorphism greater in patients with desmoplastic mesothelioma than in those with any other mesothelioma subtype. The incidence of this polymorphism was similar in all mesothelioma subtypes (range 68 - 80 %), and was comparable to the expected incidence in a normal population. A slight excess of the CC genotype in the SM group was non-significant given the small numbers in this analysis. It was interesting to note that the incidence of the codon 25 high producer polymorphism was 94% in those samples of benign pleural fibrosis that were evaluated. Although this excess of the high producer genotype in this small study did not reach statistical significance, it echoes the results of my previous study of TGF-β1 polymorphisms in pulmonary fibrosis.

The second part of the analysis was to see if any of the TGF-β1 genotypes was associated with a difference in survival. There are occasional reports of long term survivors with mesothelioma. These patients tend to be young with epithelioid tumours, however it has not yet been possible to identify any biological markers that could distinguish them from those with a poorer prognosis. The ability of TGF-β1 to promote changes in the ECM
that facilitate local invasion, and possibly also distant metastasis through its effects on angiogenesis, suggests that TGF-β₁ production might influence disease progression. Survival was known in 56 of the 60 cases of mesothelioma that had had their TGF-β₁ genotype ascertained. There was a suggestion that the CC genotype might be associated with an improved survival, but small patient numbers did not allow any robust statistical analysis between groups. There was one long term survivor in the EM group (case no 46, survival >10 years from diagnosis) who was genotype GG. The longest survivor in the DM group (case no 6, survival >15 months) was also GG. It therefore seems unlikely that the TGF-β₁ codon 25 polymorphism genotype is of any predictive value in terms of survival in patients with mesothelioma.

CONCLUSIONS

Cytokines, including TGF-β₁ have been shown to be key effectors in the development and behaviour of malignant pleural mesothelioma. In this study I investigated the potential contribution that a genetic tendency to increased TGF-β₁ production might make to the pathobiology of this tumour. My results do not support the hypotheses that excess production of TGF-β₁ secondary to the codon 25 polymorphism, either contributes to the development of malignant mesothelioma, dictates the subtype of mesothelioma developed, or predicts patient outcome.
THESIS CONCLUSIONS

In this thesis I have attempted to explore several of the more contentious areas relating to the pathobiology of malignant pleural mesothelioma. In terms of the causes of mesothelioma, the mechanisms by which amphibole asbestos induces malignant change in the pleura are becoming more clearly defined. The evidence against serpentine asbestos, SV40 and therapeutic radiation is less conclusive, and is an area that requires urgent further research. The potential threat of an even greater mesothelioma “epidemic” if SV40 is confirmed as a cause of mesothelioma remains a worry, and unless this is absolutely refuted we cannot assume that mesothelioma will no longer be a significant problem in twenty years time. A degree of complacency regarding the health effects of chrysotile, at least regarding its ability to induce mesothelioma, has only recently led to the banning of white asbestos in the UK. As asbestos is still widely used in many other countries, the world-wide incidence of mesothelioma is likely to continue rising. Furthermore, given that much of the carcinogenic potential of asbestos and erionite relate to their physical as well as chemical composition, it remains a moot point as to whether man-made fibrous minerals that have been introduced as substitutes for asbestos will eventually prove to have the same associated risks. The identification of the more commonly seen chromosomal abnormalities associated with mesothelioma appears unlikely to result in any specific screening test that might identify those at greatest risk of developing MPM.

Although it is important that we continue to investigate the mechanisms by which mesothelioma develops, for many thousands of people this knowledge is too little and too late. Significant proportions of at least two generations of men and women in the UK have been exposed to potentially carcinogenic levels of amphibole asbestos, and are at increased risk of eventually developing mesothelioma. These asbestos “victims” currently have little hope of any effective treatment or cure. Although mesothelioma research is recognised as an area of vital importance in the UK, uncertainty remains regarding the natural history of this disease, and the ability of patient-related, histopathological and biological factors to predict response to therapy and subsequent survival. Until we fully understand the relative contributions of these factors to patient outcome, there is the potential for any studies of therapy to be confounded or biased. It now seems clear that age and histology are the most important patient and tumour related factors that should be controlled for in studies of mesothelioma. This emphasizes the importance of accurate diagnosis and classification of pleural tumours. My meta-analysis of papers that have investigated the value of IHC in
mesothelioma will hopefully contribute to this importance aspect of mesothelioma pathology.

In the last twenty years there have been incredible advances in our understanding of the mechanisms by which normal and malignant cells are controlled. The critical role played by cytokines such as TGF-β1 in normal and pathological processes has been confirmed, and it is recognised that further research in this area may increase our knowledge as to how tumours develop and metastasize. I have explored two potential areas in which TGF-β1 might influence the effects of asbestos-induced disease. In the lungs TGF-β1 has been shown to promote the development of fibrosis of many different causes. In this thesis I have confirmed that DNA polymorphisms in the TGF-β1 gene that increase levels of secreted active cytokine are over-represented in patients with asbestos-related pulmonary fibrosis. In mesothelioma, we do not yet understand why some tumours are associated with the production of large amounts of desmoplastic stroma, and why these tumours have such a poor prognosis. Although I was unable to confirm a significant relationship between the codon 25 TGF-β1 DNA polymorphism, histological subtype or patient survival, the thought that polymorphisms in the genes encoding TGF-β1 or other related cytokines might in part explain some of these aspects of mesothelioma pathobiology remains an intriguing possibility. Indeed at the most recent European Mesothelioma Panel Meeting (Manchester 2003), it was decided that this was an area that merited further research.
APPENDIX ONE
IMMUNOHISTOCHEMICAL METHODS

All histochemical and immunohistochemical staining was performed according to the departmental standardised protocols, which are summarised below. Individual antibody clones and antigen retrieval methods are summarised in Table A.1 (page 266).

**Diastase-Periodic Acid-Schiff**
A coplin jar containing 50mL of deionised water was warmed to a temperature of 37°C then 0.1g diastase was added and tissue sections were incubated for 25 minutes. Sections were washed in running tap water for 10 minutes before treatment with 1% periodic acid for 10 minutes. After further washing the sections were covered with Schiff reagent for a further 10 minutes before rinsing. The sections were then washed in a 1% solution of sodium metabisulphite, washed in running water for 5 minutes, and the nuclei counterstained with Mayer’s haematoxylin. The sections were then washed in TRIS, rinsed in water, dehydrated and mounted.

**Avidin-Biotin Peroxidase Method for IHC Using the Sequenza**
Tissue sections (3μm) were mounted onto Snowcoat X-tra slides and dried overnight at 55°C. The sections were de-waxed with xylene, de-greased in IMS and washed in tap water. Endogenous peroxidase was blocked with 3% H₂O₂ for 10 minutes and the appropriate antigen retrieval performed.

Covered slides were placed in the sequenza and covered with TBS as per the manufacturers’ instructions. Diluted primary antibody (100μL) was added and incubated for 60 minutes. TBS was added to the sections and allowed to drain through, then the secondary antibody was prepared and incubated with the sections for 20 minutes. The sections were re-covered with TBS, 100μL of pre-prepared ABC reagent added and incubated for 20 minutes. After rinsing with TBS and deionised water, 100μL of 3,3’ diaminobenzidine solution was added and the sections incubated for 10 minutes. Sections were rinsed, counterstained with Mayer’s haematoxylin, washed, dehydrated and mounted.

**Antigen Retrieval Methods**
**Enzyme method**
A solution of pH8 Tris buffer-calcium chloride stock was diluted 1:10 in deionised water and warmed to 37°C in a water bath. A 0.05% solution of trypsin was prepared, added to the buffer-calcium chloride solution, and the sections added and incubated for 10 minutes.
Microwave Method
Ten ml of pH6 citrate buffer stock was added to 1 litre of deionised water in a lasagne dish, and placed in the middle of the microwave turntable. Slides were placed in alternate slots at the end of the dish. The sections were microwaved on high power for 15 minutes, then a further 15 minutes at 70% power, before being left to cool for 10 minutes. The sections were then transferred to a water bath.

Pressure Cooker Method
Two litres of deionised water was placed in the pressure cooker and 20mls citrate buffer stock added. The slides were placed in alternate spaces in a metal rack, and the pressure cooker heated up to maximum, then cooked for a further 2 minutes on minimum heat. The cooker was then cooled down in a sink with cold water until safe to open.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Clone</th>
<th>Dilution</th>
<th>Antigen Retrieval</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Becton Dickinson</td>
<td>CAM5.2</td>
<td>25μg/mL</td>
<td>Trypsin</td>
</tr>
<tr>
<td>AE1/3</td>
<td>Dako</td>
<td>AE1, AE3</td>
<td>1:50</td>
<td>Nil</td>
</tr>
<tr>
<td>CEA</td>
<td>Dako</td>
<td>11-7</td>
<td>1:50</td>
<td>Microwave</td>
</tr>
<tr>
<td>Ber-EP4</td>
<td>Dako</td>
<td>Ber-EP4</td>
<td>1:40</td>
<td>Trypsin</td>
</tr>
<tr>
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<td>Becton Dickinson</td>
<td>CD15</td>
<td>50μg/mL</td>
<td>Trypsin</td>
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<td>Pressure Cooker</td>
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Table A.1
Summary of Immunohistochemical Diagnostic Antibodies and Antigen Retrieval Methods
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