Heterogeneity and Early Diagnosis of Pleural Mesothelioma

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Abstract

Background: Diffuse pleural mesothelioma is a thoracic malignancy associated with aggressive clinical behaviour and a lack of treatment options. It is hypothesised that the diverse spectrum of observed clinical and biological characteristics in the literature could be explained by inter-tumoural and intra-tumoural heterogeneity. Consequently there is scope to improve the contemporary classification of these tumours, especially a subset of those representing minimally or superficially invasive pleural mesothelioma, allowing the disease to be diagnosed earlier in its natural history.

Materials and methods: Using a number of databases (including an institutional mesothelioma database that I retrospectively curated and prospectively maintained) and datasets linked to the diagnostic practice at an academic centre over a period of 30 years, the clinical, histopathological and molecular characteristics of pleural mesothelioma were interrogated via a multidisciplinary approach. The emphasis of the approach was on the outcomes following multimodality therapy including radical surgery, histological heterogeneity of epithelioid pleural mesothelioma and proposal of a minimal sampling requirement, histology-molecular correlation of key genomic alterations, and the development of a risk stratification system for atypical mesothelial proliferation of the pleura.

Results: The histological subtype of mesothelioma predicted outcome following multimodality therapy and influenced key surgical and oncological parameters. It was also determined that the epithelioid subtype, the most common subtype of mesothelioma, can be classified using a nuclear grading system based on the morphology of tumour cell nucleus, mitotic activity and the presence of tumour necrosis. This can be reliably performed on biopsies provided the minimum sampling requirement is met. Epithelioid mesothelioma exhibits extensive heterogeneity in architectural growth patterns and cytological features,

although most of which is a function of nuclear grade. A morphology-based classification stratified atypical mesothelial proliferation into high risk and low risk categories, the latter demonstrating significantly superior survival outcomes and supports the hypothesised existence of a minimally invasive stage of pleural mesothelioma.

Conclusions: This thesis expands the evidence base in several aspects central to the future iterations of mesothelioma classification and highlights the importance of accurate and informative histopathological diagnosis in its multidisciplinary management. Furthermore, the findings lay a cornerstone to the establishment of future academic-clinical partnerships, of multi-dimensional and multi-parametric nature, integrating the expertise of fundamental science, clinical specialities and histopathology in the research of this disease.

Declaration of originality

I declare this thesis is entirely the work of my own, except where appropriately referenced.

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List of abbreviations

5'-UTR	5'-untranslated region
AIS	Adenocarcinoma *in situ*
ALK	Anaplastic lymphoma kinase
AMP	Atypical mesothelial proliferation
ANRIL	Antisense noncoding RNA in the INK4 locus
APC	Antigen presenting cell
APC	APC regulator of Wnt signalling pathway
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
ARDS	Acute respiratory distress syndrome
ARID1A	AT-rich interaction domain 1A
ARID1B	AT-rich interaction domain 1B
ASCO	American Society of Clinical Oncology
ASS1	Argininosuccinate synthetase 1
ATM	ATM Serine/threonine kinase
BAP1	BRCA-associated protein 1
BAP1-TPDS	BAP1 tumour predisposition syndrome
BerEP4	Monoclonal antibody against epithelial cell adhesion molecule (EpCAM)

BIRC5	Baculoviral IAP repeat containing 5
B-PM	Biphasic pleural mesothelioma
BRCA1	BRCA1 DNA repair associated
BRCA2	BRCA2 DNA repair associated
BTS	British Thoracic Society
CAF	Cancer-associated fibroblasts
CAIX	Carbonic anhydrase 9
CCND1	Cyclin D1
CCND2	G_1/S-specific cyclin-D2
CD	Cluster of differentiation
CDC42	Cell division cycle 42
CDK	Cyclin-dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CDKN2B	Cyclin dependent kinase inhibitor 2B
CDX2	Caudal type homeobox 2
CEA	Carcinoembryonic antigen
CGH	Comparative genomic hybridisation
CHEK2	Checkpoint kinase 2
CI	Confidence interval

CK20	Cytokeratin 20
CK5/6	Cytokeratin 5/6
CK7	Cytokeratin 7
CLND15	Claudin 15
CNA	Copy number alterations
cnLOH	Copy number neutral LOH
CRP	C-reactive protein
CRS	Cytokine release syndrome
CT	Computerised tomography
CTLA-4	Cytotoxic-T-lymphocyte-associated antigen 4
CTLP	Chromothripsis-like pattern
DAB	Diaminobenzidine
DC	Dendritic cell
DDX3X	DEAD-box helicase 3, X-linked
DFS	Disease-free survival
DIS	Discohesive growth pattern
DNA	Deoxyribonucleic acid
EACTS	European Association for Cardio-Thoracic Surgery
EAP	Expanded access programme

EM	Electron microscopy
EMA	Epithelial membrane antigen
EML4	Echinoderm microtubule-associated protein-like 4
E-PM	Epithelioid pleural mesothelioma
EMT	Epithelial-to-mesenchymal transition
EPD	Extended PD
EPP	Extrapleural pneumonectomy
ERCC1	Excision repair cross-complementing group 1
ERS	European Respiratory Society
ESTRO	European Society for Radiotherapy and Oncology
ESTS	European Society of Thoracic Surgeons
EURACAN	European Reference Network on Rare Adult Cancers
EWRS1	EWS RNA binding protein 1
EZH2	Zeste 2 polycomb repressive complex 2 subunit
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
FFPE	Formalin-fixed paraffin-embedded
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor

FISH	Fluorescence in situ hybridisation
FOXO1	Forkhead box O1
FOXP3	Forkhead box P3
FP	Fibrinous pleuritis
FUS	FUS RNA binding protein
GA	General anaesthesia
GATA-3	GATA binding protein 3
GER	Germany
GNH	Genomic near-haploidisation
Gy	Gray
H&E	Haematoxylin & eosin
H3K27me3	Trimethylated histone H3 lysine 27
HC	Hybrid-capture (next generation sequencing)
HD	Homozygous deletion
HDAC	Histone deacetylase
HPF	High power field
HPPBP1	NEDD8 activating enzyme E1 subunit 1
HSV	Herpes simplex virus
IASLC	The International Association for the Study of Lung Cancer

IFN	Interferon
IFNA2B	IFN-alpha 2b
IHC	Immunohistochemistry
ILD	Interstitial lung disease
IMIG	International Mesothelioma Interest Group
IMRT	Intensity-modulated radiation therapy
INSM1	Insulinoma-associated protein 1
IOA	Inter-observer agreement
IPC	Indwelling pleural catheter
IRB	Institutional review board
ISG	Interferon stimulated genes
LA	Local anaesthesia
LAF	Long asbestos fibres
LATS2	Large tumour suppressor kinase 2
lncRNA	Long noncoding RNA
LOH	Loss of heterozygosity
LUAD	Lung adenocarcinoma
μm	Micrometre
m.d.	Maximum dimension

mAb	Monoclonal antibody
MAP3K7	Mitogen-activated protein kinase kinase kinase 7
MARF	Mesothelioma Applied Research Foundation
MARS	Mesothelioma and Radical Surgery randomised trial
MARS2	Mesothelioma and Radical Surgery 2
MC	Microcystic growth pattern
MCR	Macroscopic complete resection
MCS	Maximal cytoreductive surgery
MDT	Multi-disciplinary team
MEK	MAP kinase kinase
mg/L	Milligram per litre
MHC	Major histocompatibility complex
MIA	Minimally invasive adenocarcinoma
miRNA	MicroRNA
MLH1	MutL homolog 1
MM	Malignant mesothelioma
MIS	Mesothelioma *in situ*
MMR	Mismatch repair
MMT	Mesothelial-to-mesenchymal transition

MMT	Multimodality therapy
M-N score	Mitosis-necrosis score
MPP	Micropapillary growth pattern
MRD	Macroscopic residual disease
MRE11A	MRE11 homolog, double strand break repair nuclease
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSH6	MutS homolog 6
MSI	Microsatellite instability
MSLN	Mesothelin
MTA	Methyladenosine
MTAP	Methylthioadenosine phosphorylase
mTOR	Mammalian target of rapamycin
MUC4	Mucin 4
MWCNT	Multi-wall carbon nanotubes
MXRA5	Matrix remodelling associated 5
MyoD1	Myogenic differentiation 1
NCI	National Cancer Institute
NCMR	National Centre of Mesothelioma Research

NF2	Neurofibromatosis type 2
NGR	Endothelial ligand asparagine-glycine-arginine
NGS	Next generation sequencing
NK	Natural killer
NLR	Neutrophil-to-lymphocyte ratio
NLS	Nuclear localisation signal
nm	Nanometre
NOD2	Nucleotide binding oligomerization domain containing 2
NOS	Not otherwise specified
NP57	Anti-neutrophil elastase antibody
NRES	National Research Ethics Service
NSCLC	Non-small cell lung cancer
NSE	Neuron-specific enolase
NUTM1	NUT midline carcinoma family member 1
ORR	Objective response rate
OS	Overall survival
p16INK4a	CDK4 inhibitor p16-INK4
p19ARF	Tumour suppressor ARF
pAb	Polyclonal antibody

PALB2	Partner and localiser of BRCA2
PanCK	Pan-cytokeratin
PARP	Poly (ADP-ribose) polymerase
PAX8	Paired box 8
PBRM1	Polybromo 1
PCR	Polymerase chain reaction
PD	Pleurectomy and decortication
PD1	Programmed cell death protein 1
PDGF	Platelet-derived growth factor
PD-L1	Programmed death-ligand 1
PDT	Photodynamic therapy
PE	Pleural effusion
PET-CT	Positron emission tomography-computerised tomography
PFS	Progression-free survival
PI3K	Phosphoinositide 3-kinase
PM	Pleural mesothelioma
POT1	Protection of telomeres 1
PRC2	Polycomb repressive complex 2
PR-DUB	Polycomb repressive deubiquitinase complex

PRMT5	Protein arginine methyltransferase 5
PTCH1	Patched 1
PTFE	Polytetrafluoroethylene
PTM	Procedure-tract metastasis
RASSF1	Ras association domain family member 1
Rb	Retinoblastoma protein
RB1	RB transcriptional corepressor 1
RBH	Royal Brompton and Harefield Hospitals
RCC	Renal cell carcinoma
RECIST	Response Evaluation Criteria in Solid Tumours
RMH	Reactive mesothelial hyperplasia
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Radiotherapy
SDHA	Succinate dehydrogenase complex flavoprotein subunit A
SETD2	SET domain containing 2
SETDB1	SET domain bifurcated 1
SF3B1	Splicing factor 3B subunit 1

SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 4
SMARCB1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily B, member 1
S-PM	Sarcomatoid pleural mesothelioma
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
SOL	Solid growth pattern
sPD-L1	Soluble PD-L1
SUFU	Suppressor of fused homolog
SUVmax	Maximum standard unit value
SV	Structural variant
SV40	Simian virus 40
SWI/SNF	Switch/sucrose non-fermentable
TAD	Topographically-associated domain
TAZ	Tafazzin
TCGA	The Cancer Genome Atlas
TCR	T cell receptor
TED	Thromboembolism deterrent

TERT	Telomerase reverse transcriptase
T_H1	T helper type 1
TLS	Tertiary lymphoid structures
TMA	Tissue microarray
TMB	Tumour mutation burden
TMEM127	Transmembrane protein 127
T-PM	Transitional pleural mesothelioma
TNFα	Tumour necrosis factor alpha
TNM	Tumour, node, metastasis (classification system)
TP	Tubulo-papillary growth pattern
TP53	Tumour protein p53
TRAB	Trabecular growth pattern
TRACERx	TRAcking Cancer Evolution through therapy
TRAF7	TNF receptor associated factor 7
TTF-1	Thyroid transcription factor 1
TTFields	Tumour treating fields
UCH	Ubiquitin C-terminal hydrolase
UIP	Usual interstitial pneumonia
UK	United Kingdom

URHF-1	Ubiquitin-like with plant homeodomain and ring finger domains 1
USA	United States of America
VATS	Video assisted thoracoscopic surgery
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau tumour suppressor gene
VIM	Vimentin
VISTA	V-domain Ig suppressor of T cell activation
WDPMT	Well differentiated papillary mesothelial tumour
WGD	Whole genome doubling
WHO	World Health Organization
WISP	Weighed *in silico* pathology
WT1	Wilms tumour protein
YAP	Yes-associated protein 1

1 Introduction

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Following the introduction of the latest edition of the World Health Organisation (WHO) Classification of Tumours (2021), "*pleural mesothelioma*" replaces "*malignant pleural mesothelioma*" and this thesis conforms to the change in nomenclature. Unless specified, all cases of pleural mesothelioma included in this thesis refer to the diffuse rather than the localised type.

Malignant mesothelioma (MM) is an aggressive neoplasm of the mesothelium which lines the serosal surfaces in the human body including the pleura, peritoneum, pericardium and *tunica vaginalis* of the testis. Pleural mesothelioma (PM) is by far the most common form of malignant mesothelioma followed by peritoneal mesothelioma and then those at other body sites. Given the focus of this PhD, the context of this section is limited to pleural mesothelioma.

PM is a highly heterogeneous malignant disease with unique epidemiological, clinical, histopathological and molecular features. Consequently it poses substantial challenges to diagnosis, management and research. For most part of the 20th century, PM received comparatively less attention than common thoracic malignancies such as primary lung cancer, necessarily hampering the effort in improving patient outcomes. Over the past 20 years major advancements in our understanding of the disease have been achieved by the emergence of platform sciences, such as genomics; and the establishment of multi-disciplinary and often international working groups in the developed world. These have made precision oncology addressing disease heterogeneity and early diagnosis within reach, while many controversial concepts and proposals have emerged at a fast pace and continue to do so. This introductory chapter focuses on providing a contemporary and multi-disciplinary overview on the topics of tumour heterogeneity and early diagnosis in PM, and the rationale for the research work conducted and detailed within this doctoral thesis.

1.1 Epidemiology and the global disease burden of pleural mesothelioma

1.1.1 Asbestos and non-asbestos causes

PM is traditionally associated with exposure to asbestos, a class of naturally occurring silicate compounds with characteristic crystal habit (asbestiform) of fibrous appearance and elongated, thin crystals. Such association was estimated to be present in approximately 80% of cases (Rushton *et al.*, 2008). They can be further classified into commercial (chrysotile, amosite, crocidolite, anthophyllite) and non-commercial asbestos (tremolite and actinolite). The latter can also be found as a contaminant in commercial asbestos and contributes substantially to the lung fibre burden (Roggli *et al*., 2002). PM is characterised by a long latent interval, the latter being up to 40 years between first exposure and the onset of disease. This is at least in part related to the long half-life of asbestiform fibres, usually in the order of 10-20 years with steady clearance by lung resident macrophages. The biopersistence of asbestos and its downstream biological effect results in cumulative DNA damage and activation of the stromal response, including macrophages and fibroblasts, culminating in fibre-related malignant transformation (Gaudino *et al*., 2020).

The first report on the aetiological association between mesothelioma and asbestos exposure was from northwest Cape Province (South Africa) (33 patients) and was a classical example of occupational exposure via asbestos mining (Wagner *et al*., 1960). The vast majority of male mesothelioma cases (86%) were attributable to this exposure route (Rake *et al*., 2009). Similar associations have been described in well characterised epidemiological cohorts, including both occupational and para-occupational/environmental exposures, from Wittenoon Gorge (Australia) (Musk *et al*., 2020), Libby, Montana (USA) (Whitehouse *et al*., 2008) and Sivas (Turkey) (Bayram *et al*., 2013). The role of cosmetic talc, the most common form of commercial non-asbestiform fibres, as a potential causative agent is at this time highly

contentious and is not supported by fibre analysis (Roggli *et al*., 2020). Of mesothelioma cases with elevated talc levels, asbestiform fibres have been frequently found (Roggli *et al*., 2020).

The ascertainment of asbestos exposure among at-risk individuals, who have not developed asbestos-related lung diseases, might not be straightforward. This is especially true in routine clinical settings. More than 20 years since the publication of Helsinki consensus document (Henderson *et al*., 1997), mineral fibre analysis using preserved lung tissue remains the gold standard yet the assay is not widely available and reference values for threshold cut-off need to be established in individual laboratories. Microscopic evaluation for asbestiform fibres is considered highly specific but the sensitivity is exposure dosage- and pathologist-dependent. Radiological assessment for the presence of pleural plaques carries 80-90% specificity due to other aetiologies known to cause local or diffuse pleural thickening. A detailed survey of occupational history, particularly for those in high risk professions, is considered sufficient. There remains, however, a grey area with regard to brief and/or low-level exposure. In practice a multi-disciplinary approach is usually taken based on available information.

There are also non-asbestos causes of PM (Attanoos *et al*., 2018). The carcinogenic properties of non-asbestiform silicates are considered controversial, in part contributed by the fact co-existing asbestiform fibres can often be identified in lung tissue (Attanoos *et al*., 2018). Asbestiform characteristics of multi-wall carbon nanotubes (MWCNTs), an emerging industrial material with diverse potential applications, have been shown to induce PM in *in vivo* models (Huang *et al*., 2020) (Numano *et al*., 2019) (Sakamoto *et al*., 2018) (Chernova e*t al*., 2017). They are capable of inducing mesothelial cell and macrophage-driven pleural inflammation in similar fashion to asbestos (Huang *et al*., 2020) (Arnoldussen *et al*., 2018). Promoter hypermethylation of the *CDKN2A* locus, a hotspot genomic region commonly found to be deleted in PM, can be recapitulated in murine models exposed to MWCNTs

(Chernova *et al*., 2017). Its carcinogenicity can be enhanced by loss of tumour suppressors such as TP53 (Duke *et al*., 2018). Similar to silicates, MWCNTs represent a heterogenous class of compounds and carcinogenicity depends on the crystal habits (Sakamoto *et al*., 2018). Exposure to nanoscale solid particles (<100nm), given their frequent occupational co-exposure with asbestos, have been reported to not be associated with increased risk of developing PM (Manangama *et al*., 2020).

PM shows male predominance. Mesothelioma in females exhibits distinctive clinicopathological features including younger age at diagnosis (Alpert *et al*., 2020) (Vivero *et al*., 2018), low incidence of proven asbestos exposure (Malpica *et al*., 2020) (Vivero *et al*., 2018), high incidence of personal or family history of malignancy (Malpica *et al*., 2020) (Vivero *et al*., 2020) and superior overall survival (OS) (Alpert *et al*., 2020). Prior history of thoracic radiation (Cavazza *et al*., 1996), for example in the treatment of Hodgkin and non-Hodgkin lymphoma (Li *et al*., 2015) (Chirieac *et al*., 2013) (De Bruin *et al*., 2009) (Hodgson *et al*., 2007) (Teta *et al*., 2007) (Tward *et al*., 2006), breast cancer (Brown *et al*., 2007) (Deutsch *et al*., 2007) (Shannon *et al*., 1995) or testicular cancer (Travis *et al*., 2005), is an established risk factor based on a population-based database (Farioli *et al*., 2016) and there is association with unusual histological features (Chirieac *et al*., 2013). Non-therapeutic exposure to ionising radiation, such as workers in nuclear facilities, has also been shown to increase the risk of developing PM (Schubauer-Berigan *et al*., 2015). It is recently suggested, however, asbestos exposure among female mesothelioma patients might have been under-reported in the literature (Baur *et al*., 2021).

Rare association with long-standing shunt catheters was recently reported for peritoneal MM (Mujahed *et al*., 2020). The authors hypothesised a chronic inflammatory aetiology. To date, no association with chronic inflammation has been reported in the literature of PM. The aetiological effect of Simian Virus 40 (SV40) is generally regarded as insufficient, as

subsequent studies (Hmeljak *et al*., 2018) (Lundstig *et al*., 2007) (Manfredi *et al*., 2005) were unable to reproduce the initial findings (Strickler *et al*., 2001) (Shivapurkar *et al*., 1999) (Pass *et al*., 1998).

In recent years a minority of PM were identified to harbour germline mutations, mainly involving BRCA-associated protein 1 (*BAP1*) as well as other genes implicated in cell cycle regulation and DNA damage repair (further discussed in Section 1.4.7).

1.1.2 Incidence, global disease burden and the changing dynamics with asbestos ban

The rise of the incidence of PM followed the large-scale industrial utilisation of asbestos from the early to mid-1900s. Given the long latency between exposure and presentation, most developed regions banned or regulated use of asbestos, primarily concerning long asbestos fibres (LAF) based on the World Health Organisation (WHO) definition (length $\geq 5\mu m$, diameter: $< 3\mu m$ and length/diameter ratio > 3), started in the 1980s. This is likely to result in a continuous upward trend in disease incidence well into the early 21st century.

In 2011, the conservative estimate of the number of new cases worldwide was 14,200 per year (Park *et al*., 2011). The differential patterns of asbestos use in high- and low-income regions, complicated by the availability of disease registries, mean accurate estimation of PM incidence is tremendously difficult. The estimated number of deaths related to PM is between 27,000 and 40,000 per year (Global Burden of Disease, 2020) (Global Burden of Disease, 2017) (Odgerel *et al*., 2017). Following the introduction of The Asbestos Regulation in 1969, regulation of asbestos use in UK has significantly tightened. The reported annual average of mortality from PM in UK for the years 1994-2008 was 1,891 cases (Park *et al*., 2011), and this was expected to peak at approximately 2,100 cases in 2017 (Martínez-Miranda *et al*., 2016). Nonetheless the trend defies predictive models and the current incidence is estimated

at 2,500 annually. In keeping with this model, between 2016 and 2018 a total of 6,950 new cases were diagnosed (National Mesothelioma Audit, 2020). A similar trend, which is postulated to be due to longevity of the population, is seen in the United States (Kerger, 2018).

Outside the developed regions, the global incidence of PM is on the rise and is expected to reach its peak by 2020. The global disease burden is further complicated by unregulated use of asbestos in industrial applications in many developing countries. A shift in PM incidence from early-ban to late-ban countries is consequently anticipated (Kameda *et al.*, 2014) (Delgermaa *et al*., 2011). They are also likely to show unique locoregional epidemiological characteristics. For example, the Asbest cohort (Russian Federation) is comprised of an actively exposed population consisting of a substantially higher proportion of female workers (37%) with variable exposure levels (Schüz *et al*., 2020). Another example is Taiwan, characterised by late-ban (2018) and densely-populated urban residential areas surrounded by small asbestos factories of geographically-pervasive distribution (Lin *et al*., 2019).

Besides PM, it is worth noting that the other asbestos-related lung diseases, notably lung cancer (Brims *et al*., 2020) (Tamura *et al*., 2018) and asbestosis, translate into another 107,000 global deaths per year (World Health Organisation, 2014). A recent study put the estimated deaths from lung cancer at 180,000, rendering asbestos the most common aetiological agent through occupational exposure (Global Disease Burden, 2020). It is estimated that 4% of lung cancer cases in the UK are attributable to asbestos exposure (McCormack *et al*., 2014). The association between asbestos, presumably via ingestion rather than inhalation, and cancers of the alimentary tract is considered highly contentious and not substantiated by current evidence (Cheng *et al*., 2020).

Besides asbestosis, it has also been suggested asbestos exposure increases the risk of developing non-asbestosis interstitial lung disease (ILD) with a histological pattern of usual interstitial pneumonia (UIP) (Abramson *et al*., 2020) (Kawabata *et al*., 2016), although this remains a highly controversial area. A large autopsy series showed 1.3% of all asbestos-exposed ILD patients exhibited UIP pattern with fibre analysis not supporting an asbestos aetiology (Attanoos *et al*., 2016). The so-called non-asbestosis UIP could represent under-diagnosed asbestosis given the histological heterogeneity of ILD and the former group can show high levels of occupational exposure (Reynolds *et al.*, 2019). This is in turn precipitated by the recognised difficulty in establishing reliable history of asbestos exposure either from clinical consultation or histological evaluation (Barber *et al*., 2016) (Gaensler *et al*., 1991).

1.2 Histological classification of pleural mesothelioma

1.2.1 Roles of histopathology in diagnosis and prognostication

Histopathology is currently considered the gold standard in the diagnosis and prognostication of PM as recommended by the British Thoracic Society (BTS) (Woolhouse *et al*., 2018) and the American Society of Clinical Oncology (ASCO) (Kindler *et al*., 2018) guidelines. It also plays a key role in the multi-disciplinary management and research of the disease (Nicholson *et al*., 2020). By assessing tumour tissue, derived either from biopsy or less frequently surgical resection, morphological evaluation can be further complemented with ancillary tests including but not limited to immunohistochemistry (IHC), fluorescence *in situ* hybridisation (FISH) and genomic sequencing. The preserved tissue architecture in the tissue materials also holds advantages over cytology-based and tissue-free methods, as it allows the interpretation of the tumour-associated stroma. Up to date histological parameters relevant for the classification and prognostication of PM will be herein discussed.

1.2.2 Diffuse versus localised PM

Diffuse involvement of the pleura by a malignant cellular proliferation was reported as a common feature in the original northwest Cape Province cohort (Wagner *et al*., 1960). The pleura was invariably thickened, sometimes combined with a multi-nodular appearance, leading to direct anatomical disruption by compressing the lungs and pericardium. Lung involvement, distant metastasis and lymphangitic spread (*lymphangitis carcinomatosis*) were observed but infrequent. Pleural effusion was common and, in recent research, hypothesised as the result of disrupted pleural circulation secondary to the contiguous spread of tumour (Cheah *et al*., 2017) with unique biological properties.

While PM is almost exclusively diffuse in macroscopic appearance, isolated case series and recently a large multi-institutional observational study reported unique clinicopathological characteristics of localised PM (Marchevsky *et al*., 2019). They usually manifest as solitary, well defined pleural or chest wall masses, radiologically indistinct from sarcoma or carcinoma. There is minor male preponderance. Pleural effusion is less common, as is pleural thickening. The microscopic findings are the same as in diffuse mesothelioma including immunohistochemical profile, although epithelioid subtype is more common.

Given the rarity of these tumours, genomic sequencing has been performed on a limited number of cases. In an institutional series of 6 cases, two harboured *TRAF7* mutations (Hung *et al*., 2020) previously reported in well differentiated papillary mesothelial tumour (WDPMT) of peritoneal origin (Stevers *et al*., 2019). Recently two cases, initially reported as localised PM with one being pleural in origin, were found to harbour *EWRS1*/*FUS* re-arrangements (Argani *et al*., 2020) although the cases may not be mesothelioma as currently defined. Due to its overlapping radiological and histopathological features, not infrequently localised PM poses as a diagnostic mimic of pleural or chest wall sarcoma (Teo *et al*., 2010)

or carcinoma (Lin *et al*., 2013). Superior survival has been reported for localised PM undergoing surgical resection (Marchevsky *et al*., 2019) (Gelvez-Zapata *et al*., 2013). This perhaps is not surprising as the probability of achieving *bona fide* completion resection (R0) or macroscopic complete resection (MCR) is theoretically much higher in localised PM than its diffuse counterpart.

1.2.3 Histological subtypes

Early studies in the 1950s identified the presence of both epithelial-like and mesenchymal-like components in PM (McCaughey, 1958) (Campbell, 1950). Campbell and McCaughey's classifications over the next decades were consolidated and taken forward to become the three major subtypes of PM in the current diagnostic framework: epithelioid ("*tumours of epithelial character*"), sarcomatoid ("*tumours of mesenchymal type*") and biphasic ("*tumours of mixed type*").

The overall survival (OS), defined as the survival time between initial diagnosis and death, of PM ranges between 9-12 months (Beebe-Dimmer *et al*., 2016) with 1-year survival of approximately 40% (National Mesothelioma Audit, 2020). This survival figure is subtype-dependent and histological subtype is by far the most commonly adopted and robust prognostic variable in PM. Epithelioid PM (E-PM), which demonstrates tumour growth patterns similar to many malignancies of epithelial origin, is by far the most common subtype of PM (60-80%) (Nicholson *et al*., 2020) (Travis *et al*., 2015). Non-epithelioid PM comprises of sarcomatoid mesothelioma (S-PM, 10-15%) characterised by its morphological resemblance to poorly differentiated spindle cell sarcoma, and biphasic PM (B-PM, 10-15%), where within the same tumour there is co-existence of both epithelioid and sarcomatoid components. The latter is believed to represent the process of sarcomatoid transformation in a similar fashion to epithelial-to-mesenchymal transition (EMT) in epithelial malignancies

(Fassina *et al*., 2012). A diagnosis of E-PM confers better prognosis than B-PM and S-PM (Pass *et al*., 2016) (Ak *et al*., 2009) (Borasio *et al*., 2008) (Arrossi *et al*., 2008) (Flores *et al*., 2007) (Rusch and Venkatramen, 1999). The histological classification of PM is summarised in Table 1-1.

Histological subtype	Epithelioid	Biphasic	Sarcomatoid
% epithelioid component	>90%	10-90%	<10%
Median OS (months)	14.6-16.3 (Rosen *et al*., 2018, Kadota *et al*., 2011, Flores *et al*., 2007 and Borasio *et al*., 2008)	8.0-9.5 (Galateau Sallé *et al*., 2018, Flores *et al*., 2007 and Borasio *et al*., 2008)	4.5-6.1 (Flores *et al*., 2007 and Borasio *et al*., 2008)

Table 1-1 Histological classification of PM

The coexistence of at least 10% of sarcomatoid and epithelioid components is required for a tumour to be classified as biphasic.

1.2.4 Architectural growth patterns in epithelioid PM

Epithelioid PM exhibits diverse architectural patterns similar to primary adenocarcinoma of the lung. Five patterns are currently recognised by major international committees including the International Association for the Study of Lung Cancer (IASLC), the European Reference Network on Rare Adult Cancers (EURACAN), and the International Mesothelioma Interest Group (IMIG) (Nicholson *et al*., 2020): (i) tubulo-papillary, (ii) trabecular, (iii) microcystic/adenomatoid, (iv) solid and (v) micropapillary. The prognostic value of growth

patterns has been reported by several studies, where there was a trend towards more favourable prognosis for tubulo-papillary, trabecular and microcystic patterns, whilst solid and micropapillary patterns were associated with worse outcomes (Alchami *et al*., 2017) (Brčić *et al*., 2014) (Kadota *et al*., 2011). Prognostication of E-PM using growth patterns is, however, hampered by the lack of well-powered studies and the known issue of inter-observer agreement (Brčić *et al*., 2018) (Hartman *et al*., 2017) which, at least in part, was contributed by the lack of clearly defined histological criteria. Nonetheless based on available evidence, recommendation was made for pathologists to record the presence and relative abundance of each growth pattern in E-PM (Nicholson *et al*., 2020).

1.2.5 Cytological variants

At the cellular level, the majority of mesothelioma cells are morphologically indistinct from common malignancies of other lineages. Several cytological variants have been reported in the literature across all subtypes, but the data is almost exclusively limited to case reports and short case series preventing a systematic assessment of the variants, their diagnostic criteria, prognostic significance and biology. The incidence of cytological variants is currently unknown. In patients with previous history of thoracic radiation, the incidence was reported to be 32% including rhabdoid, signet ring and clear cell variants (Chirieac *et al*., 2013).

Deciduoid cell variant was initially reported in peritoneal MM as mesothelioma with "decidua-like" features due to its morphological resemblance to the modified endometrial stroma (Orosz *et al*., 1999) (Nascimento *et al*., 1994). This notion was challenged by later studies reporting its presence in pleural mesothelioma, particularly in older males (Ordóñez, 2000) (Shanks *et al*., 2000). The tumour cells contain abundant cytoplasm with centrally placed nuclei, giving rise to a "fried-egg" appearance. Ultrastructural studies demonstrated cytoplasmic accumulation of intermediate filaments whilst mesothelium-specific microvilli

were present, suggesting that for this variant the mesothelial lineage is preserved (Ordóñez *et al*., 2012) (Shia *et al*., 2002). Extensive copy number changes in keeping with mesothelioma have also been reported (Scattone *et al*., 2006), reduced SMARCB1 expression (Kawai *et al*., 2019) and found to be associated with the production of pro-inflammatory cytokines (Kimura *et al*., 2005). The prognosis appeared to be bi-modal between 7 to 23 months (Ordóñez *et al*., 2012). The aggressive subgroup exhibits marked nuclear atypia and increased mitotic activity, so it is likely the nuclear grade and not the cytological appearance that drives prognosis.

Rhabdoid cell variant, named after its resemblance to primitive rhabdomyoblasts without immunohistochemical or genomic evidence of rhabdomyoid differentiation, was first reported in a case of biphasic peritoneal MM (Matsukuma *et al*., 1996). The tumour cells contain a single eosinophilic globule with attenuated nuclei. Ultrastructural pathology revealed cytoplasmic accumulation of intermediate filaments similar to deciduoid cell variant, but with partial or complete loss of microvilli (Ordóñez *et al*., 2006). Therefore the evidence points towards this variant being one with early signs of de-differentiation. A case report also showed SMARCB1 loss (Kimura *et al*., 2018). The prognosis of rhabdoid cell variant is poor, with median OS of only 5.7 months (Ordóñez *et al*., 2006).

Signet ring cell variant is characterised by the presence of a large, unilocular cytoplasmic vacuole with attenuated, often crescent-shaped nucleus. They are negative for diastase-resistant periodic acid-Schiff reaction which aids distinguishing this variant from signet ring cell adenocarcinoma (Ordóñez, 2013) (Cook *et al*., 2000). Ultrastructural pathology shows accumulation of proteoglycan-like materials within the vacuoles (Ordóñez, 2013). The median OS of signet ring cell mesothelioma has been reported to be 15 months (Ordóñez, 2013). It requires distinction from adenomatoid tumours (Kaplan *et al*., 1996), the latter being a localised and slow growing neoplasm with excellent prognosis following surgical resection.

Clear cell variant was first described as those with optically clear cytoplasm (Ordóñez *et al*., 1996) but later reports also accepted those with foamy cytoplasm (Cavazza *et al*, 2002) (Ordóñez, 2005). Interestingly foamy cell appearance was first reported in a case of peritoneal MM more than a decade prior to these reports (Kitazawa *et al*., 1984). The apparently hollow cytoplasm was initially found to be rich in glycogen, giving rise to the description of "glycogen-rich mesothelioma" (Ordóñez and MacKay, 1999). A subsequent study showed the presence of either glycogen and/or lipid droplets (Ordóñez, 2005). Prognostic information on this variant is largely lacking, with the only report finding overall survival between 3 to 40 months among 5 patients (Ordóñez, 2005).

Small cell variant, first reported as a morphologically and immunohistochemical mimic of small cell lung cancer given its immunoreactivity to neuron-specific enolase (NSE) and CD57 (Mayall *et al*., 1991). A follow-up study including chromogranin A, a known neuroendocrine marker with higher specificity, did not found any positivity among 13 cases (Mayall and Gibbs, 1992). The lack of neuroendocrine marker expression was confirmed in later studies (Ordóñez, 2012) (Cavazza *et al*., 2002), arguing against this representing *de novo* neuroendocrine differentiation. A recent molecular study showed extensive copy number changes and *EWSR1* deletion (Hackman *et al*., 2016), but to date there has been no study undertaken to specifically investigate if there is evidence of lineage reprogramming. The median OS of small cell variant has been reported to be 8.2 months (Ordóñez, 2012). This variant was comparatively less frequently reported with a diverse spectrum of morphological features. For instance, out of the 8 cases reported by Ordóñez (Ordóñez, 2012), four showed clear cell features. Of the two cases with microscopic images presented, they could be conceivably reclassified as clear cell variants.

1.2.6 Stromal features

Mesothelioma-associated stroma exhibits morphological diversity. Myxoid stroma have been described as those with loosely arranged collagen fibrils together with a variable degree of inflammatory cell infiltration (Alchami *et al*., 2017). They have been shown to be associated with favourable prognosis. Desmoplastic features, defined as dense collagen deposition occupying more than 50% of tumour area (Wilson *et al*., 1992) often with reduced tumour cellularity, are associated with primarily sarcomatoid PM (Klebe *et al.,* 2010). This results in a histological appearance of paucicellular fibrotic pleural lesion (Mangano *et al*., 1998). It is also seen in some biphasic PM but is rare among epithelioid PM (Cantin *et al*., 1982). The prognostic impact of desmoplastic features is believed to be driven by the tumour subtype.

PM is not considered an inflammatory, or "hot", tumour in comparison with primary lung cancer for example. The prognostic relevance of the extent of host response, expressed in terms of the diffuseness and/or quality of immune cell infiltration, has been demonstrated in a limited number of studies. A high degree of lymphoplasmacytic infiltration was associated with favourable prognosis (Ujiie *et al*., 2015) (Anraku *et al*., 2008) (Hegmans *et al*., 2006). S-PM with intense lymphoplasmacytic infiltration and histiocyte-like tumour cells, termed lymphohistiocytoid mesothelioma, was initially reported as a variant of S-PM (Henderson *et al*., 1988). Subsequent studies showed the tumour cells expressed mesothelium-associated markers but not those of histiocytic lineage (Khalidi *et al*., 2000) (Kawai *et al*., 2010) (Galateau-Sallé *et al*., 2007). $CD68^{+}$ histiocytes are frequently present (Galateau-Sallé *et al*., 2007). In a separate case series, CD68 positivity was reported in the malignant population although the authors acknowledged the difficulty in interpretation given the extent of inflammation (Yao *et al*., 2004). The stringent criteria applied to this stromal variant necessarily mean only an absolute minority of PM could qualify for the diagnosis, which limits its practical utility.

1.2.7 Emerging parameters and controversies in refining current classification

1.2.7.1 Nuclear grade

Epithelioid PM represents the majority of PM cases, but a satisfactory prognostic system however has not been established. Consequently, they are treated as a single disease category both in routine clinical management, clinical trials and basic research. Despite this, it is recognised in daily practice that not all E-PM behave in the same fashion. A 3-tier grading system for E-PM based on nuclear features and mitotic activity was developed (Kadota *et al*., 2012), validated in an international multi-institutional study (Rosen *et al*., 2018) and has been shown to demonstrate prognostic value (Table 1-2). For instance, Grade III E-PM is associated with median OS of 8 months (Rosen *et al*., 2018), close to that of B-PM. Nonetheless several key questions remain.

First, the nuclear grading system requires the pathologist to search for the tumour area (≥5% of total) with the most aggressive histological features. Such probability is often a function of total tumour area available for assessment. Most patients with PM will not undergo surgical resection yet both published studies were comprised of predominantly resection specimens (Rosen *et al*., 2018) (Kadota *et al*., 2012). As a result the prognostic utility of the nuclear grading system requires validation in biopsy-heavy practice. Second, a recent report suggests the inter-observer agreement (IOA) regarding 3-tier nuclear grade is poor to fair (Cohen's κ 0-0.28) (Mlika and Mezni, 2020). A 3-tier system also complicates patient stratification and bears the risk of "ballooning" of the middle category in routine practice. Rosen *et al*. proposed using the presence of tumour necrosis to sub-classify nuclear grade II tumours, leading to a 2-tier system consists of low grade (grade I and grade II without necrosis) and

high grade (grade II with necrosis and grade III). This system has not been subjected to independent validation.

An alternative grading system incorporating Ki67 labelling index and applicable to non-epithelioid PM was also recently proposed (Pelosi *et al*., 2018). The extra requirement to provide Ki67 scores over 2,000 tumour cells and the uncertainty whether a grading system for non-epithelioid PM is warranted (which is associated with poorer prognosis to start with) will , however, likely limit adoption of this alternatively grading system.

Nuclear grade	**I**	**II**	**III**
Mitotic count per 10 high power fields (HPFs)	0-1	2-4	≥5
Nuclear size	Small	Intermediate	Large and/or bizarre and/or multinucleation
Variations in nuclear size and shape	Minimal	Intermediate	Pronounced
Nucleoli	Inconspicuous	Prominent	Macronucleoli
Frequency	39%	45%	16%
Median OS (months)	27	14	8

Table 1-2 Proposed 3-tier grading system for epithelioid PM

The criteria are summarised from Rosen *et al.* 2018. At least 5% of tumour cells are required to fulfil all the criteria in order for the tumour to be graded accordingly.

1.2.7.2 Pleomorphic features

The presence of pleomorphic features, defined as severe nuclear atypia (Ordóñez, 2012) representing at least 10% of tumour cells in E-PM (Kadota *et al*., 2011), has been found to be associated with significantly worse median OS (3.0-8.1 months) (Alchami *et al*., 2017) (Brčić *et al*., 2014) (Kadota *et al*., 2011) and early recurrence post-surgery (Kadota *et al*., 2011). Although only representing approximately 15% of cases (Kadota *et al*., 2011), this descriptor identifies a group of tumours with similar disease behaviour to S-PM. It is also associated with high metabolic uptake in line with non-epithelioid subtypes (Kadota *et al*., 2012).

The main areas of controversy surround this descriptor primarily concerns its position in the classification system. The initial study establishing the prognostic value of pleomorphic features in E-PM made the comparison with growth patterns, however, its definition is fundamentally cytological, or more precisely, a combination of nuclear features. It is therefore more appropriate to assess its prognostic utility in relation to nuclear grading system. This also argues against amalgamating E-PM with pleomorphic features into non-epithelioid PM subtypes, as subtyping of PM is entirely morphological while the former shows little evidence of sarcomatoid transformation. The prognostic relevance of pleomorphic features in non-epithelioid tumours is poorly understood.

1.2.7.3 Extent of sarcomatoid transformation and the transitional subtype

The proportion of sarcomatoid component in biphasic PM has been shown to be prognostically relevant based on two studies. Vigneswaran *et al.* employed 50% cut-off to separate resected PM into two statistically significant prognostic groups (11.8 months vs 6.6 months) (Vigneswaran *et al*., 2017). Galateau-Sallé *et al*., employed 80% cut-off in an unpublished sub-study and achieved the same objective (Galateau-Sallé *et al*., 2018) although details including the effect size were not reported in their manuscript. Nuclear atypia of the

sarcomatoid component was also shown to influence OS. These data were in keeping with those from massive parallel sequencing (discussed further in Section 1.4), where computation of transcriptomic signature stratified B-PM into epithelioid-predominant (Biphasic-E) and sarcomatoid-predominant (Biphasic-S) subgroups (Bueno *et al*., 2016), or as an epithelioid/sarcomatoid (E/S) gradient (Quetel *et al*., 2020) (Blum *et al*., 2019).

There are several unresolved issues in this matter. First, IOA with regard to the identification and quantitation of the sarcomatoid component in B-PM is a recognised challenge. Among mesothelioma experts IOA was fair to moderate (Fleiss κ 0.45-0.62) (Dacic *et al*., 2020) (Galateau-Sallé *et al*., 2018) with provision of pan-cytokeratin (AE1/AE3) immunohistochemistry in addition to haematoxylin and eosin (H&E) stained slides. Second it remains uncertain if, beyond reasonably accurate subtyping (Chirieac *et al*., 2019), quantitation of sarcomatoid component could be undertaken using biopsies. Third, the 50% cut-off threshold was not prognostically significant in a UK-based surgical series (Harling *et al*., 2019) and the 80% cut-off was not subject to independent validation to date. As a result, the latest expert consensus (Nicholson *et al*., 2020) recommended quantitation of sarcomatoid component to be restricted to surgical resection specimens despite its relatively weak evidence base. Furthermore, it recommended "*any sample can be diagnosed as biphasic mesothelioma with a comment providing the percentages of each component in the sample*." This abrogated the necessity of a cut-off threshold and consequently, at least theoretically, any presence of sarcomatoid component irrespective of the proportion of tumour cells in PM will qualify as B-PM. Given the already unsatisfactory level in B-PM, whether or not such a proposal will improve IOA as intended is highly skeptical.

In attempt to subclassify non-epithelioid PM, transitional subtype (T-PM) was introduced for tumours that have lost their epithelioid architecture and cytological appearance but are not clear-cut sarcomatoid (Galateau-Sallé *et al*., 2018). It is speculated that this represents early

sarcomatoid transformation. The prognosis of T-PM was reported to be 6.0-6.7 months (Galateau-Sallé *et al*., 2020) (Galateau-Sallé *et al*., 2018), between those of B-PM and S-PM, but no significant difference between the former and either subtypes was demonstrated. Whole transcriptomic analysis on RNA from formalin fixed paraffin embedded (FFPE) materials of a subset of these tumours, revealed T-PM clustered with S-PM (Galateau-Sallé *et al*., 2020).

As a newly proposed subtype by a single research group, T-PM awaits further validation although it has already made a strong entry in the latest expert consensus (Nicholson *et al*., 2020). The IOA for identifying transitional subtype, or component, was comparatively worse than that of sarcomatoid component (Fleiss κ 0.40) (Dacic *et al*., 2020). The lack of consensus in definition so as to distinguish transitional component from sarcomatoid component is considered a major issue in the current proposal. Although lacking frankly sarcomatous features, the loss of cohesion demonstrated by reticulin stain was distinctive compared with E-PM (Galateau-Sallé *et al*., 2018). One could also argue the reported histological criteria for transitional component fall within the morphological spectrum of S-PM i.e. malignant spindle cells without severe nuclear atypia, and that of B-PM i.e. those with low grade sarcomatoid component and high grade epithelioid component, where the latter can often exhibit nuclear pleomorphism encompassing elongation.

The estimated incidence of T-PM was 3.3% of all non-epithelioid PM or 0.65% of PM at the authors' institution (Galateau-Sallé *et al*., 2020). This suggested T-PM is considerably less common than any of the E-PM growth patterns and likewise most of the cytological variants. A total of 49 cases were published in the public domain, of which 34 have data on BAP1 IHC and *CDKN2A* FISH, and 10 have undergone RNA sequencing. At this level one would be cautious with regard to case selection bias and the lack of statistical power. The literature reflects upon such degree of uncertainty. The original report proposed transitional component

as a “pattern” implying it was a subordinate to E-PM (Galateau-Sallé *et al*., 2018). The subsequent report proposed the component to be classified as non-epithelioid PM, implying this being a novel subtype; or a subgroup of S-PM, which hinted upon a dramatic shift from the original proposal (Galateau-Sallé *et al*., 2020). The IASLC/EURACAN expert consensus took an apparent middle-ground by including the transitional “*pattern*” under “*both epithelioid and sarcomatoid types until more data is available*” (Nicholson *et al*., 2020). Whether T-PM represents a novel tumour entity with unique practice-changing properties or is merely the result of reclassifying existing entities with limited information gain remains therefore to be seen.

1.2.8 Histological heterogeneity of pleural mesothelioma

In the early days of evaluating the histological features of PM, it is admittedly somehow prophetic that Saccone and Coblenz wrote “*nothing that is characteristic for these pleural tumors can be deduced from the histologic picture*.” (Saccone and Coblenz, 1943). On one hand numerous histological features have been unravelled over the decades, each defining a subset of PM with distinct clinicopathological characteristics. On the other hand these features frequently co-exist within the same tumour, sometimes without apparent patterns of co-existence between patients, amounting to extensive inter- and intra-tumoural heterogeneity as well as diagnostic uncertainties.

Data from nuclear grading in E-PM, quantitation of sarcomatoid component in B-PM, and indeed many reported histological features of PM, have demonstrated focal presence of certain tumour subpopulation(s) within the neoplastic ecosystem. As little as 5% (nuclear grade) to 10% (sarcomatoid component in B-PM) is sufficient to determine the disease trajectory. These are not isolated findings in PM but have been similarly shown in lung cancer (Moreira *et al.*, 2020). Based on current evidence, it is not possible to ascertain if the

behaviour of the tumour is solely determined by the most aggressive morphology. In either case, the assumption of PM being a homogeneous tumour is not substantiated by histopathological evidence.

From the perspective of developmental biology, the histological heterogeneity of PM is perhaps not surprising due to the pluripotent nature of the mesothelial cells and their potential for mesothelial-to-mesenchymal transition (MMT) (Li *et al*., 2013) (Mubarak *et al*., 2012) (Yáñez-Mó M *et al.*, 2003). *In vitro* and *in vivo* studies have also shown the mesothelium is capable of undertaking lineage reprogramming, including heterologous differentiation, giving rise to mesenchymal cell types such as fibroblasts, endothelial cells and smooth muscle cells (Colunga *et al*., 2019) (Rinkevich *et al*., 2012). A significant proportion of non-epithelioid PM can express smooth muscle actin, an established marker of epithelial-to-mesenchymal transition (EMT) (Klebe *et al*., 2010) (Lucas *et al*., 2003) (Kung *et al*., 1995). The desmoplastic features observed in some non-epithelioid PM are believed to be the phenotypic manifestation of tumour cells assuming the function of fibroblasts (Cantin *et al*., 1982). This is in part supported by RNA sequencing data where high EMT signature was associated with sarcomatoid and desmoplastic PM (de Reyniès *et al.,* 2014). There is also emerging evidence of activation of cancer-associated fibroblasts (CAFs) by mesothelioma (Oraha *et al*., 2020), serving as a further addition to the complexity of the tumour.

The adult hemithorax is lined by pleura measuring approximately one square metre ($1m^2$). Only a small fraction of tumour is sampled over time and space, therefore sampling restriction becomes the overarching limitation to study efforts attempting the delineate spatial and temporal heterogeneity in PM. This is aggravated by the lack of surgical options (further discussed in Section 1.5.1) and a clearly defined targetable lesion, both of which were cardinal to the achievements of mega-scale cancer genomic studies, such as TRACERx,

deciphering the heterogeneity and evolution of lung (Jamal-Hanjani *et al*., 2017) and kidney (Turajlic *et al*., 2018a) (Turajlic *et al*., 2018b) cancers.

1.3 Current diagnostic practice and challenges

1.3.1 Optimal sampling modality and tissue requirement

Tissue sampling is currently achieved either by image-guided/thoracoscopic-guided or surgical biopsy both being recommended by major guideline committees. As non-specific presentation, such as pleural effusion and breathlessness, is common in PM together with aggressive disease progression it means definitive diagnosis is often achieved at an advanced stage. The challenges and merits concerning sampling are somehow reflected by the current TNM staging system (8th edition) such as the high incidence of upstaging at final pathological stage (23-80% depending on clinical TNM stage), a lack of definition of so-called early mesothelioma and strong surgical bias (64.5%) during its development (Pass *et al*., 2016).

Accurate multi-parametric assessment of the baseline and trajectories of lung function and functional capacity is lacking. However, based on a limited number of studies, PM patients suffer from a wide spectrum of limitations more so in relation to impaired respiratory physiology (Nagamatsu *et al*., 2018) (Bottomley *et al.*, 2007) (Nowak *et al*., 2004). In conjunction with cardiorespiratory co-morbidities indigenous to this age group, biopsies after initial clinical and radiological assessments are impossible in a considerable proportion of patients. Collectively these result in attrition at every stage of the diagnostic pathway (Figure 1-1). Within the UK, based on the National Mesothelioma Audit of 2020, 12% of patients were unable to receive a pathological diagnosis. This is likely to be an underestimate as some patients might not even enter the diagnostic pathway in the first place. Surgical biopsies,

either open or via video assisted thoracoscopic surgery (VATS) approaches, necessitate general anaesthesia. This is in contrast with image-guided/thoracoscopic-guided modalities where anaesthesia can be administered at low depth i.e. sedation, or even as local anaesthesia. Open or Video Assisted Thoracoscopic Surgery (VATS) approaches do however generate more tissue materials, occasionally as much as a mini-pleurectomy/decortication, as well as allowing direct access to pleural effusion. Hence there is a compromise between the volume of tissue acquired and the applicability within the anticipated patient population, with an additional consideration of repeated procedural risk secondary to inconclusive first biopsy.

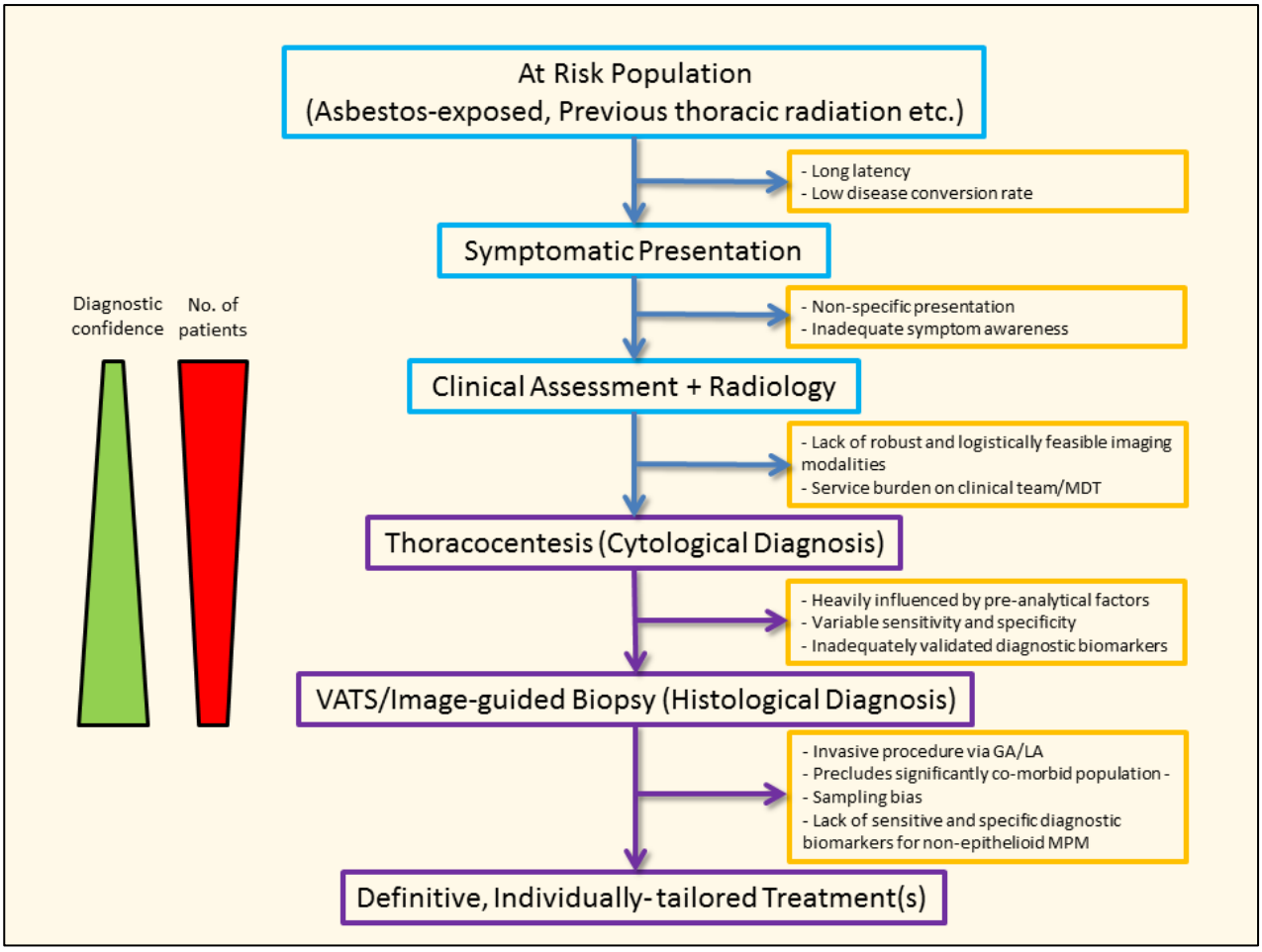


Figure 1-1 Diagnostic pathway of PM and patient attrition

The proportion of PM patients receiving definitive (high confidence) diagnosis is only a fraction of those presenting clinically. By improving the performance of pleural effusion-based diagnostics, it is anticipated more patients will be able to receive a definitive PM diagnosis and additional predictive biomarker information.

MDT, multi-disciplinary team; GA, general anaesthesia; LA, local anaesthesia; VATS, video assisted thoracoscopic surgery; PM, malignant pleural mesothelioma.

Given the intra-tumoural heterogeneity, biopsies are known to suffer from sampling bias. No published data, besides an ongoing trial (de Fonseka *et al*., 2018), exists in relation to the PM-specific diagnostic yield of various biopsy techniques in suspected patients, taking into account the heterogeneous nature of the tumour and provision of relevant prognostic parameters beyond achieving the diagnosis itself. One study involving 41 patients showed superior diagnostic yield of surgical biopsy over now-obsolete blind percutaneous needle biopsy with diagnostic yield being equivalent to CT-guided biopsy (Attanoos and Gibbs, 2008). The study however only included PM patients and was underpowered. With regard to subtyping, an early study comparing pre-surgical biopsy (thoracoscopic, needle or pleural effusion cytology) and final resection showed 59.6% overall concordance (Arrossi *et al*., 2008). More recently a study with a larger patient cohort showed 80.6% overall concordance, with higher concordance reported for the non-epithelioid subtypes (Chirieac *et al*., 2019). Regardless of approach, open or VATS, the operator remains guided by macroscopic appearance which is largely non-specific in determining the underlying histology. There is emerging evidence that high metabolic uptake (SUVmax) detected by positron emission tomography-computerised tomography (PET-CT) is associated with non-epithelioid histology (Kadota *et al*., 2012) generating potential for more targeted biopsies. Magnetic resonance imaging (MRI) has been shown to further improve the diagnostic accuracy when combined with PET-CT (Coolen *et al*., 2012). These platforms however require further validation and are associated with logistic and financial restrictions to service access.

No optimal sampling criteria concerning the size, number and anatomical locations from which samples should be taken currently exist at the time of writing. Diagnostic accuracy for subtyping was found to be improved when biopsies were taken from three sites compared with two (Chirieac *et al*., 2019). Further studies have been recommended by the latest expert consensus and until data is available from these studies, the current recommendation of a

minimum number of three sites with subpleural fat sampling has been agreed (Nicholson *et al*., 2020). To illustrate the issue of the paucity of data in this area, it is worth mentioning there is still no unified definition as to what constitutes a surgical biopsy in PM compared with various forms of surgical resection (Rice *et al*., 2011).

1.3.2 Dynamics of pleural microenvironment and the problem of assessing invasion in PM

The normal pleura comprises a single layer of mesothelial cells (mesothelium) with underlying fibroconnective tissue measuring sub-millimetre in thickness. The parietal pleura is separated from the chest wall by subpleural fat and endothoracic fascia. At the interface between pleura and subpleural fat, there are often scattered lymphoid aggregates or tertiary lymphoid structures (TLS) of uncertain biological significance. The visceral pleura is demarcated from the lung parenchyma by the internal and external elastin layers. The pleura represents a dynamic niche where both acute and chronic malignant and non-malignant processes can take place. In reactive conditions such as reactive mesothelial hyperplasia (RMH) and fibrinous pleuritis (FP), mesothelial cells can exhibit mild atypical features and histological assessment is made difficult by the presence of background inflammatory cells (Churg and Galateau-Sallé, 2012). Pleural empyema can develop when a false anatomical compartment is created with infective or inflammatory aetiologies, leading to haemorrhage, vastly increased fibrin production and destruction of the normal pleural architecture. The propensity for fibrosis secondary to external insult either by fibroblast activation, MMT (Strippoli *et al*., 2020) (Si *et al*., 2019) (Namvar *et al*., 2018) (Sandoval *et al*., 2016) (Mutsaers *et al*., 2015) or perhaps a combination of both, can result in the expansion of the sub-mesothelial compartment and entrapment of the mesothelium.

Hence, the method in establishing malignancy by assessing the invasion of cells into the surrounding stroma becomes unreliable in the setting of PM. Consequently, unequivocal evidence of invasion into the underlying anatomical structure (subpleural fat or the elastin layers, for lung invasion), is a key feature in the histological diagnosis of PM (Husain *et al.*, 2018). The only exception to this rule is the substantial presence of tumour mass with frank malignant features. This is not frequently the case in routine diagnostic practice due to the volume of biopsies and variable tumour cellularity. For the above reasons definitive diagnosis of PM is rarely a morphological one in contemporary setting, but multi-disciplinary and requires ancillary testing.

1.3.3 Diagnostic biomarkers and de-differentiation

Ancillary testing in the diagnosis of PM serves three purposes: (i) to ascertain the malignant process, (ii) to differentiate from malignancies with another organ of origin and, (iii) provide prognostic and/or predictive information.

In differentiating between PM and benign mesothelial proliferations, *CDKN2A*/p16 fluorescence *in situ* hybridisation (FISH) is currently considered the gold standard. Homozygous *CDKN2A* deletion demonstrated close to 100% specificity with variable sensitivity (45%-97%) (Hida *et al*., 2017) (McGregor *et al.*, 2017) (Wu *et al*., 2017) (Hwang *et al*., 2016) (Sheffield *et al*., 2015) (Hwang *et al*., 2014) (Monaco *et al*., 2011) (Chung *et al*., 2010) (Savic *et al*., 2010) (Kobayashi *et al*., 2008) (Illei *et al*., 2003). Its utility however is limited by its high cost and the relatively small number of FISH-capable laboratories whilst p16 IHC is considered sub-optimal (Chiosea *et al*., 2008). Methylthioadenosine phosphorylase (MTAP) was recently proposed as a surrogate marker for *CDKN2A* (Kinoshita *et al*., 2018) (Hida *et al*., 2017). Its gene (*MTAP*) occupies the same locus (9p21.3) as *CDKN2A*. An earlier FISH-based study showed 91% *CDKN2A*/*MTAP* co-deletion (Illei *et al*.,

2003). BAP1 IHC loss, defined as the loss of nuclear staining, was shown to be highly specific for PM but, in a similar fashion to *CDKN2A* deletion, its sensitivity (27-67%) (Hwang *et al*., 2016) (Sheffield *et al*., 2015) prevents PM from being safely ruled out if nuclear staining is retained. This is especially important in relation to S-PM where BAP1 loss is infrequent (0-15%) (McGregor *et al*., 2017) (Hwang *et al*., 2016), with the exception of a recent report showing 92% of S-PM exhibiting total loss BAP1 nuclear staining (Zaleski *et al*., 2020). The combination of *CDKN2A* FISH/MTAP IHC and BAP1 IHC is therefore currently recommended (Nicholson *et al*., 2020) (Husain *et al*., 2018). *CDKN2A* deletion has been shown to be an adverse prognostic variable (Hmeljak *et al*., 2019) (Dacic *et al*., 2008) (López-Ríos *et al*., 2006).

The pleura represents a common metastatic site. Primary or secondary pleural malignancies, such as metastatic carcinoma, can exhibit striking resemblance to PM of all subtypes. IMIG recommends an initial IHC panel comprised of at least one pan-cytokeratin (MNF116, AE1/AE3, Cam5.2), two mesothelium-associated markers (Calretinin, WT-1, CK5/6, D2-40), and two carcinoma markers (BerEP4, CEA, Claudin 4, TTF-1) (Husain *et al*., 2018). Secondary IHC panels can be employed subsequently to rule out primary or secondary sarcoma, lymphoma, melanoma or germ cell tumours- all of which are generally considered rare differential diagnoses. Whilst evaluating morphology and immunohistochemistry should be sufficient for diagnosing E-PM, the same cannot be said for the de-differentiated subtypes particularly S-PM. Mesothelium-associated markers tend to lose sensitivity in this setting (Marchevsky *et al*., 2017) (Klebe *et al*., 2010) (Hinterberger *et al*., 2007) (Lucas *et al*., 2003). So-called “cytokeratin-negative” S-PM (Inafuku *et al*., 2018) can be encountered in 10-30% of cases (Marchevsky *et al*., 2017) (Klebe *et al*., 2010) (Lucas *et al*., 2003). The loss of immunoreactivity to lineage markers is similarly observed in poorly differentiated neoplasms from other lineages including pleomorphic carcinoma (Marchevsky *et al*., 2017) and sarcoma

(Thway *et al*., 2016). Both B-PM and S-PM have been shown to be capable of undertaking heterologous differentiation (Klebe *et al*., 2008), a rare finding in non-epithelioid PM. Heterologous differentiation is considered a direct consequence of lineage reprogramming and often lead to *de novo* expression of mesenchymal lineage markers. As a result, the recommended IHC panels are generally less helpful and the final diagnosis is more inclined to be dependent on multi-disciplinary discussion.

The diagnosis of B-PM is at the same time straightforward and challenging. The presence of an epithelioid component is usually sufficient in establishing a diagnosis of PM, however the distinction between a sarcomatoid component and benign fibroblastic proliferation can understandably be difficult. Discordance in *CDKN2A* FISH and BAP1 IHC status between the two components has been reported, causing confusion (Galateau-Sallé *et al*., 2018). It is reasonable to hypothesise B-PM represents an intermediate state of differentiation at the height of complex tumour evolutionary events. Preliminary evidence derived *in vitro* utilising a commercial biphasic PM cell line (H2452) showed reversal of MMT where implantation of a monophasic spindle cell population recapitulated both epithelioid and sarcomatoid components (Kojima *et al*., 2020). Not only did Kojima *et al.*'s findings evidence the lineage plasticity of PM, but they also suggested the possibility of bidirectional differentiation.

Recently three novel IHC markers have been proposed to aid the histopathological diagnosis of poorly differentiated PM: GATA binding protein 3 (GATA-3), Mucin 4 (MUC4) and Endosialin (CD248). GATA-3 is an established IHC marker specific for breast and urothelial carcinoma (Davis *et al*., 2016). Positive GATA-3 staining is associated with 100% sensitivity and 85% specificity comparing S-PM to pleomorphic carcinoma (Berg and Churg, 2017). In a subsequent validation study, the sensitivity and specificity were 66% and 94% respectively (Terra *et al*., 2020). Focal GATA-3 staining, observed in approximately a third of S-PM however, was found not helpful in distinction. A recent report showed 73% sensitivity in S-

PM with a non-significant trend towards favourable OS in positive tumours (Prabhakaran *et al*., 2020). MUC4, which has been shown to have utility in differentiating E-PM from primary lung adenocarcinoma and squamous cell carcinoma (Mawas *et al*., 2018) (Llinares *et al*., 2004), when negative was associated with 100% sensitivity and 72% specificity when comparing S-PM and pleomorphic carcinoma (Amatya *et al*., 2017). For the latter, subsequent validation studies only showed 31-38% positivity hence argued against the utility proposed by the original report (Terra *et al*., 2020) (Berg *et al*., 2019). Endosialin (CD248) is a surface glycoprotein expressed on a number of poorly and undifferentiated sarcomas with up to 89% sensitivity (Thway *et al*., 2016), but data is currently lacking in PM. It is therefore of clinical relevance to evaluate the head-to-head performances of these biomarkers in well powered cohorts.

1.3.4 Pleural effusion cytology: diagnostic utility and limitations

Pleural effusion is a very common presentation in PM (Ruffie *et al*., 1989). It is easily accessible and can contain abundant tumour cells. It is also an early event, often being the first patient presentation and hence can precede definitive diagnosis by months if not years. Although used in the diagnosis of PM as early as the 1960s (Klepman, 1962), to this day pleural effusion cytology remains an adjunct to tissue biopsy in the diagnosis of PM. Currently symptomatic patients suspicious for PM often receive diagnostic thoracocentesis or "*pleural tap*", as a first-line investigation to complement tissue biopsy. In a proportion of patients tissue biopsy is not possible hence improving pleural effusion-based diagnostics has the prospect of reducing patient attrition by achieving cytology-only diagnosis. There has been published data both supporting (Muruganadan *et al*., 2017) and refuting (Pinelli *et al*., 2012) the notion that cytological diagnosis is equivalent to histology in terms of performance. Further evidence is therefore warranted while the practice of cytology-only diagnosis is

currently regarded controversial if not discouraged by major guideline committees and expert groups (Nicholson *et al*., 2020) (Husain *et al*., 2018). This is primarily due to the inability to access tissue architecture, lack of robust biomarkers, difficulty in controlling for pre-analytical variables, intermixed malignant and benign mesothelial cells, low sensitivity in non-epithelioid PM (Loveland *et al*., 2018) (Henderson *et al*., 2003), and concerns related to tumour heterogeneity. Such cautiousness is further accentuated in regions with lower pre-test probabilities of PM.

Recent studies have focused on utilising *CDKN2A*/MTAP and BAP1 as biomarkers to improve the confidence of cytological diagnosis. In a limited number of studies, the sensitivity and specificity of homozygous *CDKN2A* deletion and loss of BAP1 nuclear staining were comparable to those reported in histology samples (Cozzi *et al*., 2018) (Kinoshita *et al*., 2018) (Hiroshima *et al*., 2016) (Hwang *et al*., 2016) (Nabeshima *et al*., 2016) (Walts *et al*., 2016) (Andrici *et al*., 2015) (Cigognetti *et al*., 2015). There was only one study reporting 100% concordance for *CDKN2A* FISH (n=11) and BAP1 IHC (n=10) (Hwang *et al*., 2016). The major weaknesses of the existing literature are their retrospective nature, lack of radiology data as well as the relatively small sample sizes. There is very limited peer reviewed data available from UK-based institutions.

Pleural effusion can be seen as a naturally occurring suspension of cellular materials with a unique microenvironment. Compared with more invasive biopsy procedures, pleural effusion confers an inherent advantage of easier access which means sampling can be performed in outpatient clinics or as a day-case under local anaesthesia. Patients with high output effusions could receive indwelling pleural catheters (IPC). Not only does this allow continuous drainage and symptom relief, but also sequential sampling for disease monitoring purposes. As a source of research samples pleural effusion requires less processing steps. Hence it affords an opportunity to preserve cell integrity and molecular signatures known to be

sensitive to mechanical stress (transcriptomic and metabolic profiles). The issues of poor cellularity with or without the presence of contaminants such as erythrocytes (Schunselaar *et al*., 2017) are not specific to pleural effusions and also apply to biopsies. For pleural effusions, this can be rectified by introducing an enrichment step such as fluorescence activated cell sorting (FACS). Given its incidence among PM patients, it is fair to address pleural effusion in PM as an under-utilised source of clinical and research materials and its full potential has yet to be realised.

1.4 Molecular landscape of pleural mesothelioma

Understanding about the biology of PM has benefited significantly from research into its genetics and genomics. Following earlier efforts (Kang *et al*., 2016) (Bueno *et al*., 2010) (Dong *et al*., 2009) (Sugarbaker *et al*., 2008), four major parallel sequencing studies utilising next generation sequencing (NGS) technology have been completed over the past 5 years (Nastase *et al*., 2020) (Quetel *et al*., 2020) (Hmeljak *et al*., 2018) (Bueno *et al*., 2016) as well as one based on whole genome sequencing of primary PM cell lines (Oey *et al*., 2019). Extensive mapping of genomic and transcriptomic profiles of PM were performed and these five studies have collectively provided insights into the molecular landscape, at both DNA and RNA levels, at a greater breadth and depth. The findings from these studies form the basis of the discussion in the following sections.

1.4.1 Single nucleotide variants (SNVs)

The tumour mutation burden (TMB) of PM, derived almost exclusively from the exome regions, is generally low and typically less than two non-synonymous mutations per megabase (Oey *et al*., 2019) (Hmeljak *et al*., 2018) (Bueno *et al*., 2016). This is compared

with primary lung cancer where the mutational frequency (in terms of TMB) is often in the range of 10-100 per megabase (Lawrence *et al*., 2013).

BRCA-Associated Protein 1 (*BAP1*: 3p21.1) is a ubiquitin hydrolase initially found to interact with BRCA1, DNA repair associated (*BRCA1*) (Jensen *et al*., 1998). It is by far the most commonly mutated gene in PM (23-35%). It has dual functions depending on its nuclear or cytoplasmic localisation (Carbone *et al*., 2020). The nuclear functions of BAP1 include DNA damage repair (Parrotta *et al*., 2017) (Ismail *et al*., 2014) (Yu *et al*., 2014), cell proliferation and differentiation (Daou *et al*., 2015) (Matatall *et al*., 2013), and chromatin remodelling through the formation of polycomb repressive deubiquitinase complex (PR-DUB) (LaFave *et al*., 2015) (Sacco *et al*., 2015) (Lee *et al*., 2014). The cytoplasmic functions include promotion of apoptosis via mediating mitochondrial calcium flux (Bononi *et al*., 2017a), metabolic perturbations such as glycolysis and lactate production i.e. the Warburg effect (Bononi *et al*., 2017b), and modulation of the immune microenvironment (Shrestha e*t al.*, 2019) including interferon signalling (Yang *et al*., 2020). In pre-clinical animal models *BAP1* mutations were found to be associated with altered inflammatory response to asbestos and accelerated pathogenesis (Napolitano *et al*., 2016) associated with downregulation of retinoblastoma protein (Rb) (Xu *et al*., 2014). Mutations in *BAP1*, usually but not always, associate with loss of protein expression and/or loss of nuclear localisation of the protein product (De Rienzo *et al*., 2020) (Testa *et al*., 2011). For the latter, it is believed to be due to either the loss of C-terminal nuclear localisation signal (NLS) secondary to truncating mutations (Testa *et al*., 2011), or disruption of the ubiquitin C-terminal hydrolase (UCH) domain secondary to missense mutations. Disruption of the UCH domain results in the loss of its auto-ubiquitination property necessary for nuclear localisation (Mashtalir *et al*., 2014).

Neurofibromatosis type 2 (*NF2*: 22q11) is one of the first tumour suppressors identified with recurrent pathogenic SNVs in PM (Bianchi *et al*., 1995). The mutational frequency is

between 19-23%. *LATS2*, a downstream member of NF2 signalling pathway, has a mutational frequency of 3-10%. *NF2* mutations are frequently associated with heterozygous deletions leading to bi-allelic inactivation. Being an upstream regulator, NF2 loss was associated with Hippo pathway activation via nuclear localisation of YAP/TAZ. This subsequently results in a myriad of phenotypic manifestations including pro-proliferative effects and production of pro-inflammatory cytokines leading to modulation of the tumour microenvironment (Rehrauer *et al*., 2018). In murine asbestos models, loss of a single copy of *NF2* was found to be sufficient to sensitise the animals to asbestos exposure and promote carcinogenesis (Altomare *et al*., 2005). *NF2*-mutants were associated with worse OS (Quetel *et al*., 2020). Co-occurring alterations in both *NF2* and *LATS2* have been reported and were to be associated with more aggressive disease behaviour (Tranchant *et al*., 2017).

Mutations affecting the canonical tumour suppressors genes *TP53* (17p13.1) and *RB1* (13q14.2) have been found but are uncommon. *TP53* was found to be mutated in 6-17% of cases and associated with short median OS. *TP53*-mutants were enriched in the non-epithelioid subtypes (Bueno *et al*., 2016) and mutually exclusive with *BAP1* mutations (Quetel *et al*., 2020). In a small study (n=17), utilising a commercial hybrid capture NGS-based DNA gene panel, the frequency of *TP53* mutations was 29% although the mutations were not mutually exclusive with alterations in *BAP1*, *CDKN2A* or *NF2* also observed (Markowitz *et al*., 2020). *RB1* mutations have been reported with 1% frequency (Nastase *et al*., 2020) (Hmeljak *et al*., 2018). Mutations in the coding region of Cyclin-Dependent Kinase Inhibitor 2A (*CDKN2A*: 9p21.3) have not been observed, although one study has reported mutations in *CDKN2B*, a member of the same gene family, occupying the same locus (Quetel *et al*., 2020).

Mutations in the promoter region of telomerase reverse transcriptase (*TERT*: 5p15.33) (12%), with the majority clustered within the core promoter sequence (9.7%) with the rest in the 5'-

untranslated region (5'-UTR), were recently characterised following the initial report from the same group (Tallet *et al*., 2014). Mutations led to elevated *TERT* mRNA which was found to be associated with worse median OS (Quetel *et al*., 2020). In two separate and smaller studies *TERT* promoter mutations were detected in 10.4-11.6% of PM thus confirmed the findings of Quetel *et al.* (Campanella *et al*., 2020) (Pirker *et al*., 2020). Whether these observations in relation to TERT were a true discovery or re-discovery is a subject of debate, as TERT overexpression in PM was recognised almost two decades ago (Kumaki *et al*., 2002).

Mutations in genes involved in transcriptional regulation is a less common but still a recurrent event in PM. Histone methyltransferases, implicated in chromatin remodelling (further discussed in Section 1.4.4), SET domain containing 2 (*SETD2*: 3p21.31) and SET domain bifurcated 1 (*SETDB1*: 1q21.3) were found to harbour mutations at frequencies of 4-11% and 3-4% respectively. Genes implicated in RNA splicing, DEAD-box helicase 3, X-linked (*DDX3X*: Xp11.4) and splicing factor 3B subunit 1 (*SF3B1*: 2q33.1), were mutated in 4-5% and 1%. Of note the The Cancer Genome Atlas (TCGA) cohort did not detect any mutations in *DDX3X* but homozygous deletions only (2%).

Recently *MXRA5* and *NOD2* mutations were shown to be associated with B-PM and enriched in sarcomatoid-predominant ones (Torricelli *et al*., 2020). They were not identified in previous studies and this offered further insight into the mutational aspect of non-epithelioid PM, where data to date suggest sarcomatoid transformation is primarily the result of transcriptional regulation reflected in molecular classification systems (further discussed in Section 1.3.6). In a separate study however, the mutational frequencies of *MXRA5* in E-PM and non-epithelioid PM were found to be similar (Pagano *et al*., 2020).

Studies have found mutational signatures associated with reactive oxygen species (ROS)-induced mutagenesis (Bueno *et al*., 2016), DNA damage repair (Nastase *et al*., 2020) (Hmeljak *et al*., 2018) (Bueno *et al*., 2016), and apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) (Bueno *et al*., 2016). Outlier cases were found to harbour signatures related to mismatch repair (MMR) deficiency (Hmeljak *et al*., 2018) and immunoglobulin somatic hypermutation (Oey *et al*., 2019). Loss of MMR proteins has however, been very rarely reported in the literature (Cedrés *et al*., 2020) (Losi *et al*., 2019) (Arulananda *et al*., 2018) and microsatellite instability (MSI) similarly not found in a large tissue microarray-based study (Arulananda *et al*., 2018). Tobacco-related signature has also not been seen (Nastase *et al*., 2020) (Hmeljak *et al*., 2018) (Bueno *et al*., 2016). Mutational signatures between asbestos exposed and non-exposed individuals were not significantly different (Hmeljak *et al*., 2018) (Bueno *et al*., 2016).

Another observation, unexplained by the available evidence, relates to the long latency between asbestos exposure and presentation. Tumours with high TMB are associated with known mutagenic exposure and chronicity, exemplified by primary lung cancer (tobacco) and malignant melanoma (ultraviolet radiation) (Alexandrov *et al*., 2013). This offers a sound explanation to the low TMB observed in most paediatric malignancies. This however is not the case of PM, and suggests alternative pattern(s) of mutagenesis and/or accelerated velocity of carcinogenesis outpacing the accumulation of somatic mutations, as in a punctuated equilibrium in evolutionary genetics.

The assessment of mutational signatures in PM has two potential caveats. First, low TMB could have profound effects on signature analysis. A recent study showed in order to achieve plateau in terms of signature profile similarity (>90%) using WGS (sequencing depth 30X) as gold standard (>25,000 SNVs), between 500-1,000 mutations are required per sample (Perner *et al*., 2020). It is worth noting that no PM data were used in the development or validation of

mutational signature analysis (Alexandrov *et al*., 2013). Second, mutational signatures associated in non-coding regions of the genome has been shown to carry functional significance, in for example breast cancer (Nik-Zainal *et al*., 2016). As a result, one could hypothesise whole exome sequencing (WES) and shallow whole genomic sequencing (sequencing depth 10X) are generally inadequate for signature analysis in PM.

1.4.2 Copy number alterations (CNAs)

Gene copy number alterations are arguably more prevalent in PM compared with mutations, and the two frequently co-exist. More recent studies have reported *BAP1* alterations, by adding mutations and CNAs, amounts to 49-57% (Nastase *et al*., 2020) (Hmeljak *et al*., 2018) (Bueno *et al*., 2016) in agreement with earlier studies (Guo *et al*., 2015) (Lo Iacono *et al*., 2015) (Nasu *et al*., 2015). While mutations correlated well with the loss of nuclear BAP1 staining in an initial study (Testa *et al*., 2011), a subsequent more substantial study showed such correlation was imperfect (Nasu *et al*., 2015). Mutations in the setting of loss of heterozygosity (LOH) or copy number neutral LOH (cnLOH), the latter usually secondary to endoreduplication of the mutant allele, are likely to result in incorrect protein localisation. Homozygous *BAP1* deletion, a rare event similarly observed in peritoneal meosothelioma (Shrestha *et al*., 2019), is likely to result in complete loss of protein. Heterozygous *BAP1* deletion (loss of a single copy) and copy number gain, another rare genotype, are associated with largely unknown phenotypes. The prognostic impact of *BAP1* alterations in PM is controversial. There have been studies suggesting BAP1 loss is a favourable prognostic variable (Chou *et al*., 2017) (De Rienzo *et al*., 2016) (Farzin *et al*., 2015) possibly explained by a non-EMT phenotype (de Reyniès *et al*., 2014), whilst other studies have found *BAP1* status to be non-prognostic (Nastase *et al*., 2020) (Hmeljak *et al*., 2018).

Bueno *et al*. reported 22% of tumours that underwent copy number analysis showed *NF2* deletions. Within the TCGA cohort they were detected in 74% of cases with a roughly equal spread between homozygous and heterozygous deletions (Hmeljak *et al*., 2018), the latter frequently carrying in addition mutations. Nastase *et al*. also identified shallow (likely heterozygous) deletions of suppressor of fused homolog (*SUFU*) (21%), a member of the Hedgehog pathway. The frequency was significantly higher than that of TCGA cohort where 2% carried homozygous deletions. *SUFU* is considered a tumour suppressor, and germline mutations in *SUFU* were first detected in non-*NF2* mutated familial meningioma (Aavkko *et al*., 2012). They were later found in familial medulloblastoma (Guerrini-Rousseau *et al*., 2018) (Smith *et al*., 2014) and non-*PTCH1* driven Gorlin syndrome (Smith *et al*., 2014) (Kijima *et al*., 2012) (Pastorino *et al*., 2009). Somatic *SUFU* mutations were observed in sporadic meningiomas resulting in reduced level of the methylation marker H3K27me3 (Katz *et al*., 2018). In earlier studies of PM, Hedgehog pathway alterations were common but only mutations were identified (Lim *et al*., 2013). There has been *in vitro* and *in vivo* (murine) evidence that this pathway is active in PM, and can be inhibited pharmacologically (Meerang *et al*., 2016a) (You *et al*., 2014) (Li *et al*., 2013) (Shi *et al*., 2012).

Deletions in cyclin-dependent kinase inhibitor 2A (*CDKN2A*: 9p21.3) were one of the first copy number events revealed in PM (Prins *et al*., 1998) (Xio *et al*., 1995) (Cheng *et al*., 1994) and occur at high frequencies. In massive parallel sequencing studies it was found to be deleted in 45-56% of PM, and up to 70% in early cytogenetic studies utilising *CDKN2A* FISH (Chiosea *et al*., 2008) (Illei *et al*., 2003). There is enrichment in non-epithelioid PM and *CDKN2A* deletion has been reported to predict OS independent of subtype although *vice versa* (Hmeljak *et al*., 2020), confirming earlier results based on FISH studies (Chou *et al.*, 2017) (McGregor *et al*., 2017) (De Rienzo *et al*., 2016) (Hamasaki *et al*., 2016) (Dacic *et al*., 2008). The vast majority of *CDKN2A* deletion is homozygous; the frequency of heterozygous

deletion among all deleted cases was reported to be 12-18% (Nastase *et al*., 2020) (Hmeljak *et al*., 2018) with uncertain functional significance. Due to its proximity co-deletion of methylthioadenosine phosphorylase (*MTAP*), an enzyme involved in purine and methionine metabolism (further discussed in Section 1.4.5), was seen in 72-100% of cases.

CDKN2A encodes two protein products: p16INK4a and p19ARF. The former inhibits cyclin-dependent kinase 4/6 (CDK4/6) subsequently inducing cell cycle arrest with the latter leading to activation of p53. *CDKN2A* downregulation *in vitro* was associated with increased proliferative rate and resistance to cell death (Xu *et al*., 1999). In the non-PM setting *CDKN2A* activity collaborates with the *TP53* pathway and drives cellular senescence. Dysregulation is associated with pathology apparently unrelated to its primary function, such as fibrosis (Kim *et al*., 2013) (Kodama *et al*., 2011) (Krizhanovsky *et al*., 2008). *In vivo* studies had shown *CDKN2A* exhibits haploinsufficiency, where heterozygous mutation was sufficient for carcinogenesis (Krimpenfort *et al*., 2001) (Sharpless *et al*., 2001), although with reduced carcinogenic potential compared with homozygous mutation (Sharpless *et al*., 2001). *CDKN2A* deletion also precipitates susceptibility to carcinogens (Sharpless *et al*., 2001). This is observed in asbestos-induced PM murine models (Okazaki *et al*., 2020) (Sneddon *et al*., 2017) (Roulois *et al*., 2016) (Altomare *et al*., 2011) (Altomare *et al*., 2009). Tumour development is significantly accelerated when there is co-deletion of the other tumour suppressor genes (*BAP1*, *NF2*) (Kukuyan *et al*., 2019) or in the presence of chronic inflammation (Kadariya *et al*., 2016). Asbestos was shown to inactivate *CDKN2A in vitro* via promoter hypermethylation (Christensen *et al*., 2008) as a preceding event to deletion (Chernova *et al*., 2017). The propensity of deletion over methylation is similar to non-small cell lung cancer (Andujar *et al*., 2010), but is in contrast to many other tumour types where promoter hypermethylation is the primary mechanism of p16 loss (Zhao *et al*., 2016). It is therefore rational to hypothesise the initial molecular event leading to DNA damage

including *CDKN2A* deletion triggers a vicious cycle, sustaining or amplifying the carcinogenic potential of asbestos and/or its downstream effector responses.

Little data is available in relation to the events prior to *CDKN2A* deletion. A recent study showed an asbestos-exposed murine model exhibited self-sustained iron-induced oxidative stress mediated by macrophages, associated with localisation of *CDKN2A* locus to the nuclear A compartment (in proximity to the nuclear membrane) during G_2/M phase of the cell cycle (Ito *et al*., 2020). Compartmentalisation of gene loci (Kempfer and Pombo, 2020) within the nucleus not only influences gene expression but is also crucial to the maintenance of the chromosome. To be able to confirm their findings and investigate this phenomenon at a greater depth, advanced molecular platforms beyond dual-colour FISH used by Ito *et al*., are required, such as multiplex DNA-FISH and high-throughput conformational capture (Hi-C). It is also worth noting that phagocytic capability is not a feature exclusive to macrophages but found in most lineages including the mesothelium.

1.4.3 Structural variants (SVs)

Complex chromosomal abnormalities with subclonal changes have been recognised in PM by cytogenetic studies since more than 30 years ago (Tiainen *et al*., 1988) (Mark, 1978). These large scale structural variants are pervasive in PM and exhibit extensive heterogeneity. In recent years advanced molecular platforms have taken the investigation of chromosomal instability and alterations to the next level, achieving much greater resolution than karyotypic analysis.

Aneuploidy in PM can be complete or, predominantly, partial. The canonical deletions involving the recurrently altered genes *BAP1*, *CDKN2A* and *NF2* are examples of the latter. Complete aneuploidy, via haploidisation or tetraploidisation, is rarely reported. Three cases of PM in the TCGA cohort showed genomic near-haploidisation (GNH) where there was

>80% LOH across the genome (Hmeljak *et al*., 2018). From two external cohorts they identified a cumulative total of 32 cases. Chromosomes 5 and 7 were usually spared, and there was association with deletions or mutations involving *SETDB2* and *TP53*. They are likely to be conditional mutations as mutations in either or both genes were registered in tumours without GNH. In the cohort of Bueno *et al.* (Bueno *et al*., 2016), two cases showed predominant LOH with *NF2* mutations similar but not identical to the features of GNH. GNH is currently regarded as a rare but distinct molecular subtype but further confirmatory studies are warranted.

PM with tetraploid chromosome complement has not been reported, but hyperploidy is not uncommon. Within the TCGA cohort, approximately 22% of cases showed ploidy number >2.1 (2 being that of a diploid complement) and 15% with evidence of whole genome doubling (WGD) (Hmeljak *et al*., 2018). Out of the 8 primary PM cell lines established by Chernova *et al*., 4 were hyperploid and one had chromosome counts reaching 200 although it is not possible to conclude based on the presented evidence if it was octaploid (Chernova e*t al.*, 2016). The possibility of culture-induced hyperploidisation cannot be excluded as it can take place even in short-term culture conditions. Furthermore, it is not possible to discern if this is a subclonal event- if so, akin to the presence of pleomorphic features in PM, it will be difficult to detect without spatially resolved assay platforms.

Temporal evolution of aneuploidy, or karyotype remodelling, has been shown to occur at high frequency in other aggressive tumour types such as pancreatic ductal adenocarcinoma (Chan *et al*., 2020). This phenomenon, in the presence or absence of treatment, further amplifies the complexity of tumour genomes and has been shown to be associated with dynamic allelic dosage effect and histological subtype switching (Kalimuthu *et al*., 2020). Recently it was found that karyotype remodelling is a feature common to cancer in general (Watkins *et al*., 2020). Currently there is no data in PM or mesothelioma in general.

Three main mechanisms underpin aneuploidy in cancer cells: anaphase delay secondary to disordered spindle assembly, endoreduplication and chromoanagenesis. Given the degree of chromosomal instability in highly transformed malignancies, these processes are not mutually exclusive at cellular and colony levels. This was supported by the data on the GNH subtype where there was evidence of both haploidisation and hyperploidisation (Hmeljak *et al*., 2018). Disordered spindle assembly leads to chromosomal mislocalisation and non-modal karyotypes. At the cellular level it can result in either gain or loss of ploidy. In PM, defects in spindle assembly pathway have been identified (Szymiczek *et al*., 2017) (Suraokar *et al*., 2014). An early *in vitro* study showed that perturbations can be induced by asbestos fibres (Dopp *et al*., 1995). In renal cell carcinoma (Xiao *et al*., 2020) (Peng *et al*., 2015) and breast cancer (Zarrizi *et al*., 2014), there is an emerging role of nuclear BAP1 during mitosis that it stabilises the spindle machinery. It is therefore reasonable to hypothesise, given the majority of *BAP1* mutations result in the loss of nuclear localisation in PM, tumours with *BAP1* mutations exhibit abnormal spindle assembly in BAP1-dependent fashion.

Endoreduplication is a principal mechanism of hyperploidisation and is enabled through dysregulated cell cycle (Chibazakura *et al*., 2004) (Wuarin *et al*., 2002). The involved cells undergo DNA replication without cell division resulting in WGD and gain in ploidy. Copy number neutral LOH of *BAP1* in PM is regarded as the collective outcome of LOH followed by endoreduplication of the mutant allele. Rb is known to prevent endoreduplication (Niculescu *et al*., 1998) (Grafi *et al*., 1996) and recently, interacts with p53 (Davoli and de Lange, 2012) and p16 (Domínguez *et al*., 2015) in the context of malignant transformation. The role(s) of *CDKN2A*/*TP53*/*RB1* alterations in relation to hyperploidisation, as well as other genetic and non-genetic mechanisms, are unknown in PM.

The propensity of asbestos fibres in inducing chromosomal breakage, within the confines of technology at that time, was reported in early *in vitro* toxicology studies (Dopp and

Schiffmann, 1998) (Dopp *et al*., 1997). Chromoanagenesis, defined as large scale re-arrangements leading to the formation of structural variants with profound gain and/or loss of genomic sequences, is a rapidly expanding concept in the study of mesothelioma genomics (Yoshikawa *et al*., 2016) (Mansfield *et al*., 2019) (Oey *et al*., 2019) and cancer in general (Cortés-Ciriano *et al*., 2020). These structural variants can be subclassified into two groups: inter-chromosomal re-arrangements resulting from chromoplexy, and intra-chromosomal re-arrangements resulting from chromothripsis. The results of chromoanagenesis are highly unpredictable and large number of events can register in relatively short period of time, analogous to kataegis (localised somatic hypermutation) with respect to SNVs.

Early evidence suggesting high frequency of chromoanagenesis was reported by Bueno *et al*. where 45% (9/20) of tumours sequenced by WGS harboured re-arrangements involving either *BAP1*, *CDKN2A* or *NF2* (Bueno et al., 2016). A recent study using 22 cases of PM with paired DNA and RNA samples demonstrated a median of 41 re-arrangements per tumour (Mansfield *et al*., 2019), both intra- and inter-chromosomal. The events involved exonic and intronic regions, therefore the authors proposed that novel structural variants are likely to result in gain or loss-of-function of individual genes or gene clusters as well as the generation of neoantigens. There was no significant difference between epithelioid and non-epithelioid subtypes although the study was not sufficiently powered for this purpose. There is genomic association with *TP53* mutations, suggesting the involvement of cell cycle dysregulation. The role of *TP53* in chromoanagenesis in general was confirmed in a large international pan-cancer cohort (Cortés-Ciriano *et al*., 2020). Higher number of chromothripsis-like patterns (CTLPs) was associated with worse median OS in univariate analysis (Mansfield *et al*., 2019). Our understanding about chromoanagenesis, and SVs in general in the context of PM, is still in the early days. It is necessary to investigate these phenomena over time and space at a greater depth. In soft tissue sarcoma, the concept of "*re-*

arrangement burst" has been established (Anderson *et al*., 2018). This refers to that within a short pseudo-timeframe, a concentration of inter-chromosomal re-arrangements in certain genomic regions occurs equivalent to a molecular seismic event. It is anticipated with improved accessibility in the foreseeable future, a multi-parametric approach of high-throughput conformational capture, whole genome sequencing and optical mapping technology can leverage the advantages of multiple assay platforms (Dixon *et al*., 2018). Functional genomic studies could be subsequently employed to delineate the tumour phenotypes.

Re-arrangements involving anaplastic lymphoma kinase (*ALK*) have been reported in a small proportion of predominantly non-pleural mesothelioma arising in female patients and with earlier disease onset (Argani *et al*., 2021) (Hung *et al*., 2018). The re-arrangement frequency is estimated to be 13% in peritoneal mesothelioma (Hung *et al*., 2018) and the common re-arrangement partners are *STRN*, *TPM1* and *ATG16L1*. The majority of cases were of epithelioid subtype, exhibiting low tumour mutational burden, and appeared to be consistent with low nuclear grade. Overexpression of ALK was seen in all cases although there were variations in the immunohistochemical staining intensity and pattern. This represents a stark difference from the classical *ALK* re-arranged lung adenocarcinoma where there is an almost uniform phenotype of strong, membranous expression. Due to its low frequency, it is currently not possible to adequately power a clinicopathological study to evaluate the relationship of this genomic event with other established histological variables or its prognostic impact. *ALK* re-arrangement in PM is extremely rare based on current literature, with three cases reported all of which showed *EML4-ALK* re-arrangement (Bronte *et al*., 2020) (Hu *et al*., 2020) (Leal *et al*., 2020). Responses to ALK inhibitors such as crizotinib and anti-PD1 immunotherapy have been documented in the case reports.

1.4.4 Methylation, chromatin remodelling and other epigenetic mechanisms of gene regulation

Earlier studies revealed distinct global DNA methylation profiles of PM (Christensen *et al*., 2010), compared with benign, non-asbestos exposed pleura (Christensen *et al*., 2009). Methylation profiles also correlated with CNAs (Christensen *et al*., 2010). Promoter hypermethylation of genes involved in cell cycle regulation (*APC*, *CCND2*, *CDKN2A*, *CDKN2B*, *HPPBP1* and *RASSF1*) was a recurrent finding (Christensen *et al*., 2008). There is only one study reporting the methylome of PM to date (Blum *et al*., 2019). Whilst confirming some of the findings from earlier reports, they were able to identify distinct methylation patterns associated with tumours which were more epithelial-like or sarcomatoid-like, in keeping with the transcriptional regulatory role of this process. Correlation with molecular subtypes also depended on whether the CpG sites were located within CpG islands. Further methylome studies are required to validate these findings. Blood methylation signatures associated with PM, concerns the circulating immune cells, have received recent attention (Guarrera *et al*., 2019). This is of particular importance given the putative immunomodulatory role in carcinogenesis.

Chromatin remodelling impacts the accessibility of genome by histone modification and, at the architectural level, nucleosome organisation. Histone methyltransferases *SETD2* and *SETDB1* were mutated in approximately 5-10% of PM. SETD2 and SETDB1 are known tumour suppressors and responsible for H3K36 trimethylation (Duns *et al*., 2010) and H3K9 trimethylation (Wang *et al*., 2003) respectively. Both proteins have transcriptional repressive functions. In a *Drosophila* model, *BAP1* mutations via PR-DUB complex led to increased histone H2A ubiquitination and upregulated target gene expression (Scheuermann *et al*., 2010). The transcriptional regulatory role of BAP1 was later confirmed using *in vitro* gene

editing technology (Campagne *et al*., 2019). It was recently shown this is lineage-dependent (He *et al*., 2019). Loss of C-terminal domain, which is frequently due to the presence of truncating mutations of *BAP1*, prevents the recruitment of BAP1 to nucleosomes during H2A deubiquitination (Sahtoe *et al*., 2016). Mutations mapping outside the catalytic or C-terminal domains, was also found to prevent the assembly of PR-DUB by abolishing protein-protein interaction (Peng *et al*., 2018). Loss of BAP1 downregulates the transcription of histone deacetylase 2 (HDAC) but upregulates HDAC1 (Sacco *et al*., 2015). Reciprocal upregulation of zeste 2 polycomb repressive complex 2 subunit (EZH2), a member of polycomb repressive complex 2 (PRC2) and increased trimethylated histone H3 lysine 27 (H3K27me3) was observed when *BAP1* was deleted (LaFave *et al*., 2015). This has led to the proposals of BAP1-tagreting therapies (further discussed in Section 1.5.5). Very limited mechanistic data is available in PM. It has been shown, in murine mesothelioma models, carcinogenicity driven by PR-DUB perturbations and altered histone methylation profiles are achieved not via *BAP1* deletion alone. Concomitant *CDKN2A* and *NF2* deletions were either necessary (Badhai *et al*., 2020), or they significantly accelerated the carcinogenesis (Kukuyan *et al*., 2019).

Switch/sucrose non-fermentable (SWI/SNF) represents a multi-member chromatin remodelling complex with emerging roles in many other cancer types such as lymphoma (Yamagishi *et al*., 2019), primary thoracic sarcoma (Perrett *et al*., 2019) (Sauter *et al*., 2017) (Yoshida *et al*., 2017) or carcinoma (Agaimy *et al*., 2017) (Yoshimoto *et al*., 2015), and low grade pulmonary neuroendocrine tumours (Fernandez-Cuesta *et al*., 2014). Re-arrangements involving *PBRM1*, a member of the complex and known tumour suppressor (Varela *et al*., 2011), was reported in 3% of cases that underwent RNA sequencing (Bueno *et al*., 2016). Hypermethylation and downregulation of *PBRM1* was also found in tumours with high EMT signature (Blum *et al*., 2019). Based on IHC studies, loss of core members of SWI/SNF

complex (SMARCA4, SMARCB1, ARID1A, ARID1B) are extremely rare in PM. The reported frequency of losses was between 0-0.7% (Ren *et al*., 2020) (Ahadi and Gill, 2019). Reduced expression of SMARCB1 was observed more frequently in the deciduoid cell variant of E-PM (Kawai *et al*., 2019). There has also been a case report on SMARCB1 loss in a rhabdoid cell variant (Kimura *et al*., 2018). Due to the lack of adequately powered studies, it remains inconclusive if alterations involving SWI/SNF complex are enriched in certain subgroups of PM.

Transcriptional regulation through noncoding RNA is a fast-evolving field in genetic research, although the isolation, functional elucidation and validation pose substantial experimental challenges. Both microRNA (miRNA) and long noncoding RNA (lncRNA) have differential expression profiles in PM. Various miRNAs have been discovered over the last decade with putative roles in diagnosis, prognostication and therapy (Johnson *et al*., 2018) (Matboli *et al*., 2018) (Nicolè *et al*., 2018) (Kao *et al*., 2017) (Cheng *et al*., 2016) (Andersen *et al*., 2015) (Williams *et al*., 2015) (Andersen *et al*., 2014) (Cioce *et al*., 2014) (Matsumoto *et al*., 2014) (Yamamoto *et al*., 2014) (Reid *et al*., 2013) (Pass *et al*., 2010). The studies of Hmeljak *et al*. and Blum *et al*. are the only studies to date that have utilised multi-omic approaches to catalogue, and to a certain extent, validated the earlier findings. Both studies found associations between miRNA profiles with good/poor prognostic groups, as well as EMT signature.

Hmeljak *et al*. also surveyed lncRNA which are less frequently reported in the literature (Weber *et al*., 2020) (Kresoja-Rakic *et al*., 2019) (Matboli *et al*., 2019) (Renganathan *et al*., 2014) (Wright *et al*., 2013). Similar to the prior studies, Hmeljack *et al.* arrived at the conclusion that the abundance and diversity of lncRNAs were stratified by prognosis. Mechanistic studies are urgently required if noncoding RNAs are to take a more central position in the research of PM. This is at least in part because the transcriptional regulation of

a single gene frequently involves a number of noncoding RNAs. An example is that of *CDKN2A*. In non-PM, transcriptional silencing via antisense noncoding RNA in the INK4 locus (*ANRIL*) has been reported (Aguilo *et al*., 2011) (Cunnington *et al*., 2010) although this might be dependent upon the *rs11515* single nucleotide polymorphism (SNP) in the 3'-UTR (Royds *et al*., 2016). MicroRNA miR-24 (Lal *et al*., 2008), miR-10b (Gabriely *et al*., 2011), miR-125b (Feng *et al*., 2012), miR-517a (Feng *et al*., 2012) and miR-126 (Feng *et al*., 2012) have all been shown to downregulate *CDKN2A* expression although no data is currently available in PM.

The epigenetic regulation of gene expression in PM is likely to be multi-dimensional and of high complexity. There are processes still poorly understood in PM such as splicing alterations. Bueno *et al*. found among the few cases with *SF3B1* mutations, usage of alternative 3'-splicing sites were increased. In one case this resulted in introduction of a frameshift mutation in *MAP3K7*, the latter encodes a serine/threonine protein kinase believed to be the oncogenic driver (Bueno *et al*., 2016). Furthermore, multiple mechanisms co-exist and cooperate within the nuclear compartment. Perturbations in one, for example in the case of structural variants, will likely result in chain reactions in genetic-epigenetic interactions, resulting in the disruption of topographically-associated domain (TAD) boundaries and altered 3-dimensional configuration of the involved regions. The profound effect on gene expression is impossible to ascertain without using a multi-parametric analytical pipeline.

1.4.5 Proteome, metabolome and microbiome: unchartered territories of PM

Inter-tumoural heterogeneity in proteomic profiles of PM, studied using mass spectrometry, has revealed distinct immunophenotypes and neoantigen abundance (Lee *et al*., 2018) as well as proteins involved in metabolism and maintenance of the cytoskeleton (Giusti *et al*., 2014).

The authors were also able to identify patient subgroups who were likely to respond to immunotherapy. Nonetheless, data on the proteome of PM is still limited. Routinely available FFPE tumour sections, coupled with laser-capture microdissection, have been deemed sufficient for mass spectrometry application with additional information on spatial heterogeneity possible. The pre-processing steps, however, are highly labour-intensive (Großerueschkamp *et al*., 2017) (Hosako *et al*., 2012). Array-based methods measuring a finite number of targets have shown utility (Ostroff *et al*., 2012) and, being a more accessible technology, has led to emerging applications such as screening pleural effusions for early diagnostic biomarkers (Tsim *et al*., 2016).

Mesothelioma cells have a high level of secretory activities via exosomes (Creaney *et al*., 2017) (Manfredi *et al*., 2016). Proteomic analysis of the acellular fraction of primary PM cell culture media has revealed upregulated pathways related to proliferation, invasion, metabolic reprogramming including carbohydrate and amino acid metabolism and immunomodulation. Molecules involved in paracrine signalling to remodel the tumour matrix have also been implicated (White *et al*., 2019) (Greening *et al*., 2016) (Hegmans *et al*., 2004). In addition, blood-specific proteomic signatures have been reported (Cerciello *et al*., 2020) (Lacerenza *et al*., 2020) (Kao *et al*., 2016) (Cerciello *et al*., 2013). In a pleural effusion-based study, the secretory profiles of immunoglobulins and other mediators (together with signalling molecules involved in extracellular matrix remodelling) were shared between mesothelioma, lung cancer and breast cancer samples (Bard *et al*., 2004). This offers a glimpse into the complexity of the mesothelioma proteome, that not only it is tumour-specific but could be dependent on the niche and relative abundance of non-malignant stromal and immune cells.

Metabolic derangements and subsequent reprogramming to improve adaptability to the tumour microenvironment is a common feature in cancer cell biology. PM is not expected to be an exception (Urso *et al*., 2020). As the pleural cavity is a false anatomical compartment,

the development of PM by default encounters the challenges of hypoxia and nutritional deficit. The pleura receives dual vascular supply: the visceral pleura is normally supplied by the terminal branches of pulmonary artery (deoxygenated blood) while the parietal pleura (including part of pericardium and diaphragm) is supplied by the intercostal arteries (oxygenated blood). Any biological process within pleural effusion, if developed, depends on simple diffusion only. The association of *BAP1* mutations and the Warburg effect (Bononi *et al*., 2017b) can therefore be regarded as an evolutionary adaptation. Glucose-dependent regulation of cytosolic pH has been shown to promote cyclin D1 (*CCND1*) transcription thereby driving cell proliferation (Koch *et al*., 2020).

Genomic and transcriptomic studies have suggested putative metabolic phenotypes associated with molecular signatures. PM with argininosuccinate synthetase 1 (*ASS1*) downregulation (further discussed in Section 1.5.5) was found for a distinct class of tumours susceptible to arginine deprivation (Szlosarek *et al*., 2006). *MTAP* co-deletion in those with homozygous *CDKN2A* deletion is predicted to result in the accumulation of its enzymatic substrate, methyladenosine (MTA). This in turn inhibits protein arginine methyltransferase 5 (PRMT5), an enzyme involved in epigenetic regulation. Despite little success beyond *in vitro* activities in early studies targeting other parts of the metabolic pathway (Tang *et al*., 2012) (Lubin *et al*., 2010) (Kindler *et al*., 2009) (Chattopadhyay *et al*., 2006), it has been recently hypothesised mesothelioma with MTAP loss, as shown in *in vitro* studies utilising cell lines (Barbarino *et al*., 2020), is susceptible to PRMT5 inhibitors via synthetic lethality reported in other cancer types (Fedoriw *et al*., 2019) (Kryukov *et al.*, 2016) (Marjon *et al*., 2016) (Mavrakis *et al*., 2016). At the cellular level, the loss of *MTAP* in addition to *CDKN2A* could diversify its phenotypic manifestations including the disruption of metabolic processes in tumours (Hansen *et al*., 2019) (Tang *et al*., 2018). This however is likely to be heterogeneous and subject to the nutritional availability within the microenvironment (Sanderson *et al*.,

2019). Additionally, there has been *in vitro* evidence that altered nucleotide metabolism underpins resistance to chemotherapy (Sato *et al*., 2018).

The respiratory microbiome and its interactions with the host form a highly dynamic and convoluted ecosystem with recognised impact on the pathogenesis of respiratory diseases. The introduction of external stimuli leading to alterations in selective pressure, for example by means of tobacco smoking (Garmendia *et al*., 2012) results in the evolution of microbiome and its host interactions over time and space. It is therefore within the confines of reason that airway microbiome has been hypothesised to facilitate the uptake of asbestos fibres through airway epithelial cells (Magouliotis *et al*., 2014), the uptake being the initiating step of asbestos-induced carcinogenesis in PM. Although the role of SV40 in PM carcinogenesis is not supported by contemporary evidence, very little is known about the microbiome of PM, or the pleural microbiome in the absence of the malignant process. The pleura is considered to be aseptic until the introduction of septic agent(s) either endogenously or via instrumentation. The disruption of pleural anatomy by PM renders the pleura unlikely to be aseptic. The heterogenous nature of pleura including the abundance of contaminants and host cells (for example in the setting of pleural infection [Bedawi *et al*., 2019]) together with the lack of high throughput and high sensitivity detection methods, have become the barrier to improving our understanding about the pleural microbiome. Low throughput, culture-based species detection has been the norm. Metagenomic and single gene (for example 16S rRNA sequencing) approaches utilising NGS has transformed the research of airway microbiome (Ditz *et al*., 2020). Until its wider application in studying pleural conditions, microbiome of PM and its interactions in pathogenesis, evolution and management of the disease will remain enigmatic.

1.4.6 Proposed molecular classification systems and intra-tumoural heterogeneity

Advances of molecular knowledge about tumours have brought forward disease classifications refined by molecular characteristics. This not only improves diagnosis and prognostication, but also facilitates precision oncology by stratifying patients based on the presence of potentially targetable molecular alteration(s). Four classification systems have been developed in the last 4 years by different groups using a combination of different primary datasets and bioinformatic algorithms (Alcala *et al*., 2019) (Blum *et al*., 2019) (Hmeljak *et al*., 2018) (Bueno *et al*., 2016). All four of the systems are gene expression-based and derived from cluster analysis of a finite number of variably and highly expressed genes, a necessary step to reduce the dimensionality of data. Associations with clinical and genomic meta-data were performed subsequently to annotate the molecular clusters.

Bueno *et al*. has proposed a system comprised of 4 groups: epithelioid (cluster 1), biphasic-epithelioid (Biphasic-E, cluster 2), biphasic-sarcomatoid (Biphasic-S, cluster 3) and sarcomatoid (cluster 4). Cluster 1 is characterised by high expression levels of mesothelium-associated markers such as mesothelin (*MSLN*) and downregulation of EMT-markers. The reverse is true for cluster 3 and 4. Bueno *et al.* found statistically significant difference in median OS between cluster 1 and others, but not among individual clusters 2 to 4.

Hmeljak *et al*. has proposed a system also comprised of 4 groups: iCluster 1 to 4. iCluster 1 are mainly of epithelioid subtype, enriched for *BAP1* wildtype status, low burden of CNAs and high level of DNA methylation. iCluster 4 are mainly of non-epithelioid subtypes, enriched for *BAP1* wildtype status and homozygous *CDKN2A* deletion, and loss of mesothelium-associated markers suggesting de-differentiation. Applying univariate analysis, Hmeljak *et al.* found iClusters 1 and 4 showed significant difference in OS but this was not

seen between iClusters 1 and 2/3. In multivariate analysis, both the iCluster system, histological subtype and *CDKN2A* status were independent predictors of OS. Application of the iCluster system in the epithelioid subgroup revealed a proportion of E-PM with gene expression profiles consistent with aggressive behaviour. The findings from these two studies illustrate the complementary nature of genomic/transcriptomic studies and histopathology in the prognostication of PM.

Blum *et al*. adopted a 2-step approach when developing their classification system. First, they expanded upon a previous 2-tier system (C1, C2) (de Reyniès *et al*., 2014) by repeating cluster analysis on C1 and C2 to produce a revised system comprised of 4 groups: C1A, C1B, C2A, C2B. The gene expression profiles of these 4 groups were comparable to the Bueno *et al.* cluster 1-4 and Hmeljak *et al.* iCluster 1-4 respectively. Subsequently Blum *et al.* inferred, using their primary transcriptomic data to estimate, the relative proportion of E-PM and S-PM components (weighed *in silico* pathology, WISP). They found the relative E-score and S-score of tumours in their cohort produced a gradient from cluster C1A to C2B. By applying a S-score cut-off (22%), they produced significant prognostic stratification which was also reproducible using external datasets. The group with high S-score showed a higher level of $CD8^+$ T lymphocyte infiltration, proliferation of stromal fibroblasts and angiogenesis, as well as PD-L1 expression. In contrast, that with low S-score showed a higher level of natural killer (NK) cell infiltration and *VISTA* expression, the latter validating the findings from Hmeljak *et al*..

For all three of the above studies, internal validations were performed comparing the proposed classification system with the existing ones. The degree of concordance was generally considered satisfactory although variable when comparing individual clusters. Higher concordance was reported between clusters enriched with non-epithelioid subtypes (Bueno *et al.* cluster 4, iCluster 4, Blum *et al.* cluster C2B). These three study proposals were

followed by the study of Alcala *et al*., who proposed the molecular features of PM would be better expressed as a continuous spectrum. Instead of discrete ranks, Alecala *et al.* characterised each tumour by integrating a number of features resulting in three profiles: (1) immune "hot" microenvironment with high $CD8^+$ lymphocyte infiltration, high tumoural PD-L1 and pro-angiogenic gene expression, associated with non-epithelioid subtypes, (2) immune "cold" microenvironment with low $CD8^+$ lymphocyte infiltration and high tumoural expression of pro-angiogenic genes, associated with non-epithelioid subtypes, and (3) immune "cold" microenvironment with low $CD8^+$ lymphocyte infiltration and high tumoural expression of VISTA, associated with epithelioid subtype. Each profile was found to have a different prognostic impact. Tumours with predominant profile (1), (2) and (3) were associated with median OS of 7, 10 and 36 months respectively. They however represented a minority of the cohort, the rest showing a relatively even mixture of profiles.

The study of Alcala *et al.* offered a different and arguably novel perspective in the way that PM has highly heterogeneous vascular and immune microenvironment which was prognostically relevant. A major weakness of the study however was that although the authors made no assumption that discrete ranks exist when developing the algorithm, the study cohort of 77 cases was designed *a priori* with three highly discrete groups of PM: E-PM with prolonged median OS (42.2 months), E-PM with unusually worse median OS (5.8 months), and S-PM (4.7 months). Furthermore the identification of the so-called Achilles' heel, proposed with regard to the actionable pro-angiogenic pathways and immune microenvironment, in strict methodological sense necessitates the formation of discrete ranks. In the era of genomic medicine trade-off is inevitable between the attempt to perfectly match with clinical and genomic meta-data, and applicability in routine setting. Disease classification as an expression of continuum ceases to exist as a classification system when

one could no longer allot a particular case to a rank, within which all members exhibit high level of similarity, while sufficiently distinct from the other rank(s).

There is very limited evidence in the literature supporting PM being a polyclonal malignancy (Comertpay *et al*., 2014) but it is expected to be this type of malignancy given the degree of heterogeneity at histological level and its genomic complexity. Recently multi-region whole exome sequencing of surgically resected PM showed 80% of SNVs were shared within the same tumour while the degree of spatial heterogeneity was substantially greater for T cell receptor repertoire (Chen *et al*., 2020). Tumour sequential sampling did not reveal differential mutational patterns in *BAP1*, *NF2* or in the promoter sequence of *TERT* (Quetel *et al*., 2020). However, the sampling conducted was not systematic and the time between samples ranged between 1.1 and 161.5 months. Primary PM cell line cultures have revealed complex clonal evolution including substantial longitudinal shifts in tumour subpopulations (Oey *et al*., 2019). Variations in the mutational landscape via serial passaging have also been reported (Kanellakis *et al*., 2020). The interpretation of cell line data therefore should remain cautious as it is still uncertain if the reported propensity of hyperploidisation and introduction of novel structural variants over time among primary cell lines (Chernova *et al*., 2017) is representative of the tumour biology or due to culture artefacts or a combination of both.

Single cell sequencing is a powerful investigative tool in examining the genomic and transcriptomic profile of individual cells (Haque *et al*., 2017) (Baslan and Hicks, 2017) or nuclei (Habib *et al*., 2016) (Lacar *et al*., 2016). The latter is logistically more favourable and has shown a high degree of correlation in terms of expression data (Abdelmoez *et al*., 2018). Single cell sequencing also serves as a tool to investigate the tumour microenvironment (Stubbington *et al*., 2017) (Quatromoni and Eruslanov, 2012). Being able to perform multi-omic analyses at single cell resolution over time and space is anticipated to reveal previously unidentified molecular signatures and interactions.

1.4.7 Inherited predisposition to pleural mesothelioma

Germline mutations are found in a minority of PM patients and usually associated with strong personal and family history of malignancies. The estimated incidence based on recent large-scale sequencing studies is approximately 12% (Hassan *et al*., 2019) (Panou *et al*., 2018). Most of the implicated genes are involved in cell cycle regulation and/or DNA damage repair with the most frequent germline mutations being in *BAP1*. Other genes involved include *ATM*, *BRCA1*, *BRCA2*, *CDKN2A*, *CHEK2*, *MLH1*, *MRE11A*, *MSH6*, *PALB2*, *POT1*, *SDHA*, *TMEM127*, *TP53*, *VHL* and *WT1*.

Germline *BAP1* mutations are well recognised and the entity BAP1 tumour predisposition syndrome (BAP1-TPDS) has been coined. Likely inherited in autosomal dominant fashion (Roushdy-Hammady *et al*., 2001) (Testa *et al*., 2011), both null and missense mutations predispose individuals to a range of malignancies (Walpole *et al*., 2018) including mesothelioma (Betti *et al*., 2016) (Rai *et al*., 2016) (Wadt *et al*., 2015) (Pilarski *et al*., 2014) (Cheung *et al*., 2013) (Testa *et al*., 2011), melanoma or atypical melanocytic tumours (Hajkova *et al*., 2018) (Haugh *et al*., 2017) (Cabaret *et al*. 2017) (Betti *et al*., 2016) (McDonnell *et al*., 2016) (Turunen *et al*., 2016) (Rai *et al*., 2016) (Gerami *et al*., 2015) (Marušić *et al*., 2015) (Wadt *et al*., 2015) (Pilarski *et al.*, 2014) (Cheung *et al*., 2013) (Wiesner *et al*., 2011), basal cell carcinoma of the skin (Rai *et al*., 2016) (Mochel *et al*., 2015) (Wadt *et al*., 2015) and renal cell carcinoma (Rai *et al*., 2016) (Pilarski *et al*., 2014) (Popova *et al*., 2013). Germline *BAP1* mutations in PM are very rare (Sneddon *et al*., 2015) with an estimated incidence of 1-2% (Rusch *et al*., 2015). They are associated with younger age of disease onset but more favourable prognosis (Ohar *et al*., 2016). In keeping with the findings in animal models, PM patients carrying germline *BAP1* mutations reported lower dosage of asbestos exposure suggesting a sensitising effect (Betti *et al*., 2018).

Patients carrying germline mutations have been found to have superior overall survival when treated with platinum-based chemotherapy (Hassan *et al*., 2019). Whether or not this observed phenotype was purely dependent on germline status is uncertain, as it was demonstrated recently these tumours were associated with epithelioid subtype and, within which, low grade tumours (Chen *et al*., 2020).

1.5 Established and emerging treatment modalities

1.5.1 Surgery including maximal cytoreductive surgery

Surgery in PM is anatomically challenging due to the diffuse and infiltrative nature of the disease. Any attempt to remove the disease completely in the form of maximal debulking will involve meticulous dissection along the extrapleural plane within the diseased hemithorax, with or without resecting additional intra-thoracic structures. This invariably leads to prolonged operative time with elevated risk of haemodynamic compromise, culminating in considerable procedure-related morbidity and mortality. Furthermore such operations mandate subspecialist surgical expertise rendering the provision of service limited to a few centres. This is in keeping with the current surgical rate of 5% among UK patients, with a substantial degree of regional variation (0-13%) (National Mesothelioma Audit, 2020).

Butchart *et al*. first reported the surgical technique and outcomes in 29 patients who underwent extra-pleural pneumonectomy (EPP) (Butchart *et al*., 1976). The operation itself was in essence a composite of, total pleurectomy and decortication of the tumour (PD), intra-pericardial pneumonectomy, systematic lymph node dissection plus removal of diaphragm. In-hospital mortality was 31% and 2-year OS was 10.3%. A similar study reported by a North American group showed lower surgical mortality but almost equally poor outcome with a

median OS of 15 months (DeLaria *et al*., 1978). A multi-institutional trial conducted approximately a decade later reported 2-year OS of 33% and post-operative mortality of 15% (Rusch et al., 1991). Rusch *et al*. subsequently concluded only a small proportion of patients should be considered EPP candidates and even so, it did not appear to improve outcomes significantly compared with less radical surgical techniques, usually in form of pleurectomy and decortication. This finding was confirmed in subsequent studies (Flores *et al*., 2008) (Schipper *et al*., 2008). Significant procedure-related mortality and morbidity are still reported by more contemporary data (Burt *et al*., 2014) (Cao *et al*., 2014) (Lauk *et al*., 2014) (Stewart *et al*., 2005).

The comparable outcomes derived from EPP and PD rapidly incited discussions on whether the latter, being a lung-sparing technique, can serve as a substitute with less procedure-related complications (Rusch, 1994) (Rusch, 1993). PD, however, was associated with higher incidence of local recurrence than EPP (Stewart *et al*., 2004) (Rusch and Venkatraman, 1996), although for the latter recurrence within the ipsilateral thorax was still the most common site (Baldini *et al*., 2015) (Baldini *et al*., 1997). This coincided with the introduction of the concept of multimodality therapy (MMT) incorporating surgery, chemotherapy and radiotherapy. This was an attempt to improve the surgical outcome of EPP (Sugarbaker *et al*., 1991) (Sugarbaker *et al*., 1993a). In node-negative E-PM (N0), 2-year and 5-year OS was improved to 74% and 39% respectively in highly selected EPP patients (Sugarbaker *et al*., 1996). Median OS of 23 months was reported in a separate cohort (Weder *et al*., 2007). Studies have also shown the benefit extended to PD patients (Maggi *et al*., 2001) (Rusch *et al*., 2001) (Rusch and Venkatraman, 1996). In addition, high dose hemithoracic radiation was offered with satisfactory outcome and toxicity (Bece *et al*., 2015) (Cho *et al*., 2014) (Hill-Kayser *et al*., 2009) (Miles *et al*., 2008) (Rice *et al*., 2007) (Flores *et al*., 2006) (Ahamad e*t al*., 2003) (Yajnik *et al*., 2003), although a recent study reported high incidence of clinically

significant (grade 3-4) adverse event within the postoperative period (Cho *et al*., 2021). Among those who managed to complete the treatment protocol, median OS of 29.1 months (Krug *et al*., 2009) and 5-year disease-free survival (DFS) of 53% was achieved for N0 disease (de Perrot *et al*., 2009). However, the early success was not confirmed in the clinical trial setting (SAKK 17/04) (Stahel *et al*., 2015).

A report after the millennium demonstrated median OS of 18.1 months in patients undergoing radical or extended PD (EPD), defined as the technique aiming for complete tumour resection via the removal of pericardium and/or diaphragm (Rice *et al*., 2011), as part of MMT (Lee *et al*., 2002). Non-inferiority of EPD was demonstrated (Korston *et al*., 2017) (Batirel *et al*., 2016) (Sharkey *et al*., 2016) (Bovolato *et al*., 2014) (Luckraz *et al*., 2010) (Nakas *et al*., 2008a) (Nakas *et al*., 2008b) even in those with extensive mediastinal lymph node metastasis (N2) (Martin-Ucar *et al*., 2007). Superiority over EPP was shown in one study (Lang-Lazdunski *et al*., 2012). Across both techniques, contemporary evidence reported median OS in the range of 20-51 months (Shaikh *et al*., 2017) (Batirel *et al*., 2016) (de Perrot *et al*., 2016) (Cao *et al*., 2012) (Yan *et al*., 2011). There is, however, also evidence to suggest MMT does not improve OS (Borasio *et al*., 2008).

Outside MMT, the roles of photodynamic therapy (PDT) (Friedberg *et al*., 2011) (Friedberg *et al*., 2003), povidone-iodine pleural lavage (Lang-Lazdunski *et al*., 2011) or chemotherapy (Patel *et al*., 2019) (Burt *et al*., 2018) administered intraoperatively has also been explored. The role of PD in symptomatic control, knowing complete resection is not possible, has been demonstrated especially for E-PM (Martin-Ucar *et al*., 2001). Nevertheless, there was no survival benefit compared with talc pleurodesis based on a Phase III trial (MesoVATS) (Rintoul *et al*., 2014). Whether PD should be offered to patients with trapped lung due to pleural thickening, a group that was underrepresented in the MesoVATS study with IPC

being the comparator, is a matter of an ongoing Phase III trial (MesoTRAP) (Matthews *et al*., 2019).

Given the controversial role of EPP, the Mesothelioma and Radical Surgery randomised trial (MARS) was conducted (Treasure *et al*., 2011). Patients were randomised to receive either MMT including EPP, neoadjuvant platinum doublet chemotherapy and adjuvant hemithoracic radiation, or chemo-radiation only. No OS benefit in MMT group was demonstrated and the surgical group was associated with high incidence of procedure-related serious adverse events. This, alongside the numerous non-trial reports predating MARS data, have led to the emergence of EPD as the preferred technique (Friedberg *et al*., 2020). There are two ongoing trials aiming to investigate the role of EPD with chemotherapy versus chemotherapy alone: MARS2 (Lim *et al*., 2020) and EORTC 1205 (Raskin *et al*., 2018), with data expected to be published in the next 5 years. Given the lack of high level evidence and substantial variations in reported outcomes, it is not surprising at this point recommendations from various guideline committees vary with respect to the role of surgery. ASCO recommends, for early stage epithelioid or biphasic disease, maximal cytoreductive surgery (MCS) as part of MMT (Kindler *et al*., 2018). Joint ERS/ESTS/EACTS/ESTRO guideline considers MCS "*may be appropriate*" for highly selected patients with epithelioid or epithelioid-predominant biphasic disease, and via EPD instead of EPP (Scherpereel *et al*., 2020). BTS takes a more conservative stance than ERS/ESTS/EACTS/ESTRO and more so than ASCO, in that it recommends against offering either EPP or EPD (the latter with the exception of clinical trials) (Woolhouse *et al*., 2018).

The contemporary controversies and unanswered questions in the surgical management of PM, beyond that if surgery offers survival benefit in the first place, can be broadly classified into two categories: (i) the lack of standardisation in surgical techniques, and (ii) prognostic variables. The former is intrinsic to PM being a rare disease and that almost every surgical

centre has developed local practices suited to patient need. Such is evident in the literature and repeatedly highlighted in major consensus documents (Nicholson *et al*., 2020) (Rice *et al*., 2011). EPP and EPD are relatively well defined, but less so for PD. Specifically there is virtually no parametric distinction between PD and a large surgical biopsy. It is nevertheless certain among international experts that the predefined goal of surgery should be maximal cytoreduction. Continuous data collection at international level is warranted to facilitate harmonisation (Friedberg *et al*., 2020) (Waller *et al*., 2020).

Similar to other tumour types TNM stage influences outcome (Richards *et al*., 2010) (Flores *et al*., 2007) (Rusch and Venkatraman, 1996). From the IASLC PM database (3,101 patients), surgical resection with curative intent translates into a median overall survival (OS) of 18 months, or 30 months in early disease (T1 N0 M0) (Pass *et al*., 2016). Pathological upstaging was a common problem and found to occur in up to 80% of cases particularly in early stage disease. Lymph node metastasis is common in PM (Sugarbaker *et al*., 1999) (Pass *et al*., 1998) (Rusch and Venkatraman, 1996). Positive lymph nodes (N1/N2) have been found to be associated with worse OS in patients who underwent MMT including either EPP or PD (Sugarbaker *et al*., 2014) (Edwards *et al*., 2006) (Sugarbaker *et al.*, 1999) (Rusch and Venkatraman, 1996) (Sugarbaker *et al*., 1993b). In other studies only N2 disease was prognostic in the multivariate setting (Flores *et al*., 2008) (de Perrot *et al*., 2007). In one institutional series, lymph node status was not prognostic (Lang-Lazdunski *et al*., 2015). Together with pathological upstaging this was seen as the major limitation of the TNM system.

Subtype is a reproducible prognostic variable available to most centres. In the original report by Butchart *et al*., three long term survivors (OS 36-123 months from first presentation) were, in retrospect, epithelioid PM with prominent myxoid stroma (Butchart *et al*., 1976). For this reason, the authors recommended surgery only for patients with E-PM, a proposal that

has withstood the test of time. This was first confirmed by a prospective trial of EPP (Rusch *et al*., 1991) and then large institutional cohort studies (Sugarbaker *et al*., 2011) (Yan *et al*., 2009) (Flores *et al*., 2008) (Nakas *et al*., 2008b) (Flores *et al*., 2007) (Sugarbaker *et al*., 1999). The poor outcomes associated with non-epithelioid PM have been reflected in major guidelines, with S-PM being a universal contraindication to surgery. Nonetheless the introduction of novel prognostic variables including but not limited to nuclear grade in E-PM and the extent of sarcomatoid differentiation in non-epithelioid PM might lead to improved patient stratification. One would hypothesise low grade E-PM will derive proportionately more survival benefit than its high grade counterpart. The concept of "*good biphasic PM*", or those with a minor sarcomatoid component, is supported by limited evidence (Vigneswaran *et al*., 2017), although the absolute effect size was admittedly small despite reaching statistical significance. Molecular predictors of outcome are still in the early stages of development. Low expression level of NF2 and high expression level of BIRC5/Survivin have been associated with worse outcome, but the data in the study was derived from two historical EPP cohorts (Meerang *et al*., 2016b). The ratio of claudin 15 over vimentin (*CLDN15*/*VIM*), detected using quantitative PCR, was developed using a large surgical cohort and was shown to be predictive of outcome (De Rienzo *et al*., 2020) (Bueno *et al*., 2016).

The diffuse nature of PM meant the conventional terminologies of complete resection (R0) and residual disease (R1/2) required revision, as true R0 was highly unlikely to be achieved. Instead, nowadays macroscopic complete resection (MCR) and macroscopic residual disease (MRD) are used, the latter roughly translates into positive resection margin in conventional sense. The prognostic impact of margin status is still uncertain. Margin positivity in EPP was found to not be associated with worse OS (Sugarbaker *et al*., 1996) but this was overturned by a follow-up study from the same group (Sugarbaker *et al*., 1999) as well as an independent

study (Bölükbas *et al*., 2013). In EPD the number of studies is small, and there is evidence to support MCR being a favourable prognostic variable (Lapidot *et al*., 2020) and that it is not associated with superior overall survival (Batirel *et al*., 2018).

1.5.2 Chemotherapy

Treatment options for PM are generally limited for those ineligible for surgery. The role of pemetrexed (anti-folate) and platinum (DNA chelating agent; cisplatin or carboplatin) has been demonstrated in a Phase III trial (Vogelzang *et al*., 2003). The addition of pemetrexed was associated with significant improvement of OS (12.1 months vs 9.3 months) and PFS (5.7 months vs 3.9 months). A separate Phase III trial around the same period showed similar results, with significant OS benefit (11.4 months vs 8.8 months) (van Meerbeeck *et al*., 2005). These findings were also confirmed in a large non-randomised cohort derived from international expanded access programme (EAP) for the combination (Santoro *et al*., 2008).

Small but significant benefit was shown, for the combination of platinum doublet, both in a post-market trial (Jänne *et al*., 2006) and a systematic review pooling data from multiple studies (Green *et al*., 2007). Maintenance single agent pemetrexed after first line platinum/pemetrexed was found to not improve progression-free survival (PFS) or OS in unresectable PM patients (Dudek *et al*., 2020). No second or third line chemotherapy regimens have demonstrated efficacy in large clinical trials. Vinorelbine showed a small and non-significant OS benefit (8.5 months vs 7.6 months) compared with active symptom control alone (Muers *et al*., 2008). Docetaxel and gemcitabine combination was tolerable but the response rate was modest at best (Tourkantonis *et al*., 2011). It is therefore understandable in advanced line setting, many patients do not receive disease-specific treatment (Saddoughi *et al*., 2018).

Based on the available evidence ASCO, ERS/ESTS/EACTS/ESTRO and BTS all recommend platinum double chemotherapy as first-line treatment and given the relatively limited absolute gain in OS, also consideration for clinical trials at this stage. No recommended second-line regimen currently exists.

No predictive biomarker to chemotherapy is available in PM. Excision repair cross-complementing group 1 (ERCC1) expression, a putative predictive biomarker for chemotherapy in NSCLC despite controversies in immunohistochemical detection (Fukumoto *et al.*, 2020) (Takemoto *et al*., 2019) (Alifrangis *et al*., 2018) (Lee *et al.*, 2017) (Friboulet *et al*., 2013) (Olaussen *et al*., 2006), was found to be associated with worse OS in PM (Marino *et al*., 2020) as well as predictive of chemotherapy response (Walter *et al*., 2016) (Frischknecht *et al*., 2015) (Kao *et al*., 2013) (Ting *et al*., 2013) (Zimling *et al*., 2012). In separate studies, thymidylate synthase (TS) (but not ERCC1 status) was predictive of outcome (Zucali *et al*., 2011) (Righi *et al*., 2010). BAP1 IHC status was not predictive of response to vinorelbine (Kumar *et al*., 2019). Further research, in relation to predictive biomarkers to chemotherapy in PM, is therefore both warranted and needed.

1.5.3 Immunobiology and immunotherapy

Immunobiology and its modulation, as part of precision oncology, is a rapidly expanding field in PM. Markers of systemic inflammation such as leucocytosis (Pass *et al*., 2014) and raised neutrophil-to-lymphocyte ratio (NLR) (Kao *et al*., 2013) (Pinato *et al*., 2012) (Kao *et al*., 2010) have been shown to be adverse prognostic factors. Mesothelium plays a role in adaptive immunity, as mesothelial cells have been shown to exhibit antigen presenting cell (APC) properties (Shaw *et al*., 2016) (Mutti *et al*., 1998). PM demonstrates heterogeneity in terms of tumour infiltrating $CD4^+$ and $CD8^+$ effector T lymphocytes (Pasello *et al*., 2018) (Chee *et al*., 2017) (Awad *et al*., 2016) (Yamada *et al*., 2010), $CD20^+$ B lymphocytes (Pasello

et al., 2018), $FOXP3^+$ regulatory T lymphocytes (Chee *et al*., 2017) (Awad *et al*., 2016), $NP57^+$ neutrophils (Chee *et al*., 2017) and $CD68^+$ macrophages (Pasello *et al*., 2018) (Chee e*t al*., 2017). In particular, the M2 macrophage subtype ($CD68^+$ $CD163^+$) has been found to be associated with worse survival alone (Cornelissen *et al*., 2014) or when there is co-existing low $CD8^+$ lymphocyte infiltration (Ujiie *et al*., 2015). The favourable prognostic impact of high $CD8^+$ lymphocyte infiltration was demonstrated in the post-surgical setting (Anraku *et al*., 2015) (Yamada *et al*., 2010). In tissue biopsies high levels of regulatory T lymphocytes ($CD4^+$ $CD25^+$ $CD127^{low}$), granulocytic myeloid-derived suppressor cells ($CD11b^+$ $CD14^-$ $CD15^+$) and monocytic myeloid-derived suppressor cells ($CD11b^+$ $CD14^+$ $CD15^{low}$) were predictive of OS (Salaroglio *et al*., 2019).

The interplay between the molecular landscape of PM and its immune context is an evolving concept receiving major attention in recent years. Although PM has low TMB compared with other tumour types, mass cytometry of patient-derived samples have revealed a distinct tumour immunophenotype illustrated by differential neoantigen load, major histocompatibility complex (MHC) protein expression and immune cell infiltration (Lee *et al*., 2018). There is also genomic and transcriptomic level evidence of heterogeneity in T cell receptor (TCR) repertoire among tumour infiltrating lymphocytes (TILs) (Kiyotani *et al*., 2017). In peritoneal mesothelioma, loss of heterozygosity involving *BAP1* has been found to be associated with increased $CD3^+$ $CD8^+$ T lymphocyte infiltration defining a unique inflammatory phenotype (Shrestha *et al*., 2019).

Telomeric extension of *CDKN2A* deletion frequently involves co-deletion of type I interferon (IFN) genes (30-32%) (Delaunay *et al*., 2020) (Nastase *et al*., 2020). Utilising primary short-term culture of PM cell lines such deletion was associated with susceptibility to measles virus replication and altered expression profile of interferon stimulated genes (ISGs). Similar findings were registered in *in vitro* models of human high grade glioma (García-Romero *et*

al., 2020). Increased incidence of co-deletion has also been observed in non-epithelioid PM subtypes. Two key questions however remain as to whether type I IFN gene cluster is involved in the immune microenvironment of PM. First, data is still limited in supporting the deletion of type I IFN genes being necessary for successful viral infection. Recent success of oncolytic viral therapies has been reported in malignant melanoma (Andtbacka *et al*., 2015) (Puzanov *et al*., 2016) and to a lesser extent limited to early phase clinical studies, high grade glioma (Samson *et al*., 2018) (Geletneky *et al*., 2017). Both tumour types are considered highly aggressive and associated with high incidence of *CDKN2A* deletion alongside many other oncogenic driver events. It is therefore equally conceivable that the determinant to successful viral infection is the extent of malignant transformation. Second, it is unknown if type I IFN genes play a part in shaping and maintenance of the tumour microenvironment or if their deletion merely represents a bystander event at the absence of artificial introduction of immunogenic agents. Intrapleural administration of adenovirus vector carrying human *IFNA2B*, irrespectively of tumour type I IFN status, was sufficient to induce systemic and intra-tumoural inflammation (Sterman *et al*., 2011) (Fridlender *et al*., 2010) (Sterman *et al*., 2007) translating into prolonged OS compared with historical controls (Sterman *et al*., 2016). In another Phase I/IIa study, intrapleural administration of modified herpes simplex virus (HSV) was found associated with a proinflammatory T helper type 1 (T_H1) cytokine profile, irrespective of tumour genomic status (Danson *et al*., 2020). Recently it was reported signatures associated with ISGs downstream of type I IFN signalling is dependent on *BAP1* status (Hmeljak *et al*., 2018) (Yang *et al*., 2020). It is also worth noting direct administration of IFNα2a was associated with objective response rate (ORR) of 12% predating modern immunotherapy (Christmas *et al*., 1993). At this time therefore, the function(s) of type I IFN in the tumour environment remain to be ascertained.

Pleural effusion in PM represents a unique immune microenvironment. It shows high $CD4^+$ but not $CD8^+$ T cell infiltration compared with benign effusions (Scherpereel *et al*., 2013). Compared with systemic circulation, pleural effusion is enriched with $CD3^+$ $PD\text{-}L1^+$ cells (Khanna *et al*., 2016), as well as having a higher macrophage infiltration (Marcq *et al*., 2017), especially of the M2 subtype ($CD68^+$ $CD206^+$ $Arg1^+$) (Salaroglio *et al*., 2019). In addition, the $CD3^+$ T cell population responds to chemotherapy (Lievense *et al*., 2017) which is also seen both at the systemic and tissue level (Pasello *et al*., 2018). Recent studies have also suggested the acellular fraction of pleural effusion is biologically active. That it is pro-proliferative and pro-migratory (Cheah *et al*., 2017) as well as being capable of suppressing T lymphocyte activity (Lievense *et al*., 2016).

The monoclonal antibody against cytotoxic-T-lymphocyte-associated antigen 4 (CTLA-4), Tremelimumab, initially showed promise as a single agent (Calabrò *et al*., 2015) but subsequently did not demonstrate OS benefit in a larger study two years later (Maio *et al*., 2017). A follow-on trial evaluating the addition of another anti-CTLA4 antibody (Ipilimumab) to anti-PD1 immunotherapy (Nivolumab) showed a non-significant trend towards improved OS although at the cost of higher incidence of adverse effects (Scherpereel *et al*., 2019). This was confirmed by a single-arm study (Disselhorst *et al*., 2019). High levels of circulating NK cells were associated with prolonged OS (Sottile *et al*., 2019). CD40-activating antibody in a Phase I trial was associated with ORR of 40% but cytokine release syndrome (CRS) developed in the majority (80%) of patients (Nowak *et al*., 2015). This discouraged its further application in treatment of PM.

Anti-PD1/PD-L1 immunotherapy, which transformed practice in other malignancies, has become the focus of contemporary immunotherapy trials. KEYNOTE-028, a Phase I trial enrolling patients with PD-L1 positive (≥1% cut-off) PM, showed ORR of 20% and median OS of 18 months in patients treated with pembrolizumab (Alley *et al*. 2017). MERIT, a Phase

II study evaluating single agent nivolumab, showed ORR of 29% with median OS of 17.3 months (Okada *et al*., 2019). A separate single-centre trial showed ORR of 24% (Quispel-Janssen *et al*., 2018a). NIBIT-MESO-1, a Phase II trial evaluating the combination of tremelimumab and durvalumab (an anti-PD-L1 antibody) demonstrated ORR of 28% and median OS of 16.6 months (Calabrò *et al*., 2018). None of these trials had a comparator arm i.e. standard of care platinum doublet chemotherapy. Multiple Phase III trials for this purpose were therefore initiated: CONFIRM (Fennell *et al*., 2018; NCT03063450), CheckMate 743 (Baas *et al*., 2021; NCT02899299), and PROMISE-Meso (NCT02991482).

PROMISE-Meso was the first to release the final results which revealed no significant OS or PFS benefit in the 2nd line setting (Popat *et al*., 2020). There was also an insignificant trend towards worse OS for the pembrolizumab group. CheckMate 743 was the first trial investigating the role of dual anti-PD1/anti-CTLA4 immunotherapy in a first line setting, with the control arm receiving platinum doublet chemotherapy (Baas *et al*., 2021). Immunotherapy was associated with significant gain in OS (18.1 vs 14.1 months) and was found to be subtype-dependent. Patients with non-epithelioid disease derived substantial survival benefit (18.1 vs 8.8 months) while epithelioid disease did not show statistically significant response (18.7 vs 16.5 months). PD-L1 expression, using 1% cut-off, was not predictive of outcome. Furthermore, patients older than 75 years showed no benefit from immunotherapy. In real life practice this age group represents a considerable proportion of all patients.

It is well recognised and of practical relevance that the efficacy demonstrated in clinical trials might not fully translate in the real-world setting. The relentless trajectory of PM and the associated functional impairment mean only minority of patients can be considered fit/well enough to enter a clinical trial pending on exclusion criteria. While immunotherapy agents for PM are still in their early days, two recent studies comprised of extensively pre-treated

patients should serve as cautionary tales in this perspective. The first cohort evaluating the efficacy of single agent pembrolizumab showed ORR of 18% and median OS of 7.2 months (Metaxas *et al*., 2018). The second cohort evaluating that of single agent nivolumab showed comparable results (ORR 10%, median OS 6.7 months) (Cantini *et al*., 2020).

There is evidence, albeit almost entirely derived from early phase studies, that PD-L1 expression correlates with treatment response (Disselhorst *et al*., 2019) (Okada *et al*., 2019) (Scherpereel *et al*., 2019) (Metaxas *et al*., 2018). In other studies, there were no correlation between PD-L1 expression and treatment response (Calabrò *et al*., 2018) (Quispel-Janssen *et al*., 2018a). A variety of cut-offs were used, most commonly the 1-5% threshold range. Retrospective studies suggest PM is a low PD-L1 expressing malignancy (14-72%, using ≥1% cut-off) (Watanabe *et al*., 2018) (Nguyen *et al*., 2018) (Thapa *et al*., 2017) (Marcq *et al*., 2017) (Combaz-Lair *et al*., 2016) (Cedrés *et al*., 2015). Higher expression has been observed in biphasic and sarcomatoid subtypes (Fusco *et al*., 2020) (Brosseau *et al*., 2019) (Pasello *et al*., 2018) (Patil *et al*., 2018) (Marcq *et al*., 2017) (Combaz-Lair *et al*., 2016) (Cedrés *et al*., 2015) (Mansfield *et al*., 2014). Higher level of PD-L1 expression also appears to be correlated with T and B lymphocyte infiltration (Pasello *et al*., 2018). Tumours with ≥50% PD-L1 positive tumour cells have been reported to be as few as 2-10% (Patil *et al*., 2018) (Thapa *et al*., 2017). There is evidence of PD-L1 expression being an adverse prognostic variable (Brosseau *et al*., 2019) (Forest *et al*., 2018) (Inaguma *et al*., 2018) (Nguyen *et al*., 2018) (Marcq *et al*., 2017) (Combaz-Lair *et al*., 2016) (Cedrés *et al*., 2015) (Mansfield *et al*., 2014). Furthermore, the issue of inter-assay discordance remains (Watanabe *et al*., 2018) and consequently there is requirement for IHC harmonisation studies to be brought to the table akin to that for NSCLC (Tsao *et al*., 2018) (Rimm *et al*., 2017). Early results suggest that there is good concordance between companion 28-8 and 22C3 antibody clones (Chapel *et al*., 2019).

There are emerging biomarkers involved in modulating the immune microenvironment in PM. Recently it was shown in the NIBIT-MESO-1 study population that soluble PD-L1 (sPD-L1) level rises during treatment and low level of on-treatment sPD-L1 was predictive of prolonged survival (Chiarucci *et al*., 2020). Elevated expression of CD70, ligand of co-stimulatory molecule CD27, has been found to be associated with increased $FOXP3^+$ regulatory T cell infiltration and worse OS (Inaguma *et al*., 2020). V-domain Ig suppressor T-cell activation (VISTA), a member of the B7 gene family to which *PD-L1* belongs, was shown to be preferentially expressed in E-PM (Chung *et al*., 2020) (Muller *et al*., 2020) (Hmeljak *et al*., 2018) and associated with better OS compared with tumour with low/negative VISTA expression. In one study (Muller *et al*., 2020), VISTA was found to be an independent predictor of OS. A putative role of VISTA as a novel immune checkpoint molecule has therefore been proposed (Muller *et al*., 2020) (Hmeljak *et al*., 2018) but mechanistic studies are lacking, and to date it has not been shown to predict treatment response in any clinical trials.

Modalities beyond single agent or combined immune checkpoint inhibition represent ongoing developments in medical oncology. First-line chemo-immunotherapy was based on the premise where the abscopal effect, i.e. the pro-inflammatory aspect of chemotherapy, could be exploited by the addition of immune checkpoint inhibitors. Increased $CD4^+$ and $CD8^+$ T cell infiltration, as well as PD-L1 expression on tumour cells, have been reported post-chemotherapy (Pasello *et al*., 2018). There are several ongoing trials evaluating this question (DREAM3R, NCT04334759) (Fujimoto *et al*., 2018). DREAM was the Phase II predecessor of DREAM3R and evaluated the combination of durvalumab and platinum doublet chemotherapy and showed the combination was associated with higher ORR (48%) than immunotherapy or chemotherapy alone (Nowak *et al*., 2020). Median OS was reported to be 18.4 months however importantly the trial lacked appropriate control. No association with

PD-L1 expression (SP263 antibody clone, 1% cut-off) was found. A trial combining anti-PD1 immunotherapy and PARP inhibitor has been proposed (Passiglia *et al*., 2020).

Radio-immunotherapy, as an alternative to chemo-immunotherapy for those unsuitable to receive chemotherapy, is also being investigated (NCT04166734). The abscopal effect therein has been reported in a murine mesothelioma model (Kohno *et al*., 2020) as well as a case report (Barsky *et al*., 2019). Dendritic cell (DC) therapy, based on the hypothesis antigen presentation could be enhanced by infusing tumour lysate-primed DCs to accentuate tumour-specific $CD8^+$ lymphocytes, is currently being evaluated in a Phase II/III trial (Belderbos *et al*., 2019).

1.5.4 Radiotherapy

Radiotherapy, usually in the form of intensity-modulated radiation therapy (IMRT), is considered in the management of PM in three ways: (i) as part of MMT, (ii) as primary or definitive treatment modality and (iii) in the palliative setting. Its application as an adjuvant in MMT (discussed above in Section 1.5.3) has been limited by the controversial role of surgical resection. Hemithoracic radiation in isolation is considered unfeasible due to the disease burden in PM. Proton-based radiotherapy is an emerging modality, with unique energy dissipation profile, and serves as a safe alternative to photon-based technology (Lee *et al*., 2017). Port-site radiation, with an aim to prevent procedure-tract metastasis (PTM) after pleural intervention, was found to not demonstrate a significant decrease in the incidence in two large Phase III trials (Bayman *et al*., 2019) (Clive *et al*., 2016). The value of radiotherapy in the palliative care setting however is better established. It has demonstrated efficacy in pain control (MacLeod *et al*., 2015) as well as quality of life (McDonald *et al*., 2017).

Hence the recommendations with respect to the application of radiotherapy is region-specific. Radiotherapy was recommended by NCI/IASLC/MARF in both MMT and symptom control

settings (Gomez *et al*., 2019), while ERS/ESTS/EACTS/ESTRO and BTS only recommended radiotherapy for symptom control.

1.5.5 Targeted therapy

Exploitation of the common genomic alterations in PM (*BAP1*, *CDKN2A*, *NF2*) is currently in its early stage. In murine models *BAP1* mutations were associated with sensitivity to EZH2 inhibition (LaFave *et al*., 2015). A Phase II study evaluating Tazemetostat, a small molecule inhibitor of EZH2, is awaiting the final results (NCT02860286). Due to the roles of BAP1 in DNA repair (particularly homologous recombination repair) and its interaction with BRCA1, sensitivity to PARP inhibitors such as olaparib has been hypothesised (Yang *et al*., 2020) (Hassan *et al*., 2019). This has been further supported by *in vitro* data (Borchert *et al*., 2019) (Parrotta *et al*., 2017) (Srinivasan *et al*., 2017). There is although counter-evidence (Rathkey *et al*., 2020), including from uveal melanoma (de Koning *et al*., 2019), a tumour also known to harbour frequently *BAP1* alterations. Rucaparib, a PARP inhibitor, is currently under investigation for treatment of PM in a Phase II trial (NCT03654833).

CDK4/6 inhibitors, such as abemaciclib (NCT03654833), are being explored in PM with *CDKN2A* deletion where CDK4/6 activation is expected. *In vitro* efficacy of palbociclib, another selective CDK4/6 inhibitor, has been demonstrated using commercial and primary PM cell lines (Bonelli *et al*., 2017). The sensitivity is likely to be Rb-dependent as shown in glioblastoma (Wiedemeyer *et al*., 2010) and pancreatic ductal adenocarcinoma (Chou *et al*., 2018). In non-PM tumours *RB1* mutation or deletion (Guarducci *et al*., 2018) (Wiedemeyer *et al*., 2010), or protein level downregulation, was sufficient to escape Rb dependence (Chou e*t al*., 2018) by releasing cell cycle control. Genomic alterations of *RB1* have not been reported at high frequencies in massive parallel sequencing studies to date but protein level alterations appear to be common (Bahnassy *et al*., 2012).

Targeting the NF2 pathway is attractive for two main reasons. First, *NF2* hemizygous deletions with or without concomitant mutations are common in PM. Second, low NF2 expression has been shown to the associated with worse OS after EPP, suggesting there is a functional relevance of aberrant NF2 pathway. In the Phase I setting, focal adhesion kinase (FAK) inhibitor (GSK2256098) combined with MEK1/2 inhibitor (Tremetinib) did not result in any observable objective response but, among those with low NF2 expression there was non-significant PFS benefit. This however was not replicated in a larger Phase II study where no OS or PFS benefit was found in NF2-low patients. A potential caveat was the fact that the biomarker used for patient stratification, NF2 immunohistochemistry, was evaluated using different protocols between the studies (Fennell *et al*., 2019) (Mak *et al*., 2019) (Meerang *et al.*, 2016b).

Besides targets identified by the genomic landscape of PM, drug trials in PM can be broadly classified into those targeting (i) mesothelium-associated markers, (ii) vasculature, (iii) cell signalling pathways, (iv) metabolic vulnerabilities and (v) genomic integrity and gene regulation. Anti-mesothelin, combined with platinum doublet chemotherapy, was found to not improve OS or PFS compared with historical controls (Hassan *et al*., 2014). Anti-mesothelin conjugated to *Pseudomonas* exotoxin (SS1P) has exhibited promising activity in a Phase I study (Hassan *et al*., 2014). An improved version (LMB-100), with attenuated immunogenicity, showed limited efficacy as single agent (Hassan *et al*., 2020) but among those treated sequentially with anti-PD1 immunotherapy (Pembrolizumab) with high PD-L1 expression and increased $CD8^+$ T lymphocyte infiltration, there was a trend towards improved OS (Jiang *et al*., 2020). A cancer vaccine targeting the Wilms Tumour antigen (WT1) demonstrated efficacy in the Phase II setting with prolonged OS (22.8 months vs 18.3 months) and PFS (10.1 months vs 7.4 months) (Zauderer *et al*., 2017). The vaccine is currently being explored in combination with Nivolumab (NCT04040231).

Targeting the vasculature of PM has met with mixed success. Addition of bevacizumab, a monoclonal antibody against VEGF, to gemcitabine/platinum chemotherapy was negative (Kindler *et al*., 2012) but its addition to pemetrexed/platinum chemotherapy significantly improved OS (18.8 vs 16.1 months) despite small effect size (Zalcman *et al*., 2016). In this setting, Hippo pathway activation via *MST1* promoter methylation predicted worse outcome from treatment (Maille *et al*., 2019). Nintedanib, a multi-tyrosine kinase inhibitor, demonstrated PFS benefit in a Phase II trial (Grosso *et al*., 2017) but no OS or PFS benefit was replicated in its subsequent Phase III trial (Scagliotti *et al*., 2019). Fusion product of tumour necrosis factor alpha (TNFα) and endothelial ligand asparagine-glycine-arginine (NGR), NGR-hTNF (Gregorc *et al*., 2011), did not demonstrate OS benefit in the Phase III setting (Gregorc *et al*., 2018). Negative trial results were reported for thalidomide (Buikhuisen *et al*., 2013), axitinib, a multi-tyrosine kinase inhibitor (Buikhuisen *et al*., 2016) and a tubulin polymerisation inhibitor (BNC105P) (Nowak *et al*., 2013). The same was seen for cediranib, a VEGF/PDGF inhibitor (Tsao *et al*., 2019). Imatinib in a Phase I study demonstrated activity among those with elevated PDGF receptor expression, but the drug was poorly tolerated (Tsao *et al*., 2014). Dual regimen of bevacizumab and anti-PD1/PD-L1 immunotherapy is a subject of ongoing clinical trials (NCT03654833, NCT03762018).

Inhibition of FGFR family, where overexpression was reported in PM with growth promoting properties (Marek *et al*., 2014), was proposed as a putative therapeutic strategy. Overexpression of FGFR and/or FGF family members was also associated with *BAP1* alterations (Quispel-Janssen *et al*., 2018b). A Phase II study evaluating dovitinib, a multi-tyrosine kinase inhibitor with FGFR selectivity, showed minimal efficacy (Laurie *et al*., 2017). This was corroborated by a very recent Phase II study that did not demonstrate PFS benefit of an FGF inhibitor (AZD4547) and there was no association between BAP1 IHC and responses (Lam *et al*., 2020). However, ORR of 44% in a Phase I study was observed when

FGF inhibitor (GSK3052230) was combined with platinum double chemotherapy (van Brummelen *et al*., 2020). Consequently the verdict is still out with regard to the utility of targeting the FGF pathway in PM. Targeting the PI3K and mTOR pathways has disappointingly met with little success (Dolly *et al*., 2016).

Argininosuccinate synthetase 1 (*ASS1*) downregulation was first reported in a number of PM cell lines (Szlosarek *et al*., 2006) leading to susceptibility to arginine deprivation based on the hypothesis of synthetic lethality. Two subsequent Phase I trials from the same group evaluating pegylated arginine deiminase (ADI-PEG 20) as the arginine-depleting agent, stratified by loss of ASS1 IHC, found association with significant but modest improvement in PFS (3.2 months vs 2.0 months) but no objective response was registered (Szlosarek *et al*., 2017). Three responses were recorded in non-epithelioid PM (Beddowes *et al*., 2017). ATOMIC-Meso, a Phase II/III trial evaluating the combination of ADI-PEG 20 and platinum doublet chemotherapy is currently underway (NCT02709512).

Trabectedin, and its derivative lurbinectedin, have demonstrated promising activity in early phase trials (Cortinovis *et al*., 2020) (Metaxas *et al*., 2020). Both compounds target specific DNA sequences and have putative roles in transcriptional suppression, interference with DNA damage repair (Soares *et al*., 2011) and induction of replication stress (Tumini *et al*., 2019). Tumour treating fields (TTFields), based on the premise of disrupting the tumour mitotic machinery, reported median OS of 18.2 months in a Phase II study although there was no control arm (Ceresoli *et al*., 2019). The exact mechanism(s) by which its therapeutic effect was exerted is uncertain in PM. Disruption of cytoskeleton leading to impaired cell motility has been demonstrated *in vitro* using glioblastoma and lung cancer cell lines (Voloshin *et al*., 2020). Nanocells loaded with miR-16 demonstrated safety and possible activity in a Phase I study (van Zandwijk *et al*., 2017). Inhibition of Ubiquitin-like with plant homeodomain and ring finger domains 1 (UHRF1), a member of E3 ubiquitin ligase family involved in DNA

methylation and cell cycle regulation, has demonstrated *in vitro* and *in vivo* activity (Reardon *et al*., 2020). Virinostat, an inhibitor of HDAC, did not improve OS in Phase III trial (Krug *et al*., 2015).

The large number of clinical trials inevitably led to inter-trial competition for patients. The lack of translational portfolios incorporating histopathology and/or basic science further impaired the add-on values of these studies, especially when the vast majority turned out to be negative. This nonetheless is recognised among the leading authorities in the field. The proposal of a more multi-disciplinary approach towards the research of PM is likely to bring the idea of maximising information gain from clinical trials to full fruition.

1.6 Early diagnosis: opportunities and barriers

1.6.1 Mesothelioma *in situ* (MIS)

Whitaker *et al*. in 1992 presented a clinicopathological series of seven PM patients with areas of flat, monolayered mesothelium with cytological atypia (Whitaker *et al*., 1992). The study found adjacent foci of sub-mesothelial invasion leading to the proposal of mesothelioma *in situ* (MIS). Another 15 cases were later published by the same group (Henderson *et al*., 1998). Of the 22 published cases, all but one harboured an invasive component sufficient for a diagnosis of PM elsewhere in the same biopsy. A series of 7 cases, consisting of both *in situ* and invasive components, showed no difference in the immunohistochemical profile (Cury *et al*., 1999). A subsequent case report performed copy number analysis by comparative genomic hybridisation (CGH) and showed consistent chromosomal alterations between *in situ* and invasive components (Simon *et al*., 2005).

The diagnostic and therapeutic relevance in relation to early diagnosis was recognised. However, the propensity of benign reactive mesothelium to exhibit architectural and cytological atypia mimicking MIS, with or without superficial stromal invasion, compounded by the lack of immunohistochemical or radiological biomarkers to differentiate between malignant and non-malignant processes resulted in the conclusion MIS should not be diagnosed without a diagnosis of PM. The number of published cases was minuscule in comparison with that of PM, prohibiting the attempt to establish and validate a set of uniform diagnostic criteria. Thus, the identification of an MIS component serves little practical utility.

The concept of MIS in isolation i.e. without a concomitant diagnosis of invasive disease, was revisited with the availability of novel biomarkers of mesothelioma, *CDKN2A* and *BAP1*. Churg *et al*. presented two cases of pleural MIS showing homozygous *CDKN2A* deletion and diffuse BAP1 loss of nuclear staining (Churg *et al*., 2018). Neither case showed pleural thickening on CT or macroscopic findings during surgical biopsy. Microscopically the mesothelium was flat, monolayered with minimal cytological atypia and without sub-mesothelial invasion. One patient was alive without disease at 9 months, and the other died at 27 months without autopsy confirmation regarding the cause(s) of death. There were isolated case reports using BAP1 IHC alone (Hidaka *et al*., 2020) (Kobayashi *et al*., 2020) or MTAP IHC followed by *CDKN2A* FISH confirmation in diagnosing MIS (Minami *et al*., 2020) as well as establishing the diagnosis on pleural effusion cytology materials (Haefliger *et al*., 2020).

The largest cohort published, at the time of writing, with *CDKN2A* and BAP1 data comprised of 10 cases, of which 9 were pleural (Churg *et al*., 2020a). The inclusion criteria were (i) monolayered mesothelium with BAP1 loss of nuclear staining, (ii) no imaging and/or macroscopic evidence of invasive tumour at time of biopsy and (iii) time to PM diagnosis at least one year after the diagnosis of MIS. Of the 8 cases with sufficient tissue materials, one

showed homozygous *CDKN2A* deletion. Somatic *BAP1* mutations were detected in two cases using whole exome sequencing (Churg *et al*., 2020b) (Dacic *et al*., 2020). Progression to PM was observed in 7 cases (70%) with a median time to progression of 60 months.

The current consensus is that MIS can only be diagnosed in the correct clinical, radiological and histological context. If pleural effusion cytology demonstrates *CDKN2A* and/or *BAP1* alterations, it should prompt further biopsy. It is acknowledged that the current evidence base of MIS is limited (Nicholson *et al*., 2020).

1.6.2 Atypical mesothelial proliferation (AMP)

Mesothelial proliferations of uncertain nature have been reported since the early days when blind biopsy was standard practice (Herbert and Gallagher, 1982). Similarly encountered in the non-pleural setting (Padmanabhan *et al*., 2003) (Scurry and Duggan, 1999), these lesions exhibit a range of histological features deviating from what are expected of normal mesothelium. No definitive criteria exist however and generally these lesions represent ones suspicious for PM but insufficient for the diagnosis, nor that of MIS. Given the cytological resemblance of malignant mesothelial cells to benign ones, AMP is also used as a diagnostic category in pleural effusions. The exact incidence of AMP is unknown. In one study, it represented 10.4% of the cohort (Monaco *et al*., 2011). The largest study specific to AMP comprised of 37 cases over a period of 24 years at a tertiary institution (Pillappa *et al*., 2018). Progression to invasive mesothelioma was reported anecdotally and across a variable timeframe (Shi *et al*., 2011) (Padmanabhan *et al*., 2003) (Scurry and Duggan, 1999). At the time of writing, no genomic data for AMP is available.

All of the 34 cases available for microscopic assessment by Pillappa *et al*. lacked unequivocal invasion of suspicious cells into the subpleural fat, chest wall muscle or lung parenchyma. Most of the cases were epithelioid subtype, and a limited amount of biopsy materials were

made available for assessment. Subsequent PM diagnosis was made in 44% of patients, the majority of whom underwent surgical resection based on the degree of clinical suspicion. Median OS was 18-21 months depending on the final diagnosis, as the authors did not report the survival figures for the entire cohort. FISH studies found no homozygous *CDKN2A* deletion, only three cases of heterozygous deletion, two cases of *CDKN2A* copy number gain and one case of complex aneuploidy involving chromosome 9. BAP1 loss of nuclear staining was found in 21% of cases, almost exclusively in the cases receiving subsequent PM diagnoses.

It is reasonable to deduce based on the limited evidence that AMP does not represent a single group of disease, but a mixture of (i) under-sampled frankly invasive PM, (ii) those showing early or minimal stromal invasion, and (iii) benign mesothelium with outlier characteristics. A surgical series of 16 cases showed high degree of spatial heterogeneity in terms of invasion (Hiroshima *et al*., 2009). The authors managed to identify areas where atypical mesothelial cells were limited to the mesothelial surface or only demonstrating superficial invasion, mimicking what expected in AMP. The study cohort however was almost entirely of advanced stage diseases showing unequivocal invasion elsewhere.

Given the lack of epidemiological data it is currently not possible to estimate the breakdown of AMP into each of the probable categories. Nonetheless it should be anticipated, even after excluding the under-sampled cases, there will be significant overlap between MIS, adequately sampled PM showing minimal invasion and radiological T0 mesothelioma (Nakamura *et al*., 2020) with microscopic foci of invasion only found when total pleurectomy is performed (Hasegawa *et al*., 2012). This is in some ways analogous to the issue of adenocarcinoma *in situ* (AIS), minimally invasive adenocarcinoma (MIA) and pure lepidic pattern adenocarcinoma in lung cancer diagnosis, but without the luxury of resection specimens made available for evaluation.

1.6.3 Unresolved questions

The ability to diagnose PM early arguably offers the best opportunity to improve the long-term outcome. Unfortunately, the overwhelming majority of patients are diagnosed at later stages. Stage I disease represent 11% of the IASLC dataset (Rusch *et al*., 2012) and 17% of UK's mesothelioma patients between 2016-18 (National Mesothelioma Audit, 2020) despite patients having satisfactory access to specialist centres.

Recent resurgence in academic interest and evidence in relation to early and pre-invasive PM has led to more questions than answers. However this resurgence represents a major progression of knowledge and undoubtedly will alter the management of at least a minority of patients in a positive way, the lack of answers and consensus in several key aspects will likely introduce substantial confusions among clinicians and researchers, therefore prohibit any form of widespread application until the emergence of further evidence.

The definition of MIS was ambiguous in its first iteration. Churg *et al*. subsequently proposed a set of stringent criteria with an objective to avoid overlap with reactive mesothelial hyperplasia and frankly invasive but under-sampled PM. However, Churg *et al.*'s definition underwent subtle modifications between the three studies published at the time of writing. In their largest cohort (Churg *et al*., 2020a), two patients showed "*smooth pleural thickening*" on CT, one showed microscopic papillary architecture on the pleural surface, and at least one case showed stromal invasion despite "*the remainder of the specimen showed only flat mesothelium*". It is fair to say there has been an expansion of the definition unfortunately marred by an influx of subjective histological parameters, moving the efforts backwards in minimising ambiguity. Normal mesothelium can assume papillary architecture with stratification, which suggests there might be a papillary variant of MIS. One must be

reminded even in the assessment of histological features in invasive mesothelioma, among experts, the IOA values were disappointingly unsatisfactory.

The definition of AMP on the other hand is highly ambiguous, if it exists at all. One thing for sure is AMP currently being a more common entity than MIS and the incidence of PM will therefore need to be adjusted upwards when the usage of the former diagnosis is minimised. A central conundrum is the lack of sampling standard. Without adequate sampling of the subpleural compartment the presence and/or extent of invasion cannot be fully ascertained. The issue with the number and size of biopsies applicable to PM will inevitably apply to both MIS and AMP, in order to satisfactorily address spatial heterogeneity. Surgery including pleurectomy is unlikely to be the solution, not at least purely for the purpose of reaching a diagnosis given the procedural risks of morbidity and mortality.

All existing studies involving MIS and AMP have been retrospective in nature, meaning the so-called progression to invasive PM was subject to observer bias. Data on the temporal evolution of these lesions is lacking beyond the fact that they have the capacity to progress to invasive PM. The frequency and velocity of transformation are key to research and management, which cannot be established without a prospective-designed systematic interval sampling protocol. Furthermore, it is not known if MIS represents an early or late event. The minimal deviation of histological features from benign mesothelium suggests it could be an early event, however it is of common knowledge that histology is much less sensitive than advanced molecular platforms in identifying signatures associated with carcinogenesis. Both *CDKN2A* and *BAP1* alterations, as seen in MIS, are characteristic of invasive PM. *CDKN2A* alterations are also associated with aggressive disease behaviour. Haefliger *et al*. showed in MIS that subsequently progressed to PM, hyperploidisation as a marker of malignancy occurred in the final diagnostic sample but not in the earlier MIS sample (Haefliger *et al*.,

2020). Taken together, it remains enigmatic as to where MIS should be placed in the natural history of PM.

Finally, discussions around the topic of MIS and AMP have been almost exclusively restricted to those with epithelioid morphology. The evolutionary origin of non-epithelioid PM is not known to date. None of the published studies reported a non-epithelioid variant of MIS, neither did any of the cases exhibit features analogous to transitional PM. This will lead to the hypothesis that there is a point of divergence post-progression of MIS, when sarcomatoid transformation occur. However, Bolen *et al*. postulated an alternative origin of PM that they are derived from the sub-mesothelial fibroblasts (Bolen *et al*., 1987) (Bolen *et al*., 1986). This offers a convenient explanation to the tumour's apparently intrinsic affinity towards MMT, but also challenges the notion of MIS being the pre-malignant stage of PM, at least proposing there might be another pre-malignant entity. Based on this theory, non-epithelioid PM is the biological consequence of two possible scenarios: a sub-mesothelial evolutionary origin followed by reversed direction of differentiation to give rise to the epithelioid component, or (ii) a spatially and temporally-orchestrated collision tumour - by in large the latter is unlikely.

2 Aims

The overall aims of this thesis are to unravel the inter- and intra-tumoural heterogeneity of pleural mesothelioma (PM) using a multi-dimensional and multidisciplinary approach and, supported by these data, to determine whether a subset of atypical mesothelial proliferation (AMP) represents minimally or superficially invasive disease amenable to early diagnosis and management.

The specific aims for each chapter are therefore as follows:

Chapter 3: To investigate (1) the long-term outcome of patients with PM who have undergone multimodality therapy including radical surgery, and (2) the clinical and histological predictors of outcomes as well as key surgical and oncological parameters.

Chapter 4-6: To investigate (1) if epithelioid PM could be subclassified based on the prognostic impact of architectural and cytological features, (2) the utility of the classification system(s) in a biopsy-heavy setting, (5) development of a minimum sampling criteria in attempt to address the issues of under-sampling and spatial intra-tumoural heterogeneity, (4) the morphological characteristics and prognostic relevance of previously unreported architectural growth patterns, and (4) the inter-relationship between these tumour morphological parameters.

Chapter 7: To investigate (1) the genetic signatures of *CDKN2A* and *MTAP*, and their relationship with histopathological features in pleural mesothelioma, (2) development of MTAP immunohistochemistry, correlation with *CDKN2A*/*MTAP* genomic status, and assessment of inter-assay and inter-observer variations, (3) description of heterogeneous tumour cell expression pattern(s) of MTAP and mechanistic explanation(s).

Chapter 8: To develop (1) a novel morphology-based classification system for AMP to aid clinical management and reduce patient attrition due to grey-zone diagnosis, (2) determine whether this can be used to stratify risk of eventual diagnosis of PM, and (3) apply findings from chapter 7 to refine further any potential risk stratification, increasing diagnosis of PM at an earlier timepoint in disease evolution and reducing the need for a diagnosis of AMP.

3 Heterogeneity in Outcomes and their Predictors in Multimodality Therapy including Radical Surgery for Pleural Mesothelioma

The findings of this Chapter were presented as a poster presentation at the World Conference on Lung Cancer, 7th-10th September 2019 (Barcelona, Spain); and, in part, as a platform presentation at the 15th meeting of the International Mesothelioma Interest Group (IMIG), 7th-10th May 2021, Australia (Virtual) and the World Conference on Lung Cancer, 7th-10th September 2019 (Barcelona, Spain).

Primary data in this chapter (Sections 3.3.2-3.3.6) were collected into a database led by Professor Loic Lang-Lazdunski (Institut Universitaire de Cardiologie et Pneumologie de Quebec and Universite Laval, Canada) and contributors. Consent was obtained from patients by the surgical team before surgery to analyse their data and tissue specimens for research purposes, and also as part of a collaborative research study. The remaining sections of this Chapter were the results of my independent research.

3.1 Abstract

Background: The purpose of the study was to evaluate the long-term outcomes, safety and prognostic variables of patients receiving multimodality therapy including total pleurectomy/decortication with povidone-iodine pleural lavage, prophylactic radiotherapy and chemotherapy for pleural mesothelioma (PM).

Materials and methods: The study was a retrospective observational study of 152 consecutive (from 2004 to 2019) patients with histologically proven pleural mesothelioma treated by a specialised thoracic oncology team in London, United Kingdom. Overall survival (OS) and prognostic variables were analysed using Kaplan-Meier method, and univariate and multivariate Cox regressions.

Results: Median age at time of operation was 64 years, and the majority (70.4%) of patients had epithelioid PM. The rate of macroscopic complete resection was 58.6%, and 96.1% of patients completed multimodality therapy. With a median follow-up period of 43.2 months, median OS was 31.7 months with 1-year/2-year/5-year survival rates of 88.1%/60.1%/23.2%. 8-year and 10-year survival rates for epithelioid disease were 20.2% and 16.1% respectively. Post-operative mortality and complication rate were 0% and 42.1% (Grade III/IV: 21.8%). Second-line or third-line therapy were administered in 48.7% of patients. Multivariate analysis showed histological subtype being the solitary predictor of OS, independent of resection status, pathological stage, lymph node status and order of chemotherapy. Non-epithelioid subtype was associated with macroscopic residual disease and advanced stage.

Conclusions: Multimodality therapy including total pleurectomy and decortication is a safe and well tolerated treatment regimen in selected patients. Long-term survival is a feasible goal, and accurate histological subtyping at pre-treatment stage is crucial as it is a key determinant in surgical outcomes.

3.2 Introduction

Pleural mesothelioma (PM) is an aggressive thoracic malignancy classically associated with asbestos exposure, with rising incidence in both developing and developed regions (Alpert *et al*., 2020). The estimated deaths per annum are in excess of 27,000 globally (Global Burden of Disease, 2020), and PM is expected to be a significant health burden in the foreseeable future. For more than a decade the standard treatment for PM has been platinum doublet chemotherapy (Vogelzang *et al*., 2003), which limited absolute gain in overall survival (OS). Recent Phase III trials involving anti-angiogenic agents (Zalcman *et al*., 2016), and immunotherapy including anti-PD1 with or without anti-CTLA4 immune checkpoint inhibitors (Baas *et al*, 2021) (Scherpereel *et al*., 2019) have reported median OS approaching 18 months compared with the historical figure of 10-12 months. These have led to regulatory approvals in both the United States and Western Europe.

The roles of radical surgery in PM is controversial yet, at the same time, it represents a rapidly evolving area as the highly anticipated Phase III Mesothelioma and Radical Surgery 2 (MARS2) trial approaches completion (Lim *et al*., 2020). Utilising total pleurectomy and decortication (PD) as the surgical technique, several high-volume centres have started exploring multimodality therapy (MMT) systematically, combining radical surgery with chemotherapy and radiotherapy. Early data has shown this approach, in selected patients with good performance status, has extended median OS to approximately 30 months (Friedberg *et al*., 2012) (Lang-Lazdunski *et al*., 2012) (Bolukbas *et al*., 2011) compared with 18-24 months found in earlier surgical studies (Flores *et al*., 2008) (Lee *et al*., 2002). This is in part due to a more favourable safety profile of PD than extrapleural pneumonectomy (EPP) (Zhou *et al*., 2021) (Lang-Lazdunski *et al*., 2012) (Treasure *et al*., 2011) (Van Schil *et al*., 2010) (Flores *et al*., 2008). Simultaneously consensus was reached within the mesothelioma community in

terms of the goal of radical surgery being macroscopic tumour clearance via maximal cytoreductive surgery (MCS) (Rice *et al*., 2011). At the time, revisions to surgical resection status were proposed: macroscopic complete resection (MCR) and macroscopic residual disease (MRD). This is primarily because the traditional goal of R0 resection (microscopic clearance) in non-mesothelioma thoracic surgery is largely unachievable in diffuse PM due to the growth characteristics of the disease, with the rare exception of localised mesothelioma.

The practice of MMT however is heterogeneous both in terms of the extent of anatomical resection and combined treatment modalities. This has led to a fairly diverse spectrum of oncological outcomes and safety profiles reported in the contemporary literature, with an emphasis on relatively short-term parameters. This is further hampered by studies of limited statistical power due to the low-volume nature of mesothelioma surgery. The concept of MCR being the principal goal of surgery is also currently under contention, as is whether MRD is equivalent to outright failure of surgery (Waller *et al*., 2020) (Friedberg *et al*., 2020) (Friedberg *et al*., 2019).

In the study detailed in this chapter the aims were to report (1) long-term outcome of 152 patients underwent multimodality therapy utilising total pleurectomy and decortication (PD) with povidone-iodine intraoperative pleural lavage, chemotherapy and radiotherapy, (2) safety profile of this approach, (3) predictors of outcomes, and (4) factors influencing the achievement of macroscopic complete resection.

3.3 Materials and methods

3.3.1 Patient selection and pre-treatment evaluation

Patient recruitment was conducted via a supraregional, specialist multi-disciplinary thoracic oncology clinic in London, United Kingdom. This involves thoracic surgeons, pulmonologists, radiation and medical oncologists. All patients receiving MMT were prospectively captured in a database from September 2004, led by Professor Loic Lang-Lazdunski (Institut Universitaire de Cardiologie et Pneumologie de Quebec and Universite Laval, Canada). Primary data were extracted from this database for analysis (Sections 3.3.2-3.3.6).

A review of biopsy results and imaging was undertaken for all patients at the time of referral. All patients had chest computed tomography (CT), 18-fluorodeoxyglucose positron emission tomography computerised tomography (PET-CT), pulmonary function test and echocardiogram performed before surgery to exclude N2/N3 disease or extra-thoracic metastasis. Clinical TNM staging was performed based on RECIST 1.1 criteria.

Suitability for multi-modality treatment was determined by patient's performance status, co-morbidities, clinical TNM stage, and histological subtype. Contraindications to MMT include performance status >1, anaemia (<14.6g/dL), thrombocytopenia (≥400×103/μL), elevated white cell count (≥15.5×103/μL) and significant weight loss (>5%) (Pass *et al*., 2014) (Herndon *et al*., 1998). Previous talc pleurodesis or non-bulky N1 disease was not regarded as contraindications. Patients deemed suitable for MMT and considered resectable were offered P/D followed by prophylactic radiotherapy within 6 weeks, and adjuvant chemotherapy initiating between 6-8 weeks post-operatively. Furthermore, patients who showed response to or stable disease after neoadjuvant chemotherapy were also deemed eligible.

The treatment protocol was approved by the institutional review board and informed consent was obtained from each patient prior to each treatment step. All patients received oral and

written information about mesothelioma treatment and were offered at least two outpatient appointments to discuss their treatment plan.

3.3.2 Chemotherapy

Neoadjuvant chemotherapy consisted of pemetrexed and cisplatin/carboplatin which all patients completed within 6 weeks prior to surgery. They either completed six cycles of chemotherapy at an external institution, or three to four cycles locally followed by PET-CT evaluation for treatment response.

For patients receiving adjuvant chemotherapy, the regimen consisted of pemetrexed and cisplatin/carboplatin (from 2007 onwards) or gemcitabine and cisplatin (prior to 2007). The intent was to administer four to six cycles of chemotherapy, allowing dose reduction based upon clinical and haematological grounds.

3.3.3 Prophylactic radiotherapy

All patients were reviewed in the outpatient clinic at 4 weeks post-operatively and referred for prophylactic radiotherapy (21Gy in 3 daily fractions) on the thoracotomy, drain and previous port sites according to Boutin's protocol (Boutin *et al*., 1995).

3.3.4 Surgical protocol

All patients had epidural catheter placement, invasive arterial blood pressure and central venous pressure monitoring at time of anaesthesia. They were intubated using a double-lumen endotracheal tube, a nasogastric tube and placed in the appropriate lateral decubitus position. Prophylactic intravenous antibiotics were administered on induction. Thromboembolism deterrent (TED) stockings and intermittent pneumatic compression device were used throughout the operation.

The surgical intent in all patients was to achieve maximal cytoreduction and macroscopic complete resection (MCR). The approach was nearly always a posterolateral thoracotomy through the 5th or 6th intercostal space. Partial 6th rib resection was performed in patients with contracted hemithorax. When required, a second access thoracotomy was made in the 8th intercostal space to facilitate tumour resection in the diaphragmatic recess and/or diaphragmatic resection and grafting. In exceptional cases when invasion of great vessels was suspected, a hemi-clamshell incision was used. Total pleurectomy and decortication including pulmonary fissures was subsequently performed. Anterior mediastinal fat was usually excised en-bloc with the pleural specimen, followed by hilar and mediastinal lymphadenectomy. Extended resection to include the pericardium, lung, chest wall, great vessels and diaphragm was undertaken when necessary to achieve MCR. When the diaphragm is resected, reconstruction was performed using a large polytetrafluoroethylene (PTFE) mesh (2-mm Dual Mesh, Gore Tex, WL Gore and associates UK Ltd, Livingston, UK).

Upon completion of P/D, between 5 to 6 litres of hyperthermic pleural lavage (40-41˚C) using sterile water mixed with 10% povidone-iodine (Videne, Ecolab Ltd, UK) was undertaken. The washout was allowed to bath the pleural cavity for 5 minutes while the lung was non-ventilated, then aspirated slowly. This manoeuvre was repeated three times. Before closing, phrenic nerve block (0.5% marcaine, 5 mL) was administered and two to three large bore chest drains (28F) were placed. All patients were monitored overnight in the high dependency unit and de-escalated to thoracic surgical ward on day 1, or later if appropriate. Deep vein thrombosis prophylaxis was started the night before surgery and continued for 30 days post-operatively (subcutaneous enoxaparin 40 mg, once daily).

Grading of surgical complications was based on the Clavien-Dindo system.

3.3.5 Histopathological evaluation

All surgical resection specimens were diagnosed by a specialist pulmonary pathologist based on contemporary diagnostic and immunohistochemical criteria (Nicholson *et al*., 2020) (Husain *et al*., 2018) (Travis *et al*., 2015). TNM and final pathological stage were assigned based on the American Joint Committee on Cancer Staging Manual (8th edition). The resection status is primarily surgeon's jurisdiction based on intraoperative assessment of the extent of resection. Macroscopic complete resection (MCR) is defined as the removal of all visible tumour, while macroscopic residual disease (MRD) is defined as any amount of unresected macroscopic tumour, usually as a trade-off for unacceptable operative and/or perioperative risks.

3.3.6 Follow-up

All patients were followed-up in the specialist clinic and had PET-CT or chest CT arranged 6-monthly to monitor response to treatment or disease progression. Those relapsing or progressing following MMT were offered second- or third-line treatment either as re-challenge with platinum doublet chemotherapy, other chemotherapy regimens, immunotherapy (with single or dual immune checkpoint inhibitor), or referral for clinical trials. Radiotherapy was offered as a palliative measure when patients presented with unresectable chest wall disease, or as curative treatment for focal relapse.

Survival time was calculated from the time of initial mesothelioma diagnosis to the time of death of last follow-up. Data cut-off date was the 1st of May 2021.

3.3.7 Internal validation cohort

To perform an internal validation of the relevant findings, a cohort of 160 consecutive patients who had undergone either extended PD (EPD), or simple pleurectomy and decortication (PD) with a primary intent of disease debulking rather than MCR, from 2006-

2020 were constructed from a prospectively maintained institutional mesothelioma database, created by myself (Dr Yu Zhi Zhang), consisted of a total of 1,912 patients. Twenty-three cases (14.3%) of this database of 1,912 patients were part of the main study population, and microscopic review was performed at the time of study. As these procedures were performed across a time period during which definitions and nomenclatures regarding surgical techniques evolved, pleurectomy and decortication performed with an intent of MCR were classified as EPD even though extended resection of additional anatomical structures might not have been undertaken.

Data on clinical staging, neoadjuvant and adjuvant therapy were incomplete as those were not considered standard of care particularly for patients undergoing PD. Similarly, no discrimination was made regarding resection status, as the surgical technique of PD necessarily indicates macroscopic residual disease and is an expected outcome at the stage of pre-surgical evaluation.

3.3.8 Statistical analysis

Continuous data are reported with medians and ranges, while categorical data are reported with counts and percentages. All potential prognostic indices were measured at the time of operation and evaluated as categorical variables. Descriptive statistics were employed to analyse the demographic and clinicopathological parameters. Overall survival (OS) was estimated using the Kaplan-Meier method. A multivariate Mantel-Cox regression model was used to evaluate the effect size and statistical significance of each variable which demonstrated $P<0.05$ in univariate analysis and with ≥10 events. Two-sided chi-square or Fisher's exact test was used to evaluate associations between categorical variables. All statistical analyses were performed using SPSS 27 (IBM Corp., Armonk, NY, USA). Kaplan-Meier curves were generated using GraphPad Prism Version 8 (GraphPad Software, La Jolla

California, USA). Exact *P* values were recorded and statistical significance was defined as $P<0.05$.

3.4 Results

3.4.1 Demographic and clinicopathological characteristics

The findings are summarised in Table 3-1. From 1st October 2004 to 30th September 2019, a total of 152 patients underwent MMT including total pleurectomy and decortication. The annual caseload was 10.1 cases with four patients (2.6%) treated before 2007. Approximately 70% of referrals for surgery were not offered MMT based on imaging findings or presence of unfavourable prognostic factors. Thirty-six patients (23.7%) were alive at time of last follow-up, and the median follow-up period was 43.2 months (range 11.0-165.0). The median age was 64 years with male predominance (80.9%). Most tumours were of epithelioid subtype (70.4%) and pathological stage I/II (57.9%). Approximately a third of patients (49/152, 32.2%) had lymph node metastasis in their final resection specimen. Most patients received adjuvant chemotherapy (75.7%), and three patients did not receive prophylactic radiotherapy (their own decision) (2.0%). MCR rate was 58.6%. The completion rate for MMT was 96.1%.

Of 152 patients, 35 patients (23.0%) underwent extended resection, most commonly the diaphragm (12.5%) and pericardium (5.3%) (Table 3-2). On disease relapse or progression, a significant proportion of patients (74/152, 48.7%) received second-line or third-line treatment or were enrolled into clinical trials (Table 3-3). Immunotherapy based on anti-PD1 and/or anti-CTLA4 immune checkpoint inhibitors was offered to 10.5% and 6.5% of patients in

second- and third-line settings respectively. Two patients (1.3%) in the study population were diagnosed with drain site implants on follow-up.

Age at diagnosis, median (range)	64 (32-82)
Sex, n (%)	
Male	123 (80.9)
Female	29 (19.1)
Laterality, n (%)	
Left	61 (40.1)
Right	91 (59.9)
Chemotherapy, n (%)	
None	6 (3.9)
Neoadjuvant	31 (20.4)
Adjuvant	115 (75.7)
Histological subtype, n (%)	
Epithelioid	107 (70.4)
Biphasic	39 (25.7)
Sarcomatoid	6 (3.9)
Pathological T stage, n (%)	
T1	11 (7.2)
T2	37 (24.3)
T3	82 (54.0)
T4	22 (14.5)
Pathological N stage, n (%)	
N0/Nx	103 (67.8)
N1	17 (11.2)
N2	32 (21.0)
M stage (clinical or pathological), n (%)	
M0	151 (99.3)
M1	1 (0.7)
Final pathological stage, n (%)	
I	88 (57.9)
II	0 (0.0)
III	63 (41.4)
IV	1 (0.7)
Resection status, n (%)	
Macroscopic complete resection (MCR)	89 (58.6)
Macroscopic residual disease (MRD)	63 (41.4)

Table 3-1 Demographics and clinicopathological characteristics.

Extended resection	n (%)
Total	35 (23.0%)
Diaphragm	19 (12.5%)
Pericardium	8 (5.3%)
Chest wall	4 (2.6%)
Superior vena cava	1 (0.7%)
Lobectomy	6 (3.9%)
Bi-lobectomy	2 (1.3%)
Segmentectomy	6 (3.9%)

Table 3-2 Extended resection beyond total pleurectomy and decortication.

Treatment	Second-line, n (%)	Third-line, n (%)
No active treatment	78 (51.3)	n/a
Chemotherapy/Systemic anti-cancer therapy (SACT) †	43 (28.3)	5 (3.3)
Immunotherapy (anti-PD1 and/or CTLA-4) ‡	16 (10.5)	10 (6.5)
Radiotherapy	4 (2.6)	6 (3.9)
Stereotactic ablative radiotherapy (SABR)	2 (1.3)	3 (2.0)
Intensity-modulated radiotherapy (IMRT)	2 (1.3)	3 (2.0)
Clinical trial	11 (7.2)	0 (0.0)
Sorafenib	5 (3.3)	
Focal adhesion kinase inhibitor	2 (1.3)	
Pegylated arginine deaminase	4 (2.6)	

Table 3-3 Treatment on disease recurrence or progression.

† Second and third-line systemic chemotherapy regimens included: pemetrexed and cisplatin/carboplatin, gemcitabine and cisplatin/carboplatin, irinotecan with cisplatin and mitomycin, single agent vinorelbine, gemcitabine and metronomic cyclophosphamide.

‡ Included patients enrolled in immunotherapy-based clinical trial.

CTLA-4, cytotoxic T-lymphocyte-associated protein 4; PD1, programmed death-1.

3.4.2 Safety

With the exception of the three patients not receiving radiotherapy, six patients (3.9%) did not receive chemotherapy within the 12-week post-operative window due to clinical deterioration (*n*=4) and delayed schedule (*n*=2). No perioperative or 90-day mortality was registered.

Immediate post-operative or late complications occurred in 64 patients (42.1%), of which 11 were grade III (17.1%) and 3 were grade IV (4.7%). The most common complications were persistent air leak, hospital-acquired pneumonia and chylothorax/empyema (Table 3-4). Three out of 115 patients (2.6%) receiving adjuvant chemotherapy developed pulmonary embolism. No allergic reaction to iodine or clinical hypothyroidism was documented.

Complications	**n (%)**
Post-operative	
Persistent air leak for > 5 days	38 (25.0)
Chylothorax†	5 (3.3)
Hospital-acquired pneumonia	11 (7.2)
Pleural empyema	5 (3.3)
Atrial fibrillation	6 (3.9)
ARDS	1 (0.7)
Transient ischaemic attack	2 (1.3)
Haemorrhage requiring re-operation	2 (1.3)
Incomplete lung re-expansion‡	4 (2.6)
Late complications	
Hydropneumothorax requiring IPC	8 (5.3)
Pulmonary embolism during chemotherapy	3 (2.0)
Bronchopleural fistula after second surgery	1 (0.7)

Table 3-4 Post-operative and late complications.

† Requiring thoracotomy (*n*=3) or VATS (*n*=1) to ligate the thoracic duct.

‡ Requiring IPC (*n*=2) or VATS decortication (*n*=2).

ARDS, acute respiratory distress syndrome; IPC, indwelling pleural catheter.

3.4.3 Prognostic variables and predictors of macroscopic complete resection

The median OS of the study population was 31.7 months (Figure 3-1). At time of last follow-up, 76 patients (50.0%) had disease relapse or progression. The 1-year, 2-year and 5-year survival rates were 88.1%, 60.1% and 23.2% respectively. Long term survival was seen in a significant minority of patients, almost exclusively in those with epithelioid disease (8-year OS 20.2%, 10-year OS 16.1%) (Figure 3-2A). Univariate analysis identified histological subtype (P=8.0×10^{-6}), resection status (P=0.010), pathological stage (P=0.014), lymph node metastasis (P=0.016), and the order of chemotherapy (P=0.038) as significant predictors of overall survival (Figure 3-2) (Figure 3-3A, 3-3E). Patient age, sex and laterality were not significant prognostic variables (Figure 3-3B-D).

In multivariate analysis, histological subtype predicted OS independent of resection status, pathological stage, lymph node status and order of chemotherapy (HR 2.18, P=2.1×10^{-4}) (Table 3-5). Resection status was not shown to be an independent predictor of OS. There was interaction between histological subtype and pathological stage (P=0.004), as well as resection status (p=0.022) (Table 3-6) (Table 3-7). Non-epithelioid disease was significantly less likely to achieve MCR (44.4% vs 64.5%). These findings were reflected upon by the survival characteristics, where the achievement of MCR in non-epithelioid disease was associated with significantly superior OS (23.0 vs. 14.4 months, P=0.035), but not in epithelioid disease (P=0.312) (Figure 3-2E). An opposite trend was observed for pathological stage, where the survival advantage associated with early-stage disease was limited to epithelioid subtype, in form of a late effect from 3 years post-operatively (Figure 3-2F). Of note female patients (75.9% vs 54.5% in male) and those receiving neoadjuvant chemotherapy (71.0% vs 54.8% in adjuvant chemotherapy) were more likely to achieve MCR, however the number of cases was too small hence it is important to interpret this result with caution (Table 3-7).

A favourable prognostic group based on our findings, composed of epithelioid subtype, stage I/II disease and MCR, was associated with median OS of 42.4 months when all criteria were fulfilled (*P*=0.001) (Figure 3-4). The 1-year, 2-year and 5-year OS rates were 97.8%, 77.8% and 44.9% respectively. This group nevertheless only represented under a third of the population, and likely an absolute minority of pleural mesothelioma patients in routine clinical practice.

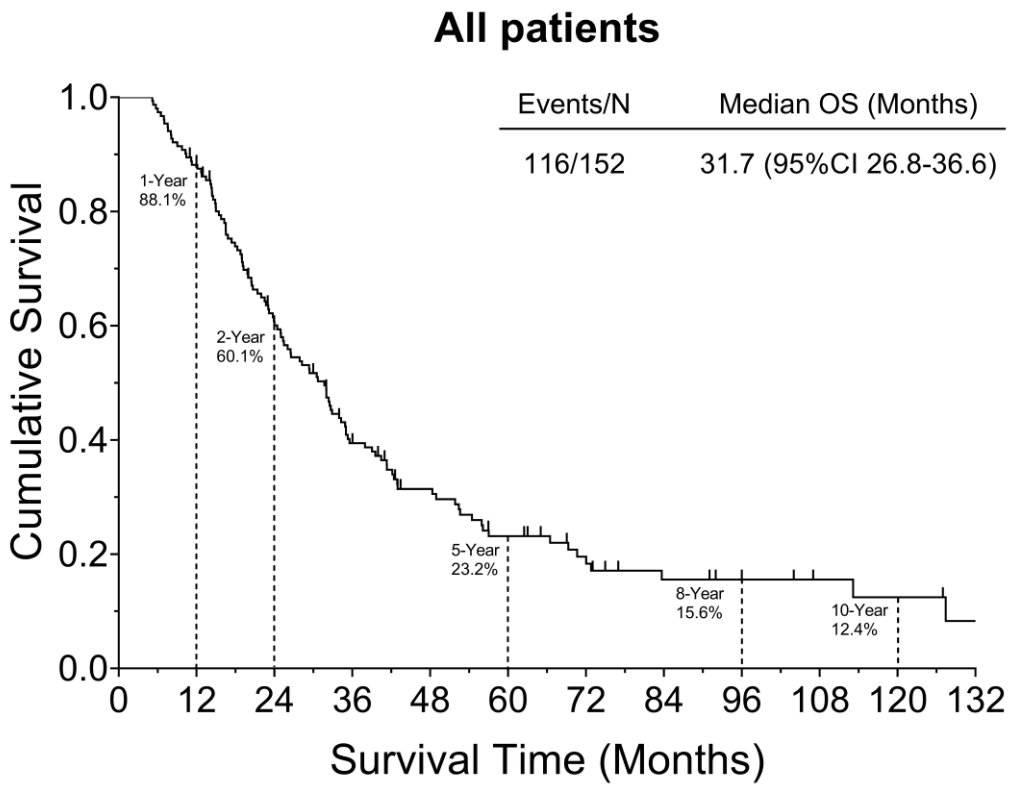


Figure 3-1 Overall survival of the study population.

CI, confidence interval; OS, overall survival.

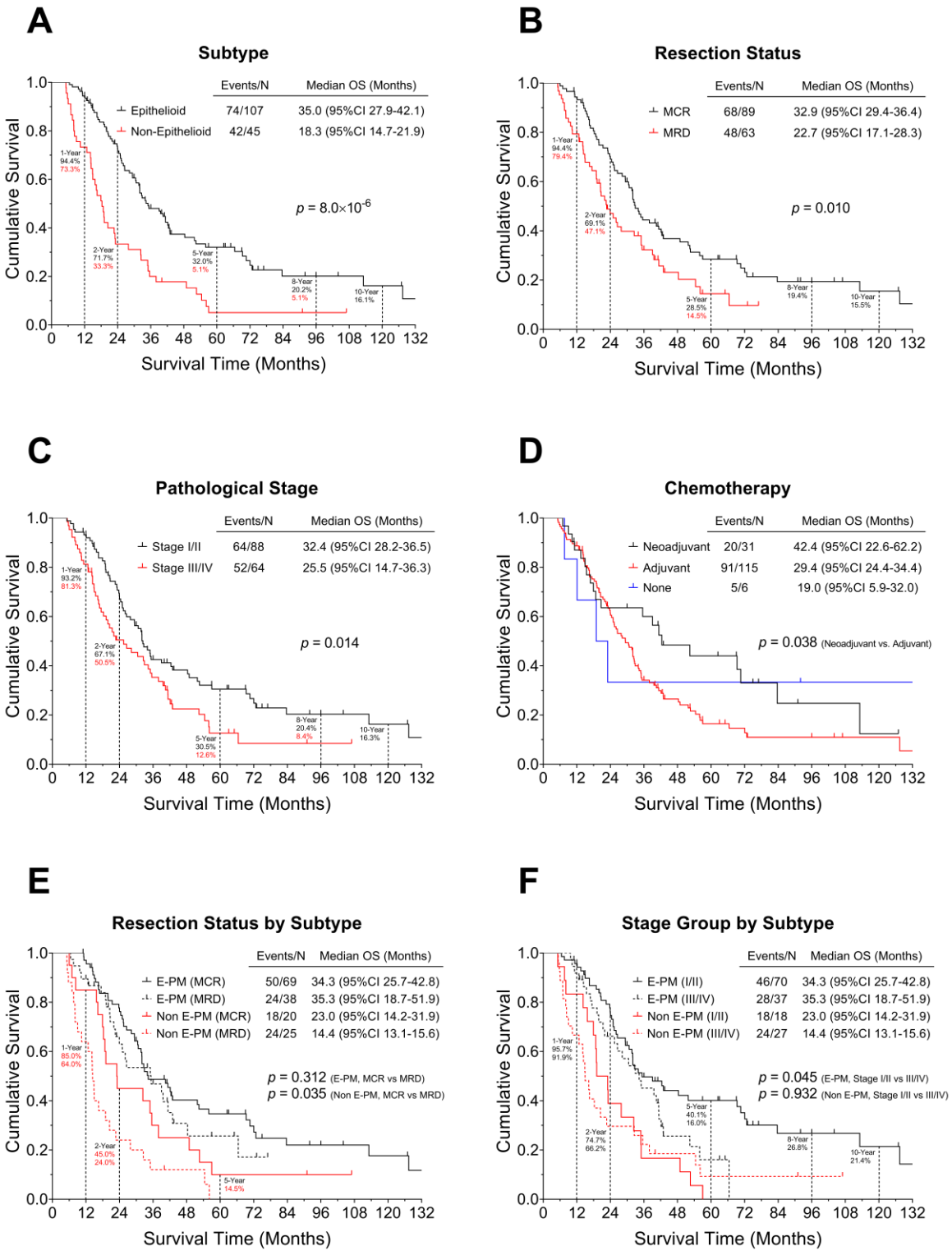


Figure 3-2 Overall survival by clinicopathological variables. **(A)** Histological subtype. **(B)** Resection status. **(C)** Pathological stage. **(D)** Administration of chemotherapy. **(E)** Resection

status stratified by histological subtype. **(F)** Pathological stage stratified by histological subtype.

CI, confidence interval; E-PM, epithelioid pleural mesothelioma; MCR, macroscopic complete resection; MRD, macroscopic residual disease; OS, overall survival.

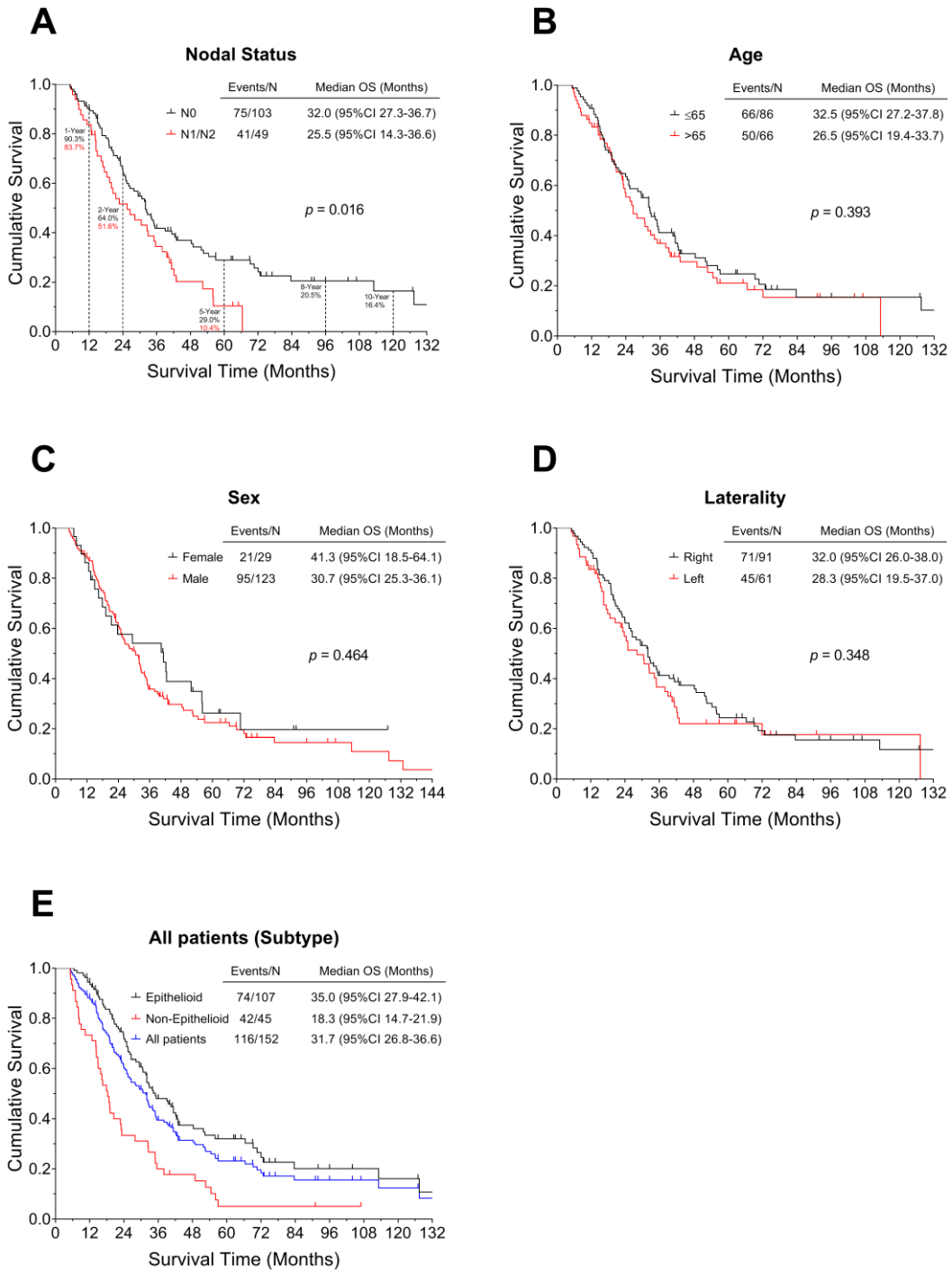


Figure 3-3 Overall survival by additional clinicopathological variables.

(A) Lymph node status. **(B)** Age. **(C)** Sex. **(D)** Disease laterality. **(E)** Global survival superimposed by histological subtype.

CI, confidence interval; OS, overall survival.

Variable	Hazard ratio	95% CI	*P*
Histological subtype			
Non-epithelioid vs Epithelioid	2.18	1.44-3.29	2.1×10^{-4}
Resection status			
MRD vs MCR	1.43	0.97-2.11	0.074
Stage group			
III/IV vs I/II	0.90	0.45-1.78	0.760
Lymph node status			
N1/N2 vs N0	1.54	0.77-3.09	0.221
Chemotherapy			
None† vs Adjuvant	1.01	0.39-2.64	0.978
Neoadjuvant vs Adjuvant	0.75	0.45-1.23	0.258

Table 3-5 Multivariate analysis of predictors of outcome following multimodality therapy.

† Less than 10 events were registered in this group.

CI, confidence interval; MCR, macroscopic complete resection; MRD, macroscopic residual disease.

Variable	Epithelioid subtype	Non-epithelioid subtype	*P*
Age ≤65 years	63 (73.3%)	23 (26.7%)	0.378
Age >65 years	44 (66.7%)	22 (33.3%)	
Male	84 (68.3%)	39 (31.7%)	0.269
Female	23 (79.3%)	6 (20.7%)	
Left-sided disease	42 (68.9%)	19 (31.1%)	0.733
Right-sided disease	65 (71.4%)	26 (28.6%)	
Neoadjuvant chemotherapy	24 (77.4%)	7 (22.6%)	0.384
Adjuvant chemotherapy	79 (68.7%)	36 (31.3%)	
Pathological N0	77 (74.8%)	26 (25.2%)	0.088
Pathological N1/N2	30 (61.2%)	19 (38.8%)	
Stage I/II	70 (79.5%)	18 (20.5%)	*0.004*
Stage III/IV	37 (57.8%)	27 (42.2%)	

Table 3-6 Association between histological subtype and clinicopathological variables (excluding resection status).

Variable	Macroscopic complete resection	Macroscopic residual disease	*P*
Age ≤65 years	53 (61.6%)	33 (38.4%)	0.380
Age >65 years	36 (54.5%)	30 (45.5%)	
Male	67 (54.5%)	56 (45.5%)	*0.038*
Female	22 (75.9%)	7 (24.1%)	
Left-sided disease	36 (59.0%)	25 (41.0%)	0.924
Right-sided disease	53 (58.2%)	38 (41.8%)	
Neoadjuvant chemotherapy	22 (71.0%)	9 (29.0%)	0.150
Adjuvant chemotherapy	63 (54.8%)	52 (45.2%)	
Epithelioid subtype	69 (64.5%)	38 (35.5%)	*0.022*
Non-epithelioid subtype	20 (44.4%)	25 (55.6%)	
Pathological N0	63 (61.2%)	40 (38.8%)	0.343
Pathological N1/N2	26 (53.1%)	23 (46.9%)	
Stage I/II	57 (64.8%)	31 (35.2%)	0.068
Stage III/IV	32 (50.0%)	32 (50.0%)	

Table 3-7 Association between resection status and clinicopathological variables.

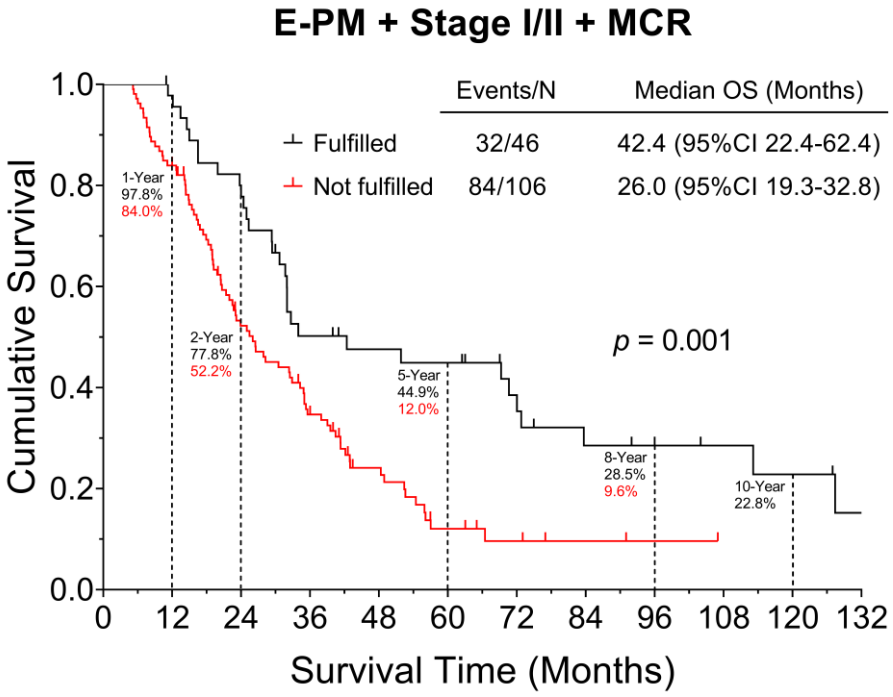


Figure 3-4 Overall survival in favourable prognostic group.

CI, confidence interval; E-PM, epithelioid pleural mesothelioma; MCR, macroscopic complete resection; OS, overall survival.

3.4.4 Internal validation cohort

The internal validation cohort consisted of 57 patients who had undergone EPD (35.6%) and 103 patients who had undergone PD. The censor fraction was 36.9% (59/160), and median OS was 19.4 months (95% CI 15.9-23.0). One-year, 2-year and 5-year survival rates were 65.7%, 38.7% and 10.7% respectively. Most cases were of epithelioid subtype (100/160, 62.5%).

In univariate analysis, epithelioid subtype and EPD was found to predict favourable survival (all $P<0.001$) (Figure 3-5A-B). Furthermore, patients with epithelioid disease derived more clinical benefit, measured in survival, from EPD compared with non-epithelioid disease ($P=0.001$) (Figure 3-5C). This confirmed the findings from the main study population. Patients with epithelioid disease who underwent PD, as opposed to EPD, showed however substantially worse survival than those in the main study population with MRD (18.1 vs 35.3 months), although the patient populations are not directly comparable. This challenges the notion, although does not provide definitive evidence, that PD is equivalent to MRD in a patient having EPD in clinical terms.

Patient age, sex and disease laterality were not significant predictors of OS (Figure 3-5D-F).

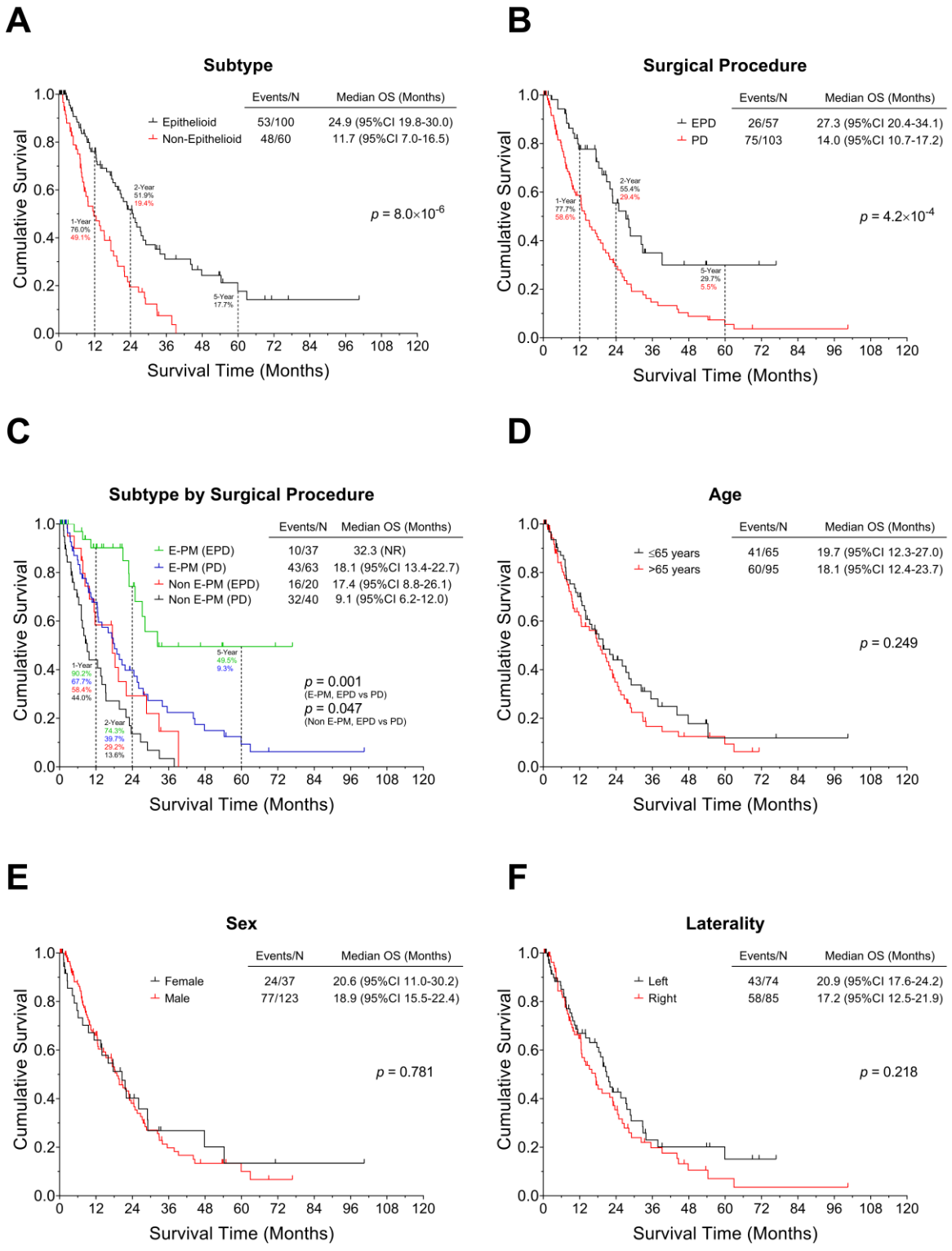


Figure 3-5 Overall survival by clinicopathological variables (Internal validation cohort).

(A) Subtype. **(B)** Type of surgical procedure. **(C)** Subtype stratified by the type of surgical procedure. **(D)** Patient age. **(E)** Sex. **(F)** Disease laterality.

CI, confidence interval; E-PM, epithelioid pleural mesothelioma; EPD, extended pleurectomy and decortication; OS, overall survival; PD, pleurectomy and decortication.

3.5 Discussion

In the present study, the local practice of multimodality therapy including total pleurectomy and decortication for pleural mesothelioma, was associated with non-inferior outcomes and safety profile to published studies in north America and Western Europe (Table 3-8). The data presented above suggest that long-term survival is a feasible treatment goal in selected patients, and supports the use of 2-year survival rate as the primary outcome of the ongoing MARS2 trial (Lim *et al*., 2020). While we confirmed histological subtype being the key determinant of overall survival, it was also shown to influence the prospect of achieving MCR as well as pathological stage. From the internal validation cohort, it could also be concluded that surgery aiming at disease debulking produces unsatisfactory and arguably unacceptable outcome in the setting of non-epithelioid subtype, given the mortality and morbidity of the procedure. This highlights the importance of accurate pre-treatment biopsy diagnosis, and ensuring adequate sampling, which is now a recognised issue (Schulte *et al*, 2021) (Chirieac *et al*., 2019), as incorrect assignment of subtype may lead to downstream treatment failures. Findings in this study echo the recent proposal of more extensive sampling for initial diagnosis (Nicholson *et al*., 2020).

The concept of trimodality therapy for PM has been replaced by multimodality therapy as individual centres introduce additional modalities based on local expertise. Compounded by a lack of high-grade evidence in this patient population, head-to-head comparison of results is challenging. In recent years, the trend is evident as pleurectomy and decortication emerges as the preferred surgical technique over EPP for its more favourable safety profile (Burt *et al*.,

2014) (Sugarbaker *et al*., 2004). The so-called maximally invasive nature of EPP has also been shown to be less permissive to further treatment modalities secondary to the risk of deteriorated performance status in a substantial proportion of patients (Cho *et al*., 2019). Efforts in improving outcomes in EPP-based MMT protocols should not be overlooked however, for example in the case of the Phase II SMART trial (neoadjuvant radiotherapy, EPP and optional adjuvant chemotherapy) where a median OS of 24.4 months has been reported (Cho *et al*., 2021).

The major limitations of the present study are its non-randomised nature, patient selection bias based on prognostic factors, and that it has not been possible to quantify the amount or nature of residual disease. The heterogeneous nature of MRD as a designator is supported by the differential outcomes comparing the main study population and the internal validation cohort, where in the latter any PD was associated by default to MRD. More importantly, the lack of standardisation of MMT in pleural mesothelioma means surgical protocols and treatment regimens enjoy a high degree of heterogeneity, the present study also contributing to the conundrum (Table 3-8).

The role of adjuvant radiotherapy is a controversial matter. Prophylactic radiotherapy with the same dosing schedule (21Gy/3) was not shown to prevent procedural site seeding nor overall survival (Bayman *et al*., 2019) (Clive *et al*. 2016). Our study population was intrinsically distinct however, in that all patients underwent a large posterolateral thoracotomy followed by a long operation with extensive manipulation and prolonged post-operative chest drainage. Whether prophylactic radiotherapy prevented chest wall recurrence in this type of setting will be a subject for future studies. The order of chemotherapy represented another contentious area, where the data presented in this Chapter showed a survival advantage of neoadjuvant chemotherapy with borderline statistical significance. This can be explained either by an increased probability of achieving a satisfactory surgical plane

of resection, and/or abscopal effect induced by the chemotherapeutic agents. The counterargument is surgical resection *per se* induces abscopal effect as shown in animal models (Predina *et al*., 2012) (Broomfield *et al*., 2005). Neither theory has attained sufficient mechanistic or experiential evidence to date in clinical setting. The ongoing EORTC 1205 trial is expected to offer insight in terms of the order of chemotherapy (Raskin *et al*., 2018).

It is important that the findings of the present study are not interpreted in isolation as the management and research of pleural mesothelioma become ever more multidisciplinary, and questions for the wider oncology community remain. First, the predictive role of recently introduced nuclear grading system for epithelioid PM (Kadota *et al*., 2012) (Rosen *et al*., 2018) (Zhang *et al*., 2020a) might explain the heterogeneity in outcomes in this subtype. Second, the issue of technical heterogeneity and evaluation of macroscopic residual disease are expected to be at least in part addressed by a recent joint NCI/IASLC/MARF proposal on mesothelioma surgery and conformation of practice (Friedberg *et al*., 2019) although rigorous validation is needed in terms of its feasibility and reproducibility. Additionally, more data is needed from non-North American or Western European regions. Third, first-line chemotherapy combined with anti-PD1 immunotherapy has shown promise in an early phase trial (Nowak *et al*., 2020). Research into the role of immunotherapy as an addition to MMT should be regarded as high priority, so does other modalities including but not limited to neoadjuvant radiotherapy similarly adopted in SMART trial (Cho *et al*., 2021), although the added complexity to treatment regimen as well as the safety profiles should be given weight in consideration.

There has been cautious optimism expressed by mesothelioma experts in the latest joint ERS/ESTS/EACTS/ESTRO and ASCO guidelines (Scherpereel *et al*., 2020) (Kindler *et al*., 2018), that surgery was recommended for selected patients with early stage, epithelioid disease and that it should be undertaken in centres of excellence and ideally within clinical

trials. The data presented in this Chapter support these guidelines. While the mesothelioma field awaits with enthusiasm the results from MARS2 and EORTC 1205 trials in the coming years, the intellectual debates around surgery are unlikely to reach a verdict. Both the proponents and opponents of mesothelioma surgery will see knowledge and practical innovations stemming from such debates, from which patients with this devastating disease will only benefit.

	Lee *et al*., 2002	Flores *et al*., 2008	Bölükbas *et al*., 2011	Lang-Lazdunski *et al*., 2012†	Friedberg *et al*., 2015	Zhou *et al*., 2021	This study
Centre	1 (USA)	3 (USA)	1 (GER)	1 (UK)	1 (USA)	1 (USA)	1 (UK)
Time period	1995-2000 (5 years)	1990-2006 (16 years)	2002-2007 (5 years)	2004-2011 (7.5 years)	2005-2010 (5 years)	2000-2019 (18.5 years)	2004-2019 (15 years)
Number of patients	29	278	35	54	38	95	152
Median follow-up (months)	9.7	17	21.7	15.7	34.4	71‡	43.2
Censor fraction (%)	19.2%	16.9%	31.4%	51.9%	36.8%	NR	23.7%
Median age (years)	69	63	65	62.5	65	65	64
Epithelioid subtype (%)	73.1%	64.0%	77.1%	66.7%	81.6%	74.7%	70.4%
Lymph node metastasis (%) *	11.5%	NR	14.3%	29.6%	73.7%	43.2%	32.2%
Extended resection (%)	30.8%	NR	11.4%	22.2%	18.4%	NR	23.0%
Chemotherapy							
None	53.8%	NR	0%	3.7%	7.9%	NR	3.9%
Neoadjuvant	0%	NR	0%	0%	26.3%	83.2%	20.4%
Adjuvant	46.2%	NR	100%	96.3%	65.8%	NR	75.7%
Radiotherapy							
None	7.7%	NR	2.9%	0%	100%	NR	2.0%
Neoadjuvant	0%	NR	0%	0%	0%	NR	0%
Adjuvant	92.3% (median 41.4Gy, EBRT)	NR	97.1% (EBRT 50.4Gy, 14.3%); (21Gy/3, 82.9%)	100% (21Gy/3)	0%	52.6% (EBRT)	98.0% (21Gy/3)
Additional modality	Intraoperative RT (58.6%)	n/a	n/a	Povidone-iodine lavage (100%)	Photodynamic therapy (100%)	n/a	Povidone-iodine lavage (100%)
Post-operative complications (%)	30.8%	14.7%	20.0%	27.7%	68.4%	NR	42.1%
Postoperative death (%)	7%	4.7%	2.9%	0%	2.6%	0%	0%
MMT completion rate (%)	NR	57.9%	94.3%	96.3%	NR	NR	96.1%
MCR (%)	65.4%	NR	51.4%	57.4%	97.4%	90.5%	58.6%
Median OS (months)	18.1	23 (Stage I/II)	30.0	23	31.7	18	31.7
1-year survival (%)	64.4%	NR	69%	81.9%	NR	NR	88.1%
2-year survival (%)	32.2%	NR	50%	49.0%	NR	NR	60.1%
5-year survival (%)	12.3%	NR	NR	30.1%	NR	NR	23.2%

Table 3-8 Published studies of multimodality therapy including total pleurectomy and decortication and comparison with the current study.

† All patients underwent P/D were included in the current study. This study also included 22 patients receiving EPP as the surgical modality.

‡ This study included 187 patients underwent EPP and did not differentiate follow-up period based on procedure type.

* Pathological TNM stage not used due to TNM7 classification used in earlier studies.

EBRT, external beam radiotherapy; GER, Germany; NR, not reported; RT, radiotherapy; USA, United States of America; UK, United Kingdom.

3.6 Conclusions

This study has shown that P/D with hyperthermic pleural lavage with povidone-iodine, prophylactic chest wall radiotherapy and systemic chemotherapy is a safe and well tolerated form of multimodality therapy for pleural mesothelioma. Medium-term (>2 years) survival was seen in the majority of patients, with long-term (>5 years) survival observed in a significant minority. Further research is needed to improve pre-treatment evaluation, optimisation of the choices and schedules of non-surgical modalities and harmonisation of surgical protocols and reporting.

4 Refining the Classification of Epithelioid Pleural Mesothelioma: Nuclear Grade

This chapter has been presented as a platform presentation at the United States and Canadian Academy of Pathology (USCAP) annual meeting, 16th-21st March 2019 (National Harbour, USA) and published as Zhang YZ, Brambilla C, Molyneaux PL, Rice A, Robertus JL, Jordan S *et al*. Utility of Nuclear Grading System in Epithelioid Malignant Pleural Mesothelioma in Biopsy-heavy Setting: An External Validation Study of 563 Cases. Am J Surg Pathol. 2020; 44:347-356. doi: 10.1097/PAS.0000000000001416.

This was also published in part, as Schulte JJ, Chapel DB, Attanoos R, Brcic L, Burn J, Butnor KJ *et al*. Comparison of Nuclear Grade, Necrosis, and Histologic Subtype Between Biopsy and Resection in Pleural Malignant Mesothelioma. Am J Clin Pathol. 2021 May 12:aqab054. doi: 10.1093/ajcp/aqab054.

4.1 Abstract

Background: Nuclear grading systems for epithelioid pleural mesothelioma (PM) have been proposed but it remains uncertain if they could be applied in a biopsy-heavy setting.

Materials and methods: This study was an independent, external validation study using 563 consecutive cases of epithelioid PM diagnosed at the Royal Brompton and Harefield Hospitals between 2003 and 2017, of which 87% of patients underwent biopsies only. Multiple proposed nuclear grading systems were evaluated for their prognostic performance and the impact of tissue sampling.

Results: The median number of sites sampled was one, with median maximum tissue dimension of 17mm (Biopsy) and 150mm (Resection). The median overall survival (OS) was 14.7 months. The frequencies of Grade I, II and III tumours were 31% (132/563), 52% (292/563) and 17% (94/563) respectively. Grade I tumours were associated with the most favourable median OS (24.7 months) followed by grade II (12.7 months) and III (7.2 months). 2-tier nuclear grade separated tumours into low grade (19.3 months) and high grade (8.9 months). In multivariate analysis, 3-tier nuclear grade, 2-tier nuclear grade and mitosis-necrosis score predicted OS independent of age, procedural type, solid-predominant growth pattern, necrosis and atypical mitosis (all $P<0.001$ except 2-tier nuclear grade, $P=0.001$). In the scenario of a single site biopsy with tissue dimension less than or equal to 10mm, none but age ($P=0.002$) were independently predictive. The data also suggested that sampling three sites or a maximum tissue dimension of at least 20mm from a single site is optimal for nuclear grade assessment.

Conclusions: This study, using a biopsy-heavy cohort, has confirmed the utility of nuclear grade in epithelioid PM provided that the tissue sample meets minimum dimensional criteria.

4.2 Introduction

As discussed in Chapter 1, pleural mesothelioma (PM) is a rare pleural malignancy and by far the most common type of mesothelioma affecting approximately 30000 to 40000 patients globally (Odgerel *et al*, 2017) (Global Burden of Disease, 2016) with the majority (74-80%) attributable to asbestos exposure (Rushton *et al*., 2008). The prognosis of PM is poor, with reported overall survival (OS) between 9-12 months in the Western world (National Mesothelioma Audit, 2018) (Beebe-Dimmer *et al*., 2016). Even for the minority of patients eligible for surgical resection, OS varies between 10-35 months depending on pathological stage (Nelson *et al*., 2017) (Verma *et al*., 2017) (Lang-Lazdunski *et al*., 2015) (Rusch *et al*., 2012) (Rice *et al*., 2007). Histological subtype is an established and robust predictor of survival to date and is used in both routine care and patient stratification in research studies. Literature evidence, however, is mounting with regard to epithelioid PM representing a spectrum of tumours with variable clinical behaviour rather than being a homogeneous group. The median OS for pleomorphic epithelioid PM for instance, has been reported to be around 8 months (Alchami *et al*., 2017) (Kadota *et al*., 2011) whilst epithelioid PM with microcystic-predominant growth pattern in a myxoid stroma was associated with median OS of 24 months (Alchami *et al*., 2017). There are promising therapeutic options being explored for PM (see Chapter 1, Section 1.5) encompassing radical surgery, targeted therapy and immunotherapy (Treasure *et al*., 2011) (Lang-Lazdunski *et al.*, 2015) (Alley *et al*., 2017) (Shaikh *et al*., 2017) (Fennell *et al*., 2018) (Metaxas *et al*., 2018) (Ricciardi *et al*., 2018) (Tsao *et al*., 2018) (Scherpereel *et al*., 2019). Among the ongoing trials, the majority of enrolled patients have epithelioid PM.

The search for robust predictors of survival, which will subsequently allow better stratification of patients and comparison between results from different studies, is limited by

several factors. First, there is a considerable degree of practice variation in the radiological staging for PM (Kindler *et al*., 2018) (Woolhouse *et al*., 2018). Second, pathological staging suffers from heavy selection bias as most PM patients are ineligible for radical surgery. Third, unlike lung cancer, comprehensive multi-omics studies in mesothelioma have only started to be reported in the last few years (Blum *et al*., 2019) (Mansfield *et al*., 2019) (Hmeljak *et al*., 2018) (Bueno *et al*., 2016). Consequently histopathological predictors, ideally validated in biopsy setting, remain attractive in pre-treatment evaluation.

A 3-tier nuclear grading system was developed in 2012 using 232 cases of epithelioid PM based on the extent of nuclear atypia and mitotic activity (Kadota *et al*., 2012b). It was subsequently validated using an international, multi-institutional cohort of 776 cases (Rosen *et al*., 2018). The proportion of biopsy-only patients was 5% and 27% respectively. It demonstrated a superior degree of separation between the best and worst prognostic groups compared with established predictors such as age and sex in multivariate models. A recent study including 87 cases of epithelioid PM also validated the nuclear grading system although the proportion of biopsies was undefined (Forest *et al*., 2018). Furthermore, Rosen *et al*. proposed two alternatives to the 3-tier nuclear grade: a simplified 2-tier nuclear grade by computing 3-tier nuclear grade and necrosis, and mitosis-necrosis (M-N) score based on mitotic activity and the presence of necrosis.

The aims of the present study were (1) to externally validate the 3-tier nuclear grading system, M-N scoring system and 2-tier nuclear grading system using a large, biopsy-heavy cohort, (2) to evaluate the discriminative power of nuclear grade with regard to grade III versus pleomorphic PM, and (3) evaluate importance of single versus multiple sites and biopsy size in the value of grading.

4.3 Materials and methods

4.3.1 Study population

Six hundred and thirty-two consecutive cases of histologically confirmed epithelioid PM were identified from between 2003 and 2017 at the Royal Brompton and Harefield Hospitals. Approximately 70 new cases of PM are diagnosed and/or undergo cytoreductive surgery annually. An institutional mesothelioma database was retrospectively curated and prospectively maintained by myself (Dr Yu Zhi Zhang) in conjunction with the National Centre of Mesothelioma Research (NCMR; National Heart & Lung Institute, Imperial College London). This database was central to the work conducted within this Chapter and the subsequent Chapters contained within this thesis. Institutional review board (IRB) approval was obtained for this study. Clinical and histopathological information were collected from the database, with additional outcome data retrieved from the electronic patient record, National Health Service Spine repository, surgical records and regional thoracic oncology service. Fifty-one cases classified as pleomorphic epithelioid PM were excluded from the main study, but they served as a comparator. A further 18 cases were excluded because they were: unavailable for histological assessment (n=12), post-mortem diagnoses (n=2), contained less than 100 viable tumour cells for assessment (n=2), diagnosis had been made solely on pleural effusion cell block (n=1), or a definitive diagnosis was achieved only after extensive multidisciplinary team discussion (n=1). The final study cohort size was therefore 563 cases.

Clinical variables included in this study were age, sex, laterality of disease, date and type of procedure, number of sites sampled, and date of death or last follow-up. Survival time was defined as time (measured in months) between the date of initial procedure from which a definitive diagnosis of PM was made and the date of death or last follow-up. Information in

relation to asbestos exposure, TNM staging, metabolic uptake and chemotherapy/radiotherapy in either neoadjuvant or adjuvant setting were not included in this study as these were incompletely recorded in the electronic patient record. Censor fraction in this study was 23% (131/563).

4.3.2 Microscopy and imaging

Microscopic assessment was performed using a Nikon Eclipse Ci-L microscope (Nikon Corporation, Japan) with a field area measuring 0.24mm^2 per high power field (HPF, ×400 magnification). Microscopic images in 300dpi TIF format were taken from representative cases using a Nikon Digital Sight DS-L3 camera (Nikon Corporation, Japan), annotated using the GNU Image Manipulation Program 2.10.10 (http://www.gimp.org, retrieved on 20.04.2019).

4.3.3 Histopathological assessment

All cases were diagnosed by at least one specialised pulmonary pathologist using the current histological and immunohistochemical criteria (Husain *et al*., 2018) (Travis *et al*., 2015). Maximum tissue dimension as a routinely recorded item was taken from the pathology gross descriptions. Histopathological parameters were assessed and recorded by myself, blinded to outcome. Problematic cases (1.6%) were jointly reviewed with a second pathologist in the Department of Histopathology, Royal Brompton and Harefield Hospitals (Dr Cecilia Brambilla, Dr Alexandra Rice, Dr Jan Lukas Robertus, Professor Andrew G Nicholson). All available haematoxylin & eosin (H&E) sections were reviewed, with an average of 3.9 sections per case (range: 1-47). Quality control parameters were recorded for the presence of crush/cauterization artefacts representing more than 50% of tissue area, and less than 300 (but more than 100) viable tumour cells present. The criteria for nuclear atypia and mitotic activity were taken from earlier studies (Rosen *et al*., 2018) (Kadota *et al*., 2012b) (Table 4-

1). Mitosis scores were subsequently assigned: 1 (0-1 per 10 HPF), 2 (2-4 per 10 HPF), 3 (≥5 per 10 HPF). 3-tier nuclear grade was computed by adding nuclear atypia score (1-3) to mitosis score (1-3): Grade I (2-3), grade II (4-5), and grade III (6). Representative images illustrating the relevant nuclear features are shown in Figure 4-1.

Growth patterns were evaluated using the current diagnostic criteria (Travis *et al*., 2015) and a solid pattern was defined as sheets and/or nests of cohesive tumour cells without otherwise discrete architectural patterns. A predominant pattern was defined as the most abundant, frequently but not necessarily representing >50% of the entire tumour. Necrosis was assessed under ×400 magnification and recorded as present or absent. No further stratification by the extent of necrosis was performed. M-N score (0, I, II) was computed by adding the scores based on the presence (1) or absence (0) of necrosis, and a mitotic count of ≥5 (1) or less than 5 (0). 2-tier nuclear grade was computed using necrosis to sub-classify grade I and II tumours: low grade included all grade I and grade II tumours without necrosis, and high grade includes grade II with necrosis and all grade III tumours.

Nuclear atypia	Score 1 (Mild)	Score 2 (Moderate)	Score 3 (Severe)
Nuclei	Small Uniform Round	Intermediate Limited anisonucleosis Limited pleomorphism	Large Anisonucleosis Pleomorphism Multinucleation
Nucleoli	Inconspicuous	Conspicuous	Prominent Multi-nucleoli
Chromatin quality	Fine granular	Coarse	Coarse
Other features	-	-	Intra-nuclear inclusion Irregular nuclear contour Tumor giant cells Cell-in-cell

Table 4-1 Assessment criteria for nuclear atypia.

Evaluation of nuclear atypia was performed across the whole tumour and a threshold of ≥5% tumour cells was employed based on the worst (highest score) area. For example, a tumour with 5% of tumour cells sufficing the criteria of score 3 and 60% of cells sufficing that of score 2 will be assigned a nuclear atypia score of 3.

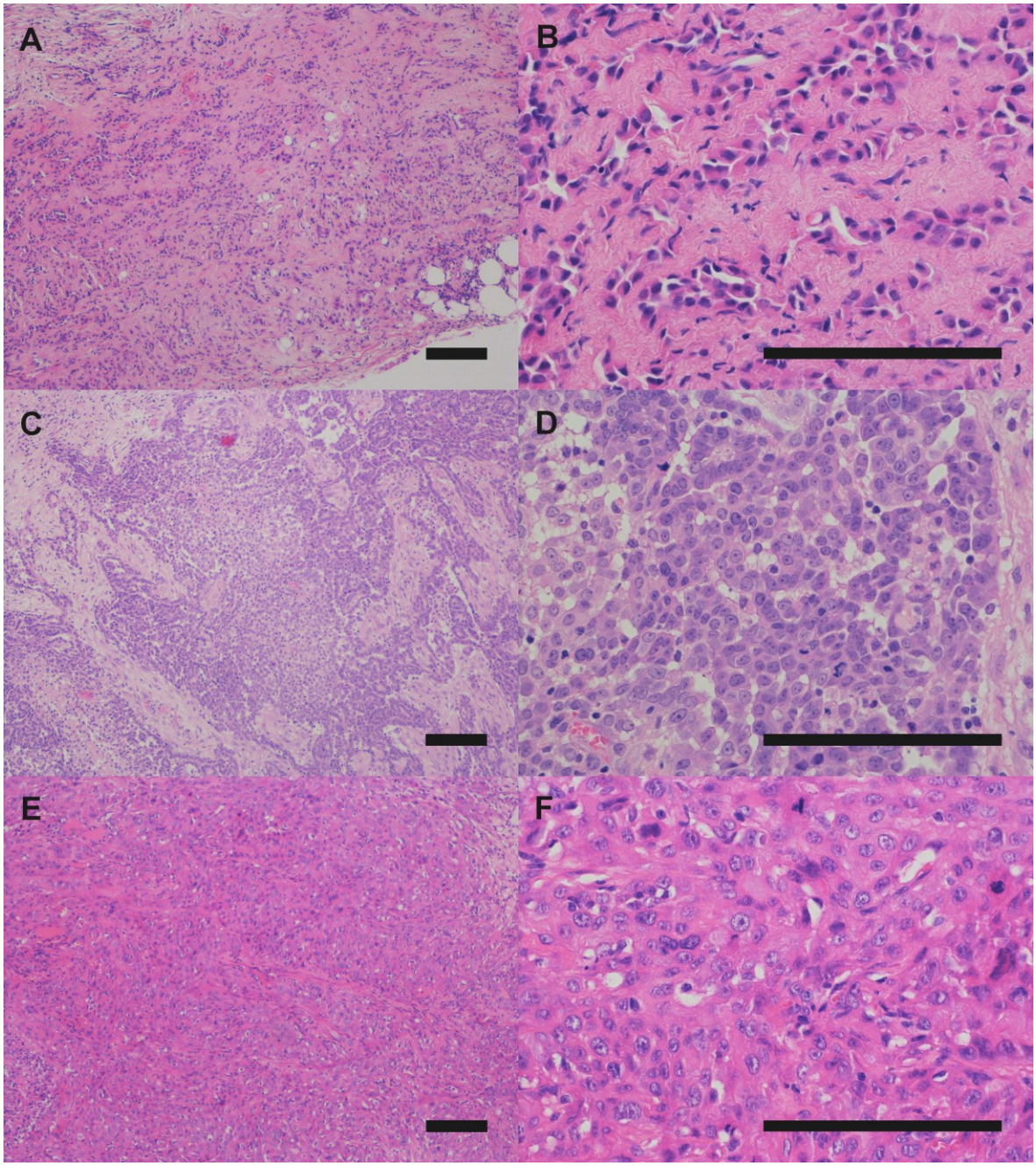


Figure 4-1 Nuclear features of epithelioid pleural mesothelioma. **(A)** Low grade tumour (nuclear grade I) with tubulo-papillary predominant growth pattern. **(B)** Tumour cells show features of mild nuclear atypia: small, uniform nuclei with fine chromatin pattern, and no nucleoli are seen. **(C)** High grade tumour **(**nuclear grade II with necrosis) with predominantly tubulo-papillary growth pattern, with solid and micropapillary secondary patterns. **(D)** Tumour cells show features of moderate nuclear atypia: the nuclei are enlarged with

increased nuclear-cytoplasmic ratio. Anisonucleosis and pleomorphism are apparent, with conspicuous nucleoli. **(E)** Nuclear grade III tumour with predominantly solid growth pattern. **(F)** Tumour cells show features of severe nuclear atypia: large and pleomorphic nuclei with prominent nucleoli and occasionally multiple nucleoli. Tumour giant cells are seen very focally. In tumours with ≥10% of tumour nuclei showing similar features, it would be sufficient to assign the diagnosis of epithelioid PM with pleomorphic features.

Scale bar = 100μm.

4.3.4 Statistical analysis

Descriptive statistics were employed to analyse the baseline demographic and clinicopathological parameters. Two-sided chi-square or Fisher's exact test was used to evaluate associations between categorical variables. Wilcoxon rank sum test was used for continuous variables. Kruskal-Wallis test was used to evaluate differences on continuous variables by categorical variables. OS was estimated using the Kaplan-Meier method. Exact *P* values were recorded and $P<0.05$ denotes statistical significance. Multivariate Mantel-Cox regression model was used to evaluate the effect size and statistical significance of each variable which demonstrated $P<0.05$ in univariate analysis. Proportional hazard assumption was confirmed using log-log and residual plots. All statistical analyses were performed using SPSS 24 (IBM Corp., Armonk, NY, USA). Kaplan-Meier curves were generated using GraphPad Prism Version 8 (GraphPad Software, La Jolla California, USA).

4.4 Results

4.4.1 Demographic and clinicopathological characteristics

The median age of patients was 69.1 years (range 32-91), with 75.1% (423/563) being male. Eighty-seven % (490/563) of patients underwent biopsy only, whilst 11.6% (65/563) underwent limited (PD) or extended pleurectomy and decortication (EPD), 0.9% (5/563) underwent extrapleural pneumonectomy (EPP), and 0.5% (3/563) underwent other procedures. Extensive crush and/or cauterisation artefacts occupying more than 50% of tissue area were present in 3.7% of cases (21/563), and in 7.5% (42/563) there were less than 300 but more than 100 viable tumour cells available for evaluation.

Solid-predominant disease (213/563, 37.8%) and necrosis (190/563, 33.7%) were present in a significant minority of patients. The frequencies of nuclear grade I, II, III tumours were 31.4% (177/563), 51.9% (292/563), and 16.7% (94/563) respectively (Figure 4-2A). Of Grade I and grade II tumours 10.2% (18/177) and 26.4% (77/292) respectively were associated with necrosis (Figure 4-2C). For M-N score, 51.5% (290/563) were assigned score of 0, 27.5% (155/563) score of I, and 17.0% (118/563) score of II (Figure 4-2B). Using the 2-tier grading system, 65.0% (366/563) of tumours were of low grade and 35.0% (197/563) were of high grade (Figure 4-2D). Pleomorphic epithelioid PM represented 8.3% (51/614) of the epithelioid PM cohort.

The median OS of the study cohort was 14.7 months (95% confidence interval 12.9-16.4 months). Univariate analysis showed age, type of procedure, solid-predominant growth pattern, and necrosis as predictors of OS (all $P<0.001$) (Table 4-2) (Figure 4-3). Lymphatic and vascular invasion were present in 8.3% (47/563) and 7.5% (42/563) of cases. Neither were statistically significant predictors of survival. Higher incidence of lymphovascular invasion was observed in resected cases (Table 4-3).

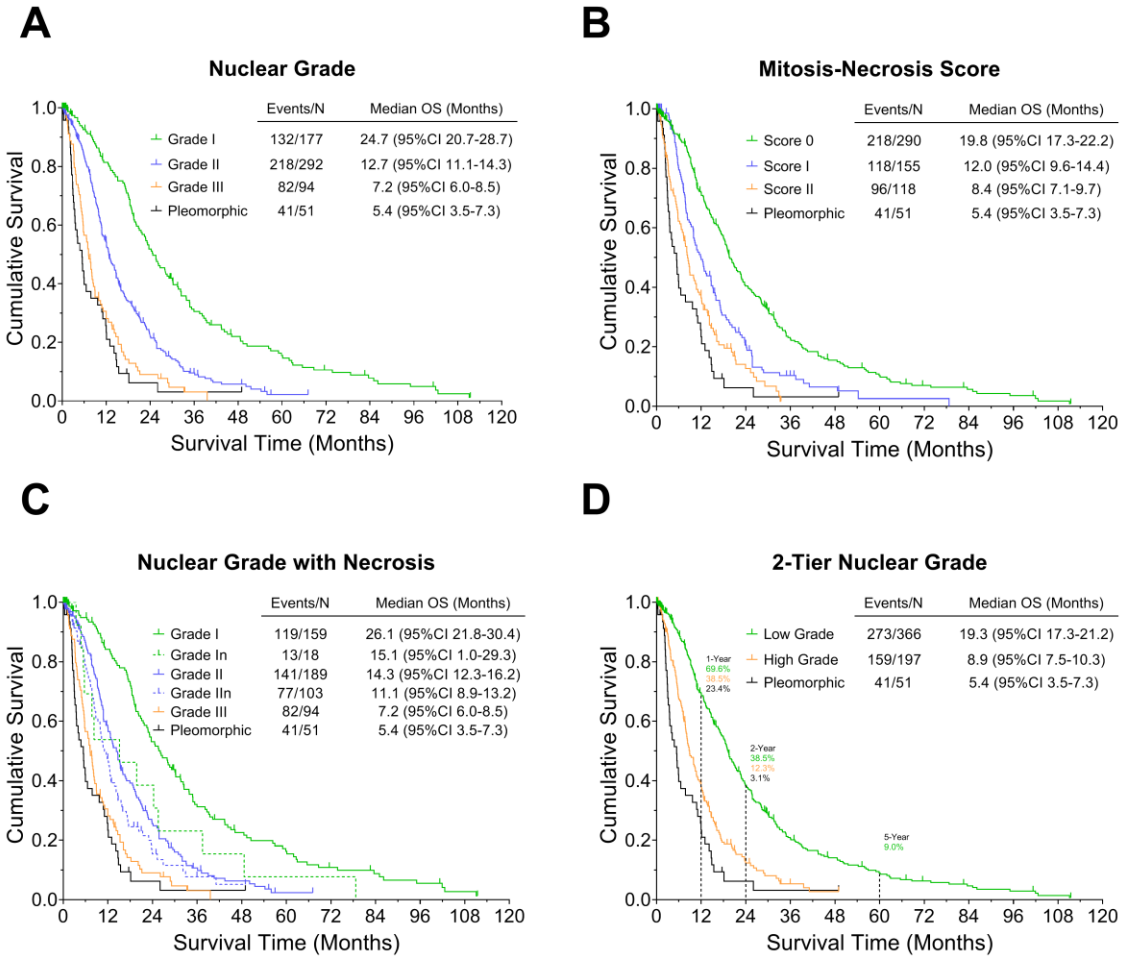


Figure 4-2 Overall survival by 3-tier nuclear grade, mitosis-necrosis score, 3-tier nuclear grade subclassified by the presence of necrosis, and 2-tier nuclear grade. **(A)** Nuclear grade I tumours showed the most favourable survival followed by grades II and III. **(B)** Mitosis-Necrosis score 0 tumours showed the most favourable survival followed by score I and II. **(C)** The presence of necrosis had an adverse prognostic impact on the survival of patients with nuclear grade I and II tumours. **(D)** Low grade tumours showed more favourable survival than high grade tumours.

Variable	Patients (%)	Median OS (months)	*P*
All Patients	563 (100.0)	14.7	-
Age (Years)			
≤65	193 (34.3)	18.2	5.0×10^{-6}
>65	370 (65.7)	12.7	
Sex			
Male	423 (75.1)	14.7	0.469
Female	140 (24.9)	14.7	
Laterality			
Left	227 (40.3)	15.6	0.053 (Left vs right)
Right	331 (58.8)	13.8	
Not documented	5 (0.9)	-	
Procedure			
Biopsy	490 (87.0)	13.2	1.1×10^{-5} *(Biopsy vs resection)*
PD or EPD	65 (11.6)	25.3	
EPP	5 (0.9)	28.4	
Other procedures	3 (0.5)	32.9	
Solid-predominant growth pattern			
Yes	213 (37.8)	10.5	3.9×10^{-7}
No	350 (62.2)	18.0	
Necrosis			
Present	190 (33.7)	9.3	2.8×10^{-11}
Absent	373 (66.3)	18.5	
Lymphatic invasion			
Present	47 (8.3)	16.8	0.169
Absent	516 (91.7)	14.6	
Vascular invasion			
Present	42 (7.5)	15.6	0.303
Absent	521 (92.5)	14.6	

Table 4-2 Univariate analysis in predicting overall survival by clinicopathologic factors.

EPP, extrapleural pneumonectomy; EPD, extended pleurectomy and decortication; PD, pleurectomy and decortication; OS, overall survival.

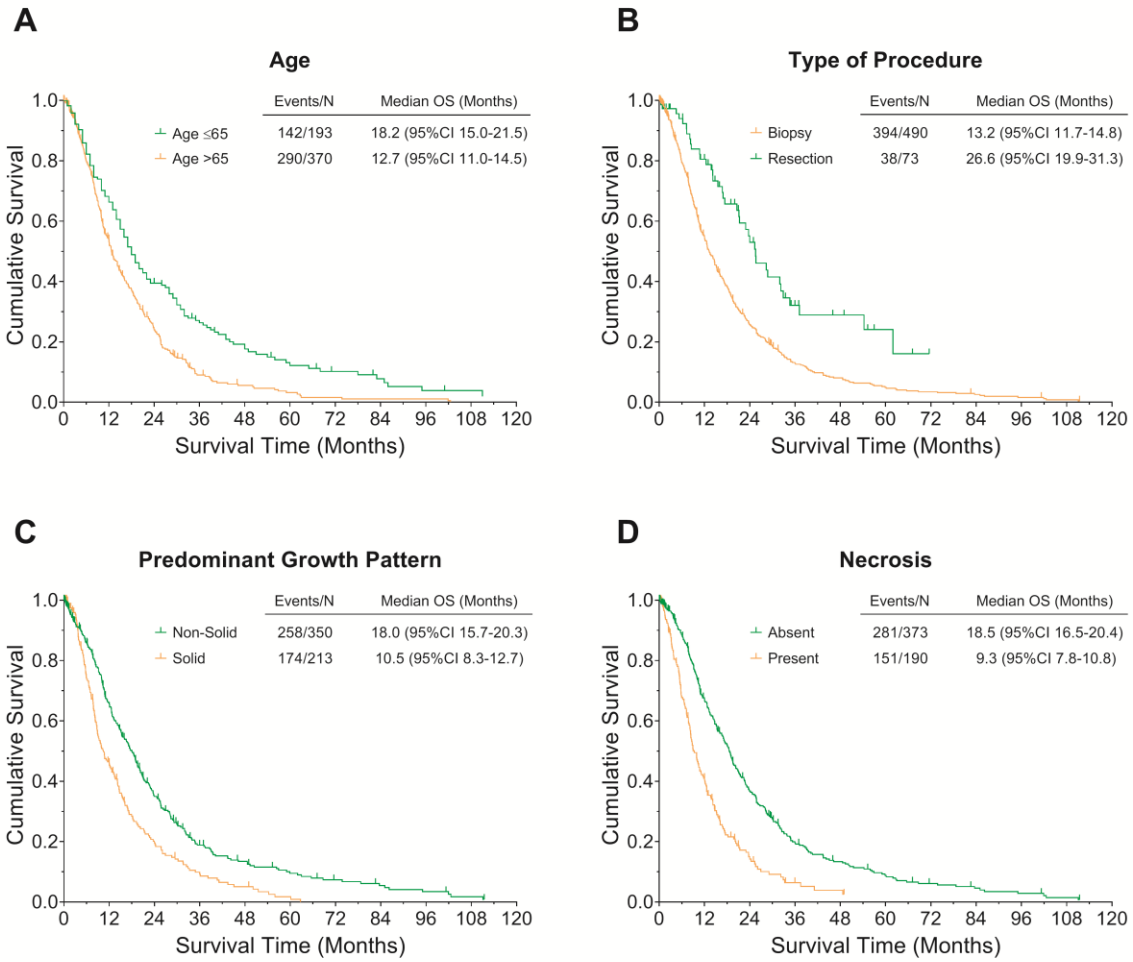


Figure 4-3 Overall survival by clinicopathologic variables. In univariate analysis, **(A)** age≤65 years, **(B)** surgical resection, **(C)** non solid-predominant growth pattern and **(D)** absence of necrosis were favourable prognostic variables.

CI, confidence interval; OS, overall survival.

Procedure	Patients (%)	No Invasion	Lymphatic invasion	Vascular invasion	Both	*P*
Biopsy	490 (87.0)	468 (95.5)	7 (1.4)	10 (2.0)	5 (1.0)	2.9×10^{-39} (Lymphatic)
Resection	73 (13.0)	30 (41.1)	16 (21.9)	8 (11.0)	19 (26.0)	7.7×10^{-25} (Vascular)

Table 4-3 Detection of lymphatic and vascular invasion by type of procedure.

4.4.2 All grading systems predicted survival in univariate and multivariate analyses

Univariate analysis of nuclear features demonstrated nuclear atypia, mitotic count, and atypical mitosis as predictors of OS (all $P<0.001$) (Table 4-4) (Figure 4-4). Nuclear grade I tumours were associated with the most favourable median OS of 24.7 months, followed by grades II (12.7 months) and III (7.2 months) ($P<0.001$) (Figure 4-2A). Pleomorphic epithelioid PM had the worst OS (5.4 months) but was not statistically significant compared with grade III tumours ($P=0.208$). No significant difference in mitotic activity was seen ($P=0.547$) (Figure 4-5). Based upon the M-N scoring system, a score of 0 was associated with the most favourable median OS (19.8 months) followed by score I (12.0 months) and II (8.4 months) ($P<0.001$) (Figure 4-2B). Collectively the findings of this study were congruent with previously published studies.

The presence of necrosis modified the prognosis of nuclear grade I (15.1 months vs 26.1 months, $P=0.053$) and II (11.1 months vs 14.3 months, $P=0.058$) tumours with borderline statistical significance (Figure 4-2C). Subsequently, 2-tier nuclear grade resulted in satisfactory separation in terms of median OS between low grade (19.3 months), high grade (8.9 months) and pleomorphic (5.4 months) E-PM ($P<0.001$) (Figure 2D). The 2-year survival of low grade, high grade and pleomorphic E-PM were 38.5%, 12.3% and 3.1%

respectively. Long term (5-year) survival was seen only in low grade E-PM and represented 9% of the population.

In multivariate analysis, 3-tier nuclear grade, M-N score and 2-tier nuclear grade predicted OS independent of age, type of procedure, necrosis, solid-predominant growth pattern and atypical mitosis (all $P<0.001$ except 2-tier nuclear grade, $P=0.001$) (Table 4-5). The constituents of nuclear grade, namely nuclear atypia and mitotic count were also independent predictors of OS (all $P<0.001$). On the other hand, solid-predominant growth pattern ($P=0.725$) and atypical mitosis ($P=0.640$) did not predict OS independent of age, type of procedure, necrosis, 3-tier nuclear grade and atypical mitosis. Laterality of disease, although demonstrated borderline statistical significance in univariate analysis ($P=0.053$), was not shown to be an independent predictor in multivariate setting ($P=0.977$). Three-tier nuclear grade created a greater degree of prognostic separation among the three grading systems.

Variable	Patients (%)	Median OS (months)	*P*
Nuclear atypia			
Score 1 (Mild)	71 (12.6)	24.3	*Reference*
Score 2 (Moderate)	378 (67.1)	15.5	*0.002*
Score 3 (Severe)	114 (20.3)	7.8	5.3×10^{-12}
			(3 vs 2: 2.4×10^{-11}*)*
Mitotic count			
Score 1 (0-1)	178 (31.6)	23.7	*Reference*
Score 2 (2-4)	182 (32.3)	12.8	3.7×10^{-8}
Score 3 (≥5)	203 (36.1)	10.0	7.9×10^{-18}
			(3 vs 2: 0.002)
Atypical mitosis			
Yes	398 (70.7)	12.0	1.5×10^{-7}
No	165 (29.3)	20.3	
3-tier nuclear grade			
I	177 (31.4)	24.7	*Reference*
II	292 (51.9)	12.7	5.4×10^{-12}
III	94 (16.7)	7.2	1.0×10^{-22}
			(III vs II: 6.6×10^{-7}*)*
Mitosis-Necrosis score			
Score 0	290 (51.5)	19.8	*Reference*
Score I	155 (27.5)	12.0	3.7×10^{-7}
Score II	118 (21.0)	8.4	3.1×10^{-16}
			(II vs I: 0.003)
2-tier nuclear grade			
Low grade	366 (65.0)	19.3	*Reference*
Grade I	159 (28.2)	26.1	-
Grade I with necrosis	18 (3.2)	15.1	-
Grade II	189 (33.6)	14.3	-
High grade	197 (35.0)	8.9	4.5×10^{-16}
Grade II with necrosis	103 (18.3)	11.1	-
Grade III	94 (16.7)	7.2	-

Table 4-4 Univariate analysis in predicting overall survival by nuclear features.

OS, overall survival.

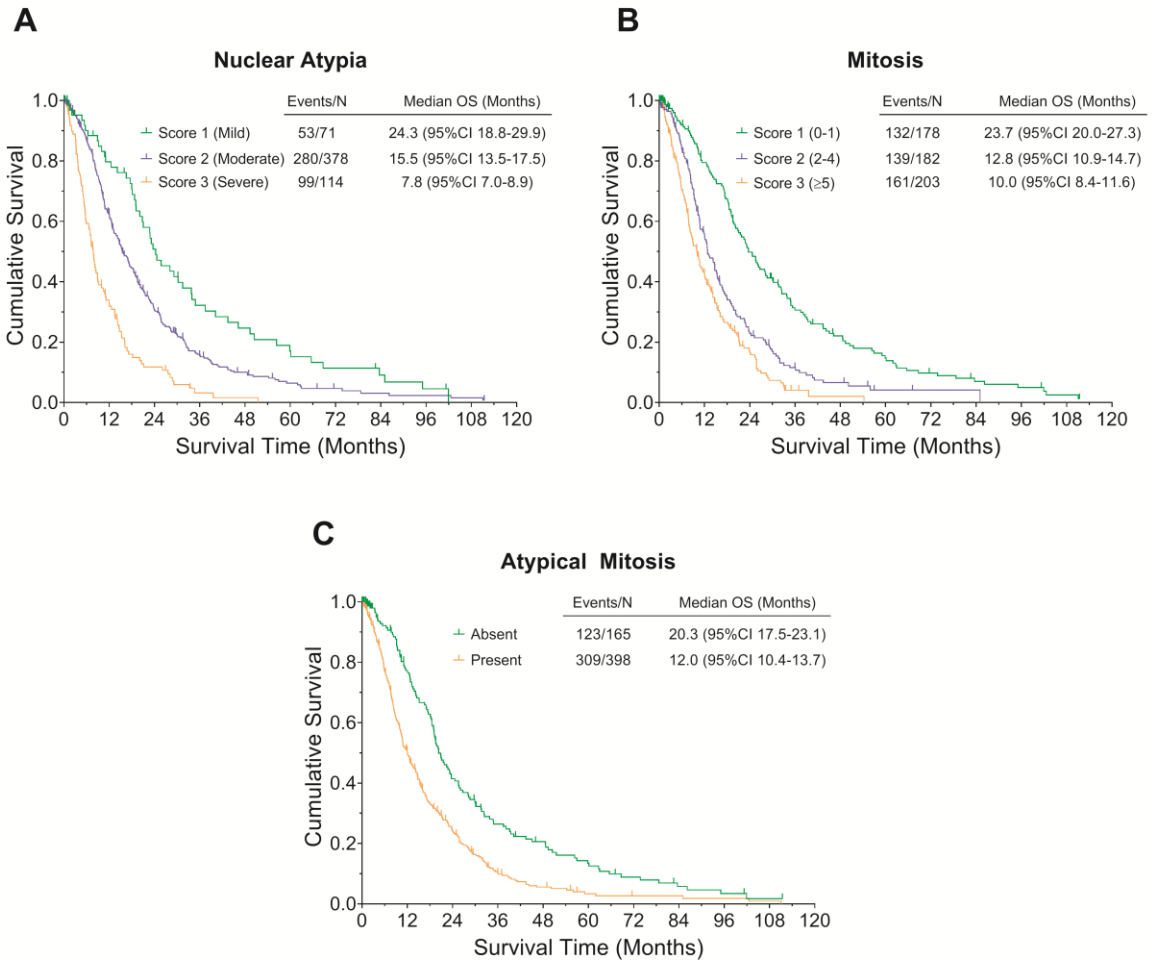


Figure 4-4 Overall survival by nuclear features. **(A)** Nuclear atypia score of 1 was associated with the most favourable prognosis followed by score 2 and 3. **(B)** Mitotic score 1(0-1 per 10 HPF) was associated with the most favourable prognosis followed by score 2 (2-4 per 10 HPF) and score 3 (≥5 per 10 HPF). **(C)** The absence of atypical mitosis was associated with more favourable prognosis.

CI, confidence interval; HPF, high power field; OS, overall survival.

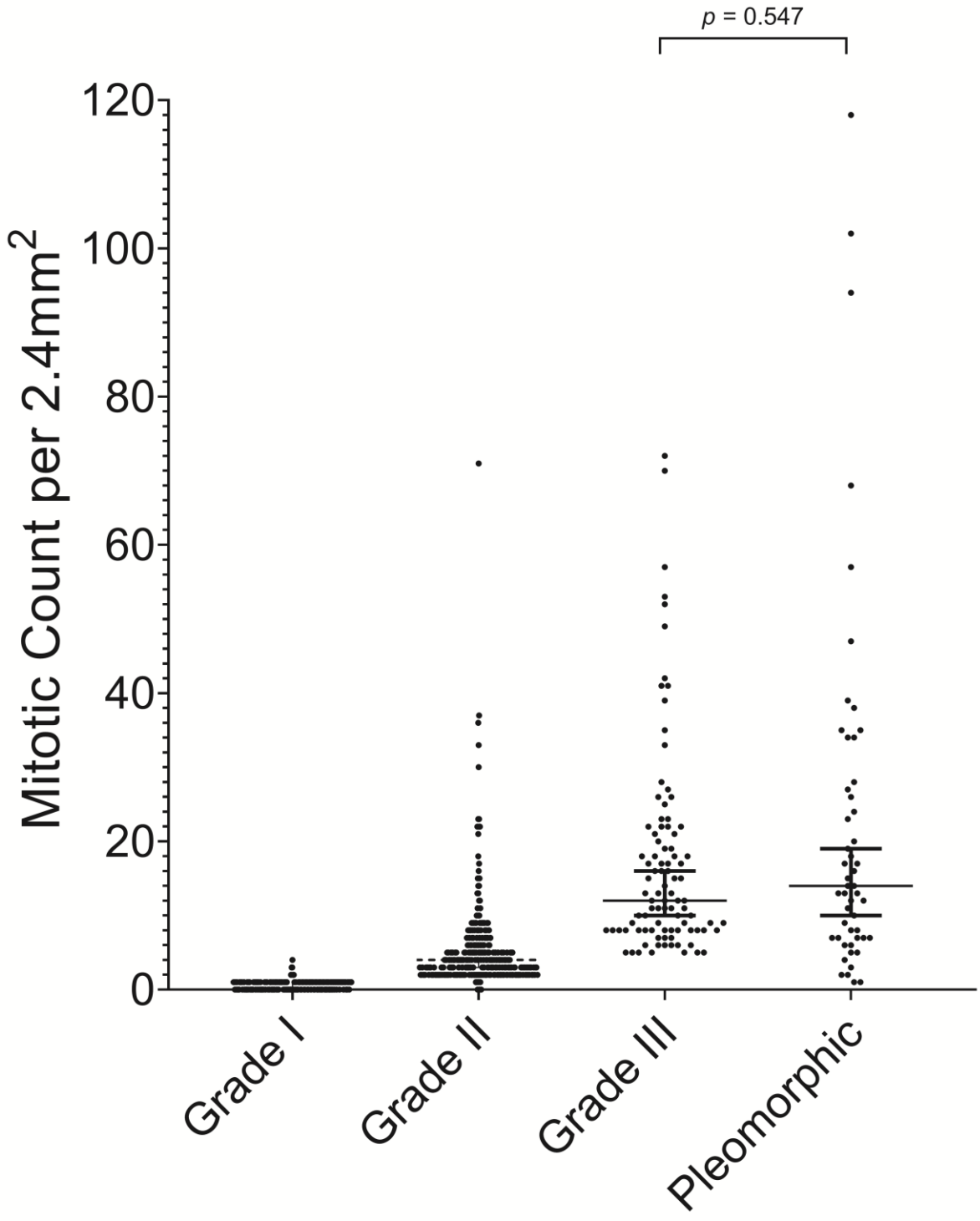


Figure 4-5 Mitotic activity by nuclear grade. No significant difference was found comparing the mitotic activity between nuclear grade III and pleomorphic epithelioid pleural mesothelioma.

Variable	Hazard ratio	95% CI	*P*
Age			
>65 vs ≤65 years	1.46	1.19-1.81	2.8×10^{-4}
Procedure			
Resection vs biopsy only	0.30	0.21-0.43	4.8×10^{-11}
Predominant growth pattern			
Solid vs non-solid	1.03	0.83-1.27	0.799
Necrosis			
Present vs absent	1.72	1.35-2.20	3.9×10^{-5}
Nuclear atypia			
Score 2 vs 1	1.43	1.04-1.95	0.027
Score 3 vs 1	2.29	1.56-3.38	2.8×10^{-5}
Mitotic count			
Score 2 vs 1	2.19	1.55-3.09	4.0×10^{-6}
Score 3 vs 1	3.00	2.07-4.34	2.2×10^{-9}
Atypical mitosis			
Present vs absent	0.95	0.71-1.27	0.713
Nuclear grade			
II vs I	2.56	1.87-3.49	1.6×10^{-9}
III vs I	3.77	2.52-5.63	2.6×10^{-11}
Mitosis-Necrosis score			
I vs 0	1.65	1.22-2.23	0.001
II vs 0	2.38	1.49-3.78	7.1×10^{-5}
2-tier nuclear grade			
High grade vs Low grade	2.02	1.33-3.07	0.001

Table 4-5 Multivariate analysis in predicting overall survival.

CI, confidence interval.

4.4.3 Association between nuclear grade and clinicopathological variables

Next the association between nuclear grade and other clinicopathological variables was explored. Higher nuclear grades were associated with solid-predominant growth pattern, necrosis, atypical mitosis, lymphatic and vascular invasion (all $P<0.001$) (Table 4-6). No association was seen with patient age ($P=0.600$), sex ($P=0.092$), or laterality of disease ($P=0.101$). The strong association between nuclear grade and M-N score reported previously was confirmed ($P=1.6\times10^{-61}$) (Table 4-7).

Interestingly higher nuclear grades were more frequently encountered in larger resection specimens (P=0.003) although the absolute number of cases was small (Table 4-6). Spatial and temporal heterogeneity are recognized but under-investigated phenomena in PM with uncertain implications in disease behaviour and management. Due to the small number of patients undergoing surgical resection after the first biopsy, or receiving sequential biopsies, only an underpowered analysis using our study cohort was possible. Thirty-three biopsy-resection pairs were identified (5.9%), of which 14 were discordant (42.4%). The vast majority (78.6%, 11/14) of tumours were upgraded in the subsequent surgical resection specimen.

Variable	All patients	Grade I	Grade II	Grade III	*P*
All Patients (%)	563 (100.0)	177 (31.4)	292 (51.9)	94 (16.7)	-
Age (years)					
Median	70	71	69.5	70	0.600
Range	32-91	41-88	37-90	32-91	
Sex (%)					
Male	423 (75.1)	137 (77.4)	209 (71.6)	77 (81.9)	0.092
Female	140 (24.9)	40 (22.6)	83 (28.4)	17 (18.1)	
Laterality (%)					
Left	227 (40.3)	80 (45.7)	117 (40.3)	30 (32.3)	0.101
Right	331 (58.8)	95 (54.3)	173 (59.7)	63 (67.7)	
Not documented	5 (0.9)	-	-	-	-
Procedure (%)					
Biopsy	490 (87.0)	169 (95.5)	242 (82.9)	79 (84.0)	*0.003*
PD or EPD	65 (11.6)	7 (4.0)	43 (14.7)	15 (16.0)	
EPP	5 (0.9)	1 (0.5)	4 (1.4)	0 (0.0)	
Other procedures	3 (0.5)	0 (0.0)	3 (1.0)	0 (0.0)	
Solid-predominant growth pattern (%)					
Yes	213 (37.8)	32 (18.1)	120 (41.1)	61 (64.9)	9.6×10^{-14}
No	350 (62.2)	145 (81.9)	172 (58.9)	33 (35.1)	
Necrosis (%)					
Present	189 (33.6)	18 (10.2)	103 (35.3)	69 (73.4)	3.1×10^{-25}
Absent	374 (66.4)	159 (89.8)	189 (64.7)	25 (26.6)	
Lymphatic invasion (%)					
Present	47 (8.3)	1 (0.6)	30 (10.3)	16 (17.0)	4.0×10^{-6}
Absent	516 (91.7)	176 (99.4)	262 (89.7)	78 (83.0)	
Vascular invasion (%)					
Present	42 (7.5)	3 (1.7)	24 (8.2)	15 (16.0)	9.2×10^{-5}
Absent	521 (92.5)	174 (98.3)	268 (91.8)	79 (84.0)	
Atypical mitosis (%)					
Present	398 (70.7)	41 (23.2)	265 (90.8)	92 (97.9)	3.1×10^{-62}
Absent	165 (29.3)	136 (76.8)	27 (9.2)	2 (2.1)	

Table 4-6 Distribution of clinicopathological variables by nuclear grade.

EPP, extrapleural pneumonectomy; EPD, extended pleurectomy and decortication; PD, pleurectomy and decortication.

	Patients (%)	Nuclear Grade				
		Grade I N (%)		Grade II N (%)		Grade III N (%)
		N (+)	N (-)	N (+)	N (-)	
Mitosis-Necrosis Score						
0	290	159 (54.8)		131 (45.2)		
		0 (0.0)	159 (54.8)	0 (0.0)	131 (45.2)	0 (0.0)
I	155	18 (11.6)		111 (71.6)		
		18 (11.6)	0 (0.0)	53 (34.2)	58 (37.4)	26 (16.8)
II	118	0 (0.0)		50 (42.4)		
		0 (0.0)	0 (0.0)	50 (42.4)	0 (0.0)	68 (57.6)

Table 4-7 Association between nuclear grade and mitosis-necrosis score.

N, necrosis; (+), present; (-), absent.

4.4.4 Impact of small biopsies on the performance of grading systems

In the study cohort, the median number of sites sampled was one (range 1-20) with median maximum tissue dimensions of 17mm for biopsies (range 2-140mm) and 150mm for resections (range 40-350mm) (Figure 4-6A). Of all biopsies 19.4% (95/490) were taken from a single site with a maximum dimension of less than or equal to 10mm (median: 8mm), of which 10.5% (10/95) were estimated to contain between 100 and 300 viable tumour cells.

All three grading systems independently predicted survival in single site biopsy setting (all $P<0.05$), but with reduced degree of separation (Table 4-8). Restraining the analysis to, both 3-tier nuclear grade and M-N score did not discriminate survival differences between nuclear grade II versus III ($P=0.468$) (Figure 4-7A) or score I versus II respectively ($P=0.175$) (Figure 4-7B). Although predictive in univariate analysis ($P=9.8\times10^{-5}$) (Figure 4-6B), 2-tier nuclear grade was not shown to be an independent predictor of OS in the multivariate setting ($P=0.572$) (Table 4-9). Single site and ≤10mm scenario should be considered as the minimum

tissue requirement for nuclear grade assessment. Age was the only statistically significant predictor in such circumstance (P=0.002).

Finally, the relationship between the sampling parameters and the performance of the 2-tier nuclear grade was investigated. The pleomorphic E-PM cohort was included in this part of analysis. A grade-shift phenomenon was observed, where more high-grade diseases were detected as maximum dimension increased (P=0.017) (Figure 4-6C) or more anatomical sites being sampled (P=2.2×10^{-4}) (Figure 4-6D). The relatively small number of cases with 3 or more sampled sites or ≥30mm in maximum dimension in the study cohort did not permit more extensive analysis. Using hazard ratio and P values derived from multivariate analysis as surrogates, the incremental improvement in the performance plateaued out when the number of sampled sites and maximum tissue dimension reached 3 or 20mm respectively (Figure 4-8). This as the optimal sampling standard based on available evidence is therefore proposed by this present study.

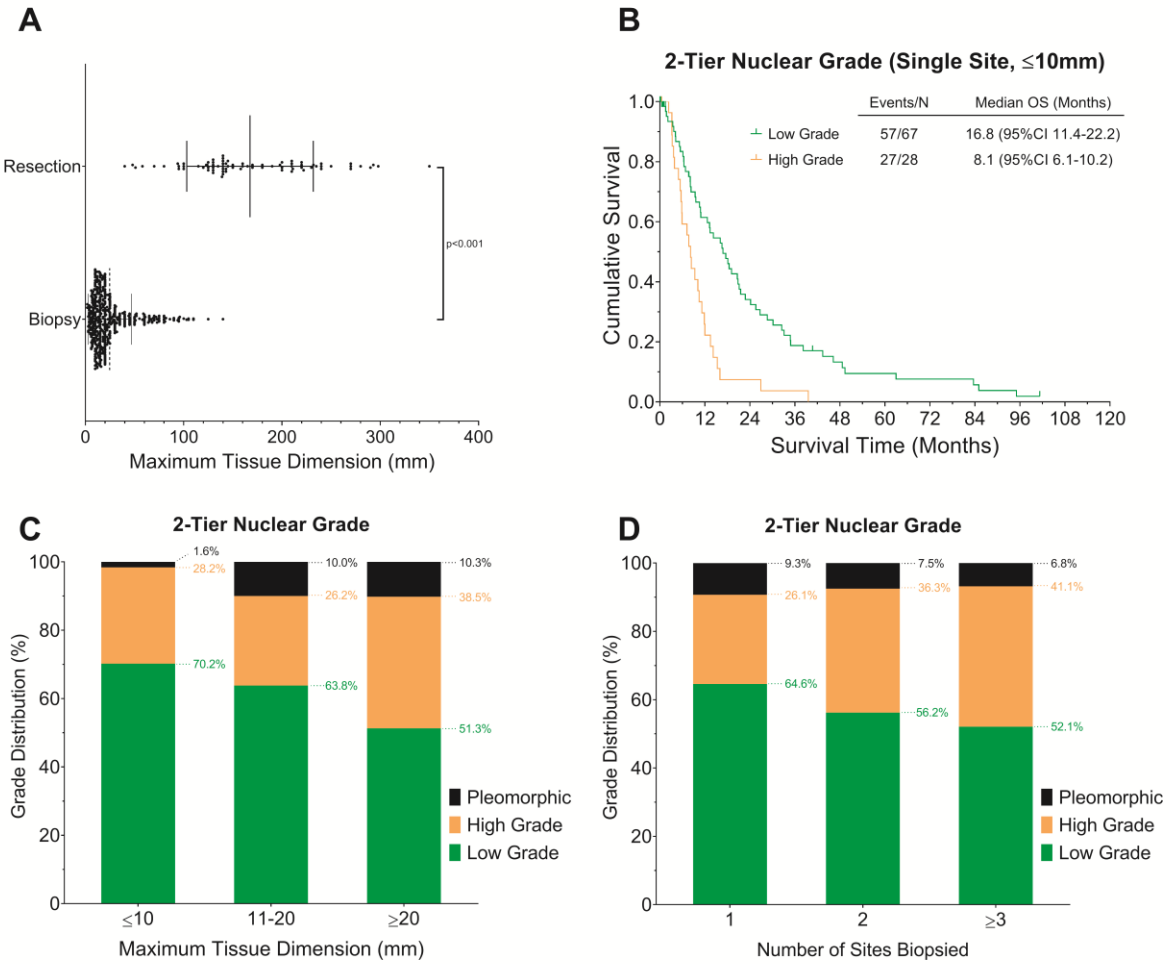


Figure 4-6 Utility of nuclear grade in small biopsy setting. **(A)** The distribution of maximum tissue dimension between biopsies and resections. **(B)** 2-tier nuclear grade, **(C)** high grade diseases were detected at higher frequencies with increasing maximum tissue dimension. **(D)** high grade diseases were detected at higher frequencies with more sites being sampled.

Variable	Hazard ratio	95% CI	*P*
Age			
>65 vs ≤65 years	1.31	0.97-1.77	0.074
Predominant growth pattern			
Solid vs non-solid	1.17	0.88-1.55	0.290
Necrosis			
Present vs absent	2.13	1.51-3.01	2.8×10^{-5}
Nuclear atypia			
Score 2 vs 1	1.55	1.01-2.40	*0.046*
Score 3 vs 1	2.23	1.31-3.81	*0.003*
Mitotic count			
Score 2 vs 1	2.09	1.31-3.32	*0.002*
Score 3 vs 1	2.80	1.67-4.67	8.7×10^{-5}
Atypical mitosis			
Present vs absent	0.83	0.57-1.21	0.333
Nuclear Grade			
II vs I	2.26	1.52-3.37	5.8×10^{-5}
III vs I	3.04	1.77-5.20	5.1×10^{-5}
Mitosis-Necrosis score			
I vs 0	2.04	1.36-3.04	*0.001*
II vs 0	2.08	1.10-3.95	*0.025*
2-tier nuclear grade			
High grade vs Low grade	1.87	1.11-3.14	*0.019*

Table 4-8 Multivariate analysis in predicting overall survival (single site biopsy).

CI, confidence interval.

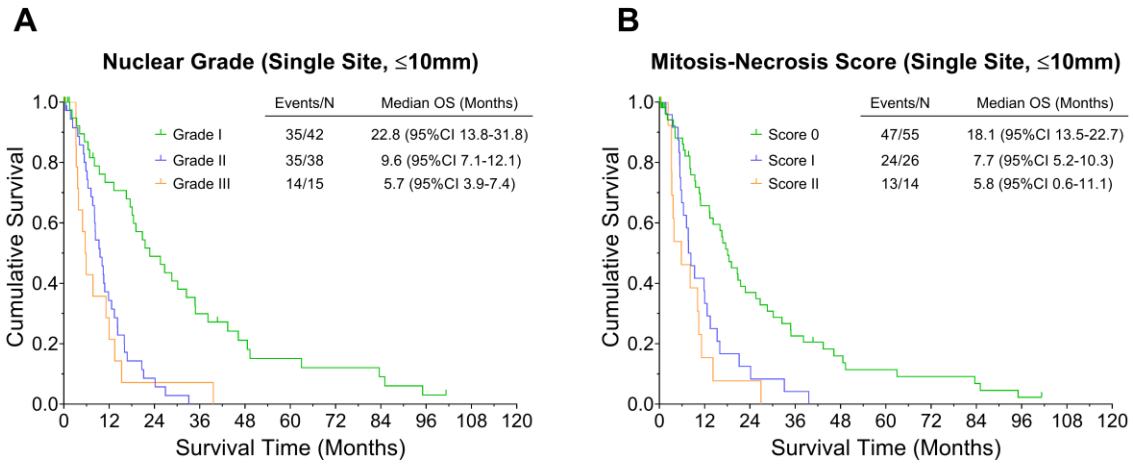


Figure 4-7 Performance of 3-tier nuclear grade and mitosis-necrosis score in single site, ≤10mm setting. **(A)** 3-tier nuclear grade discriminated survival difference between grades I and II/III, but not between grades II and III. **(B)** M-N score discriminated survival difference between scores 0 and I/II, but not between grades I and II.

CI, confidence interval; OS, overall survival.

Variable	Hazard ratio	95% CI	*P*
Age			
>65 vs ≤65 years	2.30	1.34-3.94	*0.002*
Predominant growth pattern			
Solid vs non-solid	1.34	0.80-2.25	0.267
Necrosis			
Present vs absent	1.55	0.67-3.55	0.303
Nuclear atypia			
Score 2 vs 1	1.62	0.80-3.27	0.182
Score 3 vs 1	2.99	1.23-7.27	*0.015*
Atypical mitosis			
Present vs absent	1.31	0.76-2.26	0.339
2-tier nuclear grade			
High grade vs Low grade	1.27	0.55-2.92	0.572

Table 4-9 Multivariate analysis in predicting overall survival (single site biopsy, maximum dimension ≤10mm).

CI, confidence interval.

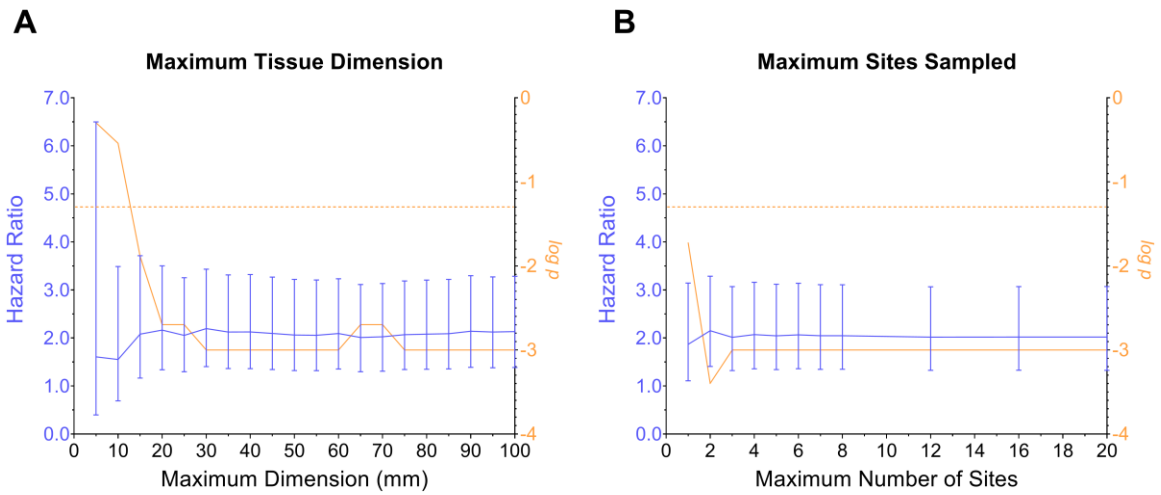


Figure 4-8 Association between hazard ratio/*P* values (2-tier nuclear grade) and maximum tissue dimension/number of sites sampled. **(A)** Hazard ratio reached plateau with maximum tissue dimension of 20mm. **(B)** Hazard ratio reached plateau with three or more sites being sampled. Error bar denotes 95% confidence interval and the dotted orange line denotes *P*=0.05 (log transformed).

4.5 Discussion

In this study the utility of epithelioid PM grading systems proposed by other groups has been validated using a biopsy-heavy cohort (Kadota *et al*., 2012b) (Rosen *et al*., 2018). The demographic and clinicopathologic characteristics of the study cohort were comparable, in terms of patient sex, proportion of tumours with solid-predominant growth pattern and necrosis (Table 4-10). The mean age of the patient population (69.1 years), although slighter older in comparison, is expected of the UK mesothelioma population (74.8 years) according to the latest UK national cancer audit (National Mesothelioma Audit, 2018). The incidences of lymphatic and vascular invasion were lower than earlier report (44% and 23%) (Kadota *et al*., 2011) and it is hypothesised that this was due to the limited nature of biopsy materials.

Both 3-tier and 2-tier nuclear grades in the present study served as independent predictors of OS. A key question thereby arises: which grading system should be adopted in clinical practice for patient stratification in future basic and clinical research? From this study's data, each have their strengths and weaknesses. The 3-tier nuclear grade demonstrates superior discriminative power. Inter-observer agreement between grade III tumour and pleomorphic epithelioid PM could however be of concern, with the latter reported to represent 8-15% of the epithelioid subtype (Alchami *et al*., 2017) (Kadota *et al*., 2011). Although Rosen *et al*. validated the grading systems as a multi-institutional study and their findings were suggestive of inter-departmental consistency, study specifically investigating the inter-observer agreement in the assignment of nuclear grade, together with the potential patterns of disagreement, is lacking. This should be a focus of future research using an adequately powered cohort. The 2-tier nuclear grade is simpler to implement by segregating patients into two groups instead of three, and allows better separation from pleomorphic epithelioid PM. It also facilitates clinical studies by condensing the number of patient categories, hence improving the statistical power. This could be of practical relevance as PM is a relatively rare malignancy with a difficult to recruit patient population, at least in part due to its aggressive clinical phenotypes. It is worth mentioning that the M-N score confers the advantage of eliminating the potential caveat of inter-observer disagreement concerning nuclear atypia, nonetheless the diagnosing pathologist is still required to perform whole slide screening to identify the area for precise mitotic count. Overall, the data from this study adds to the evidence base for guideline development in this regard.

The guidance on the optimal number and size of biopsies available from major guideline committees and international expert consortia is currently a topic of high priority relevant for both pathologists and operators. It is not uncommon for pathology departments to receive materials taken from a single anatomical site that measured ≤10mm. In this setting and in this

study all grading systems lost predictive value in multivariate analysis. This is not a high hurdle to reach in routine diagnostic practice therefore it should not be interpreted as the optimal standard but an absolute minimum requirement. In the present study cohort the performance of the 2-tier nuclear grade started to plateau out with 3 sampled sites or a maximum tissue dimension of 20mm. Below such cut-off values, there was a clear benefit in obtaining more tissue in terms of detecting high grade disease. Such notion was supported by the results from a previous study of 305 cases of biopsy-EPP where more non-epithelioid disease was detected in the resection specimen (Bueno *et al*., 2004). The present study findings however have to be interpreted with caution as there was likely operator bias i.e. the biopsies were not taken in blind fashion. Specimen size is a parameter that could be easily measured or estimated during sampling procedures. A recommendation of sampling three sites in a recent consensus paper (Nicholson *et al*., 2020) is an important step forward, however many questions remain. Future audits are advocated as are prospective cohort studies across centres to compare best practice versus a more extensive sampling protocol, whether it is of random, template or targeted nature. In the long term these studies will prove pivotal in addressing the correlation between diagnostic adequacy, including but not limited to grading, and the number and size of diagnostic specimens.

The present study has several major weaknesses. Firstly, it is retrospective in nature, with incomplete data on staging and treatment including chemotherapy. Secondly, it was not possible to compare the discriminative power of the grading systems with TNM staging due to incomplete data. It could only be inferred from results reported by Kadota *et al*. (Kadota *et al*., 2012b) where the difference in OS between Stage I and IV disease was 7 months hence TNM staging is a weaker prognostic variable than any of the grading systems. The future work includes the association of nuclear grade with growth patterns (Kadota *et al*., 2011), cytological variants (Ordóñez, 2013) (Ordóñez, 2012a) (Ordóñez, 2012b), stromal features

(Klebe *et al*., 2010) (Galateau-Sallé *et al*., 2007) and genomic signatures via deep sequencing using a mesothelioma-specific gene panel.

	RBH (This study)	**Kadota *et al*., 2012**	**Rosen *et al*., 2018**
n	563	232	776
Age (Mean, range)	69 (32-91)	64 (29-85)	65 (29-91)
Sex (M:F)	75:25	72:28	79:21
Procedure			
Biopsy	490/563 (87.0%)	11/232 (4.8%)	206/776 (26.5%)
PD or EPD	65/563 (11.6%)	91/232 (39.2%)	269/776 (34.7%)
EPP	5/563 (0.9%)	130/232 (56.0%)	253/776 (32.6%)
Others	3/563 (0.5%)	0/232 (0%)	48/776 (6.2%)
No. of H&E sections reviewed	3.9 (1-47)	9 (1-43)	3 (2-20)
Solid-predominant growth pattern	213/563 (37.8%)	n/a	275/776 (35.4%)
Necrosis	190/563 (33.7%)	n/a	235/776 (30.3%)
Nuclear grade, median OS (months)			
Grade I	24.7	28	27
Grade II	12.7	14	14
Grade III	7.2	5	8
Mitosis-Necrosis score, Median OS (months)			
0	19.8	-	22
I	12.0	-	14
II	8.4	-	10

Table 4-10 Comparison of demographic and clinicopathologic characteristics with previous studies.

RBH, Royal Brompton and Harefield Hospitals; M, male; F, female; PD, pleurectomy and decortication; EPD, extended pleurectomy and decortication; EPP, extra-pleural pneumonectomy; H&E, hematoxylin & eosin; OS, overall survival.

4.6 Conclusion

An independent external validation of the 3-tier nuclear grading system for epithelioid PM, and the derived 2-tier grading system using a large, biopsy-heavy cohort has been performed. In addition, a minimum sampling standard for accurate evaluation of nuclear grade is proposed.

5 Refining the Classification of Epithelioid Pleural Mesothelioma: Pleomorphic Features and Architectural Growth Patterns

This chapter has been presented in part as a platform presentation at the World Conference on Lung Cancer, 7th-10th September 2019 (Barcelona, Spain), platform presentation at the British Thoracic Society Winter Meeting, 4th-6th December 2019 (London) and published as Zhang YZ, Brambilla C, Molyneaux PL, Rice A, Robertus JL, Jordan S *et al*. Presence of pleomorphic features but not growth patterns improves prognostic stratification of epithelioid malignant pleural mesothelioma by 2-tier nuclear grade. *Histopathology*. 2020;77:423-436. doi: 10.1111/his.14127.

5.1 Abstract

Background: Nuclear grade has been recently validated as a powerful prognostic tool in epithelioid pleural mesothelioma (E-PM). Other histological parameters including pleomorphic features and growth patterns have however also been shown to exert prognostic impact based on published literature. The primary aims of this study were (1) to externally validate the prognostic utility of pleomorphic features in E-PM and (2) to investigate if the evaluation of growth pattern improves the prognostic stratification by 2-tier nuclear grade.

Materials and methods: This was a follow-up study of the previous study on nuclear grading systems (Chapter 4) and included 614 consecutive cases of E-PM from RBH over a period of 15 years, including 51 with pleomorphic features. Growth patterns were subclassified by predominant and secondary patterns, as well as high-grade (solid, micropapillary) and low-grade (all others). The prognostic significance of cribriform and well differentiated papillary mesothelial tumour (WDPMT)-like features, and intratumoural heterogeneity of growth patterns were also assessed.

Results: E-PM with pleomorphic features showed significantly worse overall survival compared to those without (5.4 months vs 14.7 months). Tumours with predominantly micropapillary pattern showed the worst survival (6.2 months) followed by solid (10.5 months), microcystic (15.3 months), discohesive (16.1 months), trabecular (17.6 months) and tubulo-papillary (18.6 months). Sub-classification of growth patterns into high- and low-grade led to good separation of overall survival (10.5 months vs. 18.0 months) but did not predict survival independent of 2-tier nuclear grade. A composite score comprised of growth pattern and 2-tier nuclear grade did not improve prognostication compared with nuclear grade alone. Intra-tumoural heterogeneity in growth patterns is ubiquitous.

Conclusions: The findings of the study support incorporating E-PM with pleomorphic features in the epithelioid subtype as a highly aggressive variant distinct from 2-tier nuclear grade. E-PM demonstrate extensive heterogeneity in growth patterns but they do not offer additional prognostic utility to 2-tier nuclear grade, hence the latter remains as the principal prognostic variable in this subtype.

5.2 Introduction

Epithelioid pleural mesothelioma (E-PM) morphologically resembles primary thoracic epithelioid malignancies, particularly non-small cell lung cancer, with the propensity to form diverse growth patterns (Nicholson *et al*., 2020) (Husain *et al*., 2018) (Travis *et al*., 2015). The prognostic impact of these patterns has been investigated in a limited number of studies (Alchami *et al*., 2017) (Brčić *et al*., 2014) (Kadota *et al*., 2011) although none have fully explored the relationship with nuclear grade, the latter having only been recently proposed and validated as a robust prognostic tool (Nicholson *et al*., 2020) (Zhang *et al*., 2020a) (Rosen *et al*., 2018) (Kadota *et al*., 2012b). Data is also lacking in relation to the extent of intratumoural heterogeneity of growth patterns and the association with nuclear grade and prognosis.

Distinct from pattern- and nuclear grade-based classification, E-PM with pleomorphic features (also termed pleomorphic E-PM) has been reported as an uncommon and aggressive variant of pleural mesothelioma (Alchami *et al*., 2017) (Brčić *et al*., 2014) (Kadota *et al*., 2011) (Ordóñez, 2012c) (Galateau-Sallé *et al*., 2010). The incidence is between 5.1-14.7% of all E-PM, and median OS has been reported between 3.0-8.1 months. The relatively small number of published cases (103 cases over the past 10 years) poses challenges to the ongoing debate within major guideline committees in relation to its classification, as to whether it should be regarded as a variant under the epithelioid or non-epithelioid subtype.

The primary aims of this study were (1) independent validation of the prognostic impact of pleomorphic features in E-PM and (2) evaluation of the prognostic impact of growth patterns, alone or in combination with 2-tier nuclear grade. The secondary aims of the study were (1) characterisation of intratumoural heterogeneity of growth patterns by assessing predominant and co-existing secondary patterns, (2) investigation of the association of growth patterns

with 2-tier nuclear grade and (3) evaluation of the prognostic impact of three growth patterns in addition to those currently recognised, and which were identified during review of the cohort: discohesive, cribriform and well differentiated papillary mesothelial tumour (WDPMT)-like features (Churg *et al*., 2014).

5.3 Materials and methods

5.3.1 Study population

A previously characterised cohort (Chapter 4) (Zhang *et al*., 2020a) of 614 consecutive cases of histologically confirmed epithelioid PM between 2003 and 2017 from RBH was used for this study. Institutional review board (IRB) approval was obtained. Additional outcome data were retrieved from the National Health Service Spine repository, surgical records and regional thoracic oncology service. Median OS was defined as time (measured in months) between the date of initial procedure from which a definitive diagnosis of PM was made, and the date of death or last follow-up. Censor fraction in our study was 23% (141/614).

5.3.2 Microscopy and imaging

Microscopic assessment was performed using a Nikon Eclipse Ci-L microscope (Nikon Corporation, Japan) with a field area measuring 0.24mm^2 per high power field (HPF, ×400 magnification). Microscopic images in 300dpi TIF format were taken from representative cases using a Nikon Digital Sight DS-L3 camera (Nikon Corporation, Japan), annotated using GNU Image Manipulation Program 2.10.10 (http://www.gimp.org, retrieved on 20.04.2019).

5.3.3 Histopathological assessment

All cases were diagnosed by at least one specialist pulmonary pathologist using current histological and immunohistochemical criteria (Nicholson *et al*., 2020) (Husain *et al*., 2018) (Travis *et al*., 2015). Histopathological parameters were assessed and recorded independently, blinded to outcome. All available haematoxylin and eosin (H&E) sections were reviewed, with an average of 3.9 sections per case (range: 1-47). Recognised growth patterns (solid, tubulo-papillary, trabecular, micropapillary, microcystic) and the presence of pleomorphic features were evaluated using the current diagnostic criteria. A minimum of 10% of tumour cells exhibiting severe nuclear atypia was required for the diagnosis of E-PM with pleomorphic features. For this reason, in this study, it was not regarded as a growth pattern. Representative images illustrating the growth patterns are shown in Figure 5-1.

Discohesive pattern was considered morphologically distinct from micropapillary pattern that tumour cell clusters consisted of no more than three cells, and frequently manifest as a single cell infiltration in variable stroma. Cribriform pattern was defined as tumours with atypical glandular architecture, either sieve-like or showing distorted back-to-back glands. It resembles the cribriform pattern seen in lung adenocarcinoma (Moreira *et al*., 2014), but without the nuclear attenuation to distinguish it from the microcystic pattern (Table 5-1) (Figure 5-2). WDPMT-like was defined using published criteria for WDPMT (Table 5-2) (Churg et al., 2014), after a *de novo* pleural WDPMT had been ruled out and unequivocal subpleural invasion confirmed histologically (Figure 5-3).

Predominant pattern was defined as the most abundant pattern, usually but not always representing >50% of the entire tumour. Secondary pattern was defined as any non-predominant pattern representing at least 5% of the tumour. All secondary patterns were recorded. High-grade pattern was defined by us, as no prior study has done so, as solid or micropapillary patterns, and low-grade pattern was defined as any other pattern. A pattern-nuclear grade composite score was generated by giving one point to predominant growth

pattern (high-grade = 1, low-grade = 0) and nuclear grade (high-grade = 1, low-grade = 0) then adding both components to give a total score 0-2.

Data on patient age, surgical resection, 2-tier nuclear grade, necrosis and atypical mitosis, which were established as independent predictors of OS in E-PM, were extracted from the previous study (Chapter 4).

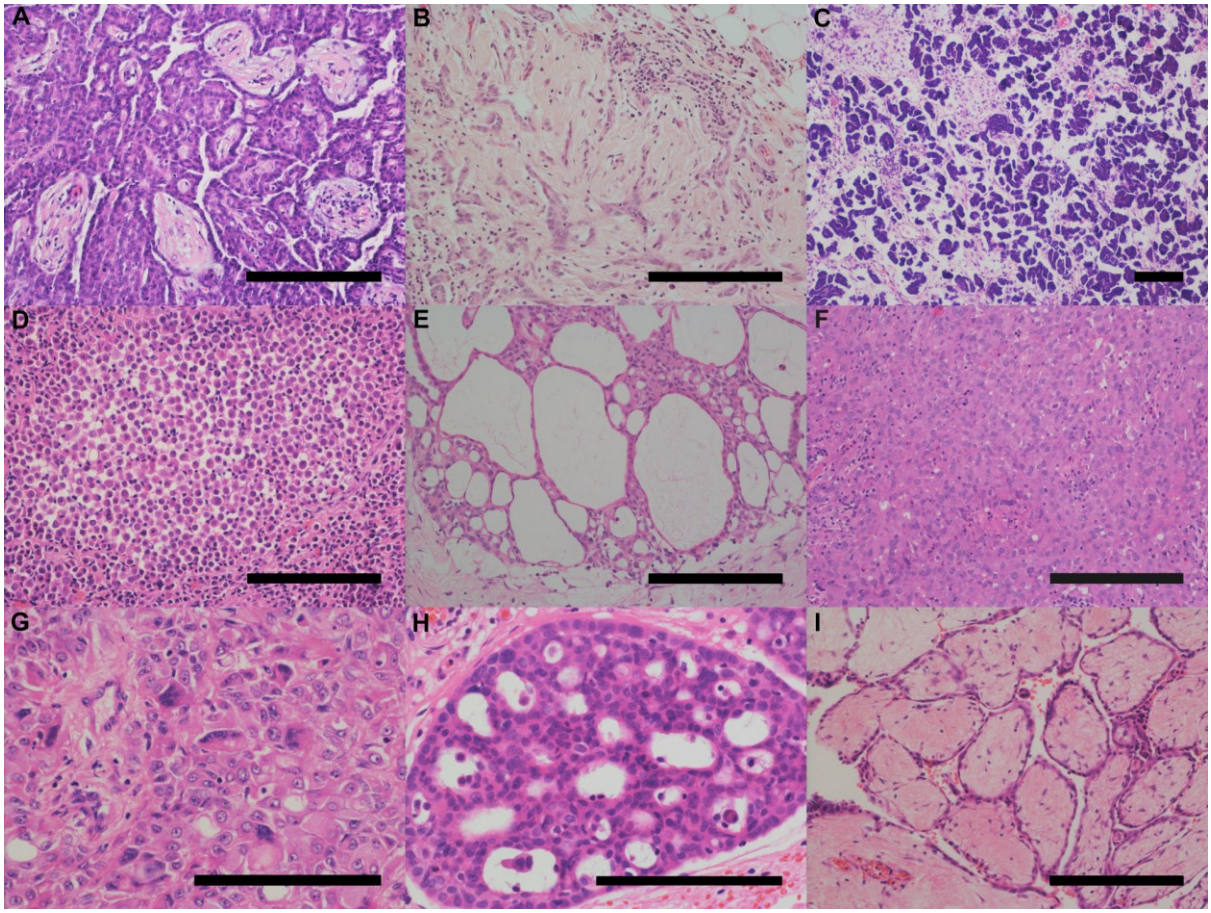


Figure 5-1 Growth patterns of epithelioid pleural mesothelioma. **(A)** Tubulo-papillary growth pattern: this is a combination of frequently co-existing tubular (single luminal spaces allowing branching) and papillary patterns. **(B)** Trabecular growth pattern: tumour cells are arranged in linear fashion with or without formation of arcades. **(C)** Micropapillary growth pattern: this pattern resembles "miniaturised" papillary pattern but without its fibrovascular

core. In the context of the present study, a group of ≥4 cells was required to fulfil the diagnostic criteria. **(D)** Discohesive growth pattern: tumour cells frequently show single cell infiltration, occasionally forming clusters of no more than 3 cells. **(E)** Microcystic growth pattern: tumour cells with attenuated nuclei form dilated glandular spaces. These can be nested so give rise to sieve-like appearance. This pattern was also known as adenomatoid growth pattern given the resemblance to adenomatoid tumours **(F)** Solid growth pattern: tumour cells form cohesive nests and nodules without discernible architecture. **(G)** Pleomorphic features. Tumour cells exhibit severer atypia with features including multinucleation and tumour giant cells **(H)** Cribriform growth pattern: the lack of intervening stroma and nuclear attenuation distinguish it from tubulo-papillary and microcystic patterns respectively. **(I)** WDPMT-like features: the image shows an area indistinguishable from WDPMT but there was unequivocal invasion elsewhere in the tumour (Figure 5-3).

Scale bar = 100μm.

Histological features	**Cribriform**	**Tubular**
Architecture	Nodular, sieve-like or Back-to-back glands (Fusion)	Single lumen Allows “branching”
Intervening stroma	Absent	Present
Nuclear grade	Any	Any
Attenuated nuclei	Absent	Absent

Table 5-1 Assessment criteria for cribriform growth pattern.

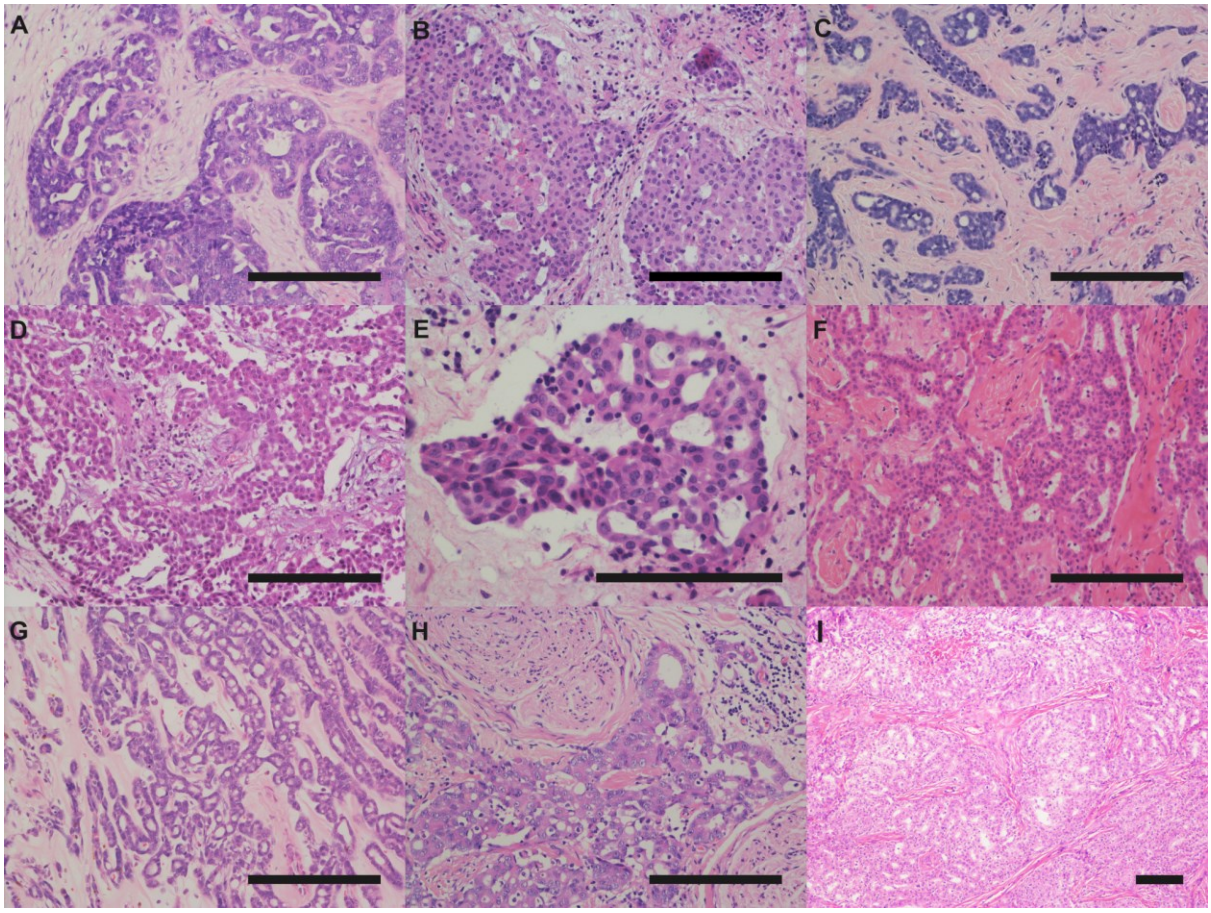


Figure 5-2 Cribriform growth pattern in epithelioid PM.

Two types of cribriform growth patterns were identified. In the first type, the atypical glandular architecture exhibits sieve-like appearance but without nuclear attenuation and gland dilatation seen in microcystic growth pattern **(A, B, E, I)**. In the second type, there are back-to-back glands with distortion **(C, D, F, H)**, occasionally arranged in "string-of-beads" fashion **(G)**. In both types, no intervening stroma was present within the architecture.

Scale bar = 100μm.

Histological features	WDPMT-like	Papillary
Architecture	Broad, well-formed papillae Allows "compression crowding"	Variable sizes & shapes "Budding"‡
Stroma	Myxoid	Fibrovascular ± Hyalinisation
Nuclear stratification	Absent (Single layer)	Usually present
Nuclear grade	Low-grade (Grade I)	Any
Mitotic count †	Typically absent	Any

Table 5-2 Assessment criteria for WDPMT-like features.

† Per 10 HPF.

‡ This refers to the arborising morphology commonly seen in this growth pattern, where smaller and variably sized papillae stem out from larger papillae. The budding appearance is usually better appreciated with cross-sectioning.

HPF, high power field; WDPMT, well differentiated papillary mesothelial tumour.

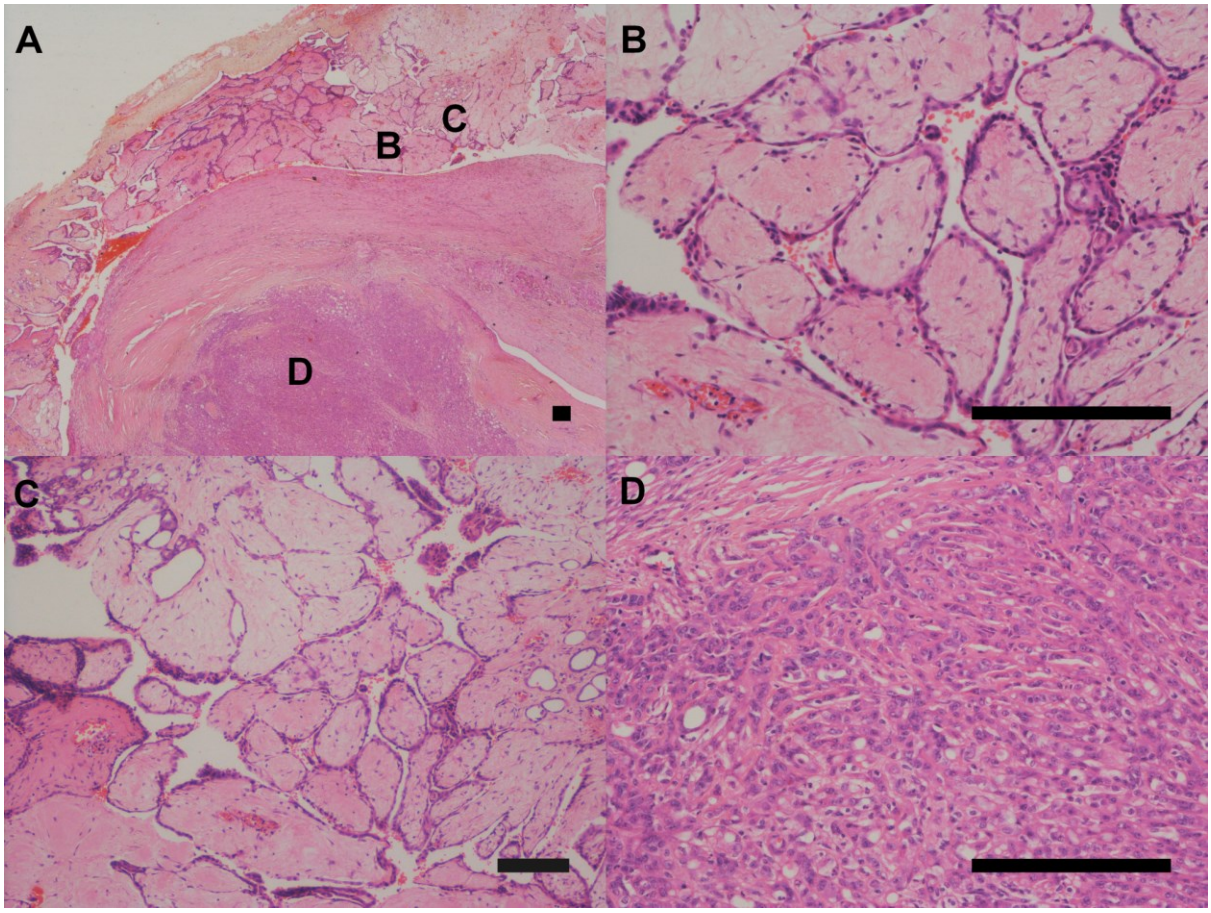


Figure 5-3 Example of invasive E-PM with WDPMT-like features.

(A) At low power magnification (×20), the area of WDPMT-like features, towards the surface of the sample, is clearly separated from the unequivocally invasive component with predominantly solid growth pattern. **(B)** WDPMT-like feature (×200) indistinguishable from WDPMT under this magnification. **(C)** There are adjacent invasive foci with predominantly microcystic growth pattern. **(D)** Solid-predominant growth pattern with focal tubulo-papillary pattern, set in a fibrotic stroma.

Scale bar = 100μm.

5.3.4 Statistical analysis

Descriptive statistics were employed to analyse the baseline demographic and clinicopathological parameters. Two-sided chi-square or Fisher's exact test was used to evaluate associations between categorical variables. Kruskal-Wallis test was used to evaluate differences on continuous variables by categorical variables. OS was estimated using the Kaplan-Meier method. Exact *P* values were recorded and $P<0.05$ denotes statistical significance. Multivariate Mantel-Cox regression model was used to evaluate the effect size and statistical significance of each variable which demonstrated $P<0.05$ in univariate analysis and with ≥10 events. All statistical analyses were performed using SPSS 26 (IBM Corp., Armonk, NY, USA). Kaplan-Meier curves were generated using GraphPad Prism Version 8 (GraphPad Software, La Jolla California, USA).

5.4 Results

5.4.1 Demographic and clinicopathological characteristics

The median age of patients was 70 years (range 32-91) with 75.6% (464/614) being male and 57.8% (355/614) having right-sided disease. Eighty-seven % (534/614) of patients, with no documented history of neoadjuvant therapy, underwent biopsy only via video-assisted thoracoscopic surgery (VATS). This is defined as cases not having undergone maximal cytoreductive surgery (MCS) and generally comprised a lesser amount of tissue (Nicholson et al., 2020) (Rice et al., 2011). E-PM with pleomorphic features represented 8.3% (51/614) of the study population. The median OS of the study population was 13.5 months (95% confidence interval (CI) 12.2-14.8 months).

After excluding those with pleomorphic features, tubulo-papillary was the most common predominant growth pattern (47.8%, 269/563), followed by solid (37.7%, 212/563), trabecular (8.2%, 46/563), discohesive (4.5%, 25/563), micropapillary (1.1%, 6/563) and microcystic (0.9%, 5/563). High-grade predominant pattern was present in 38.7% (218/563) and low-grade in 61.3% (345/563).

As described in the previous study (Chapter 4), necrosis and atypical mitoses were present in 37.5% (230/614) and 73.0% (448/614) of cases respectively. Lymphatic and vascular invasion were present in 9.1% (56/614) and 8.1% (50/614) of cases although due to the biopsy-heavy setting of the RBH service, these percentages should be considered to be underestimates of the real incidence.

5.4.2 Pleomorphic features in E-PM predicts unfavourable prognosis in univariate and multivariate settings

E-PM with pleomorphic features was associated with significantly worse median OS (5.4 months) than those with predominant tubulo-papillary (18.6 months), trabecular (17.6 months), discohesive (16.1 months) and solid (10.5 months) patterns (all $P<0.001$) (Table 5-3) (Figure 5-4A). A non-significant trend towards worse OS for E-PM with pleomorphic features was also observed when compared to microcystic (15.3 months, $P=0.069$) and micropapillary (9.4 months, $P=0.075$) patterns although this was likely due to the limited number of cases in these latter two categories.

Tumours with pleomorphic features were associated with one- and two-year survival rates of 23.4% and 6.2% respectively (Figure 5-4B). The majority (82.4%, 42/51) of tumours consisted of between 10 and 20% of tumour cells exhibiting severe nuclear atypia (median 15%, range 10-50%) (Figure 5-5A). Using a 20% cut-off, no significant difference in median OS ($P=0.423$) was observed (Figure 5-5B).

In multivariate analysis, pleomorphic features predicted survival independent of age, type of procedure, necrosis, atypical mitosis and growth patterns (P<0.001 vs tubulo-papillary pattern, P=0.007 vs solid pattern) (Table 5-4). This confirmed the findings from Kadota *et al*. (Kadota *et al*., 2011). Furthermore if instead of growth pattern, nuclear grade is employed as a covariate, pleomorphic features remained independently prognostic (HR 3.01 vs low-grade, P<0.001; HR 1.47 vs high-grade, P=0.029) (Table 5-5).

Variable		Patients (%)	Median OS (months)	*P*
All Patients		614 (100.0)	13.5	-
Predominant growth pattern				
	Tubulo-papillary	269 (43.8)	18.6	6.8×10^{-13}
	Trabecular	46 (7.5)	17.6	3.2×10^{-4}
	Discohesive	25 (4.1)	16.1	*0.001*
	Microcystic	5 (0.8)	15.3	0.069
	Solid	212 (34.5)	10.5	4.7×10^{-5}
	Micropapillary	6 (1.0)	9.4	*0.075*
	Pleomorphic	51 (8.3)	5.4	*Ref.*
Grade (growth pattern) †				
	Low-grade	345 (61.3)	18.0	*Ref.*
	High-grade	218 (38.7)	10.5	1.0×10^{-6}
Composite score †				
	Score 0	271 (48.1)	19.8	*Ref.*
	Score 1	169 (30.0)	13.4	*0.001*
	Score 2	123 (21.8)	8.1	1.0×10^{-17}
Predominant-secondary growth patterns †				
Low-grade (predominant) + Low-grade (secondary)		123 (21.9)	20.8	*Ref.*
Low-grade (predominant) + High-grade (secondary)		222 (39.4)	15.3	*0.026*
High-grade (predominant) + Low-grade (secondary)		197 (35.0)	12.0	6.0×10^{-6}
High-grade (predominant) + High-grade (secondary)		21 (3.7)	5.7	3.5×10^{-10}
Cribriform growth pattern ‡				
	Absent	214 (79.6)	18.9	*Ref.*
	Predominant	9 (3.3)	28.4	0.672
	Secondary	46 (17.1)	12.8	0.952
WDPMT-like features ‡				
	Present	11 (4.1)	78.7	*0.001*
	Absent	258 (95.9)	18.0	*Ref.*

Table 5-3 Univariate analysis in predicting overall survival.

† Epithelioid PM with pleomorphic features were excluded.

‡ Only tumours with tubulo-papillary predominant growth pattern were included.

EPP, extrapleural pneumonectomy; EPD, extended pleurectomy and decortication; PD, pleurectomy and decortication; OS, overall survival.

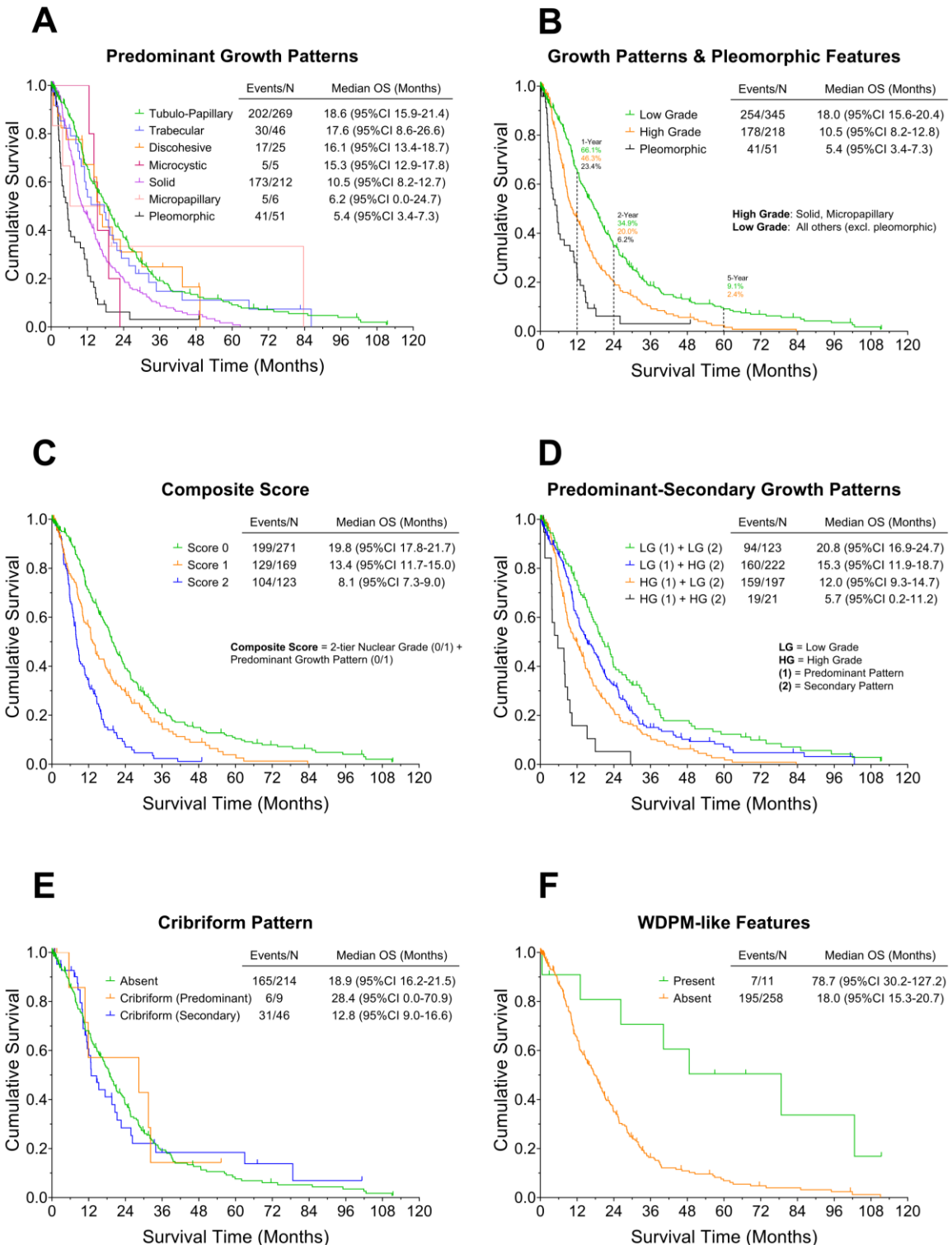


Figure 5-4 Overall survival by growth patterns. **(A)** Predominant growth patterns showed different survival characteristics. **(B)** Prognostic stratification was achieved by classifying predominant growth patterns into high-grade and low-grade. Pleomorphic features were associated with significant worse survival. **(C)** Composite score offered superior separation

of median OS in univariate setting. **(D)** Evaluation of secondary patterns further modified prognostic stratification by high-grade and low-grade growth patterns. **(E)** Presence of cribriform pattern did not influence median OS. **(F)** E-PM with WDPMT-like features was associated with superior median OS compared with tubulo-papillary-predominant tumours without.

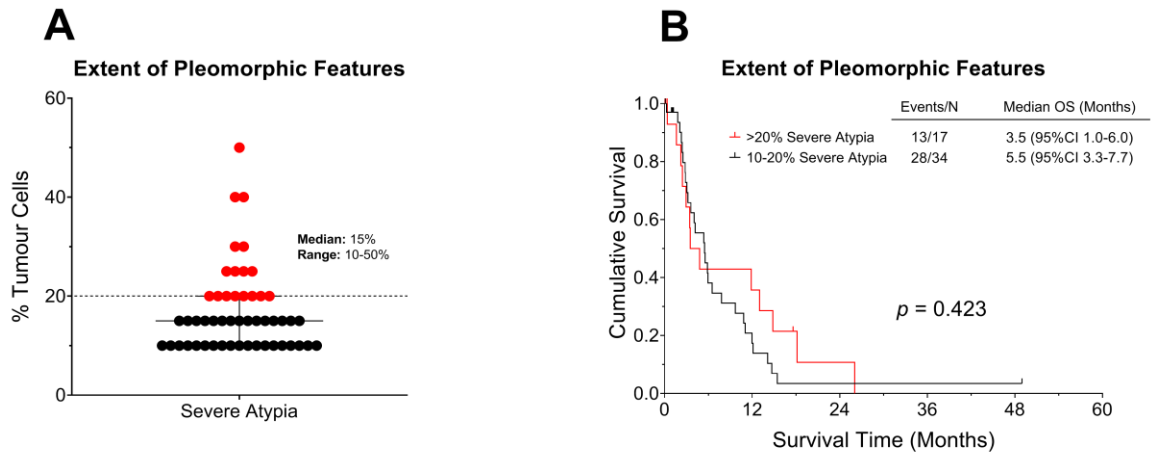


Figure 5-5 Extent of pleomorphic features and association with survival.

(A) Distribution of percentages of tumour cells exhibiting severe atypia in pleomorphic epithelioid PM. **(B)** Univariate analysis in predicting overall survival by the extent of pleomorphic features in pleomorphic epithelioid PM.

Variable	Hazard ratio	95% CI	*P*
Age			
>65 vs ≤65 years	1.39	1.14-1.70	*0.001*
Procedure			
Resection vs Biopsy only	0.33	0.24-0.46	5.6×10^{-11}
Necrosis			
Present vs Absent	2.10	1.67-2.63	1.6×10^{-10}
Atypical mitosis			
Present vs Absent	1.71	1.36-2.16	4.0×10^{-6}
Predominant growth pattern			
Solid vs Pleomorphic	0.62	0.44-0.88	*0.007*
Tubulo-papillary vs Pleomorphic	0.51	0.35-0.74	4.0×10^{-4}
Trabecular vs Pleomorphic	0.65	0.39-1.08	0.098
Micropapillary vs Pleomorphic	0.79	0.30-2.08	0.637
Microcystic vs Pleomorphic	0.92	0.36-2.39	0.871
Discohesive vs Pleomorphic	0.57	0.32-1.03	0.061

Table 5-4 Multivariate analysis in predicting overall survival (Pleomorphic features as a function of growth pattern).

CI, confidence interval.

Variable	Hazard ratio	95% CI	*P*
Age			
>65 vs ≤65 years	1.38	1.13-1.69	*0.002*
Procedure			
Resection vs Biopsy only	0.31	0.23-0.43	3.0×10^{-12}
Necrosis			
Present vs Absent	1.28	0.89-1.83	0.180
Atypical mitosis			
Present vs Absent	1.54	1.22-1.95	3.3×10^{-4}
2-tier nuclear grade			
High-grade vs Low-grade	2.03	1.39-2.98	2.8×10^{-4}
Pleomorphic features			
Present vs Low-grade	3.01	1.92-4.73	2.0×10^{-6}
Present vs High-grade	1.47	1.04-2.09	*0.029*

Table 5-5 Multivariate analysis in predicting overall survival (Pleomorphic features as a function of nuclear features).

CI, confidence interval.

5.4.3 E-PM demonstrates extensive heterogeneity in growth patterns

Next the heterogeneity in growth patterns in E-PM was explored by assessing the diversity and abundance of its secondary patterns. Growth patterns were found to frequently co-exist as a mixture of high- and low-grade patterns. For example, 77.8% (165/212) of solid-predominant tumours had a tubulo-papillary secondary pattern, whereas 56.1% (151/269) of tubulo-papillary-predominant tumours had a solid secondary pattern (Table 5-6). Tumours with a secondary pattern i.e. pure growth pattern was seen in only 9.6% (54/563) of cases, in contrast to a significant minority showing at least three co-existing secondary patterns (Table 5-7). A reduced degree of heterogeneity was seen for those with solid-predominant growth pattern (P=0.002), but not for those with 2-tier nuclear grade (P=0.122) (Table 5-8). The number of secondary growth patterns had no significant impact on survival (Table 5-9).

The number of resections in the study population was limited (n=80 decreasing to 73 after excluding pleomorphic E-PM). The biopsy-only cohort was associated with a fewer number of detected secondary patterns (P<0.001) suggestive of sampling bias (Table 5-10). There was however, no significant difference with regard to the detection of high-grade/low-grade predominant pattern (P=0.400) (Table 5-11). Overall, these findings echo a recently published multi-institutional study that reported moderate agreement in the assignment of subtype, presence of necrosis and nuclear grade between biopsies and subsequent resections (Schulte *et al*., 2021).

Predominant growth pattern	Secondary growth pattern					
	SOL (%)	TP (%)	TRAB (%)	MPP (%)	MC (%)	DIS (%)
SOL (*n*=212)	-	165 (77.8)	88 (41.5)	30 (14.2)	7 (3.3)	58 (27.4)
TP (*n*=269)	151 (56.1)	-	107 (39.8)	87 (32.3)	34 (12.6)	106 (39.4)
TRAB (*n*=46)	28 (60.9)	40 (87.0)	-	5 (10.9)	1 (2.2)	11 (23.9)
MPP (*n*=6)	5 (83.3)	5 (83.3)	2 (33.3)	-	1 (16.7)	3 (50.0)
MC (*n*=5)	1 (20.0)	5 (100.0)	4 (80.0)	0 (0.0)	-	3 (60.0)
DIS (*n*=25)	15 (60.0)	19 (76.0)	10 (40.0)	13 (52.0)	6 (24.0)	-

Table 5-6 Co-existence of growth patterns in epithelioid PM.

DIS, discohesive pattern; MC, microcystic pattern; MPP, micropapillary pattern; PM, pleural mesothelioma; TP, tubulo-papillary pattern; TRAB, trabecular pattern; SOL, solid pattern.

No. of secondary growth patterns	All patients (%)	SOL (%)	TP (%)	TRAB (%)	MPP (%)	MC (%)	DIS (%)
0	54 (9.6)	21 (9.9)	31 (11.5)	1 (2.2)	0 (0.0)	0 (0.0)	1 (4.0)
1	189 (33.6)	84 (39.6)	86 (32.0)	14 (30.2)	0 (0.0)	1 (20.0)	4 (16.0)
2	187 (33.2)	69 (32.5)	86 (32.0)	22 (47.8)	2 (33.3)	1 (20.0)	7 (28.0)
≥3	133 (23.6)	38 (17.9)	66 (24.5)	9 (19.6)	4 (66.7)	3 (60.0)	13 (52.0)

Table 5-7 Heterogeneity in growth patterns in epithelioid PM.

DIS, discohesive pattern; MC, microcystic pattern; MPP, micropapillary pattern; PM, pleural mesothelioma; TP, tubulo-papillary pattern; TRAB, trabecular pattern; SOL, solid pattern.

No. of secondary growth patterns	Low-grade (%)	High-grade (%)	*P*
0	40 (74.1)	14 (25.9)	0.122
1	123 (65.1)	66 (34.9)	
2	128 (68.4)	59 (31.6)	
3	53 (58.2)	38 (41.8)	
4	19 (50.0)	19 (50.0)	
5	3 (75.0)	1 (25.0)	

Table 5-8 Distribution of 2-tier nuclear grade by the number of secondary growth patterns.

OS, overall survival.

No. of secondary growth patterns	Patients (%)	Median OS (months)	*P*
0	54 (9.6)	15.3	*Ref.*
1	189 (33.6)	14.3	0.164
2	187 (33.2)	14.8	0.179
3	91 (16.2)	16.6	0.817
4	38 (6.7)	15.6	0.792
5	4 (0.7)	10.8	0.349

Table 5-9 Univariate analysis in predicting overall survival by number of secondary growth patterns.

OS, overall survival.

No. of secondary growth patterns	All patients (%)	SOL (%)	TP (%)	TRAB (%)	MPP (%)	MC (%)	DIS (%)
0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
1	8 (11.0)	3 (13.6)	5 (12.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
2	24 (32.9)	5 (22.7)	16 (40.0)	0 (0.0)	1 (33.3)	0 (0.0)	2 (28.6)
≥3	41 (56.1)	14 (63.7)	19 (47.5)	0 (0.0)	2 (66.7)	1 (100.0)	5 (71.4)

Table 5-10 Heterogeneity in growth patterns in epithelioid PM (Resections).

DIS, discohesive pattern; MC, microcystic pattern; MPP, micropapillary pattern; PM, pleural mesothelioma; TP, tubulo-papillary pattern; TRAB, trabecular pattern; SOL, solid pattern.

Predominant growth pattern	All patients (%)	Biopsy-only (%)	Resection (%)
Low-grade	345 (61.3)	297 (60.6)	48 (65.8)
High-grade	218 (38.7)	193 (39.4)	25 (34.2)

Table 5-11 Detection of high-grade/low-grade predominant growth pattern by type of procedure.

5.4.4 Evaluation of growth pattern does not offer additional prognostic utility to 2-tier nuclear grade

Univariate analysis showed high-grade predominant pattern was associated with worse median OS compared with its low-grade counterpart (10.5 months vs 18.0 months, P<0.001) (Table 5-3) (Figure 5-4B). The composite score provided further improvement where score 2 was associated with the worst median OS (8.1 months) (P<0.001 vs score 0) followed by score 1 (13.4 months) (P=0.001 vs score 0) and 0 (19.8 months) (Figure 5-4C). In multivariate analysis, however, the composite score exhibited inferior prognostic separation (HR 1.65, score 2 vs 0) compared with the 2-tier nuclear grade (HR 2.02, high-grade vs low-

grade) (Zhang *et al*., 2020a), despite the composite score being an independent prognostic variable (P=0.010) (Table 5-12).

Incorporating secondary growth patterns as a modifier to predominant growth pattern led to statistically significant prognostic groups (Table 5-3) (Figure 5-4D) in the univariate analysis. In the multivariate setting, however, only a pure high-grade pattern (3.7%, 19/563) predicted survival independent of 2-tier nuclear grade and other established prognostic variables (P<0.001) (Table 5-13). The presence of any high-grade pattern (≥5% of tumour) irrespective of other co-existing patterns showed a trend (non-significant) towards worse OS (P=0.061) (Table 5-14).

Within the study population, patients who underwent surgical resection were a statistically underpowered and clinically heterogeneous group in relation to surgical techniques and administration of adjuvant treatment. Two-tier nuclear grade and pleomorphic features remained predictive of OS in the univariate setting (Figure 5-6). A non-significant trend towards worse survival comparing pleomorphic and high-grade E-PM was observed (5.9 months vs 20.6 months, P=0.085). Pleomorphic features also predicted OS independent of high-grade/low grade predominant growth patterns (P=0.009) (Table 5-15). Given the inherent limitations to this subpopulation these findings should be interpreted with caution. The prognostic impact of pleomorphic features relative to high nuclear grade in the setting of surgical resection in particular warrants independent validation using an adequately powered and homogeneous surgical cohort.

Variable	Hazard ratio	95% CI	*P*
Age			
>65 vs ≤65 years	1.39	1.16-1.77	*0.001*
Procedure			
Resection vs Biopsy only	0.33	0.23-0.47	6.1×10^{-10}
Necrosis			
Present vs Absent	1.73	1.27-2.37	*0.001*
Atypical mitosis			
Present vs Absent	1.59	1.25-2.01	1.2×10^{-4}
Composite score			
Score 1 vs 0	1.27	0.99-1.63	0.058
Score 2 vs 0	1.65	1.13-2.41	*0.010*

Table 5-12 Multivariate analysis in predicting overall survival (Composite score).

CI, confidence interval.

Variable	Hazard ratio	95% CI	*P*
Age			
>65 vs ≤65 years	1.45	1.18-1.79	4.8×10^{-4}
Procedure			
Resection vs Biopsy only	0.31	0.22-0.44	9.9×10^{-11}
Necrosis			
Present vs Absent	1.29	0.87-1.94	0.209
Atypical mitosis			
Present vs Absent	1.44	1.13-1.84	*0.003*
2-tier nuclear grade			
High-grade vs Low-grade	1.95	1.28-2.96	*0.002*
Growth pattern			
Low-grade (predominant) + High-grade (secondary) vs Low-grade (predominant) + Low-grade (secondary)	1.28	0.98-1.67	0.074
High-grade (predominant) + Low-grade (secondary) vs Low-grade (predominant) + Low-grade (secondary)	1.26	0.95-1.68	0.110
High-grade (predominant) + High-grade (secondary) vs Low-grade (predominant) + Low-grade (secondary)	2.67	1.59-4.49	2.1×10^{-4}

Table 5-13 Multivariate analysis in predicting overall survival (Predominant-secondary growth patterns).

CI, confidence interval.

Variable	Hazard ratio	95% CI	*P*
Age			
>65 vs ≤65 years	1.45	1.18-1.79	4.8×10^{-4}
Procedure			
Resection vs Biopsy only	0.31	0.22-0.44	5.8×10^{-11}
Necrosis			
Present vs Absent	1.29	0.87-1.92	0.210
Atypical mitosis			
Present vs Absent	1.44	1.13-1.84	*0.003*
2-tier nuclear grade			
High-grade vs Low-grade	1.95	1.28-2.95	*0.002*
Growth pattern			
Any high-grade vs Low-grade (predominant) + Low-grade (secondary)	1.27	0.99-1.63	0.061
High-grade (predominant) + High-grade (secondary) vs Low-grade (predominant) + Low-grade (secondary)	2.67	1.59-4.49	2.0×10^{-4}

Table 5-14 Multivariate analysis in predicting overall survival (Prognostic impact of the presence of any high-grade growth pattern).

CI, confidence interval.

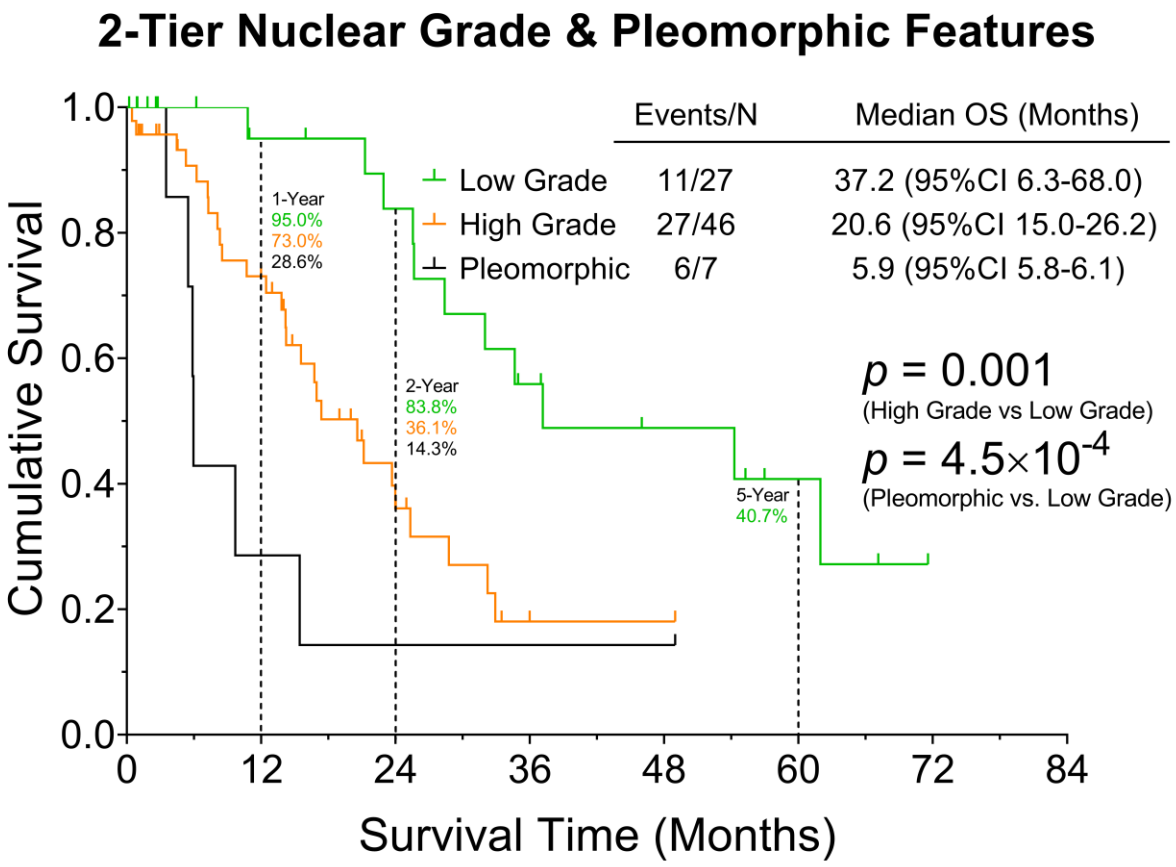


Figure 5-6 Univariate analysis in predicting overall survival by 2-tier nuclear grade and pleomorphic features (Resections only).

Among those underwent resections, low nuclear grade was associated with the most favourable overall survival, followed by high nuclear grade and pleomorphic E-PM.

Variable	Hazard ratio	95% CI	*p*
2-tier nuclear grade			
High-grade vs Low-grade	2.95	1.32-6.62	*0.009*
Growth pattern			
High-grade vs Low-grade	1.64	0.83-3.24	0.157

Table 5-15 2-tier nuclear grade predicted OS independent of high/low-grade predominant pattern (Resections).

CI, confidence interval.

5.4.5 WDPMT-like but not cribriform growth pattern modified survival in E-PM with predominant tubulo-papillary pattern

Cribriform pattern was seen in 20.4% of tumours with a predominant tubulo-papillary pattern (55/269). No significant prognostic impact was observed for predominant (P=0.672) or secondary cribriform patterns (P=0.952) in the univariate setting compared with tubulo-papillary-predominant epithelioid PM without such growth pattern (Table 5-3) (Figure 5-4E).

WDPMT-like features pattern was rarely seen and represented just 4.1% of cases (11/269). Ten of the 11 cases were of low nuclear grade. Significantly prolonged median OS was seen for WDPMT-like features compared with those without such features (78.7 months vs. 18.0 months, P=0.001) (Table 5-3) (Figure 5-4F). The small number of cases (n=11) however precluded multivariate analysis.

5.4.6 Interaction between pleomorphic features, growth patterns and clinicopathological variables

Finally, the interaction between pleomorphic features, growth patterns and other clinicopathological variables was investigated. E-PM with pleomorphic features were primarily solid-predominant (84.3%, 43/51). They retained expression of mesothelium-associated immunohistochemical markers (Calretinin, Wilms tumour protein, cytokeratin 5/6) typically expressed by E-PM (Table 5-16). They were associated with a higher frequency of necrosis and atypical mitosis (all P<0.001) (Table 5-17). Higher incidence of lymphovascular invasion was seen in micropapillary and discohesive patterns, as well as those with pleomorphic features (all P<0.001). High-grade growth patterns were associated with high nuclear grade (P<0.001) although such was not exclusive. Progressive transformation towards high nuclear grade, with an increasing proportion of high-grade growth pattern, was observed (P<0.001) (Table 5-18).

With regard to sampling criteria, pleomorphic features were detected at higher frequency with increasing maximum tissue dimension (*P*=0.016) (Figure 5-7B) but not the number of biopsy sites (*P*=0.842) (Figure 5-7A). This served as further supportive evidence to the proposed extensive sampling recommendations (Nicholson *et al*., 2020) (Zhang *et al*., 2020a) (Chirieac *et al*., 2019). Neither sampling criteria had significant impact on the detection of high-grade growth patterns.

Immunohistochemistry	Test rate (%)	Positive (%)	Focal (%)	Negative (%)
Calretinin	51 (100.0)	38 (74.5)	10 (19.6)	3 (5.9)
WT-1	14 (27.5)	7 (50.0)	5 (35.7)	2 (14.3)
CK5/6	47 (92.2)	30 (63.8)	12 (25.5)	5 (10.6)
Thrombomodulin	4 (7.8)	3 (75.0)	1 (25.0)	0 (0.0)
EMA	15 (29.4)	9 (60.0)	6 (40.0)	0 (0.0)
Pan-CK	20 (39.2)	20 (100.0)	0 (0.0)	0 (0.0)
BerEP4	47 (92.2)	1 (2.1)	5 (10.6)	41 (87.3)
TTF-1	39 (76.5)	0 (0.0)	0 (0.0)	39 (100.0)
CEA	38 (74.5)	0 (0.0)	0 (0.0)	38 (100.0)

Table 5-16 Immunoprofile of pleomorphic epithelioid PM (*n*=51).

BerEP4, monoclonal antibody against epithelial cell adhesion molecule (EpCAM); CEA, carcinoembryonic antigen; CK5/6, cytokeratin 5/6; EMA, epithelial membrane antigen; PM, pleural mesothelioma; Pan-CK, pan-cytokeratin; TTF-1, thyroid transcription factor 1; WT-1, Wilms tumour protein.

Variable	All Patients	PLEO	SOL	TP	TRAB	MPP	MC	DIS	*P*
All Patients (%)	614 (100.0)	51 (8.3)	212 (34.5)	269 (43.8)	46 (7.5)	6 (1.0)	5 (0.8)	25 (4.1)	-
Age (years)									
Median	70	69	70	70	68	58	71	71	0.084
Range	32-91	37-88	39-91	41-90	46-88	48-67	61-82	32-83	
Sex (%)									
Male	464 (75.6)	41 (80.4)	165 (77.8)	200 (74.3)	29 (63.0)	4 (66.7)	5 (100.0)	20 (80.0)	0.277
Female	150 (24.4)	10 (19.6)	47 (22.2)	69 (25.7)	17 (37.0)	2 (33.3)	0 (0.0)	5 (20.0)	
Laterality (%)									
Left	254 (41.4)	27 (52.9)	90 (42.5)	101 (37.5)	19 (41.3)	1 (16.7)	1 (20.0)	15 (60.0)	
Right	355 (57.8)	24 (47.1)	121 (57.1)	166 (61.7)	26 (56.5)	5 (83.3)	4 (80.0)	9 (36.0)	0.187
Not documented	5 (0.8)	0 (0.0)	1 (0.4)	2 (0.8)	1 (2.2)	0 (0.0)	0 (0.0)	1 (4.0)	
Procedure (%)									
Biopsy	534 (87.0)	44 (86.3)	190 (89.6)	229 (85.1)	46 (100.0)	3 (50.0)	4 (80.0)	18 (72.0)	
PD or EPD	72 (11.7)	7 (13.7)	21 (9.9)	35 (13.0)	0 (0.0)	2 (33.3)	1 (20.0)	6 (32.0)	4.3×10^{-4}
EPP	5 (0.8)	0 (0.0)	0 (0.0)	4 (1.5)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	
Other procedures	3 (0.5)	0 (0.0)	1 (0.5)	1 (0.4)	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.0)	
Necrosis (%)									
Present	230 (37.5)	41 (80.4)	112 (52.8)	61 (22.7)	6 (13.0)	3 (50.0)	1 (20.0)	6 (24.0)	1.3×10^{-19}
Absent	384 (62.5)	10 (19.6)	100 (47.2)	208 (77.3)	40 (87.0)	3 (50.0)	4 (80.0)	19 (76.0)	
Lymphatic Invasion (%)									
Present	56 (9.1)	9 (17.6)	18 (8.5)	20 (7.4)	1 (2.2)	3 (50.0)	0 (0.0)	5 (20.0)	4.5×10^{-4}
Absent	558 (90.9)	42 (82.4)	194 (91.5)	249 (92.6)	45 (97.8)	3 (50.0)	5 (100.0)	20 (80.0)	
Vascular Invasion (%)									
Present	50 (8.1)	8 (15.7)	15 (7.1)	15 (5.6)	2 (4.3)	4 (66.7)	0 (0.0)	6 (24.0)	8.2×10^{-8}
Absent	564 (91.9)	43 (84.3)	197 (92.9)	254 (94.4)	44 (95.7)	2 (33.3)	5 (100.0)	19 (76.0)	
Atypical Mitosis (%)									
Present	448 (73.0)	50 (98.0)	178 (84.0)	176 (65.4)	23 (50.0)	4 (66.7)	2 (40.0)	15 (60.0)	
Absent	166 (27.0)	1 (2.0)	34 (16.0)	93 (34.6)	23 (50.0)	2 (33.3)	3 (60.0)	10 (40.0)	6.4×10^{-10}
Nuclear Grade (%) †									
Low-grade	366 (59.6)	-	92 (43.4)	210 (78.1)	39 (84.8)	3 (50.0)	4 (80.0)	18 (72.0)	2.2×10^{-14}
High-grade	197 (32.1)	-	120 (56.6)	59 (21.9)	7 (15.2)	3 (50.0)	1 (20.0)	7 (28.0)	

Table 5-17 Distribution of clinicopathological variables by predominant growth pattern.

DIS, discohesive pattern; EPP, extrapleural pneumonectomy; EPD, extended pleurectomy and decortication; MC, microcystic pattern; MPP, micropapillary pattern; PD, pleurectomy and decortication; PLEO, pleomorphic; SOL, solid pattern; TP, tubulo-papillary pattern; TRAB, trabecular pattern.

† Epithelioid PM with pleomorphic features does not receive nuclear grade.

Growth pattern	Patients (%)	Low-grade	High-grade
Low-grade (1) + Low-grade (2)	123 (21.8)	116 (94.3%)	7 (5.7%)
Low-grade (1) + High-grade (2)	222 (39.5)	155 (69.8%)	67 (30.2%)
High-grade (1) + Low-grade (2)	197 (35.0)	88 (44.7%)	109 (55.3%)
High-grade (1) + High-grade (2)	21 (3.7)	7 (33.3%)	14 (66.7%)

Table 5-18 Distribution of 2-tier nuclear grade by predominant-secondary growth patterns.

(1) = predominant growth pattern, (2) = secondary growth pattern. OS, overall survival.

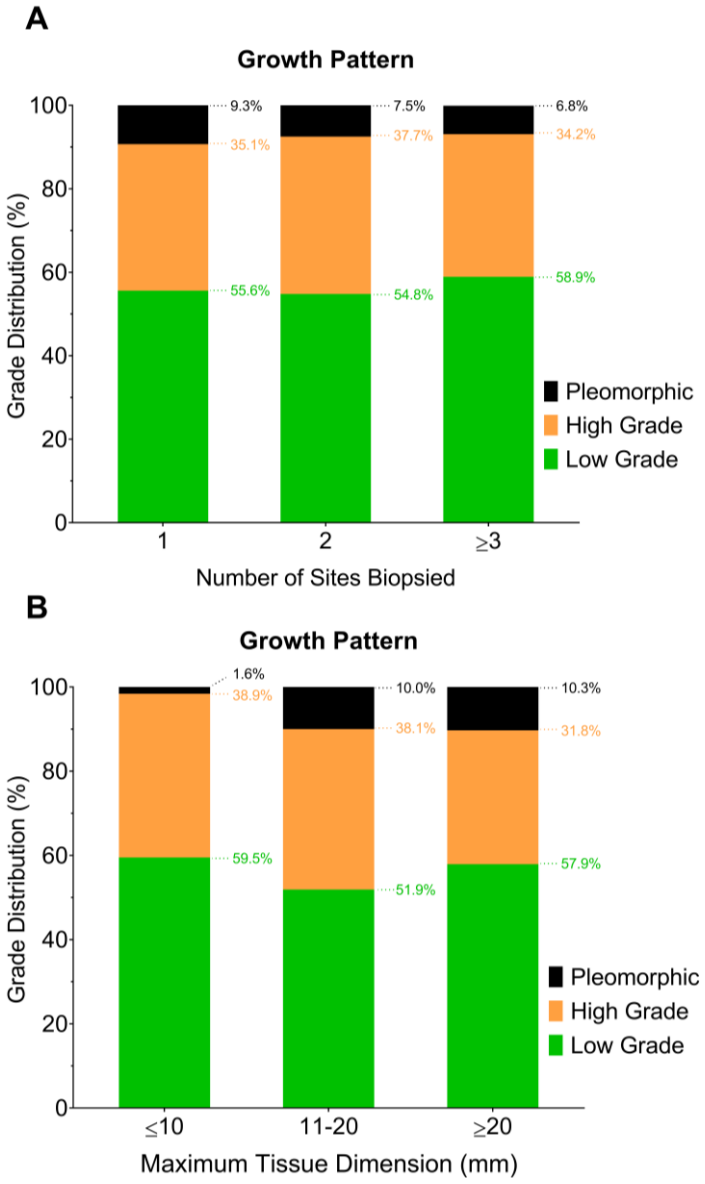


Figure 5-7 Effect of sampling criteria on the detection of pleomorphic features and growth patterns.

(A) The number of sampling sites had no significant impact on the detection of pleomorphic features and high-grade growth patterns. **(B)** Pleomorphic features were detected at higher frequencies with increasing maximum tissue dimension. No impact on the detection of high-grade growth pattern was seen.

5.5 Discussion

In this study the impact of other histological parameters beyond nuclear grade on the prognosis of epithelioid PM and how they interact with nuclear grade was assessed. First findings from the previous reports on E-PM with pleomorphic features, showing dramatically worse prognosis than the historical figures on E-PM, were validated (Table 5-19). Furthermore statistically significant inferior median OS in this rare group of tumours versus both low- and high-grade E-PM was observed. Consequently its prognostic impact and clinicopathological features warrant a distinct diagnostic category. Based on available evidence from the literature and the present study, E-PM with pleomorphic features are thought to be an aggressive variant of E-PM rather than non-epithelioid subtype, as the preservation of mesothelial lineage is still evident despite suggestions of early de-differentiation (Table 5-20). Although it is not possible to exclude sampling error with absolute certainty, as 44 out of 51 cases did not undergo resection or autopsy based on available information, no cases with pleomorphic features were associated with sarcomatoid morphology elsewhere in the RBH series. Therefore, pleomorphic features as a suffix, equivalent to 2-tier nuclear grade as the diagnosis is based on nuclear features alone, is proposed as pleomorphic E-PM has a significantly worse prognosis than conventional high-grade E-PM (Zhang *et al*., 2020a). Based on the findings from this study and the prior study (Chapter 4), E-PM could be reliably sub-classified using biopsies into statistically and

clinically relevant groups of low-grade (59.6%, median OS 19.3 months), high-grade (32.1%, median OS 8.9 months) and pleomorphic (8.3%, median OS 5.4 months).

Second, whilst confirming the survival characteristics conferred by growth patterns in reported series (Table 5-21), the work presented shows a combined approach (utilising growth pattern and 2-tier nuclear grade) did not improve prognostic stratification. This is an important negative finding, as it suggests 2-tier nuclear grade alone remains the prognostic tool of choice for E-PM other than the addition of pleomorphic E-PM. The extensive intratumoural heterogeneity observed offers a possible explanation: not only could high-grade and low-grade growth patterns co-exist, but tumour cells exhibiting high nuclear grade could commit to low-grade growth patterns and *vice versa*. This extended the previous observation where higher nuclear grade was associated with tumours with solid-predominant growth pattern (Zhang *et al*., 2020a) (Rosen *et al*., 2018). Evaluation of growth pattern is still of value however, for example in aiding accurate diagnosis and identification of growth patterns associated with lymphovascular invasion.

Third, this Chapter's findings on cribriform pattern and WDPMT-like features warrant external validation as they might represent uncommon groups of tumours with unique survival and biological characteristics. The presence of the latter should also be considered as a potential diagnostic pitfall in mesothelioma especially in the small biopsy setting, albeit rarely encountered, highlighting the importance of correlation with clinicoradiological and intraoperative findings. Of note, although the authors studied only cases of peritoneal origin, true WDPMT has been shown to harbour mutations in *CDC42* or *TRAF7* (Stevers *et al*., 2019), in contrast to *BAP1* and *CDKN2A* alterations frequently registered in PM (Hmeljak *et al*., 2018) (Bueno *et al*., 2016). An evolutionary link between WDPMT and subsequent malignant transformation to diffuse invasive mesothelioma has also been proposed (Lee *et al*., 2018). As no genomic data are available for pleural WDPMT nor those with WDPMT-

like features in this study, it is uncertain if the latter represents a biologically distinct entity, or early diffuse mesothelioma characterised by minimal or superficial stromal invasion.

The present study has two major limitations. First, it is retrospective, and it was not possible to investigate the impact of additional parameters (such as tumour metabolic uptake (Kadota *et al*., 2012) associated with pleomorphic features and could only focus on than prognosis. Second it was not possible to evaluate response to treatment including maximal cytoreductive surgery as well as chemotherapy, in relation to pleomorphic features and growth patterns. In addition, the investigation of the impact of nuclear grade and pleomorphic features on the outcomes following surgery was underpowered, and this is worthy of further investigation using a homogeneous cohort of surgical patients.

Future work therefore includes the genetic and epigenetic basis, as well as the evolution of pleomorphic features in E-PM compared with those showing low- or high-nuclear grades. Evaluation of the prognostic impact of pleomorphic features in non-epithelioid PM (biphasic, sarcomatoid), and assessment of inter-observer agreement which is known to be problematic (Brčić *et al*., 2018) (Hartman *et al*., 2017). The questions around growth patterns in E-PM could also be revisited when deeper understanding and novel tools to spatially deconvolute the cellular and molecular signatures of co-existing growth patterns have been acquired. This is because for such morphologically distinct tumour architectures, a homogenous biological process is highly implausible.

	RBH (This study)	Kadota *et al*., 2011	Alchami *et al*., 2017	Brčić *et al*., 2014	Ordóñez, 2012	Galateau-Salle *et al*., 2010 ‡
n	51	34	10	5	10	44
Incidence among E-PM	8.3%	14.7%	8.9%	5.1%	n/a	n/a
Age (Median, range)	69 (37-88)	67 (35-81)	n/a	n/a	67 (56-74)	n/a
Sex (M:F)	80:20	82:18	n/a	n/a	100:0	n/a
Laterality (L:R)	53:47	44:56	n/a	n/a	50:40 †	n/a
Lymphatic invasion	18%	68%	n/a	n/a	n/a	n/a
Vascular invasion	18%	59%	n/a	n/a	n/a	n/a
Median OS (Months)	5.4	8.1	8.0	3.0	7.3	7.0

Table 5-19 Comparison of demographic and clinicopathological characteristics with previously published cohorts of pleomorphic epithelioid PM.

E-PM, epithelioid pleural mesothelioma; L, left; M, male; F, female; OS, overall survival; R, right; RBH, Royal Brompton and Harefield Hospitals.

† One case was diagnosed as peritoneal malignant mesothelioma.

‡ The cohort was published as a conference abstract. The exact number of cases classified as epithelioid subtype was not available.

For	Against
Inferior OS compared with epithelioid PM	Cytomorphology lacks sarcomatoid features
High metabolic uptake in keeping with non-epithelioid PM	Retained expression of mesothelium-associated IHC biomarkers
Association with solid growth pattern (indication of early de-differentiation)	Presence of microvilli under EM

Table 5-20 Arguments for and against pleomorphic epithelioid PM being incorporated into non-epithelioid PM subtype.

EM, electron microscopy; IHC, immunohistochemistry; PM, pleural mesothelioma; OS, overall survival; SUVmax, maximum standardised uptake value.

	RBH (This study)	Kadota *et al*., 2011	Alchami *et al*., 2017	Brčić *et al*., 2014
Tubulo-papillary	18.6	17.9	17.0	13-25.5 ‡
Trabecular	17.6	24.9	-	11.0
Discohesive	16.1	-	-	-
Microcystic	15.3	- †	24.0	25.5
Solid	10.5	13.7	14.0	8.5
Micropapillary	6.2	15.8	6.0	11.0

Table 5-21 Comparison with previously published studies on the prognostic impact of growth patterns by overall survival.

OS, overall survival; RBH, Royal Brompton and Harefield Hospitals.

† Microcystic growth pattern was detected in 18.5% of cases but the overall survival was not reported.

‡ The authors reported overall survival figures for tubulo-papillary (25.5 months) and tubular (13 months) separately.

5.6 Conclusions

In this study the adverse prognostic impact of pleomorphic features in E-PM has been confirmed, and that it should be regarded as a rare but highly aggressive variant. E-PM exhibits extensive heterogeneity in terms of architectural growth patterns, and the evaluation of growth patterns carries prognostic utility. It does not, however, improve stratification based on the current 2-tier nuclear grade.

6 Refining the Classification of Epithelioid Pleural Mesothelioma: Cytological Variants

This chapter has been presented as a platform presentation at the 15th meeting of the International Mesothelioma Interest Group (IMIG), 7th-10th May 2021, Australia (Virtual).

6.1 Abstract

Background: Cytological variants of epithelioid pleural mesothelioma (E-PM) are well recognised yet, unlike nuclear grade and growth patterns, their incidence and prognostic impact are less well studied. It is also uncertain if prognostic relevance is independent of nuclear grade.

Materials and methods: A previously characterised cohort of 614 cases of E-PM diagnosed over a 15-year period, for which clinicopathological parameters including 2-tier nuclear grade had been evaluated, was retrospectively reviewed. The diagnostic criteria for clear cell, signet ring, deciduoid, rhabdoid and small cell variants were extracted from the literature. No minimal cut off threshold was employed. Survival analysis was performed using the Kaplan-Meier method after excluding cases of E-PM with pleomorphic features (n=51) due to its low incidence and known aggressive behaviour.

Results: Cytological variants were seen in 32.4% of all cases (199/614), more commonly seen in high-grade and pleomorphic E-PM (P<0.001). In non-pleomorphic E-PM, the presence of clear cell variant was associated with worse median overall survival (OS) than those without (12.0 vs. 15.3 months, P<0.028), and predicted survival independent of age, type of procedure, atypical mitosis and 2-tier nuclear grade (HR 1.40, P=0.012). Rhabdoid cell variant was rare and almost exclusively seen in high-grade and pleomorphic E-PM (P=1.9×10^{-18}). It also exhibited highly aggressive behaviour (median OS 5.2 vs. 15.3 months, P=2.4×10^{-5}). Deciduoid cell variant was the most common cytological variant and associated with high-grade and pleomorphic E-PM (P=2.2×10^{-16}), but not prognostically significant in the univariate setting (Median OS 12.4 vs. 15.2 months, P=0.225). The perceived prognostic impact, as reported in the literature, was associated with nuclear grade. Small cell variant was the least common and the two cases in the study cohort were histologically high-grade (OS

3.3 and 3.9 months respectively). Signet ring cell variant was not shown to be prognostically significant (17.6 vs 14.7 months, *P*=0.894) and was seen across nuclear grades (*P*=0.474).

Conclusions: The present study has shown that cytological variants are not uncommon in epithelioid PM. Clear cell variant was found to be an adverse independent predictor of overall survival but the effect size was small. The adverse prognostic impact of deciduoid and rhabdoid cell variants was a function of nuclear grade. Based on available evidence, recording the presence of cytological variants is best considered an optional item in routine diagnosis. The findings of the present study warrant independent validation.

6.2 Introduction

The classification of epithelioid PM is refined by nuclear grade, either using the 2-tier or 3-tier system, providing a robust and validated tool in its prognostication (Kadota *et al*., 2012) (Rosen *et al*., 2018) (Zhang *et al*., 2020a). Whilst the presence of pleomorphic features confers significantly worse prognosis than high-grade E-PM, the evaluation of architectural growth patterns does not provide additional prognostic value (Chapter 5) (Zhang *et al*., 2020b). Cytological variants in epithelioid PM, primarily concerning the cytoplasmic appearance of individual tumour cells, represents an area of mesothelioma research where there is a paucity of data derived from well powered and systematic studies. The incidence was reported as high as 32% (Chirieac *et al*, 2013), however the literature is heavily biased, being predominantly case reports and short series. A diverge range of overall survival have been associated with clear cell and deciduoid cell variants (Ordóñez *et al*., 2012) (Ordóñez, 2005). Rhabdoid cell and small cell variants based on current published literature appear to be associated with worse prognosis (Ordóñez, 2012) (Ordóñez *et al*., 2006). There has been a suggestion of interaction between nuclear features and the prognosis of deciduoid cell variant (Ordóñez *et al*., 2012), but the observation preceded the introduction of nuclear grading systems and no subsequent study has been undertaken to investigate this question.

It is conceivable that morphological heterogeneity in cytological variants underpins distinct biological characteristics which in turn determines the clinical behaviour of the tumour. This chapter therefore aims to systematically investigate (1) the incidence of known cytological variants (clear cell, signet ring cell, deciduoid cell, rhabdoid cell and small cell) in epithelioid PM, (2) establish their prognostic relevance and (3) establish what relationships exist with the 2-tier nuclear grade.

6.3 Materials and Methods

6.3.1 Study population

The study population comprised 614 consecutive cases of histologically confirmed epithelioid PM between 2003 and 2017, characterised for two previous studies (Chapters 4 and 5) (Zhang *et al*., 2020a) (Zhang *et al*., 2020b). Survival data was updated with a new data cut-off date of 30th September 2019. The censor fraction was 11.9% (73/614). Due to the small number of pleomorphic E-PM in the study population (*n*=51), and given it is a known aggressive cytological variant, they were excluded from the survival analysis. Institutional review board (IRB) approval was obtained for the study.

6.3.2 Microscopy and imaging

The instrument and methodology were unchanged from the previous (Chapters 4 and 5) (Zhang *et al*., 2020a) (Zhang *et al*., 2020b). Microscopic assessment was performed using a Nikon Eclipse Ci-L microscope (Nikon Corporation, Japan) with a field area measuring 0.24mm^2 per high power field (HPF, ×400 magnification). Microscopic images in 300dpi TIF format were taken from representative cases using a Nikon Digital Sight DS-L3 camera (Nikon Corporation, Japan), annotated using GNU Image Manipulation Program 2.10.10 (http://www.gimp.org, retrieved on 20.04.2019).

6.3.3 Histopathological assessment

All cases were previously diagnosed by at least one specialist pulmonary pathologist using current histological and immunohistochemical criteria (Nicholson *et al*., 2020) (Husain *et al*., 2018) (Travis *et al*., 2015). Parameters in relation to the cytological variants were assessed and recorded, blinded to patient outcome. Each cytological variant was recorded in bimodal fashion (present versus absent), as well as in 5% increments. A 1% cut-off threshold was

employed for tumours with any amount of convincing tumour cell subpopulation exhibiting features of the corresponding cytological variant. This usually represented at least 100 tumour cells. For challenging cases, joint review with a specialist pulmonary pathologist was sought to reach a consensus.

Clear cell variant was defined as tumour cells with optically clear or fully vacuolated cytoplasm, a central and non-attenuated nucleus, in any growth pattern (Figure 6-1). Signet ring cell variant was defined as those containing a single, optically clear cytoplasmic vacuole with a displaced and attenuated nucleus (Figure 6-2). The vacuoles were Periodic acid–Schiff (PAS) stain negative. The lack of architecture distinguished this variant from the microcystic/adenomatoid growth pattern in epithelioid PM. Deciduoid cell variant showed morphological resemblance to decidualised endometrial stromal cells. The tumour cells contained abundant homogeneous eosinophilic cytoplasm allowing peripheral clearance, accentuated cytoplasmic membranes often resembling those seen in plant cells, and centrally placed nuclei. This often gave them a "fried egg" appearance (Figure 6-3). Rhabdoid cell variant was defined as tumour cells with a single, pale eosinophilic cytoplasmic globule with displaced nucleus/nuclei (Figure 6-4). The possibility of rhabdomyosarcomatous differentiation was ruled out based on negative skeletal muscle lineage markers. Small cell variant was defined as consisted of small round blue cells with increased nuclear to cytoplasmic ratio, hyperchromatic nuclei with inconspicuous nucleoli, nuclear moulding and high mitotic activity (Figure 6-5). The expression of mesothelium-associated markers was preserved, and cells were negative for specific neuroendocrine lineage markers (Chromogranin A, Synaptophysin).

6.3.4 Statistical analysis

Descriptive statistics were employed to analyse the baseline demographic and clinicopathological parameters. Two-side chi-square or Fisher's exact test was used to evaluate associations between categorical variables. OS was estimated using the Kaplan-Meier method. Exact *P* values were recorded and $P<0.05$ denotes statistical significance. Multivariate Mantel-Cox regression model was used to evaluate the effect size and statistical significance of each variable which demonstrated $P<0.05$ in univariate analysis and with at least 10 events. All statistical analyses were performed using SPSS 26 (IBM Corp., Armonk, NY, USA). Kaplan-Meier curves were generated using GraphPad Prism Version 8 (GraphPad Software, La Jolla California, USA).

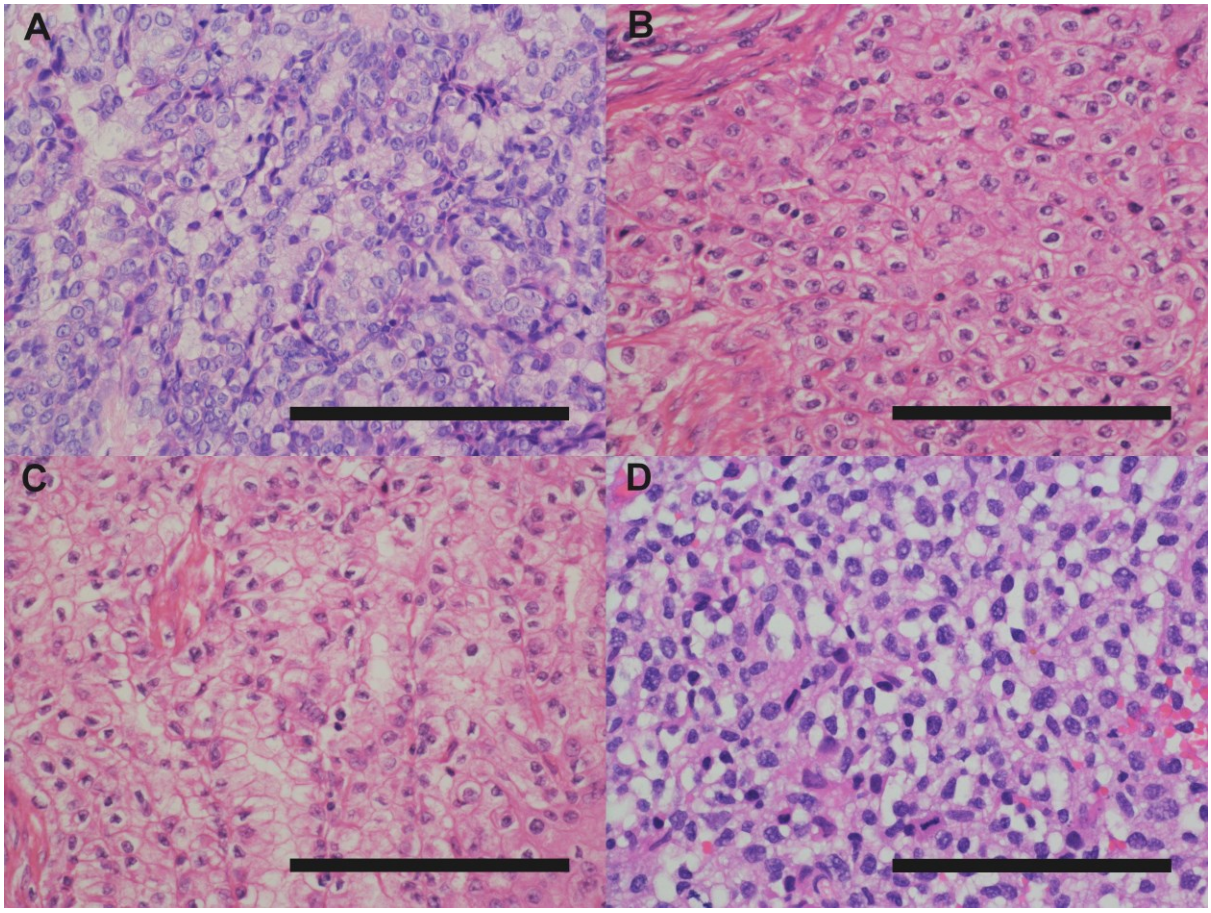


Figure 6-1 Clear cell variant of epithelioid PM.

(A-D) The tumour cells have optically clear or vacuolated cytoplasm and are often a mixture of both. Delicate intervening stroma can be seen although this is not an essential feature.

Scale bar = 100µm.

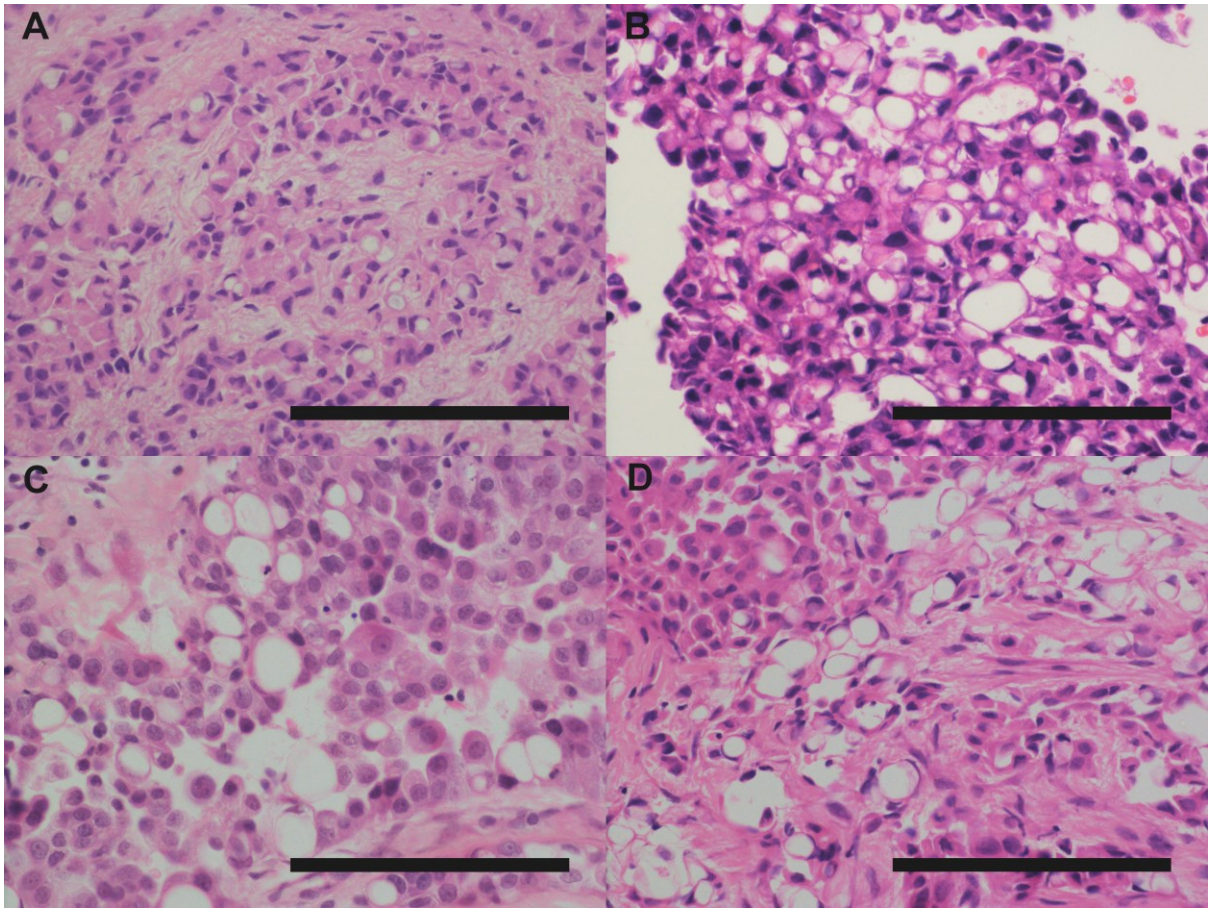


Figure 6-2 Signet ring cell variant of epithelioid PM.

(A-D) The tumour cells have single, optically clear cytoplasmic vacuoles, and often crescent-shaped nuclei. The vacuoles are generally round to oval and, although the cells can be larger than a standard epithelioid PM cell, architecture suggestive of microcystic growth pattern is absent.

Scale bar = 100μm.

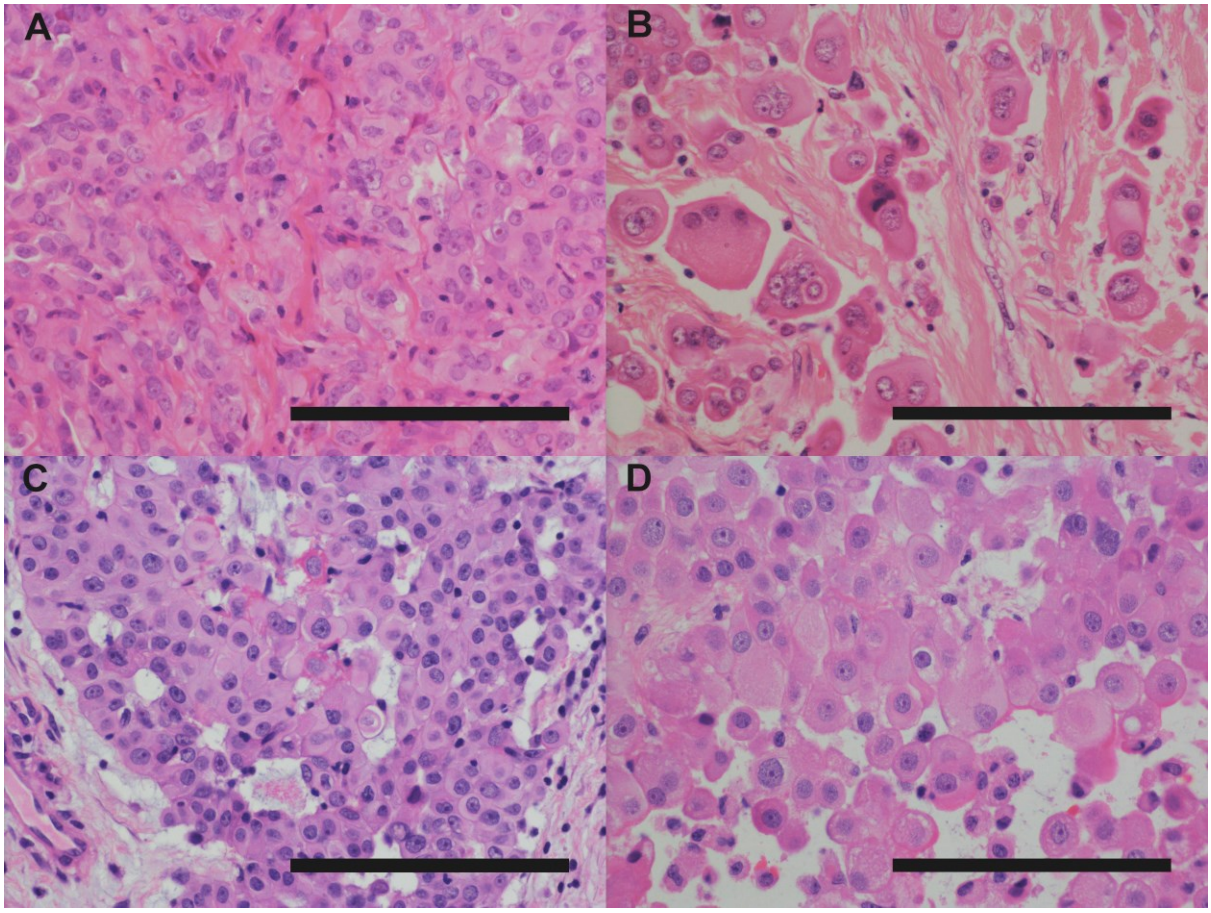


Figure 6-3 Deciduoid cell variant of epithelioid PM.

(A-D) The tumour cells contain abundant eosinophilic cytoplasm, with centrally placed nuclei and accentuated, plant cell-like plasma membrane. Co-existence with rhabdoid cell variant is focally seen in **(B)**. Peripheral clearance of the cytoplasm is an acceptable feature **(D)**.

Scale bar = 100μm.

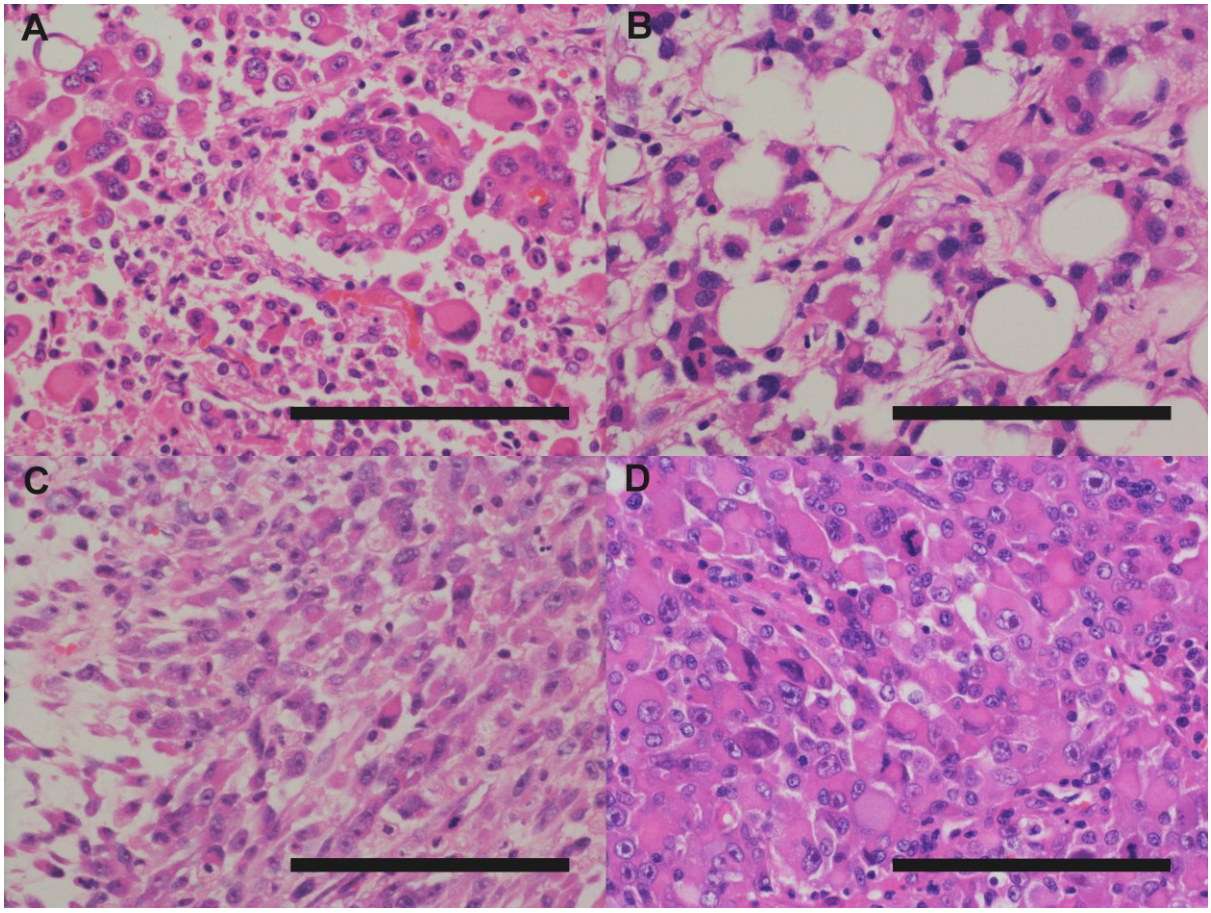


Figure 6-4 Rhabdoid cell variant of epithelioid PM.

(A-D) The tumour cells contain pale eosinophilic globules and displaced nuclei. The tumours are generally high-grade or with pleomorphic features. Mitotic activity is high and frequently present in atypical forms **(D)**.

Scale bar = 100µm.

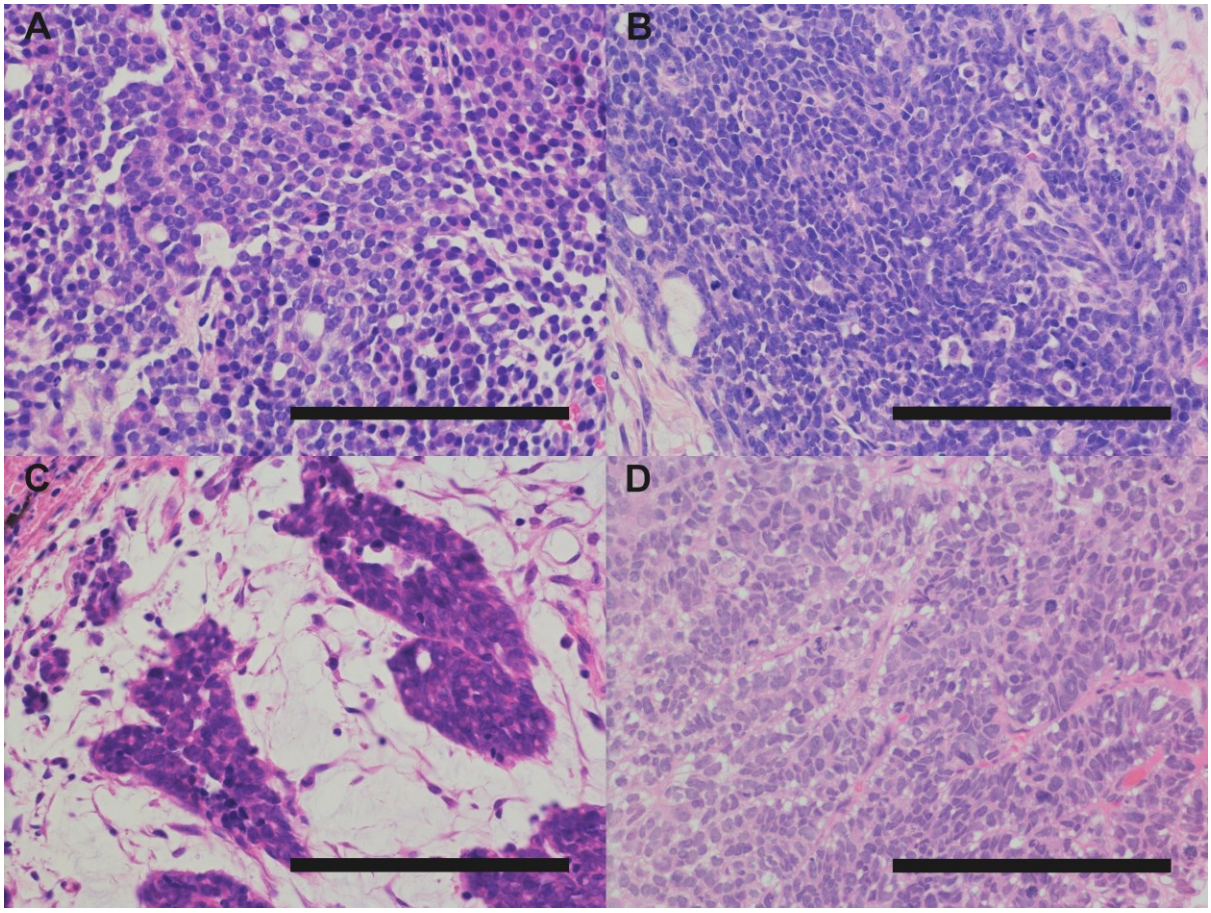


Figure 6-5 Small cell variant of epithelioid PM.

(A-D) The tumour cells are smaller than a classical epithelioid PM cell and contain hyperchromatic nuclei with inconspicuous nucleoli. Nuclear moulding is easily appreciated. The presence of spindle cells is an acceptable feature **(B-D)**. Some tumours cells can contain clear cytoplasmic vacuoles **(D)**, a feature not seen in small cell carcinoma. This could lead to an initial impression of clear cell variant, however, the mitotic activity in small cell variant is generally >20 per 10 HPFs, a level much higher than even high-grade epithelioid PM.

Scale bar = 100µm.

6.4 Results

6.4.1 Clinicopathological characteristics

The baseline demographic profile was identical to previously published studies (Chapters 4 and 5) (Zhang *et al*., 2020a) (Zhang *et al*., 2020b). Using a 1% cut-off threshold, 32.4% (199/614) of cases consisted of at least one variant, and they generally represent less than 10% of the tumour suggesting there is a strong element of intratumoural heterogeneity (Table 6-1). For this reason, subsequent analysis of the prognostic impact was based on this cut-off. The deciduoid cell variant was the most common (105/614, 17.1%), followed by clear cell variant (80/614, 13.0%), signet ring cell variant (33/614, 5.4%), rhabdoid cell variant (20/614, 3.3%) and finally small cell variant (2/614, 0.3%). The co-existence of cytological variants was present in an absolute minority of cases (Table 6-2). Of those with at least one cytological variant, 19.1% (38/199) showed two co-existing variants, and 1.0% showed three co-existing variants. The extent of co-existence was notably less frequent than that of growth patterns (Zhang *et al*., 2020b).

Cytological variants were more commonly seen in high-grade and pleomorphic E-PM (P=3.3×10^{-9}). This pattern was also observed in deciduoid (P=2.2×10^{-16}) and rhabdoid cell variants (P=1.9×10^{-18}). Deciduoid cell variant, representing ≥50% of tumour cells, was found in eight cases with all but one being pleomorphic E-PM. Both cases of small cell variant were high-grade tumours, but the number of cases in this present study was too small to reach statistical power. No grade preference was seen for clear cell (P=0.354) and signet ring cell (P=0.474) variants. For deciduoid cell variant, no variance in incidence based on gender was found (male incidence: 16.6% vs female incidence: 18.7%, P=0.259), or patient age (≤65 years vs >65 years, P=0.609).

Cytological variant	Total (≥1%)	Total (≥10%)	Low-grade (*n*=366) †	High-grade (*n*=197) †	Pleomorphic (*n*=51) †	*P* †
Total *	199 (32.4%)	77 (12.5%)	88 (24.0%)	78 (40.0%)	33 (64.7%)	3.3×10^{-9}
Clear cell	80 (13.0%)	23 (3.7%)	42 (11.4%)	31 (15.7%)	7 (13.7%)	0.354
Signet ring cell	33 (5.4%)	10 (1.6%)	22 (6.0%)	10 (5.1%)	1 (2.0%)	0.474
Deciduoid cell	105 (17.1%)	44 (7.2%)	33 (9.0%)	44 (22.3%)	28 (54.9%)	2.2×10^{-16}
Rhabdoid cell	20 (3.3%)	2 (0.3%)	2 (0.5%)	10 (5.1%)	8 (15.7%)	1.9×10^{-18}
Small cell	2 (0.3%)	1 (0.2%)	0 (0.0%)	2 (1.0%)	0 (0.0%)	-

Table 6-1 Incidence of cytological variants in epithelioid PM and association with nuclear grade and pleomorphic features.

* Presence of any cytological variant (≥1, multiple variants can co-exist).

† Based on ≥1% cut-off.

Cytological variant	Co-existing cytological variant				
	Clear cell (%)	Signet ring cell (%)	Deciduoid cell (%)	Rhabdoid cell (%)	Small cell (%)
Clear cell (*n*=80)	-	7 (8.8)	18 (22.5)	0 (0.0)	1 (1.3)
Signet ring cell (*n*=33)	7 (21.2)	-	5 (15.2)	1 (3.0)	0 (0.0)
Deciduoid cell (*n*=105)	18 (17.1)	5 (9.5)	-	11 (10.5)	0 (0.0)
Rhabdoid cell (*n*=20)	0 (0.0)	1 (5.0)	11 (55.0)	-	0 (0.0)
Small cell (*n*=2)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	-

Table 6-2 Co-existence of cytological variants in epithelioid PM.

The presence of cytological variants was assessed based on ≥1% cut-off.

6.4.2 Prognostic impact of cytological variants

Univariate survival analysis showed the presence of clear cell (median OS 12.0 vs 15.3 months, P=0.028) or rhabdoid cell (5.2 vs 15.3 months, P=2.4×10^{-5}) variants were adverse prognostic variables (Figure 6-6). Both cases of small cell variants were of highly aggressive clinical phenotypes, with OS of 3.3 and 3.9 months respectively. Signet ring (P=0.894) and deciduoid cell (p=0.225) variants were not found to be significant prognostic variables. The prognostic impact of deciduoid cell variant was dependent on the nuclear grade (Figure 6-6D).

In multivariate analysis, clear cell variant predicted OS independent of established prognostic variables including age, type of procedure, necrosis, atypical mitosis and 2-tier nuclear grade (HR 1.40, P=0.012) (Table 6-3). Using the same set of covariates, rhabdoid cell variant however was not an independent predictor of OS (P=0.606) (Table 6-4).

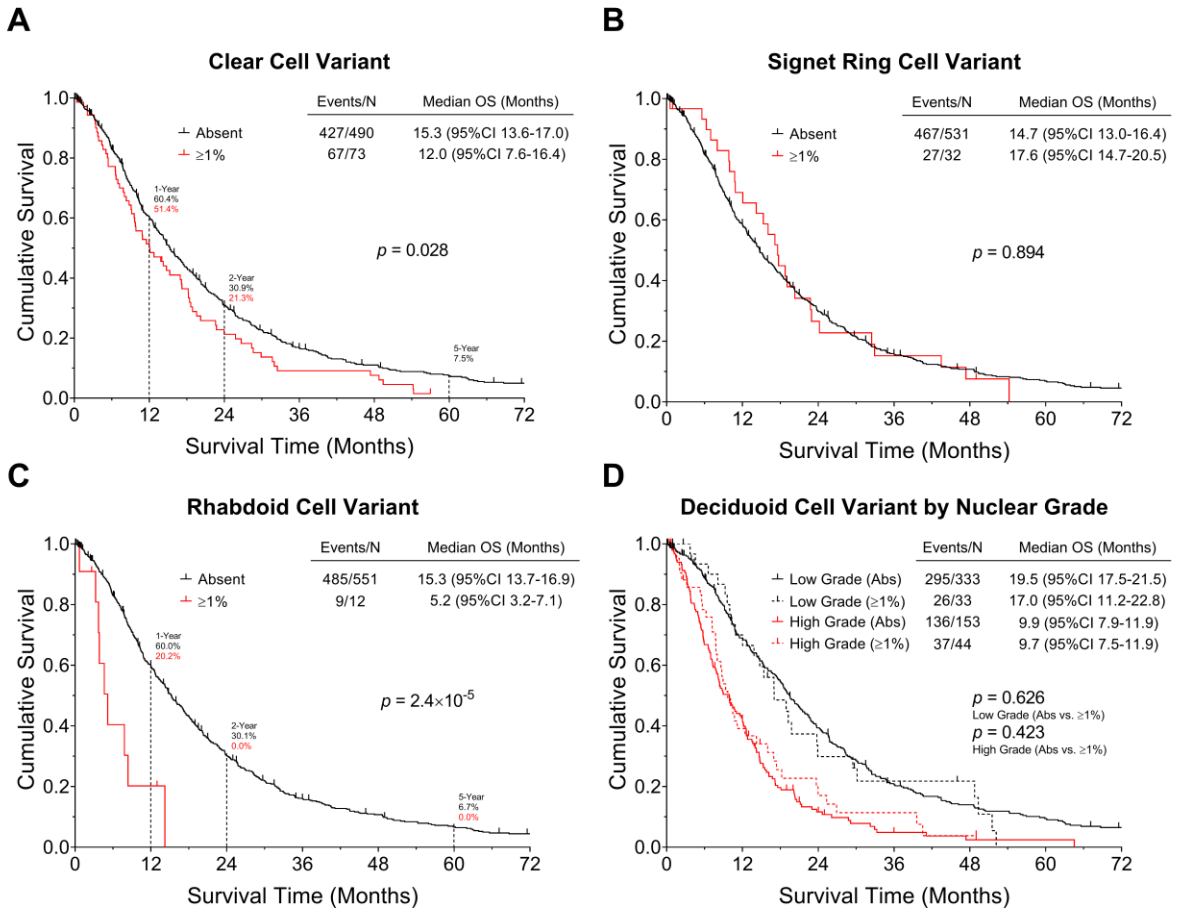


Figure 6-6 Univariate survival analysis of the prognostic impact of cytological variants in non-pleomorphic E-PM.

(A) Clear cell variant, **(B)** signet ring cell variant, **(C)** rhabdoid cell variant and **(D)** deciduoid cell variant stratified by 2-tier nuclear grade. Abs, absent; CI, confidence intervals; OS, overall survival.

Variable	Hazard Ratio	95% CI	*P*
Age >65 vs. ≤65 years	1.40	1.15-1.71	*0.001*
Procedure Resection vs. Biopsy only	0.32	0.23-0.44	2.8×10^{-12}
Necrosis Present vs. Absent	1.12	0.78-1.62	0.533
Atypical mitosis Present vs. Absent	1.59	1.27-1.98	4.5×10^{-5}
2-tier nuclear grade High-grade vs. Low-grade	1.96	1.45-3.11	1.2×10^{-4}
Clear cell variant Present vs. Absent	1.40	1.08-1.81	*0.012*

Table 6-3 Multivariate analysis in predicting overall survival (Clear cell variant).

CI, confidence intervals.

Variable	Hazard Ratio	95% CI	*P*
Age >65 vs. ≤65 years	1.34	1.12-1.61	*0.002*
Procedure Resection vs. Biopsy only	0.36	0.26-0.48	2.0×10^{-11}
Necrosis Present vs. Absent	1.19	0.86-1.64	0.292
Atypical mitosis Present vs. Absent	1.56	1.25-1.94	6.9×10^{-5}
2-tier nuclear grade High-grade vs. Low-grade	1.94	1.37-2.75	2.0×10^{-4}
Rhabdoid Cell Variant Any vs. Absent	0.86	0.48-1.53	0.606

Table 6-4 Multivariate analysis in predicting overall survival (Rhabdoid cell variant).

CI, confidence intervals.

6.5 Discussion

This study offers a further expansion of the concept of inter- and intratumoural heterogeneity of E-PM beyond nuclear grade and growth patterns- that cytological variants are not uncommonly seen in this type of malignancy. The variants show strong associations with nuclear grade both in terms of incidence and prognostic impact. Clear cell variant is an independent prognostic variable based on available evidence, however, the clinical significance of this is uncertain given that the effect size is relatively small. These findings warrant independent validation.

The findings of the study are relevant to contemporary diagnosis and research of PM in three aspects. First, the presence of cytological variants is associated with diagnostic challenges, more so in the small biopsy setting or when clinical and radiological information is not made available to the pathologist at time of histopathological assessment. To differentiate them from the morphological mimics, however, will likely require no more than a few widely available immunohistochemical or molecular markers (Table 6-5). Second, the prognostication of E-PM is centred around nuclear grade, and this is of particular relevance for deciduoid cell variant where this present study has confirmed the earlier observations by Ordóñez *et al*. (Ordóñez et al., 2012). A recent discussion was raised by Aujayeb and Taylor (Aujayeb and Taylor, 2020) (Taylor *et al*., 2019), implying deciduoid cell variant confers worse prognosis on its own thereby challenging the notion that this variant bears no prognostic relevance (Nicholson *et al*., 2020). At that point, it was likely that the observation was the result of nuclear grade bias, since reviewing the presented pictomicrograph and microscopic description (Taylor *et al*., 2019) the tumour was at least high-grade (grade II with necrosis) epithelioid PM, and if representative of the entire tumour, one with pleomorphic features. In the present study population, 1-year survival of deciduoid variant in

low- and high-grade E-PM was 70.0% and 39.2% respectively, and did not differ significantly from tumours without. In a large study (*n=164*) of peritoneal mesothelioma, a predominant deciduoid cell component was found to predict OS independent of nuclear grade (Malpica *et al*., 2021). Nonetheless the data from the Malpica *et al.* study requires cautious interpretation because the level of statistical significance was borderline and the clinical characteristics of peritoneal and pleural mesothelioma are radically different. Tumours with predominantly deciduoid cell component was rarely seen in the cohort of the present study, meaning the question on whether there is a dosage effect will be a subject for further investigation.

Third, the clinical significance of clear cell is likely small, and small cell variant appears extremely rare despite its highly aggressive clinical phenotype. In addition, among a minority of cases there are co-existing variants which are of uncertain clinical significance. The findings from this study support that the recording of cytological variants should be optional in routine diagnostic practice and depends on the interests and capacity of individual centres. However, it is arguably a good practice to document their presence similar to growth patterns as outlined by the latest WHO classification allowing further assessment in relation to its prognostic and predictive utility. This is in contrast to nuclear grade where the evidence supports it being a key or mandatory item to be recorded.

The major weakness of this study was the lack of molecular correlation. Pathogenic mutation in the gene encoding Von Hippel-Lindau Tumour Suppressor (*VHL*: 3p25.3) was found in a case of peritoneal mesothelioma with clear cell variant (Smith-Hannah and Naous, 2019). Clear cell renal cell carcinoma, including those with sarcomatoid transformation and rhabdoid features, are commonly associated with alterations in *BAP1* and *CDKN2A* (Bakouny *et al*., 2021) (Ricketts *et al*., 2018). Alterations in these two genes are also common in PM. In deciduoid cell variant, SMARCB1 (*SMARCB1*: 22q11.23) expression was frequently reduced

or lost (Kawai *et al*., 2019), and early comparative genomic hybridisation (CGH) data has shown high frequency of copy number losses in regions encoding *CDKN2A* (9p), *RB1* and *BRCA2* (13q) (Scattone *et al*., 2006). It is reasonable to hypothesise there are distinct genomic and transcriptomic profiles in these variants in conjunction with metabolic and epigenetic transcriptional reprogramming. Collectively the cytological variants likely represent complex tumour biology, to be deciphered utilising a myriad of multi-omic and experimental approaches.

6.6 Conclusions

To conclude, in relation to the aims of this Chapter, cytological variants are not uncommon in epithelioid PM, and are more common in high-grade tumours. The clear cell variant is an independent predictor of survival but the effect size is small. The prognostic impact of deciduoid and rhabdoid cell variants is a function of nuclear grade. Therefore at this point and based on the results presented, it is concluded that the recording of cytological variants should be best regarded as an optional item in routine diagnostic practice.

Cytological variant	Common morphological mimic(s)	Distinguishing features, IHC-based or molecular markers†
Clear cell	- Clear cell renal cell carcinoma - Clear cell LUAD	1. Standard mesothelioma IHC panel 2. TTF-1, BerEP4, Napsin A (to rule out lung adenocarcinoma) 3. CD10, RCC antigen, PAX8, CAIX (to rule out renal cell carcinoma)
Signet ring cell	- Microcystic/adenomatoid growth pattern - Signet ring adenocarcinoma - Entrapped adipocytes	1. Microcystic pattern has >1 tumour cell per open space. The cystic space often shows significant expansion. 2. CK7, CK20, CDX2, PAX8, WT-1 (to rule out common sites of signet ring adenocarcinoma) 3. Adipocytes are found at the advancing edge of the tumour. With significant disease burden they tend to be absent in the tissue sample.
Deciduoid cell	- Decidualised endometrial stromal cells - Rhabdoid cell variant - Squamous cell carcinoma (non-keratinising)	1. The presence of nuclear atypia is not a feature of pleural deciduosis. 2. Rhabdoid cell variants are characterised by the presence of well-defined cytoplasmic pale eosinophilic globules. 3. Strong p63 or p40 expression (but not CK5/6) is not expected in mesothelioma
Rhabdoid cell	- Rhabdomyosarcoma - Rhabdomyosarcomatous differentiation in PM - Other high-grade neoplasms with rhabdoid features	1. Pan-cytokeratin, Desmin, Myogenin, MyoD1, *FOXO1* re-arrangement (to rule out rhabdomyosarcoma) 2. Absence of myoid lineage marker expression excludes rhabdomyosarcomatous differentiation in PM 3. Heterologous differentiation is almost never seen in epithelioid PM, and most likely represents under-sampled non-epithelioid disease 4. Low threshold for specialist referral in case of poorly or undifferentiated neoplasms with rhabdoid features.
Small cell	- Small cell carcinoma - High-grade lymphoma with pleural involvement - Carcinoma with *NUTM1* re-arrangement	1. TTF-1, CD56, Synaptophysin, Chromogranin A, INSM1, *TP53*/*RB1* status (to rule out small cell carcinoma) 2. CD20, CD45, CD79a, CD138 (to rule out lymphoma) 3. p63/p40, *NUTM1* status by IHC, FISH or RNA sequencing (to rule out carcinoma with *NUTM1* re-arrangement, although pleural involvement is considered extremely uncommon)

Table 6-5 Common morphological mimics of cytological variants.

BerEP4, antibody clone against epithelial cell adhesion molecule (EpCAM); CAIX, carbonic anhydrase 9; CD, cluster of differentiation; CDX2, caudal type homeobox 2; CK5/6, cytokeratin 5/6; CK7, cytokeratin 7; CK20, cytokeratin 20; FISH, fluorescence *in situ* hybridisation; *FOXO1*, forkhead box O1; INSM1, insulinoma-associated protein 1; LUAD, lung adenocarcinoma; MyoD1, myogenic differentiation 1; NUTM1, NUT midline carcinoma family member 1; PAX8, paired box 8; RCC, renal cell carcinoma; TTF-1, thyroid transcription factor 1; WT-1, Wilms Tumour Protein.

† With extensive clinical and radiological information, diagnosis can be reliably achieved in most cases without ancillary testing.

7 Correlation between *CDKN2A*/*MTAP* Genomic Status, MTAP Immunohistochemistry and Histopathological Features in Pleural Mesothelioma

This chapter has been presented as a platform presentation at as a platform presentation at the European Congress of Pathology, 5th-9th December 2020, Glasgow (Virtual); and published in part, as Nastase A, Mandal A, Lu SK, Anbunathan H, Morris-Rosendahl D, Zhang YZ *et al*. Multiple therapeutic pathways in malignant mesothelioma identified by genomic mapping. *medRxiv* 2020.01.23.20018523; doi: https://doi.org/10.1101/2020.01.23.20018523.

Primary genomic data on *CDKN2A* and *MTAP* copy number status (section 7.4.3 and 7.4.4) was generated prior to this study, and the analysed data was provided by Professor Miriam F Moffatt and Professor William OCM Cookson (National Centre for Mesothelioma Research, National Heart and Lung Institute, Imperial College London). The experimental components of MTAP immunohistochemistry protocol development (section 7.3.2) were performed by Mr Alexander Bowman and Ms Toyin Adefila-Ideozu (Respiratory Biomedical Research Unit, Royal Brompton Hospital) for 2G4 clone, and Mr Jamie Hughes (Department of Histopathology, Royal Brompton and Harefield Hospitals) for EPR6893 clone. The assessment of stained tissue sections during assay development was performed by myself and Professor Andrew G Nicholson (Department of Histopathology, Royal Brompton and Harefield Hospitals). The remaining sections of this Chapter were the results of my independent research.

7.1 Abstract

Background: Deletion of cyclin dependent kinase inhibitor 2A (*CDKN2A*)/p16 tumour suppressor is one of the hallmark genomic events in the pathogenesis of pleural mesothelioma (PM). Methylthioadenosine phosphorylase (MTAP) has emerged as a potential cost-effective surrogate marker based on the high frequency of co-deletion. MTAP immunohistochemistry (IHC) has shown good correlation with *CDKN2A* FISH, however correlation with *CDKN2A*/*MTAP* genomic status by next generation sequencing (NGS), and to lesser extents, inter-assay and inter-observer agreement are currently uncertain.

Materials and methods: This was a single-institutional validation study of MTAP IHC for routine diagnostics using whole exome sequencing (WES) and single nucleotide polymorphism (SNP) genotyping as gold standard. Twenty-seven histologically confirmed PM with matched frozen and FFPE tissue were included. Inter-assay agreement was performed for two commercial clones (EPR6893 & 2G4). Inter-observer agreement was performed between four pulmonary pathologists.

Results: *CDKN2A* deletion was detected in 55.6% (15/27) of cases, 48.1% (13/27) being homozygous. Both cases with *CDKN2A* heterozygous deletion showed *MTAP* heterozygous deletion and led to loss of MTAP staining. Copy number discordance between *CDKN2A* and *MTAP* was found in two cases with *CDKN2A* homozygous deletion. Overall concordance between NGS and MTAP IHC (EPR6893) was 73.1%. By assigning focal staining as positive, sensitivity and specificity were 81.8% and 66.7% respectively. The two antibody clones demonstrated good inter-assay agreement (Cohen's kappa = 0.718). Inter-observer agreement was moderate to good (Fleiss' kappa = 0.650). Focal staining pattern and discordant staining patterns in biphasic PM were observed, neither was informative in

determining *CDKN2A* status. IHC utilising EPR6893 clone abrogated the problem of weak staining intensity reported in previous literature utilising 2G4 clone.

Conclusions: MTAP IHC showed reasonable concordance with *CDKN2A*/*MTAP* genomic status whilst a confirmatory assay by FISH or NGS is advisable especially outside the correct clinical context. EPR6893 and 2G4 clones could be used interchangeably with moderate to good inter-observer agreement.

7.2 Introduction

Recent advances in massive parallel sequencing of pleural mesothelioma (PM) have identified recurrent deletions of cyclin dependent kinase inhibitor 2A (*CDKN2A*)/p16 tumour suppressor as one of the hallmark genomic events in its pathogenesis (Hmeljak *et al*., 2018) (Bueno *et al*., 2016). This has subsequently generated substantial effort in utilising *CDKN2A* genomic status in the diagnosis and prognostication of PM, for the latter *CDKN2A* loss is associated with non-epithelioid subtypes and worse overall survival (OS).

Unlike BRCA-associated protein 1 (BAP1) where immunohistochemistry (IHC) for the protein correlates strongly with loss of *BAP1* gene expression, fluorescence *in situ* hybridisation (FISH) is currently regarded the gold standard in diagnosis for assessment of *CDKN2A* status (Hida *et al*., 2016) (Sheffield *et al*., 2015) (Chiosea *et al*., 2010). IHC for p16 has shown variable sensitivity (71-88%) and specificity (51-100%) (Hida *et al*., 2017) (Krasinskas *et al*., 2010) (Chiosea *et al*., 2008) (Dacic *et al*., 2008), and a report using quantitative PCR as gold standard showed whilst 7 out of 8 primary PM cell lines harboured *CDKN2A* deletion, convincing p16 loss was seen in only one case (Chernova *et al*., 2016). However methylthioadenosine phosphorylase (*MTAP*), encoding an essential enzyme and proposed tumour suppressor (Schmid *et al*., 2000) involved in the salvage pathway of adenosine monophosphate synthesis, occupies the same gene locus (*9p21.3*) as *CDKN2A* over a 200kb segment. Co-deletion is frequent based on FISH studies (92-100%) (Krasinkas *et al*., 2010) (Illei *et al*., 2003) and therefore this has led to its emergence as a surrogate marker for *CDKN2A* status with a diagnostic sensitivity and specificity, indicated by MTAP loss, of 42-76% and 77-100% respectively, which can be further improved when combined with BAP1 IHC (Table 7-1).

Over the past 4 years there have been several published studies utilising various types of tissue materials assessing the concordance between *CDKN2A* status, exclusively via FISH, and MTAP immunohistochemistry in mostly pleural mesothelioma (Table 7-2). MTAP IHC demonstrated satisfactory assay concordance (80-95%), sensitivity (75-100%) and specificity (67-94%). On the other hand several questions remain with limited available data to date. Firstly, data on the correlation between MTAP IHC and *CDKN2A* status determined by next generation sequencing (NGS) is lacking. Secondly, there are uncertainties in relation to inter-assay agreement although inter-observer agreement was recently reported to be excellent (Chapel *et al*., 2020). Thirdly, performance of MTAP IHC in biopsy-heavy setting is crucial in determining its utility as it will likely suffer from low cellularity, intra-tumoural heterogeneity and variable staining intensities, the latter two were already reported in a significant minority of cases (4-45%) (Berg *et al*., 2018) (Kinoshita *et al*., 2018a) (Zimling *et al*., 2012), so was the deterioration in assay performance in a biopsy-subgroup analysis although the number of cases was limited (Chapel *et al*., 2020).

In this study we aim to address these questions using two independently developed in-house MTAP IHC assays, and correlation of MTAP IHC with a previous comprehensive genomic and transcriptomic study of PM.

	Study cohort	Origin	Subtype	Tissue materials (PM)	MTAP immunohistochemistry	Diagnostic performance†[a]
Zimling *et al*., 2012	*n*=138 PM: 99 Benign: 38	Pleural	E: 58 B: 29 S: 12 NOS: 0	Resection: 0 Biopsy: 138 PE cell block: 34 TMA (1mm ×4-8)	Rabbit anti-human pAb (ProteinTech Group, Inc.)** Dilution: 1:800 Platform: Dako Autostainer PLUS Heterogeneity or weak: 45.4%	<u>Biopsy</u> Sensitivity: 65% Specificity: 77% <u>PE cell block</u> Sensitivity: 71% Specificity: 90%
Hida *et al*., 2017	*n*=76 PM: 51 Benign: 25	Pleural	E: 44 B: 7 S: 0 NOS: 0	Resection: 35 Biopsy: 16 PE cell block: 0 Whole slide staining	Rabbit anti-human mAb (Abcam)‡ Dilution: 1:500 Platform: Dako Omnis Cut-off: ≥67.8% loss	Sensitivity: 45.1% (82.4%) Specificity: 100% (80%)
Kinoshita *et al*., 2018a	*n*=66 PM: 45 Benign: 21	Pleural	E: 42 B: 3 S: 0 NOS: 0	Resection: 0 Biopsy: 0 PE cell block: 45 Whole slide staining	Mouse anti-human mAb (2G4, Abnova) Dilution: 1:100 Platform: Not specified Cut-off: ≥50% loss	Sensitivity: 42.2% (77.8%) Specificity: 100% (100%)
Berg *et al*., 2018	*n*=37 MM: 20 Benign: 17	Pleural (35) Peritoneal (2)	E: 20 B: 0 S: 0 NOS: 0	Resection: Not specified Biopsy: Not specified PE cell block: Not specified TMA	Mouse anti-human mAb (2G4) Dilution: 1:100 Platform: Dako Omnis Cut-off: ≥50% loss*	Sensitivity: 65% (90%) Specificity: 100% (100%)
Kinoshita *et al*., 2018b	*n*=47 PM: 30 Benign: 17	Pleural	E: 0 B: 12 S: 18 NOS: 0	Resection: Not specified Biopsy: Not specified PE cell block: 0 Whole slide staining	Mouse anti-human mAb (2G4) Dilution: 1:100 Platform: Not specified Cut-off: ≥50% loss	Sensitivity: 82.8% (90%) Specificity: 100% (100%)
Yoshimura *et al*., 2019	*n*=67 PM: 38 Benign: 29	Pleural	E: 27 B: 6 S: 5 NOS: 0	Resection: 24 Biopsy: 13 Autopsy: 1 PE cell block: 0 Whole slide staining	Mouse anti-human mAb (2G4) Dilution: 1:100 Platform: Not specified Cut-off: ≥50% loss	Sensitivity: 47.3% (73.7%) Specificity: 100% (100%)

Hamasaki *et al*., 2019	*n*=93 PM: 73 Benign: 20	Pleural	E: 68 B: 5 S: 0 NOS: 0	Resection: 0 Biopsy: 0 PE cell block: 93 Whole slide staining	Mouse anti-human mAb (2G4) Dilution: 1:100 Platform: Not specified Cut-off: ≥50% loss	<u>Loss = Cytoplasmic loss</u> Sensitivity: 50.7% Specificity: 100% <u>Loss = Nuclear and cytoplasmic loss</u> Sensitivity: 49.3% Specificity: 100%
Kinoshita *et al*., 2020	*n*=74 PM: 47 Benign: 27	Pleural	E: 32 B: 5 S: 10 NOS: 0	Resection: Not specified Biopsy: Not specified PE cell block: Not specified Whole slide staining	Mouse anti-human mAb (2G4) Dilution: 1:100 Platform: Not specified Cut-off: ≥50% loss	Sensitivity: 70.2% (89.4%) Specificity: 100% (100%)
Hiroshima *et al*., 2020	*n*=153 PM: 62 Benign: 36 Other cancers: 55	Pleural	E: 24 B: 9 S: 0 NOS: 29	Resection: 0 Biopsy: 0 PE cell block: 62 Whole slide staining	Mouse anti-human mAb (DA2) Dilution: 1:200 Platform: Leica BOND Max or BOND III Cut-off: ≥75% loss	Sensitivity: 76.2% (84.4%) Specificity: 100% (100%)

Table 7-1 Published studies using MTAP immunohistochemistry for the diagnosis of malignant mesothelioma.

† Sensitivity and specificity were calculated based on the loss of MTAP staining. ‡ No further information on the product specification was provided. * No preset cut-off was used by the authors. The lowest percentage loss of staining in categorically MTAP negative cases was 50%. [a] Sensitivity and specificity when MTAP IHC were combined with BAP1 IHC are included in brackets.

E, epithelioid (subtype); FISH, fluorescence *in situ* hybridisation; mAb, monoclonal antibody; MM, malignant mesothelioma; PM, pleural mesothelioma; MTAP, methylthioadenosine phosphorylase; NOS, not otherwise specified; pAb, polyclonal antibody; PE, pleural effusion; S, sarcomatoid (subtype); TMA, tissue microarray.

	Study cohort	Origin	Subtype	Tissue materials (PM)	*CDKN2A* detection	MTAP detection	Diagnostic performance‡
Hida *et al*., 2017	*n*=76 PM: 51 Benign: 25	Pleural	E: 44 B: 7 S: 0 NOS: 0	Resection: 35 Biopsy: 16 PE cell block: 0 Whole slide staining	FISH Cut-off: ≥11% HD	Rabbit anti-human mAb† Dilution: 1:500 Platform: Dako Omnis Cut-off: ≥67.8% loss	Concordance: 84.3% Sensitivity: 100% Specificity: 74.2%
Kinoshita *et al*., 2018a	*n*=66 PM: 45 Benign: 21	Pleural	E: 42 B: 3 S: 0 NOS: 0	Resection: 0 Biopsy: 0 PE cell block: 45 Whole slide staining	FISH Cut-off: ≥10% HD	Mouse anti-human mAb (2G4) Dilution: 1:100 Platform: Not specified Cut-off: ≥50% loss Heterogeneity or weak: 4.9%	Concordance: 80% Sensitivity: 100% Specificity: 67.9% Concordance with tissue diagnosis: 100% (*n*=30)
Berg *et al*., 2018	*n*=37 MM: 20 Benign: 17	Pleural (35) Peritoneal (2)	E: 20 B: 0 S: 0 NOS: 0	Resection: Not specified Biopsy: Not specified PE cell block: Not specified TMA (1mm ×1-2)	FISH Cut-off: ≥20% HD Not assessable: 15%	Mouse anti-human mAb (2G4) Dilution: 1:100 Platform: Dako Omnis Cut-off: ≥50% loss*	Concordance: 82.3% Sensitivity: 80% Specificity: 83.3%
Kinoshita *et al*., 2018b	*n*=47 PM: 30 Benign: 17	Pleural	E: 0 B: 12 S: 18 NOS: 0	Resection: 0 Biopsy: 0 PE cell block: 73 Whole slide staining	FISH Cut-off: ≥10% HD	Mouse anti-human mAb (2G4) Dilution: 1:100 Platform: Not specified Cut-off: ≥50% loss Not assessable: 4.3%	Concordance: 93.1% Sensitivity: 100% Specificity: 92.6%
Hamasaki *et al*., 2019	*n*=93 PM: 73 Benign: 20	Pleural	E: 68 B: 5 S: 0 NOS: 0	Resection: 0 Biopsy: 0 PE cell block: 93 Whole slide staining	FISH Cut-off: ≥10% HD	Mouse anti-human mAb (2G4) Dilution: 1:100 Platform: Not specified Cut-off: ≥50% loss	Loss = Cytoplasmic loss Concordance: 90.4% Sensitivity: 100% Specificity: 84.1% Loss = Nuclear and cytoplasmic loss Concordance: 89.0% Sensitivity: 100% Specificity: 81.8%
Chapel *et al*.,	*n*=119	Pleural	E: Not	Resection: 40	FISH	Clone 1: Mouse anti-human	Inter-observer agreement

2020[a]	PM: 99 Benign: 20		specified B: Not specified S: Not specified NOS: Not specified	Biopsy: 18 PE cell block: 8 Not specified: 33 Whole slide staining: 66 TMA: 33 (1mm ×1-2)	Cut-off: ≥10-20% HD (centre-dependent)	mAb (2G4) Clone 2: Mouse anti-human mAb (42-T) Clone 3: Mouse anti-human mAb (DA2) Dilution: 1:100 (42-T), 1:100 (DA2), 1:50-100 (2G4) Platform: Leica Bond Max or RX Cut-off: Pathologist judgement Heterogeneity: 4.0% Weak staining: 11.1%	Fleiss' κ: 0.82 (95% CI 0.74-0.90) <u>Inter-assay agreement</u> Cohen's κ: 0.39-0.89 (3 centres) <u>All materials</u> Concordance: 85.7% Sensitivity: 95.8% Specificity: 78.1% <u>Biopsies</u> Concordance: 76.5% Sensitivity: 100% Specificity: 66.7% <u>PE cell blocks</u> Concordance: 85.7% Sensitivity: 100% Specificity: 75%
Kinoshita *et al*., 2020	*n*=74 PM: 47 Benign: 27	Pleural	E: 32 B: 5 S: 10 NOS: 0	Resection: Not specified Biopsy: Not specified PE cell block: Not specified Whole slide staining	FISH Cut-off: ≥10% HD	Mouse anti-human mAb (2G4) Dilution: 1:100 Platform: Not specified Cut-off: ≥50% loss	Concordance: 91.7% Sensitivity: 100% Specificity: 91.7%
Brich *et al*., 2020	*n*=75 MM: 75 Benign: 0	Peritoneal	E: 71 B: 2 S: 2 NOS: 0	Resection: 75 Biopsy: 0 PE cell block: 0 Whole slide staining: 23 TMA: 52 (×4-12)	FISH Cut-off: ≥30% HD	Mouse anti-human mAb (2G4) Dilution: 1:200 Platform: Ventana BenchMark ULTRA Cut-off: ≥50% loss	Concordance: 93.8% Sensitivity: 97.0% Specificity: 86.7%
Chapel *et al*., 2021	*n*=40 PM: 40 Benign: 0	Pleural	E: 28 B: 10 S: 2 NOS: 0	Resection: 34 Biopsy: 6 PE cell block: 0 Whole slide staining	HC-NGS: 29 SNP array: 7 Both: 4	Mouse anti-human mAb (42-T) Dilution: 1:75 Platform: Not specified Cut-off: >50% loss	<u>*CDKN2A* HD only</u>[b] Concordance: 62.5% Sensitivity: 66.7% Specificity: 53.8% <u>Heterozygous deletion + HD</u> Concordance: 72.5%

						Heterogeneity: 22.5% Weak staining: 17.5%	Sensitivity: 100% Specificity: 59.3%
Berg *et al*. 2020	*n*=36 PM: 21 Benign: 15	Pleural	E: Not specified B: Not specified S: Not specified NOS: Not specified	Resection: 0 Biopsy: 0 PE cell block: 39 Whole slide staining	FISH Cut-off: ≥20% HD	Mouse anti-human mAb (2G4) Dilution: 1:100 Platform: Not specified Cut-off: ≥75% loss Heterogeneity: 14.3%	Concordance: 71.4% Sensitivity: 75% Specificity: 66.7%
Hiroshima *et al*., 2020	*n*=153 PM: 62 Benign: 36 Other cancers: 55	Pleural	E: 24 B: 9 S: 0 NOS: 29	Resection: 0 Biopsy: 0 PE cell block: 62 Whole slide staining	FISH Cut-off: ≥15% HD	Mouse anti-human mAb (DA2) Dilution: 1:200 Platform: Leica BOND Max or BOND III Cut-off: ≥75% loss	Concordance: 95% Sensitivity: 100% Specificity: 94.1%

Table 7-2 Published studies correlating *CDKN2A* genomic status with MTAP immunohistochemistry.

† No further information on the product specification was provided. ‡ Sensitivity is calculated, among cases with retained FISH signals or copy number-neutral *CDKN2A* status, as (IHC positive cases) / [(IHC positive cases) + (IHC negative cases)]; and specificity is calculated, among cases with *CDKN2A* HD or *CDKN2A* copy number loss, as (IHC negative cases) / [(IHC positive cases) + (IHC negative cases)]. * No preset cut-off was used by the authors. The lowest percentage loss of staining in categorically MTAP negative cases was 50%. [a] Includes previously published cohorts (Berg *et al*., 2018) (Hida *et al*., 2017). [b] Calculated using binary categorisation of *CDKN2A* status into homozygous deletion (HD) and non-HD (includes both copy number neutral state and heterozygous deletion).

CDKN2A, cyclin dependent kinase inhibitor 2A; E, epithelioid (subtype); FISH, fluorescence *in situ* hybridisation; HC-NGS, hybrid-capture next generation sequencing; HD, homozygous deletion; mAb, monoclonal antibody; MM, malignant mesothelioma; PM, pleural mesothelioma; MTAP, methylthioadenosine phosphorylase; NOS, not otherwise specified; pAb, polyclonal antibody; PE, pleural effusion; S, sarcomatoid (subtype); TMA, tissue microarray.

7.3 Materials and methods

7.3.1 Study population

Twenty-seven cases of pleural mesothelioma diagnosed between 2005-2011 at the Royal Brompton and Harefield NHS Foundation Trust (London, UK) were included, all of which were part of a previous genomic and transcriptomic study (Nastase *et al*., 2020) and underwent routine diagnostic procedures or surgical resection with no prior chemotherapy or radiotherapy. Research ethics approval was obtained for the previous study (NRES: 10/H0504/9, 10/H0504/29). Formalin-fixed paraffin-embedded (FFPE) tissue blocks were prepared after mirrored fresh frozen samples were taken for molecular analysis. Histological diagnosis, tumour cellularity and purity (cut-off: ≥30%) were confirmed by a specialist pulmonary pathologist. Histopathological and clinical data including 2-tier nuclear grade (Zhang *et al*., 2020a) and presence of pleomorphic features (Zhang *et al*., 2020b) were extracted from an institutional mesothelioma database. Median OS was defined as time (measured in months) between the date of initial procedure from which a definitive diagnosis of PM was made, and the date of death or last follow-up. Censor fraction in our study was 7.4% (2/27).

7.3.2 Immunohistochemistry

4μm whole slide FFPE tumour sections from the same tissue block used for molecular analysis were stained for haematoxylin & eosin (H&E) and MTAP. IHC protocols for MTAP 2G4 (NovusBio, UK) (Nastase *et al*., 2020) and EPR6893 (Abcam, UK) clones were independently developed separated by a period of 8 months (2018-2019), with no team cross-over or prior knowledge. For 2G4 protocol, staining was undertaken on Ventana BenchMark ULTRA (Ventana Medical Systems Inc., USA). Antigen retrieval was performed using CC1 buffer (Ventana Medical Systems Inc., USA) with 5% goat serum, pH 8.55, at 100ºC for 32

minutes. This was followed by antibody incubation (1:200) at 37ºC for 32 minutes. For EPR6893 protocol, staining was undertaken on Leica BOND-III (Leica Biosystems, UK). Antigen retrieval was performed using ER2 buffer (Leica Biosystems, UK), pH 9.0, at 99ºC for 30 minutes. This was followed by antibody incubation (1:1000) at room temperature for 15 minutes. Both protocols utilised diaminobenzidine (DAB) amplification system. Weak haematoxylin counterstain and mounting were performed as per routine protocol. For both clones, external positive control tissue was renal tubular epithelium with internal positive controls being lymphocytes and stromal fibroblasts.

IHC staining was evaluated independently by each scorer blinded to the clinical and molecular data. The results were categorised as "positive", "negative" or "focal" based on pathologist's judgement. No pre-set cut-off for diffuseness was employed, but all scorers were asked to provide staining percentages of positive tumour cells in 5% increments. For 2G4, unanimous results were obtained after a joint review between 2 scorers (Nastase *et al*., 2020). For EPR6893, such were obtained from 5 scorers, upon which inter-observer agreement was also based. Scores on 2G4 and EPR6893 clones were generated independently with a wash-out period of 12 months.

Microscopic images in 300dpi TIF format were taken from representative cases using a Nikon Eclipse Ci-L microscope (Nikon Corporation, Japan) linked to a Nikon Digital Sight DS-L3 camera (Nikon Corporation, Japan), annotated using GNU Image Manipulation Program 2.10.10 (http://www.gimp.org, retrieved on 20.04.2019).

7.3.3 Next generation sequencing

The methodology has been described elsewhere (Nastase *et al*., 2020). In summary, genomic DNA was isolated from frozen tumour tissues according to manufacturer instructions (Qiagen DNA and RNA extraction kits, Qiagen, Germany). DNA yield and purity were assessed, and

subsequently hybridised using SureSelectXT Target Enrichment System for the Illumina Paired-End Multiplex Sequencing protocol (Illumina, UK). The captured libraries were whole-exome sequenced on an Illumina HiSeq2000 according to standard protocols. Copy number analysis was also performed using the Human Infinium Omni-Express-Exome v1.3 and v1.4 Bead Chips (Illumina, UK) arrays containing >950k markers.

7.3.4 Statistical analysis

Descriptive statistics were employed to analyse the baseline demographic and clinicopathological parameters. Two-sided chi-square test or Fisher's exact test was used to evaluate associations between categorical variables. Sensitivity was calculated as the percentage of MTAP positive cases among those with neutral *CDKN2A* copy number. Specificity was calculated as the percentage of MTAP negative (loss) cases among those with *CDKN2A* deletion (homozygous or heterozygous). For practical purposes, the diagnostic performance based on loss of MTAP staining can be gathered by swapping the sensitivity and specificity values calculated therein. Inter-assay and inter-observer agreements were assessed using Cohen's and Fleiss' κ, respectively. The strength of agreement based on κ values was categorised as follows: poor (<0.20), fair (0.21-0.40), moderate (0.41-0.60), good (0.61-0.80), excellent (0.81-1.00). OS was estimated using the Kaplan-Meier method and univariate analyses were performed for categorical variables with ≥10 events. Exact *P* values were recorded and $P<0.05$ denotes statistical significance. Multivariate analyses were not performed due to the small number of cases in our study. All statistical analyses were performed using SPSS 26 (IBM Corp., Armonk, NY, USA). Kaplan-Meier curves were generated using GraphPad Prism Version 8 (GraphPad Software, La Jolla California, USA).

7.4 Results

7.4.1 Patient characteristics

A summary of the characteristics of the study population is outlined in Figure 7-1A. The median age of our study population was 66 years (range: 50-81 years) with male preponderance (77.8%, 21/27). Right-sided disease represented 63% (17/27) of cases. The vast majority of patients (88.9%, 24/27) underwent biopsy only. Information on asbestos exposure was available on 14 patients (51.8%), of whom 13 (92.9%) were positive. With regard to the tissue dimension, 44.4% (12/27) had a maximum dimension of ≥20mm.

The tumour subtype was predominantly epithelioid (74.0%, 20/27), the rest being biphasic (26.0%, 7/27). Among the epithelioid disease, 11 were low grade (Grade I: 4, Grade II without necrosis: 7), 8 were high grade (Grade II with necrosis: 4, Grade III: 4) and one was with pleomorphic features. The median OS of all patients was 10.3 months (95% CI 1.9-18.7 months), with 1-year, 2-year and 5-year survival rates of 48%, 32% and 12% respectively (Figure 7-1B). Overall this was a small but representative study population of PM in the developed regions.

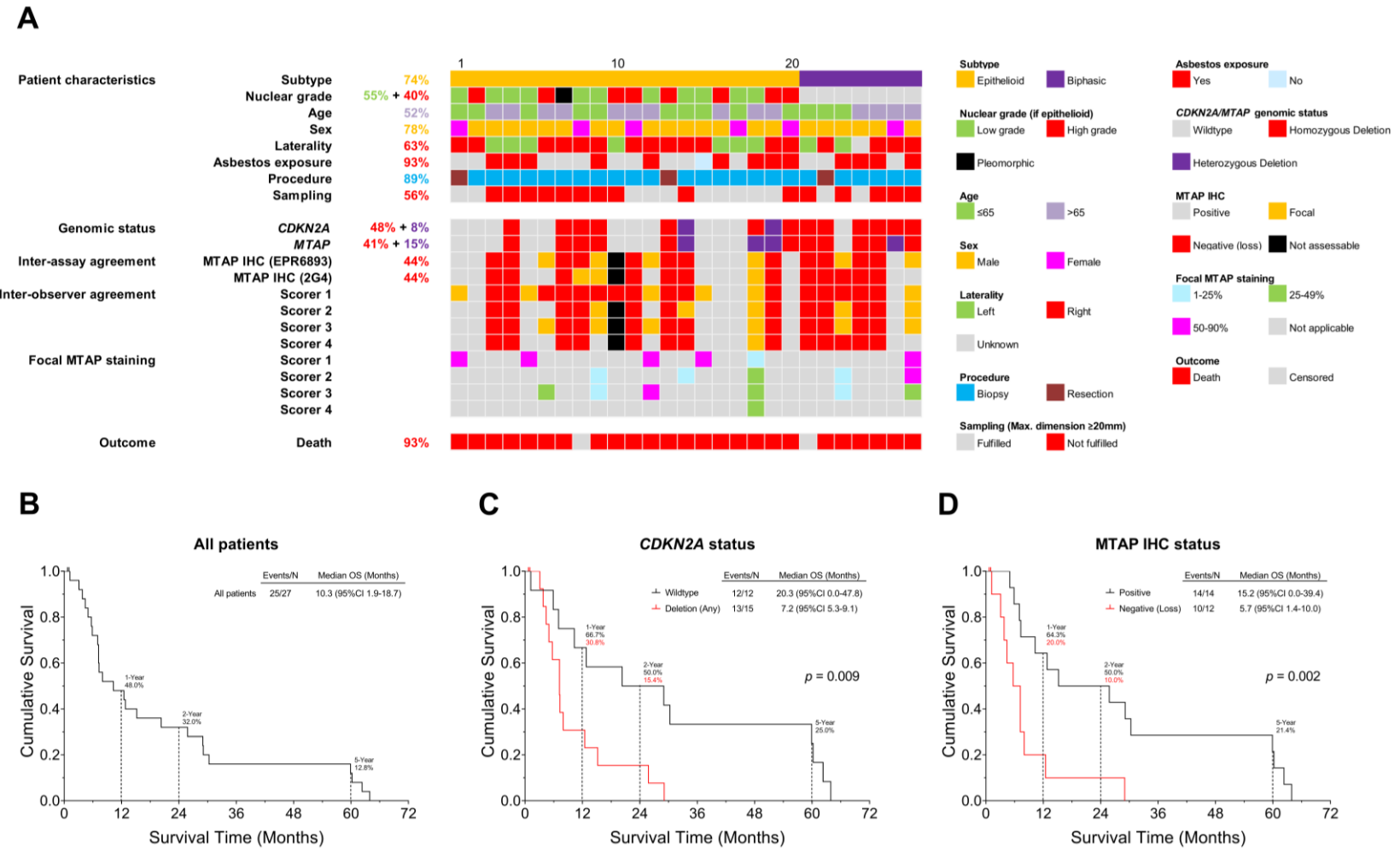


Figure 7-1 Characteristics of the study population, and prognostic impact of *CDKN2A* deletion (NGS) and MTAP loss (IHC). **(A)** Patient characteristics (*top panel*), correlation between *CDKN2A* genomic status and MTAP IHC status, inter-assay and inter-observer agreement (*middle panel*). **(B)** Overall survival of the study population. **(C)** Overall survival by *CDKN2A* genomic status. **(D)** Overall survival by MTAP (EPR6893) status.

7.4.2 Inter-assay and inter-observer agreement

The inter-assay agreement was good (Cohen's κ 0.718, 95% CI 0.508-0.927, *P*<0.001) (Figure 7-1A), and demonstrates that both assays can be employed interchangeably. It was noted the 2G4 clone generally produced stronger staining intensity but also with higher background (Figure 7-2C, 2F), a finding commented upon by previous studies (Chapel *et al*., 2020) (Kinoshita *et al*. 2018a). The EPR6893 clone on the other hand produced weaker staining intensity but allowed more clear-cut distinction (Figure 7-2B, 2E). The overall inter-observer agreement was moderate to good (Fleiss' κ 0.650, 95% CI 0.646-0.653, *P*<0.001) (Figure 7-1A) (Table 7-3). Out of the 27 cases, the scoring category in 5 (18.5%) were regarded as "focal" by at least two scorers and a total of 6 cases (22.2%) showed focal staining pattern after reaching consensus during review. Among all scorers, "focal" category was associated with at least 10% loss of MTAP staining in tumour cells and a wide range of values were recorded (10-99%) (Figure 7-1A). This pattern was prone to greater interobserver disagreement (Fleiss κ 0.333). One case (Case 10) was deemed not assessable among all scorers after review based on low tumour cellularity, and was therefore removed from subsequent analyses.

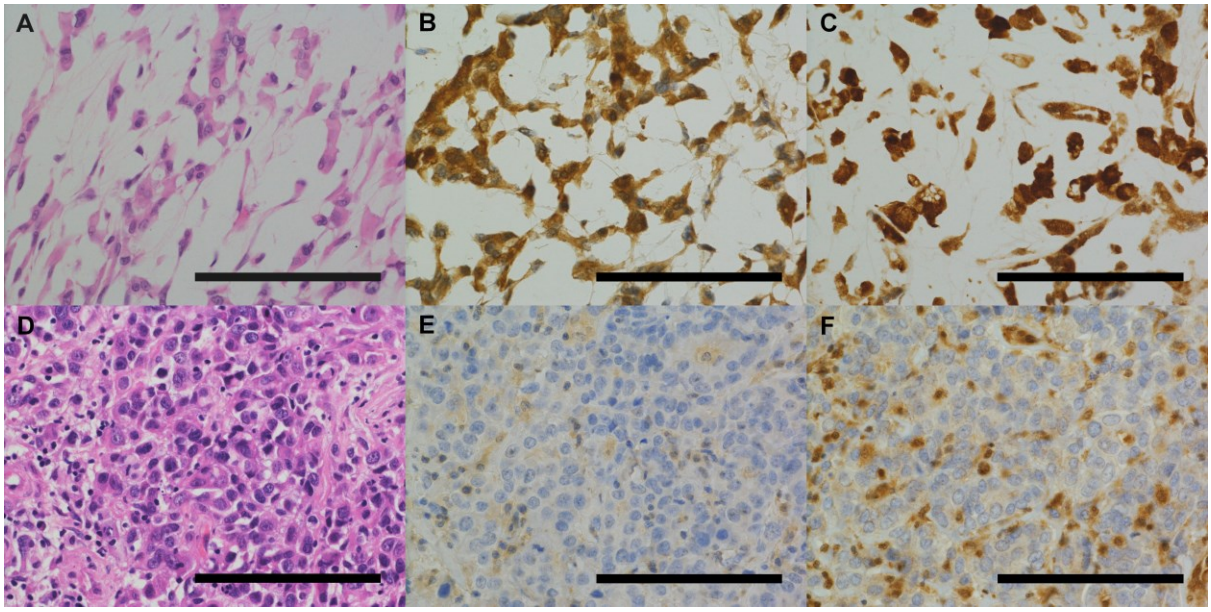


Figure 7-2 MTAP immunohistochemistry. **(A-C)** Tumour with positive MTAP staining, **(A)** H&E, **(B)** with EPR6893 clone, **(C)** with 2G4 clone. **(D-F)** Tumour with negative MTAP staining, **(D)** H&E, **(E)** with EPR6893 clone, **(F)** with 2G4 clone. Scale bar = 100µm.

	Fleiss' κ	**95% CI**	***P***
Overall	0.650	0.646-0.653	*<0.001*
Scoring categories			
Positive	0.676	0.671-0.681	
Focal	0.333	0.328-0.338	*<0.001*
Negative	0.802	0.797-0.807	

Table 7-3 Inter-observer agreement on MTAP IHC (EPR6893).

CI, confidence interval.

7.4.3 Correlation between *CDKN2A*/*MTAP* genomic status and MTAP immunohistochemistry

The genomic status of *CDKN2A* and *MTAP* were determined using a combination of whole exome sequencing (WES) and single nucleotide polymorphism (SNP) genotyping. *CDKN2A* deletion was detected in 55.6% of cases (15/27) and was enriched among biphasic subtype (85.7% vs. 45.0% among epithelioid subtype, $P<0.001$) (Figure 7-1A). Eleven cases presented with homozygous co-deletion of both genes, two with heterozygous co-deletion, and two with *CDKN2A* homozygous deletion and *MTAP* heterozygous deletion. No *CDKN2A*/*MTAP* mutation, or aneuploidy involving chromosome 9 were present in our study population. Within the epithelioid subtype, there was a non-significant trend that *CDKN2A* deletion was more frequently detected in Grade III/pleomorphic disease compared with Grade I and II ($P=0.205$) (Table 7-4).

After review 12 out of 26 cases (46.2%) were considered to show MTAP loss by immunohistochemistry (EPR6893). Given there is insufficient literature on the genomic correlate of focal MTAP staining pattern, it was regarded as "positive" at time of data analysis. The overall assay concordance was 73.1%, and positive MTAP staining predicted copy number neutral *CDKN2A* status with sensitivity of 81.8% and specificity of 66.7% (Table 7-5). A non-significant trend was observed towards discordant results when the maximum tissue dimension was <20mm ($P=0.391$) (Table 7-6). For 2G4 clone the assay concordance, sensitivity and specificity were 65.4%, 72.7% and 60.0% respectively (Table 7-7). While both cases with *CDKN2A* heterozygous deletion without copy number disparity with *MTAP* showed diffuse loss of MTAP staining, the other two cases with heterozygous *MTAP* deletion (Cases 18 and 27) but *CDKN2A* homozygous deletion showed at least focal MTAP retention.

CDKN2A homozygous deletion (7.2 months vs. 20.3 months, *P*=0.009) or loss of MTAP staining (EPR6893) (5.9 months vs. 15.2 months, *P*=0.002) alone predicted worse median OS in keeping with their prognostic implications reported in the literature (Figure 7-1C-D).

Histology	***n***	***CDKN2A* deletion**	***P***
Epithelioid	20	9 (45.0%)	-
Grade I	4	1 (25.0%)	-
Grade II	11	5 (45.5%)	-
Grade III/Pleomorphic	5	3 (60.0%)	0.205
Biphasic	7	6 (85.7%)	*<0.001* (vs. Epithelioid)

Table 7-4 Association of *CDKN2A* genomic status with histology including 2-tier nuclear grade.

	Neutral *CDKN2A* copy number (NGS)	***CDKN2A* deletion (NGS)**
MTAP positive	9	5
MTAP negative (Loss)	2	10
		Sensitivity: 81.8% Specificity: 66.7%

Table 7-5 Assay performance of MTAP IHC (EPR6893).

	≥20mm	<20mm
Concordance	10	9
Discordance	2	5
		$P = 0.391$

Table 7-6 Association between assay discordance (NGS vs. MTAP EPR6893) and maximum tissue dimension.

	Neutral *CDKN2A* copy number (NGS)	*CDKN2A* deletion (NGS)
MTAP positive	8	6
MTAP negative (Loss)	3	9
		Sensitivity: 72.7% Specificity: 60.0%

Table 7-7 Assay performance of MTAP IHC (2G4).

7.4.4 Unusual MTAP staining patterns and assay discordance

We encountered unusual patterns of focal MTAP staining (Chapel *et al*., 2021) (Berg *et al*., 2020) (Chapel *et al*., 2020) (Zimling *et al*. 2012) and weak staining intensity (Chapel *et al*., 2021) (Chapel *et al*., 2020) (Kinoshita *et al*. 2018a) as described by earlier reports. Of the 6 cases with focal MTAP staining, all showed staining intensity above the positive internal control. There was no morphological evidence suggesting sarcomatoid transformation. A representative case (Case 18) is shown in Figure 7-3A-C. Three cases had *CDKN2A*/*MTAP* co-deletion (3/6, 50%).

One of the 27 cases (Case 14) stained with 2G4 clone showed convincing weak staining intensity as described by earlier reports (Figure 7-3D-F). The tumour cells stained diffusely negative using EPR6893 clone, and NGS confirmed *CDKN2A*/*MTAP* heterozygous co-deletion.

In biphasic PM (B-PM) there have been anecdotal observations of discordant *CDKN2A* status between the epithelioid and sarcomatoid components, within the mesothelioma community although no data was made available in the public domain at this point. Discordant MTAP status by IHC has been reported recently (Schulte *et al*., 2020). While there was general concordance, two of the seven cases of B-PM in our study showed focally retained MTAP with diffuse loss in the epithelioid component (Figure 7-4). It has to be noted that these isolated observations were made either in the presence of stromal fibroblasts (Case 23, Figure 7-4I-L) or intense host response (Case 27, Figure 7-4M-P), both of which could complicate histological interpretation. Case 23 was *CDKN2A* copy number neutral while Case 27 harboured *CDKN2A* homozygous deletion with *MTAP* heterozygous deletion. The sequencing platform used in this study does not allow differential evaluation of genomic states in individual tumour cells.

We then evaluated the 7 cases with IHC-NGS assay discordance which was more frequent than reported in the published literature. Their pre-analytical variables and genomic findings are summarised in Table 7-8. None of the MTAP stained cases showed edge effect, high background or processing artefacts (e.g. folded sections). Cases 18 and 27 showed the unusual copy number states of *CDKN2A* homozygous deletion and *MTAP* heterozygous deletion. All but two cases failed to meet the minimum sampling criteria proposed by the recent IASLC/EURACAN consensus document (≥3 sites) (Nicholson *et al*., 2020) or an alternative recommendation proposed by our previous study when the former cannot be fulfilled (≥20mm maximum dimension from at least one site) (Zhang *et al*., 2020a). This raised the possibility of discordance due to intra-tumoural heterogeneity, but there was no definitive evidence as none of the patients received surgical resection or interval biopsies. The remaining case (Case 11) was an adequately sampled high grade E-PM with high tumour cellularity showing diffuse loss of MTAP (Figure 7-5). NGS showed *CDKN2A*/*MTAP* copy

number neutral states. We were unable to explain the observed discordance based on available data.

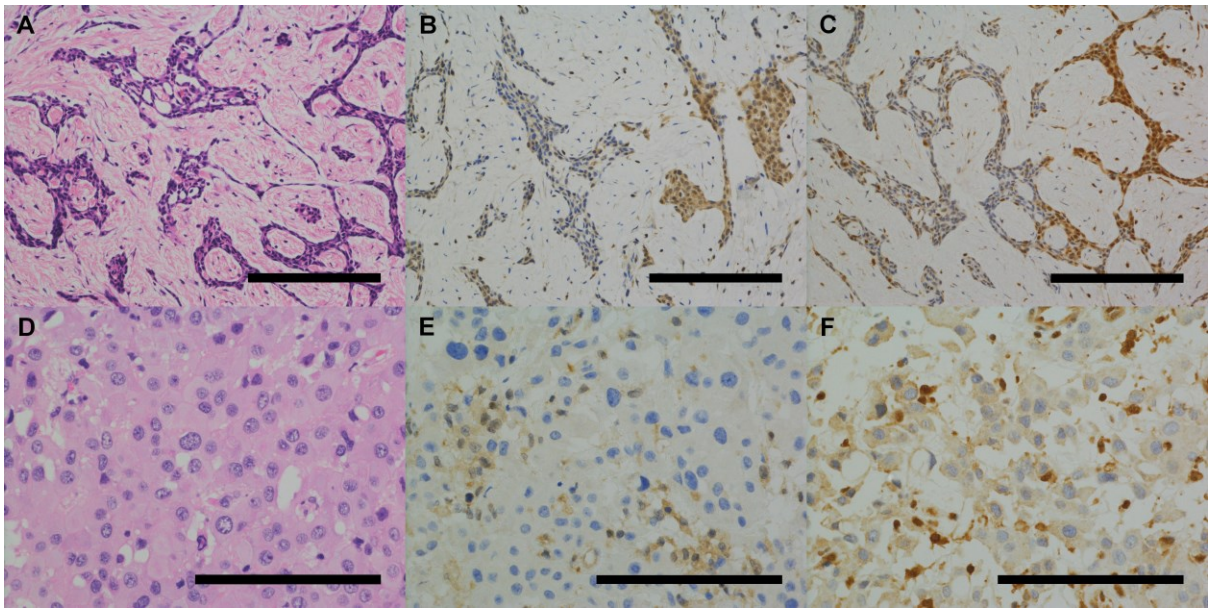


Figure 7-3 Representatives of focal and weak MTAP staining. **(A-C)** Tumour with focal MTAP staining (Case 18), **(A)** H&E, **(B)** with EPR6893 clone, **(C)** with 2G4 clone. **(D-F)** Tumour with weak MTAP staining (Case 14), **(D)** H&E, **(E)** with EPR6893 clone, noting the tumour cells were diffusely negative, **(F)** with 2G4 clone, noting tumour cells stained with a lower intensity than internal positive control (lymphocytes). Scale bar = 100µm.

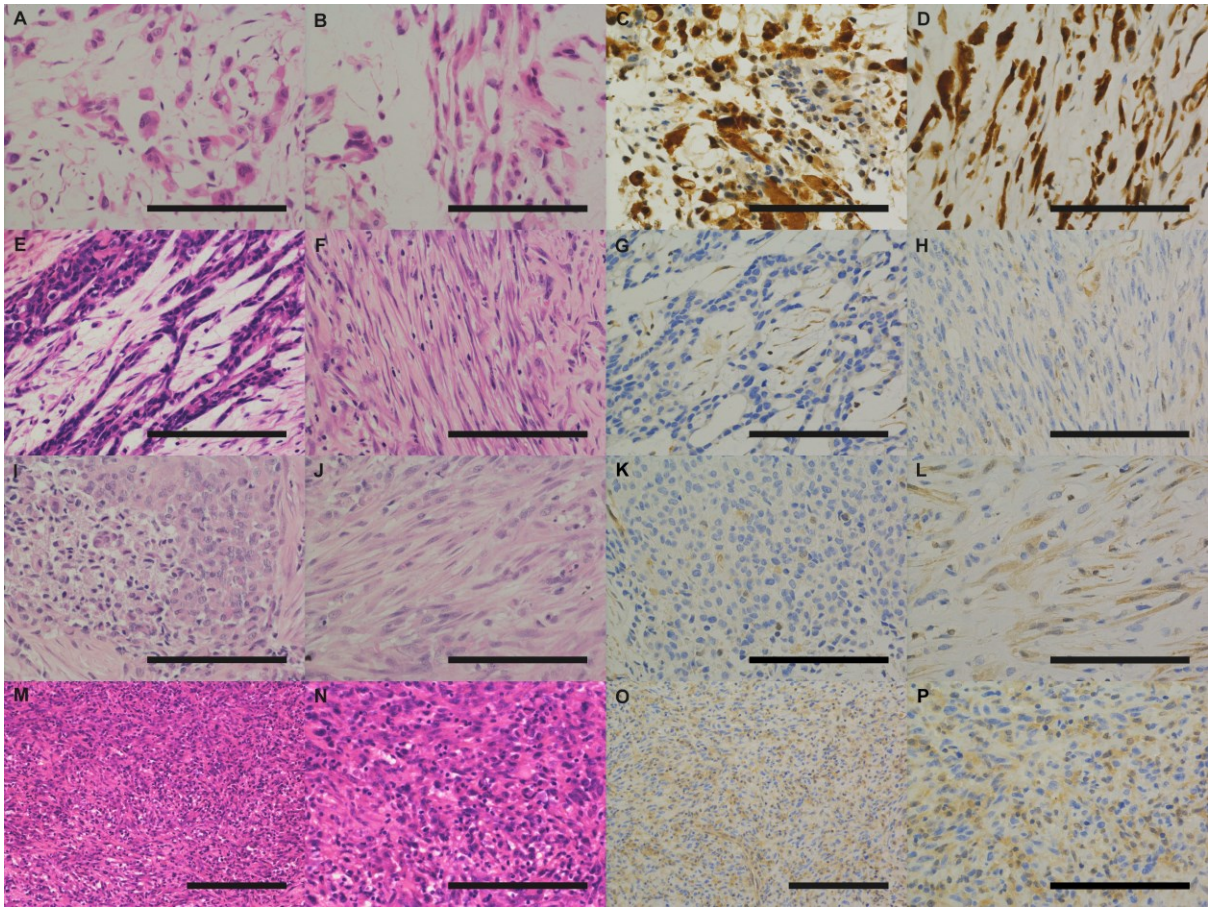


Figure 7-4 MTAP immunohistochemistry in biphasic PM. **(A-D)** MTAP-positive B-PM (Case 26). Both the epithelioid **(A, C)** and sarcomatoid **(B, D)** components were diffusely positive for MTAP. **(E-H)** MTAP-negative B-PM (Case 24). Both the epithelioid **(E, G)** and sarcomatoid **(F, H)** components were diffusely negative for MTAP. **(I-J)** A case of discordant MTAP staining (Case 23), with diffusely negative MTAP in the epithelioid component **(I, K)** and focal positivity in the sarcomatoid component **(J, L)**. **(M-P)** A case of discordant MTAP staining with intense host response (Case 27). **(M-N)** The epithelioid and sarcomatoid components were intermixed with predominantly lymphocytic inflammatory infiltration. **(O-P)** MTAP staining was predominantly lost, but focal staining was retained in the sarcomatoid component. Scale bar = 100μm.

Case ID	Subtype	Max. dimension (mm)†	*CDKN2A* copy number	*MTAP* copy number	MTAP IHC
3	Epithelioid, Low grade	12, 2	Neutral	Neutral	Negative
9	Epithelioid, Low grade	15	HD	HD	Focal (20% positivity)
11	Epithelioid, High grade	30	Neutral	Neutral	Negative
18	Epithelioid, Low grade	40, 35, 20, 15	HD	Heterozygous	Focal (30% positivity)
20	Epithelioid, High grade	10	HD	HD	Positive
26	Biphasic	15	HD	Heterozygous	Positive
27	Biphasic	15	HD	HD	Focal (30% positivity)

Table 7-8 Cases with discordant *CDKN2A*/*MTAP* copy number states and their MTAP IHC status.

† If more than one site were sampled, the maximum dimension of biopsy from each site is given.

HD, homozygous deletion.

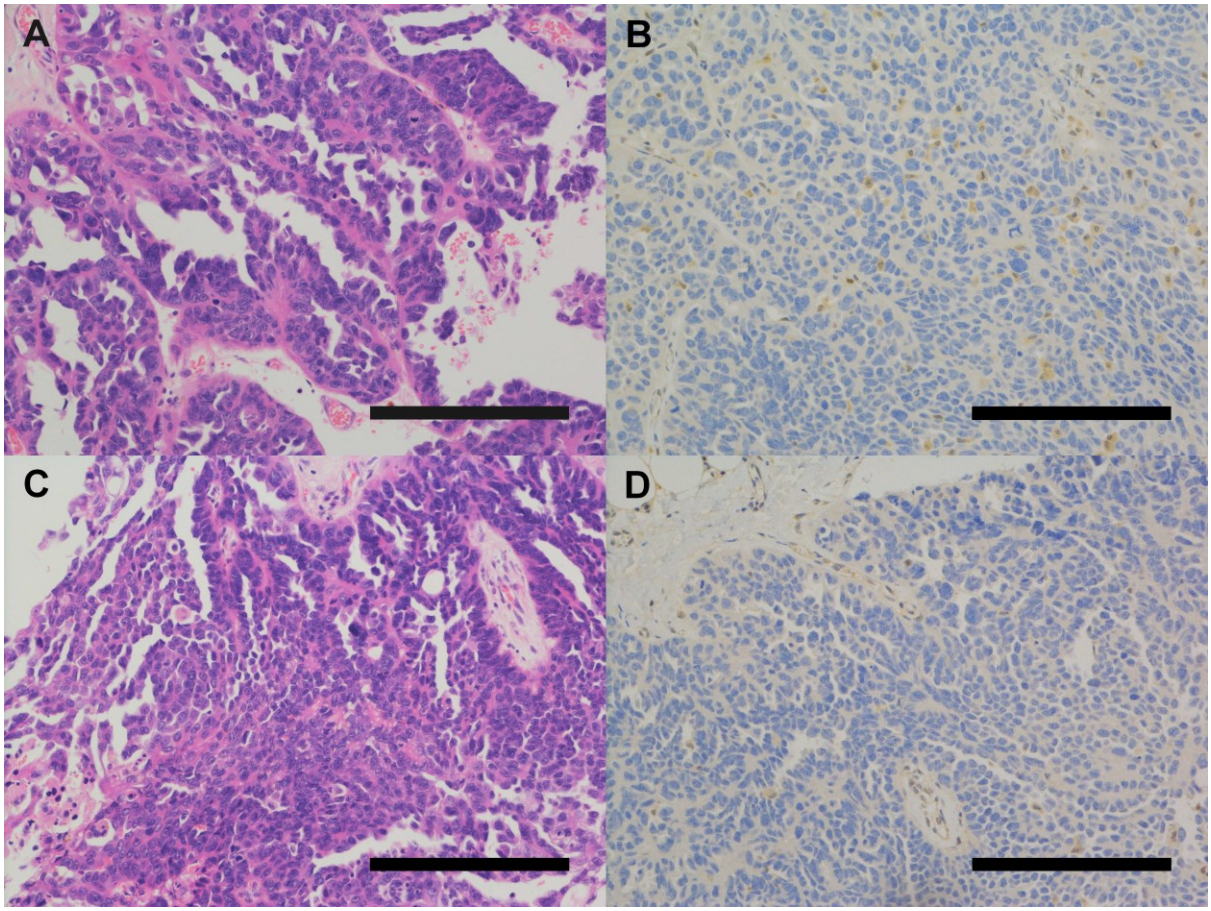


Figure 7-5 *CDKN2A*/*MTAP* copy number neutral high grade E-PM with diffuse loss of MTAP staining (Case 11). **(A, C)** H&E with conspicuous nuclear pleomorphism, **(B, D)** diffuse loss of MTAP staining. Scale bar = 100μm.

7.5 Discussion

This study reports reasonable but imperfect assay concordance (73%) between *CDKN2A*/*MTAP* genomic status by NGS, and MTAP immunohistochemistry. This is broadly in agreement with a recent, similarly-designed study (Chapel *et al*., 2021). The inter-assay agreement between two commercial MTAP antibody clones was good (Cohen's κ 0.718), and inter-observer agreement was moderate to good (Fleiss' κ 0.650). These were comparable to a recent multi-institutional study (Chapel *et al*., 2020). It is conceivable that the lower inter-

observer agreement in our study was due to the higher incidence of focal MTAP staining (22.2% vs 12.4%), which is prone to disagreement. In addition our study provides further evidence in the aspects of challenging scenarios including unusual MTAP staining patterns and correlation with histopathological features.

MTAP IHC is being rapidly incorporated into the routine diagnostics in centres with mesothelioma workload, and there are emerging applications in pleural effusion cytology (Berg *et al*., 2020) (Chapel *et al*., 2020) (Hiroshima *et al*., 2020) (Hamasaki *et al*., 2019) (Siddiqui *et al*., 2019) (Kinoshita *et al*., 2018a) (Kinoshita *et al*., 2018b) in place of *CDKN2A* FISH (Chevrier *et al*., 2020) (Hida *et al*., 2015) (Illei *et al*., 2003) and diagnosis of early disease such as mesothelioma *in situ* (MIS) (Haefliger *et al*., 2020) (Minami *et al*., 2020). *MTAP* itself currently presents as a putative therapeutic target since its co-deletion with *CDKN2A* could diversify the phenotypic manifestation of the latter by introducing metabolic disruption (Hansen *et al*., 2019) (Tang *et al*., 2018). Mesothelioma cell lines with *MTAP* knock-down were susceptible to inhibition of protein arginine methyltransferase 5 (PRMT5), an enzyme involved in epigenetic regulation via the accumulation of MTAP substrate, methyladenosine (MTA) (Barbarino *et al*., 2020). Such synthetic lethality has been previously reported in other cancer types (Zhang *et al*., 2020) (Fedoriw *et al*., 2019) (Kryukov *et al*., 2016) (Marjon *et al*., 2016) (Mavrakis *et al*., 2016) therefore it is hypothesised MTAP loss in PM could be exploited in a similar fashion. This then raises the possible scenario of *MTAP* testing outside the scope of determining *CDKN2A* status.

Our results suggest MTAP IHC can be reliably developed and interpreted to determine *CDKN2A*/*MTAP* genomic status, but its practical utility needs to take into context several caveats of the assay. Firstly, our data does not support MTAP IHC as a substitute for NGS in determining *CDKN2A*/*MTAP* genomic status. In other words, it remains a candidate surrogate marker until further evidence. Nonetheless, based on available evidence, we advocate MTAP

IHC as a screening assay due to its cost-effectiveness and accessibility. A confirmatory assay (NGS or FISH) is advised for cases with negative or focal staining, similarly for discordant staining in non-epithelioid PM. This is of particular relevance in the setting of biomarker-stratified trials or treatment as patient eligibility might be dictated by biomarker status. For purely diagnostic purposes however, MTAP IHC *per se* should be sufficient as the diagnostic approach is taken pragmatically in combination with morphology and other immunohistochemical biomarkers.

Secondly, the rare occurrence of weak staining encountered when using 2G4 clone appeared to be assay-dependent but might also indicate unusual genotype. The index case in our study harboured *CDKN2A*/*MTAP* heterozygous co-deletion in contrast to the 11 cases included in Chapel et al., all of which were associated with *CDKN2A* homozygous deletion detected by FISH. In practical setting and based on available evidence, this staining pattern (or loss of staining when using EPR6893 clone) signifies copy number loss in *CDKN2A*/*MTAP* locus and amounts to an unequivocal evidence of malignancy.

Thirdly, focal staining should be commented upon while the results should be regarded as inconclusive. While this still shifts the diagnostic confidence alongside other clinicopathological variables, until the emergence of further evidence, we advise against diagnosing and/or prognosticating PM based on focal MTAP staining. This equally applies to B-PM with discordant staining, mainly in the scenarios of intermixed epithelioid and sarcomatoid components, and when there is low confidence in distinguishing tumour cells from stromal cells.

We believe our results, particularly the higher degree of IHC-NGS discordance compared with published literature, are relevant to the clinical and research communities of PM for several reasons. Firstly, the complexity of the genomic architecture of the hotspot region

9p21 might yet to be unravelled. In a considerable minority of *CDKN2A*-deleted pancreatic adenocarcinoma, there was no *MTAP* co-deletion (Barrett *et al*., 2017). Fine mapping of the telomeric extension of *9p21* deletions in lung cancer and glioma found variable deletion of *MTAP* exons associated with immunogenic protein degradation products (Schmid *et al*., 2000). We did not find incomplete deletion of *MTAP* in our study, and to our knowledge no high resolution data is currently available for PM. We did however detect a minority of cases harbouring heterozygous *CDKN2A*/*MTAP* co-deletions or copy number disparity between the two genes. Heterozygous *CDKN2A* deletions have been reported in studies of pleural (Nastase *et al*., 2020) (Hmeljak *et al*., 2018) (Illei *et al*., 2003) and peritoneal mesothelioma (Brich *et al*., 2020). The phenotype(s) of heterozygous *MTAP* deletion and the compound genotypes resultant of the large scale genomic alterations affecting *CDKN2A* and its adjacent telomeric segment, are largely unknown. Functional genomic studies utilising a well-powered and annotated dataset is necessary to investigate if the diploid heterozygous state described in our study results in low level transcriptional and/or translational activity, or whether it represents a computational reflection of copy number clonality.

Secondly, the multitude of factors culminated in the assay discordance warrants a systematic assessment before drawing a conclusion regarding its performance. Intra-tumoural heterogeneity in MTAP, as illustrated by the focal staining pattern, and the compound effects in the setting of biopsies, are like to be further complicated by variations in the technical parameters used in assay development and pathologist's thresholds (Table 7-2). These pre-analytical and analytical variables might also influence the performance of NGS, concerning which data on PM is still limited.

Finally, while our data must be interpreted with caution due to the lack of power, stratification of the incidence of *CDKN2A* deletion by nuclear grade in the epithelioid subgroup showed a progressive shift towards high grade (Grade III) and pleomorphic

diseases. Although less frequent than its non-epithelioid counterparts, a significant proportion of epithelioid PM are known to harbour *CDKN2A* deletions (Nastase *et al*., 2020) (Hmeljak *et al*., 2018) (Bueno *et al*., 2016) and our findings rationalised the hypothesis that this hallmark genomic event is associated with nuclear grade (Zhang *et al*., 2020a). Our observations on the discordant MTAP staining in biphasic PM could be due to the presence of intervening stromal fibroblasts, often difficult to discern from the malignant spindle cell population even by experienced mesothelioma pathologists, or *bona fide* complex clonal architecture with tumour cell subpopulations harbouring differential *CDKN2A*/*MTAP* states.

Our study has several limitations. There was no head-to-head comparison with other molecular platforms including FISH and quantitative PCR (Cheng *et al*., 2020), also p16 IHC given it showed variable assay performance and numerous commercial clones are currently available. In addition our study was performed on a small number of cases and NGS was not performed on FFPE materials for which the readout would be of greater practical relevance. We were not able to evaluate the frequencies and phenotypes of promoter methylation of either *CDKN2A* (Kobayashi *et al*., 2008) or *MTAP* locus, as the latter is recognised in other cancer types (Watanabe *et al*., 2009) (Behrmann *et al*., 2003) although currently not evidenced in PM (Bosio *et al*., 2018). A similar statement can be made in case of transcriptional silencing via long non-coding RNA (lncRNA) such as *ANRIL* mapped to the *CDKN2A* locus (Aguilo *et al*., 2011). These however represent the focus of our future work.

A diagnostic algorithm combining MTAP IHC and NGS, the former as a cost-effective screening tool with reliable readout and fast turnaround time, leverages upon the strengths of both assay platforms. This however, require the practising pathologists to be informed of the assay performance, and in the setting of NGS being unavailable, provide the multidisciplinary team with an alternative predictive tool. Our study provided early data should this approach be adopted. Independent validation of our findings on a larger scale is necessary, and

advanced, high resolution molecular platforms including but not limited to multi-region single cell hybrid sequencing and/or *in situ* sequencing will likely to unravel a wealth of novel mechanisms beyond our observations. This, in turn, will undoubtedly crosspollinate routine diagnostics and accelerate the progress at which patients with this disease of dismal prognosis benefit from targeted therapies, and also allow diagnosis at an earlier stage of disease.

7.6 Conclusions

In this study MTAP immunohistochemistry showed reasonable but imperfect concordance with *CDKN2A*/*MTAP* genomic status. EPR6893 and 2G4 antibody clones can be used interchangeably with moderate to good inter-observer agreement. We observed focal MTAP staining pattern which is not informative in determining *CDKN2A* status based on available evidence. Therefore confirmatory assay either by FISH or NGS is advisable especially when the histological or immunohistochemical features are outside the correct clinical context.

8 Atypical Mesothelial Proliferation of the Pleura: Multidisciplinary Approach, Prognostic Stratification and Proposal of Minimally Invasive Pleural Mesothelioma

This chapter has been presented in part as a platform presentation at the European Congress of Pathology, 5th-9th December 2020, Glasgow (Virtual).

8.1 Abstract

Background: Atypical mesothelial proliferation (AMP) of the pleura poses challenges to diagnosis and patient management as it encompasses a spectrum of benign and malignant mesothelial lesions. Along with cases limited by sampling error, it also likely includes those exhibiting morphological features of pleural mesothelioma (PM) yet showing insufficient invasion into underlying structures. This study reviewed cases classified as AMP, with a view to improving early diagnosis of PM using a multidisciplinary approach.

Materials and methods: This study was retrospective including 131 consecutive cases of histologically confirmed AMP diagnosed at the RBH (2005-2019), of which 35 (26.5%) had subsequent biopsies showing definitive PM. Clinicopathological data were retrieved from the institutional mesothelioma database. Prognostic variables predictive of overall survival (OS) were identified using univariate and multivariate Cox regression analyses. Statistical significance was denoted as $P<0.05$.

Results: Atypical mesothelial proliferations had comparable demographic profile to invasive PM. Multivariate analysis showed lack of subpleural structure sampling ($P=0.010$) and severe nuclear atypia ($P=0.001$) were independent predictors of worse median OS. Cases, inclusive of all subtypes, fulfilling the criteria of (i) adequate subpleural sampling either without invasion or invasion limited to fibrous pleura, (ii) no severe nuclear atypia, (iii) no necrosis, were associated with significantly improved median OS compared with those without (24.4 vs 12.8 months, $P=4.0\times10^{-6}$). Epithelioid-restricted criteria led to improved stratification in terms of median OS (33.4 vs 11.9 months, $P=8.6\times10^{-8}$).

Conclusions: Morphology-based criteria are proposed to stratify AMP into high and low risk groups. It is likely the former was the result of insufficient sampling whilst the latter

represented minimally invasive PM. To refine these criteria, further investigation is needed including the use of BAP1/MTAP immunohistochemistry.

8.2 Introduction

Atypical mesothelial proliferation (AMP) of the pleura represents a diagnostic term used to report cases that are characterised by morphological suspicion of pleural mesothelioma (PM) but are not unequivocally malignant. This is usually due to a lack of sufficient invasion into subpleural structures and/or a lack of architectural and cytological and atypia. The exact incidence of pleural AMP is unknown, as its usage likely reflects to a degree the experience of the pathologist, and the management options for patients are then driven by the lack of definitive diagnosis. The latter is usually due to the need for further sampling or observation leading to delay in diagnosis if the case is a PM, or more advanced disease by the time of diagnosis. Of the 34 cases reported by Pillappa *et al*., the largest series to date including 21 cases originated from the pleura (Pillappa *et al*., 2018), it has been suggested that some cases could be precursor PM including but not limited to mesothelioma *in situ* (MIS). The recently revised definition of MIS is expected to delineate a proportion of these cases. Due to the stringent clinical and histological criteria, the need remains however to minimise cases that would otherwise be classified as AMP and to achieve definitive diagnosis in cases that are PM with minimal invasion and/or minimal atypia.

It is recognised that AMP is not a single entity but encompasses (1) under-sampled invasive PM, (2) minimally or superficially invasive PM without invasion of the subpleural structures, and (3) reactive mesothelial processes with atypia overlapping with those features seen in PM. Hence the aim of this present study was to minimise the need for AMP to be employed as a diagnostic category by using morphological features alongside recently available biomarkers, and to identify the subset of AMP representing early invasive disease thereby enabling PM to be diagnosed earlier in its natural history.

8.3 Materials and Methods

8.3.1 Study population

Archived cases of AMP were identified using a prospectively maintained institutional mesothelioma database, which included a total of 1,912 cases of histologically confirmed mesothelioma and AMP between 1991-2019 at the Royal Brompton and Harefield Hospitals (London, UK). Institutional review board (IRB) approval was obtained for the study. Additional radiological and outcome data were retrieved from the National Health Service Spine repository, electronic patient records and regional thoracic oncology. Median OS was defined as time (measured in months) between the date of initial procedure, from which a diagnosis of AMP was made, and the date of death or last follow-up. The time to PM diagnosis was defined as that between the dates of initial procedures from which the diagnoses of AMP and subsequent PM were made, respectively.

A total of 131 consecutive cases of AMP from 2005 to 2019 were included in this study (denoted as Group 1), of which 53 cases received a subsequent histological diagnosis of PM (denoted as Group 2). There were no missing cases in either study cohorts. All cases show radiological and/or macroscopic disease during intra-operative evaluation without features of frank invasion. None of the cases was definitive for mesothelioma, nor fulfilled the current definition of MIS (Churg *et al*., 2020a) (Churg *et al*., 2020b). Censor fraction in the study was 32.8% (43/131).

The role of BAP1 and MTAP immunohistochemistry was explored using a prospective cohort of 31 consecutive patients diagnosed between 1st January 2020 and 31st July 2021. These were either from referral service (n=15) or local practice (n=16). All cases qualified for the diagnosis of AMP prior to immunohistochemistry. BAP1 loss was defined as the loss of nuclear staining, and MTAP loss as the loss of both nuclear and cytoplasmic staining as

described previously (Chapter 7, Section 7.3.2). Focal loss was regarded as positive. A case was considered sufficient for upgrade or re-classification as pleural mesothelioma when there was either BAP1 or MTAP loss, or both.

8.3.2 Microscopy and imaging

Microscopic assessment was performed using a Nikon Eclipse Ci-L microscope (Nikon Corporation, Japan) with a field area measuring 0.24mm^2 per high power field (HPF, ×400 magnification). Microscopic images in 300dpi TIF format were taken from representative cases using Nikon Digital Sight DS-L3 camera (Nikon Corporation, Japan) and were annotated using the GNU Image Manipulation Program 2.10.10 (http://www.gimp.org, retrieved on 20.04.2019).

8.3.3 Histopathological assessment

All cases were diagnosed by at least one specialist pulmonary pathologist and all cases fell short of a definitive diagnosis of PM using current histological and immunohistochemical criteria (Nicholson *et al*., 2020) (Husain *et al*., 2018) (Travis *et al*., 2015). Unequivocal invasion of subpleural structures was lacking by histopathological assessment in all cases. Pre-analytical (Table 8-1) and histopathological parameters were determined *a priori*. They were assessed and recorded independently by myself, blinded to outcome. All available haematoxylin & eosin (H&E) sections were reviewed, with an average of 2.6 sections per case (range: 1-13). The average number of sections including immunohistochemistry was 12.8 sections.

Histological subtyping of AMP was performed in accordance with that of invasive PM with the exception of sarcomatoid subtype, where the nomenclature “spindle cell subtype” was used. Immunohistochemistry (IHC) including mesothelium-associated markers and pan-

cytokeratin were reviewed whenever available to facilitate subtyping. The depth of invasion was defined as the deepest anatomical compartment into which atypical mesothelial cells were seen. Superficial invasion, or that of atypical cells abutting the subpleural structures, was allowed. The stroma, defined as the predominant form, was classified as myxoid (loosely fibrotic), cellular fibrosis (established fibrosis with conspicuous fibroblasts), pleural plaque-like (resembling pleural plaques but with atypical mesothelial cells) or desmoplasia (paucicellular atypical cell population set in densely fibrotic stroma). The growth patterns of surface mesothelium were subdivided into linear (a single layer of mesothelial cells), complex (any other growth pattern), or absent. The growth patterns and its grading of the invasive component and the evaluation of nuclear atypia, mitotic activity and nuclear grade were performed according to previous studies (Chapters 4-5) (Zhang *et al*., 2020a) (Zhang *et al*., 2020b). Invasive growth patterns and nuclear grade were recorded only for the epithelioid subtype. The presence of cytological variants was documented based on the criteria outlined previously (Chapter 6, Section 6.3.3).

8.3.4 Statistical analysis

Descriptive statistics were employed to analyse the baseline demographic and clinicopathological parameters. Two-sided chi-square test or Fisher's exact test was used to evaluate associations between categorical variables. OS (Group 1) and time to PM diagnosis (Group 2) were estimated using the Kaplan-Meier method. Exact *P* values were recorded and $P<0.05$ denotes statistical significance. Multivariate Mantel-Cox regression model was used to evaluate the effect size and statistical significance of each variable that demonstrated $P<0.05$ in univariate analysis and with ≥10 events.

The principal criteria for risk stratification of AMP was generated based on a combination of independent predictors of OS. Further computation of this criteria to restrict the low risk

group to epithelioid subtype was determined *a priori*, to align the histopathological features of this group to that of MIS. Further computation generated alternative criteria based on practical utility, in order to rectify the subjectivity of the pathologist assessment of the histological findings. In all cases, a two-tier risk stratification was subsequently achieved based on the fulfilment of the criteria. All statistical analyses were performed using SPSS 26 (IBM Corp., Armonk, NY, USA). Kaplan-Meier curves were generated using GraphPad Prism Version 8 (GraphPad Software, La Jolla California, USA).

Parameter	Variables	Notes
Sampling	≥3 sites	Recommended by EURACAN/IASLC proposal (Nicholson *et al*., 2020). Evidenced by two independent studies on nuclear grading systems (Zhang *et al*., 2020a) (Chirieac *et al*., 2019).
	≤3 sites, tissue m.d. ≥20mm	Secondary sampling recommendation by one study on nuclear grading systems (Zhang *et al*., 2020a).
	1-2 sites, tissue m.d. <20mm	-
	Single site, tissue m.d. <10mm	At this level nuclear grading systems were shown to lose its prognostic power (Zhang *et al*., 2020a).
Subpleural structure sampling	Present Absent	Defined as the presence of subpleural fat or anatomical structures.
Orientation	Orientated Not orientated	Defined as clear demonstration of the anatomical plane of mesothelium, submesothelial stroma and subpleural structures on the same section.
Cellularity of atypical cells	>300 cells 100-300 cells <100 cells	Defined as the number of cells per section.
Background inflammation or haemorrhage	Present Absent	Defined as >50% involvement by area of section.
Crush or cauterisation artefacts	Present Absent	Defined as >50% involvement by area of section.

Table 8-1 Assessment of pre-analytical variables.

EURACAN, European Reference Network on Rare Adult Cancers; IASLC, The International Association for the Study of Lung Cancer; m.d., maximum dimension.

8.4 Results

8.4.1 Demographic and clinicopathological characteristics

Atypical mesothelial proliferation was a recurrent diagnostic challenge (Figure 8-1). Between 1991 and 2019 there were 281 cases of AMP, of which 76 cases (27.0%) had subsequent diagnosis of PM. This translated into a historical prevalence of 14.7% (281/1,912) of all malignant or suspicious pleural mesothelioma. In other words, 10.7% of cases (205/1,912) were assigned AMP without subsequent malignant diagnosis, one per 9.1 cases of PM.

For the population (*n*=131) within this study, the median age was 72 years (range 46-89 years) with male preponderance (74.8%) (Table 8-2). Among those with documented occupational history, asbestos exposure was found in 65.6% (59/90). Significant comorbidities in at least one organ system (respiratory, cardiovascular, renal or diabetes) were present in 33.6% of patients (44/131). Pleural effusion was almost an invariable presentation. The majority of patients had radiological evidence of pleural thickening. All patients were found to have macroscopic pleural disease intraoperatively. A minority of patients showed systemic inflammatory profile indicated by elevated neutrophil-lymphocyte ratio (≥5, 29.0%) and serum C-reactive protein (>10mg/L, 48.9%).

Only 22% of cases (29/131) had undergone the recommended sampling standard proposed by EURACAN/IASLC (Nicholson *et al*., 2020). In 18.3% of these cases subpleural structures were absent in the tissue biopsy (24/131) (Table 8-3). Lack of tissue orientation was a recognised issue (39.7%, 52/131). In a minority of cases the cellularity of the atypical proliferation was low (<100 cells). Background inflammation or haemorrhage (48.9%, 64/131), and crush or cauterisation artefacts occupying more than 50% of tissue area were present in a significant proportion of cases (17.6%, 23/131).

The majority of cases were of epithelioid subtype (62.6%, 82/131), and comprised of either linear (58.0%, 76/131) and/or tubulo-papillary (40.5%, 53/131) surface growth patterns (Figure 8-2). For the epithelioid subtype the invasive component mainly showed discohesive or tubulo-papillary growth patterns, both predominant and secondary. This differed from previous data on invasive epithelioid PM, where the most common growth patterns were found to be tubulo-papillary and solid and discohesive growth pattern represented an absolute minority (Chapter 5) (Zhang *et al*., 2020b). Cytological variants were found but at a lower frequency compared with invasive epithelioid PM (17.5%, 23/131). Of note, rhabdoid and small cell variants, both known to be highly aggressive, had a cumulative frequency of only 2.3% (3/131). In relation to stroma, the most commonly seen was that of cellular fibrosis (69.5%, 91/131), followed by desmoplasia (19.1%, 25/131), myxoid (6.1%, 8/131) and pleural plaque-like (5.3%, 7/131).

The immunoprofile of AMP was comparable to that of invasive PM (Table 8-4). Loss of expression of both mesothelium-associated markers and pan-cytokeratin was seen in spindle cell subtype, also observed in sarcomatoid PM. The degree of subtype concordance among those with subsequent PM diagnosis was very high (94.3%, 50/53) (Table 8-5), compared with the recent literature (Schulte *et al*., 2021) (Chirieac *et al*., 2019).

The median follow-up interval was 12.0 months and the median OS of the study population was 19.2 months (95% CI 14.6-23.7 months). For patients with subsequent diagnosis of PM, 74% received the diagnosis within 12 months of their initial biopsy that was deemed to be AMP (39/53).

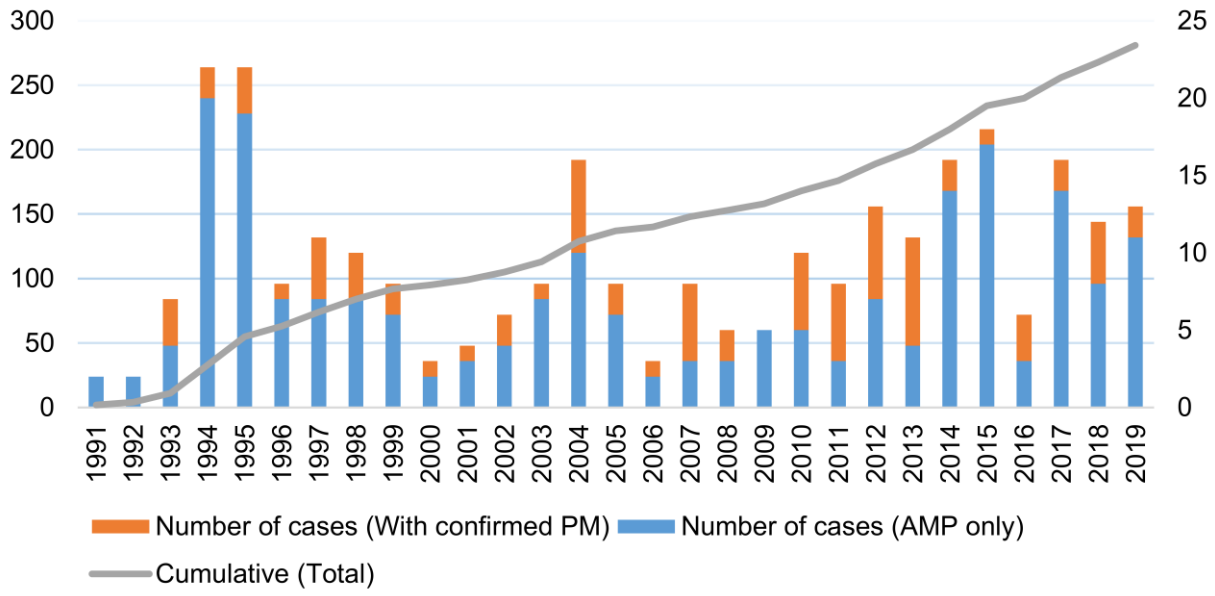


Figure 8-1 Diagnostic burden of atypical mesothelial proliferation over 29-year period.

Left axis: Cumulative number of diagnosis. Right axis: Annual caseload. Trend line denotes the cumulative number of diagnosis.

AMP, atypical mesothelial proliferation; PM, pleural mesothelioma.

Variable		No. (%)
Age, median (range), years		72.0 (46-89)
Sex		
	Male	98 (74.8)
	Female	33 (25.2)
Laterality		
	Left	54 (41.2)
	Right	76 (58.0)
	Unknown	1 (0.8)
Asbestos exposure		
	Present	59 (45.0)
	Absent	31 (23.7)
	Unknown	41 (31.3)
History of malignancy		
	Present	25 (19.1)
	Absent	80 (61.1)
	Unknown	26 (19.8)
Family history of malignancy		
	Present	8 (6.1)
	Absent	97 (74.0)
	Unknown	26 (19.9)
Comorbidities		
	Chronic respiratory disease	11 (8.4)
	Chronic cardiovascular disease	24 (18.3)
	Chronic kidney disease	5 (3.8)
	Diabetes	19 (14.5)
	Unknown	26 (19.8)
Pleural effusion		
	Present	127 (96.9)
	Absent	4 (3.1)
Pleural thickening		74 (56.5)
	Present	10 (7.6)
	Absent	47 (35.9)
	Unknown	
Systemic Neutrophil-Lymphocyte Ratio (NLR), median (range)		3.63 (1.1-17.6)
	<5	82 (62.6)
	≥5	38 (29.0)
	Unknown	11 (8.4)
Serum C-Reactive Protein (CRP), median (range, mg/L)		16 (0-329.0)
	≤10	43 (32.8)
	>10	64 (48.9)
	Unknown	24 (18.3)
Follow-up interval, median (range), months		12.0 (0-123.0)
Overall survival, median (95% CI), months		19.2 (14.6-23.7)
Censor fraction		43 (32.8)

Table 8-2 Clinicopathological characteristics.

CI, confidence interval; mg/L, milligram per litre.

Variable	No. (%)
Sampling	
≥3 sites	29 (22.1)
1-2 sites, ≥20mm m.d.	62 (47.3)
1-2 sites, <20mm m.d.	25 (19.1)
Single Site, ≤10mm m.d.	15 (11.5)
Subpleural structure sampling	
Present	107 (81.7)
Absent	24 (18.3)
Orientation	
Orientated	79 (60.3)
Not orientated	52 (39.7)
Cellularity (atypical)	
>300	97 (74.0)
100-300	25 (19.1)
<100	9 (6.9)
Background inflammation/haemorrhage	
Present	64 (48.9)
Absent	67 (51.1)
Crush/cauterisation artefacts	
Present	23 (17.6)
Absent	108 (82.4)

Table 8-3 Pre-analytical variables.

m.d., maximum dimension.

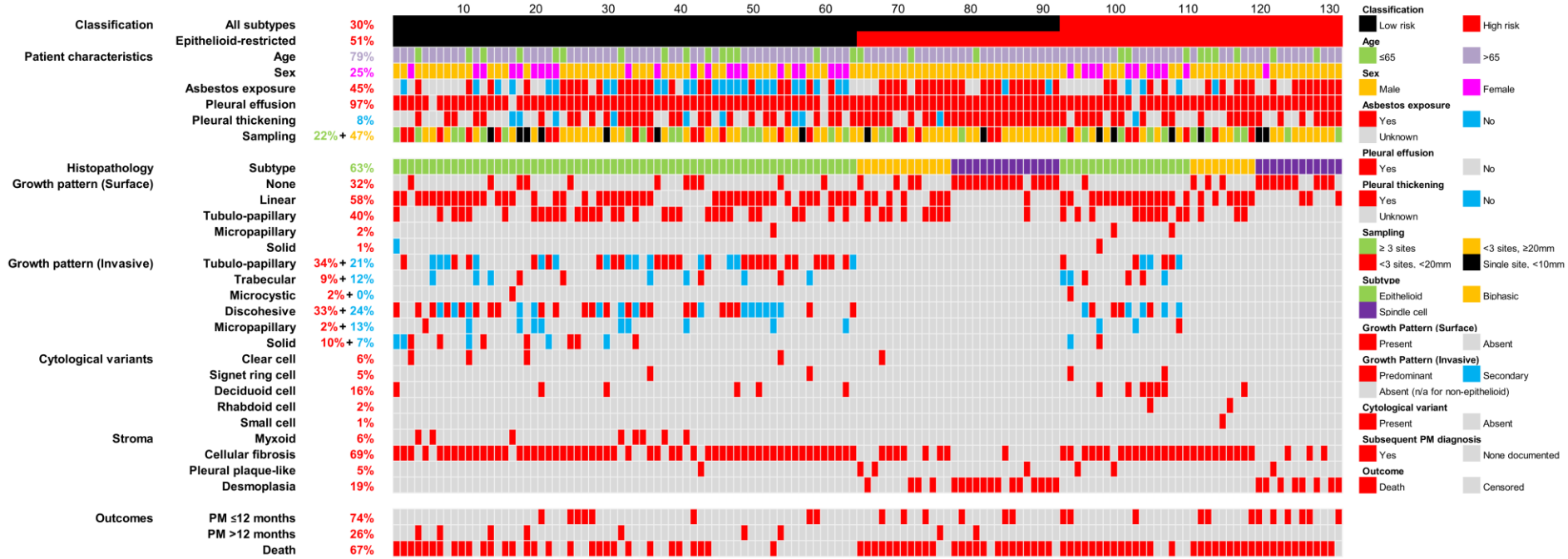


Figure 8-2 Clinical and histopathological features of atypical mesothelial proliferation.

The percentages of patients receiving subsequently PM diagnosis stratified by time interval were based on its total number (*n*=53) instead of the number of AMP in the study population.

PM, pleural mesothelioma.

IHC		Epithelioid AMP (*n*=82)	Biphasic AMP (*n*=22)	Spindle cell AMP (*n*=27)
Calretinin	Test rate (%)	66/82 (80.5%)	22/22 (100.0%)	15/27 (55.6%)
	Results: Positive/Focal/Negative (%)	61/2/3 (92.4%/2.4%/5.2%)	14/7/1 (63.6%/31.8%4.6%)	3/7/5 (20.0%/46.7%/33.3%)
WT-1	Test rate (%)	18/82 (22.0%)	8/22 (36.4%)	3/27 (11.1%)
	Results: Positive/Focal/Negative (%)	16/2/0 (88.9%/11.1%/0%)	7/1/0 (87.5%/12.5%/0%)	1/2/0 (33.3%/66.7%/0%)
Cytokeratin 5/6	Test rate (%)	35/82 (42.7%)	10/22 (45.5%)	6/27 (22.2%)
	Results: Positive/Focal/Negative (%)	29/3/3 (82.9%/8.6%/8.5%)	6/3/1 (60.0%/30.0%/10.0%)	0/1/5 (0.0%/16.7%/83.3%)
Pan-cytokeratin	Test rate (%)	60/82 (73.2%)	18/22 (81.8%)	21/27 (77.8%)
	Results: Positive/Focal/Negative (%)	60/0/0 (100%/0%/0%)	18/0/0 (100%/0%/0%)	12/7/2 (57.1%/33.3%/9.6%)

Table 8-4 Immunoprofile of atypical mesothelial proliferation.

Pan-cytokeratin IHC represented antibody cocktails and the choices were based on the individual practice, including MNF116, AE1/AE3 or Cam5.2. The cut-off threshold for focal staining varied between pathologists but generally referred as <50% tumour cell positivity.

AMP, atypical mesothelial proliferation; IHC, immunohistochemistry; WT-1, Wilms Tumour protein.

		Final diagnosis		
		Epithelioid	Biphasic	Spindle cell
AMP diagnosis	Epithelioid	25	0	0
	Biphasic	1	10	1
	Spindle cell	0	1	15

Table 8-5 Subtype concordance among patients with subsequent PM diagnosis.

AMP, atypical mesothelial proliferation; PM, pleural mesothelioma.

8.4.2 Prognostic variables

Univariate analysis showed sex, presence of subpleural structure in the tissue sample, subtype, presence of severe nuclear atypia, mitotic count and stroma predicted poor survival following a categorisation as AMP (Figure 8-3 and Table 8-6). Significantly worse prognosis was seen in the setting of superficial invasion of subpleural structures, with stromal invasion as reference (P=0.002). In epithelioid AMP, 2-tier nuclear grade (using criteria from epithelioid PM composed from the degree of nuclear atypia, mitotic activity and necrosis), but not growth pattern of the invasive component, was a significant prognostic variable (Table 8-7).

Multivariate analysis showed the presence of subpleural structures and the absence of severe nuclear atypia predicted favourable prognosis independent of sex, depth of invasion, subtype, mitotic activity and stroma (Table 8-8). Borderline statistical significance was reached for sex and stroma.

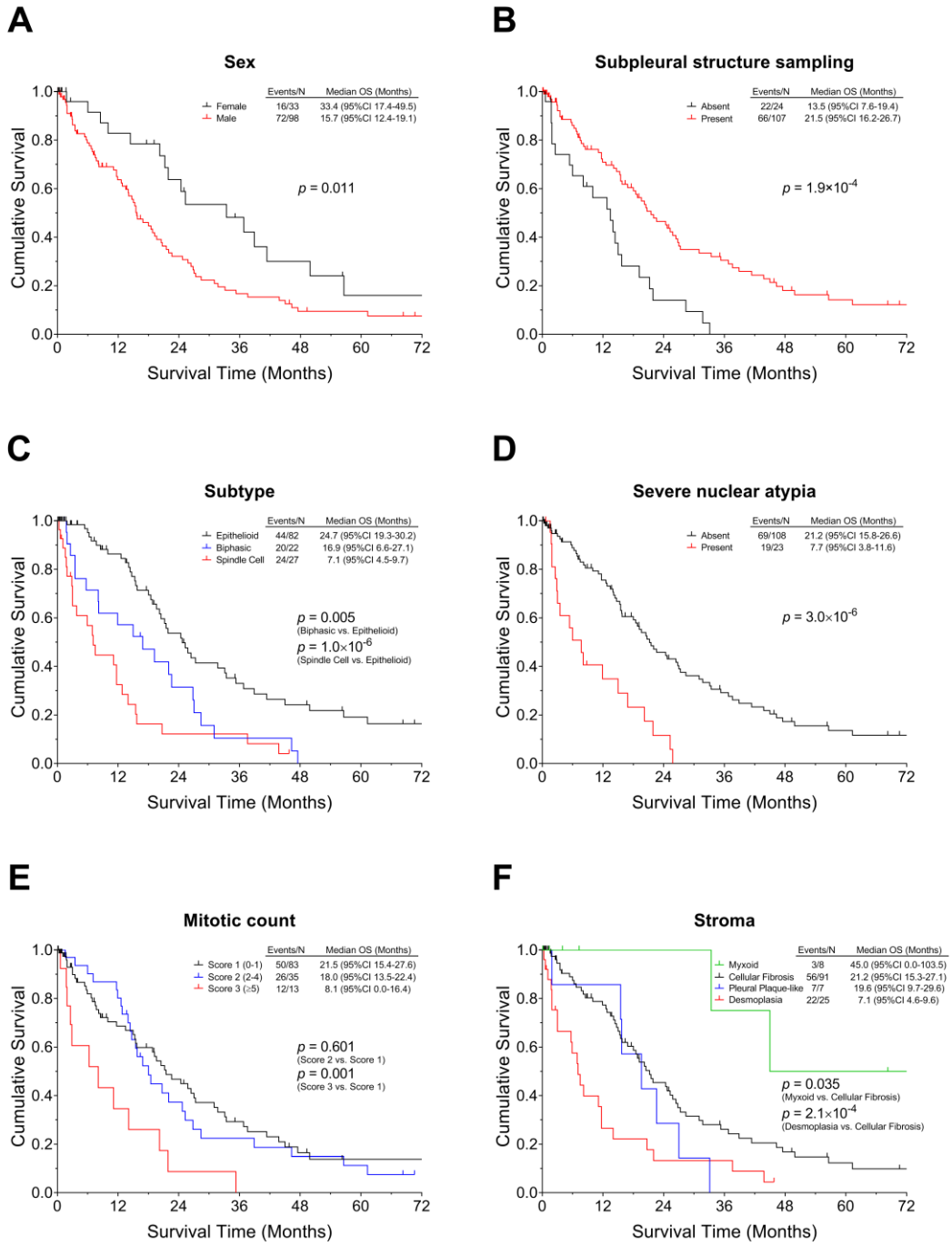


Figure 8-3 Univariate analysis of variables predicting overall survival (Group 1).

Male sex **(A)**, lack of subpleural structure sampling **(B)**, non-epithelioid subtype **(C)**, presence of severe nuclear atypia **(D)**, high mitotic count (≥5/10HPF) **(E)** and desmoplasia **(F)** were associated with worse median OS.

CI, confidence interval; OS, overall survival.

Variable	Median OS (Months)	95% CI	*P*
Age			
≤65 years	24.4	16.4-32.5	Ref.
>65 years	18.0	13.5-22.5	0.118
Sex			
Female	33.4	17.4-49.5	Ref.
Male	15.7	12.4-19.1	0.011
Laterality			
Left	15.7	10.2-21.3	Ref.
Right	20.2	16.9-23.6	0.838
Asbestos exposure			
Absent	24.7	17.4-32.1	Ref.
Present	16.9	12.3-21.4	0.317
History of malignancy			
Absent	18.5	13.6-23.4	Ref.
Present	35.3	6.6-64.0	0.109
Comorbidity			
None	22.6	8.9-36.4	Ref.
≥1	18.0	11.9-24.0	0.070
Subpleural structure sampling			
Absent	13.5	7.6-19.4	Ref.
Present	21.5	16.2-26.7	1.9×10^{-4}
Orientation			
Not orientated	15.3	13.4-17.3	Ref.
Orientated	22.6	15.2-30.0	0.135
Cellularity (Atypical cells)			
>300 cells	19.2	14.1-24.3	Ref.
100-300 cells	19.6	12.7-26.5	0.836
<100 cells	10.0	2.3-17.7	0.920
Inflammation/haemorrhage			
Absent	18.8	13.6-24.0	Ref.
Present	20.7	12.6-28.8	0.298
Crush/cauterisation artefacts			
Absent	19.2	13.9-24.4	Ref.
Present	18.8	15.4-22.2	0.581
Depth of invasion			
Surface only	31.7	1.9-61.5	Ref.
Stromal	20.5	16.9-24.1	0.791
Superficial invasion of subpleural structure	11.1	2.1-20.1	0.686 (*0.030* vs stromal)
Subtype			
Epithelioid	24.7	19.3-30.2	Ref.
Biphasic	16.9	6.6-27.1	*0.005*
Spindle cell	7.1	4.5-9.7	1.0×10^{-6}
Nuclear Atypia			
Score 1 (Mild)	22.6	10.7-34.5	Ref.
Score 2 (Moderate)	20.7	17.0-24.4	0.810
Score 3 (Severe)	7.7	3.8-11.6	*0.001*
Severe nuclear atypia			
Absent	21.2	15.8-26.6	Ref.

Present	7.7	3.8-11.6	3.0×10^{-6}
Mitotic activity			
Score 1 (0-1)	21.5	15.4-27.6	Ref.
Score 2 (2-4)	18.0	13.5-22.4	0.601
Score 3 (≥5)	8.1	0.0-16.4	*0.001*
Atypical mitosis			
Absent	20.5	10.3-30.7	Ref.
Present	18.0	12.9-23.0	0.343
Surface growth pattern			
Linear	22.6	12.7-32.5	Ref.
Complex	20.5	17.0-24.0	0.617
No surface mesothelium	11.7	4.7-18.7	0.094
Cytological variants			
Absent	19.2	14.2-24.1	Ref.
Present	15.8	7.6-24.0	0.918
Stroma			
Myxoid	45.0	0.0-103.5	Ref.
Cellular fibrosis	21.2	15.3-27.1	0.035
Pleural plague-like	19.6	9.7-29.6	*0.002*
Desmoplasia	7.1	4.6-9.6	*0.005*

Table 8-6 Univariate analysis of variables predicting overall survival (Group 1).

Mitotic activity was evaluated across 10 high power fields (HPF) in the area of highest activity, in accordance with the criteria for nuclear grade assignment.

CI, confidence interval; OS, overall survival; Ref., reference.

Variable	Median OS (Months)	95% CI	*P*
2-tier nuclear grade			
Low grade	25.3	20.3-30.4	Ref.
High grade	5.4	1.8-8.9	1.0×10^{-6}
3-tier nuclear grade			
Grade I	27.0	18.3-35.7	Ref.
Grade II	16.9	13.1-20.6	0.052
Grade III	8.1	0.0-19.9	2.4×10^{-4}
Growth pattern (Invasive)			
Low grade	22.0	17.3-26.7	Ref.
High grade	27.3	0.0-56.6	0.567

Table 8-7 Univariate analysis of variables predicting overall survival (Group 1, epithelioid subtype).

CI, confidence interval; OS, overall survival; Ref., reference.

Variable	Hazard Ratio	95% CI	*P*
Sex			
Male vs Female	1.92	0.98-3.76	0.056
Subpleural structure sampling			
Present vs Absent	0.43	0.23-0.82	*0.010*
Depth of invasion			
Stromal vs Surface only	1.26	0.33-4.76	0.732
Superficial invasion of subpleural structure vs Surface only	2.53	0.54-11.8	0.237
Subtype			
Biphasic vs Epithelioid	1.26	0.65-2.47	0.495
Spindle cell vs Epithelioid	1.70	0.54-5.34	0.360
Severe nuclear atypia			
Present vs Absent	3.21	1.64-6.30	*0.001*
Mitotic activity			
Score 2 vs Score 1	1.21	0.72-2.04	0.468
Score 3 vs Score 1	1.62	0.75-3.48	0.222
Stroma			
Cellular fibrosis vs Myxoid	3.51	0.82-15.0	0.090
Pleural plaque-like vs Myxoid	5.06	0.89-28.9	0.068
Desmoplasia vs Myxoid	5.59	0.93-33.6	0.060

Table 8-8 Multivariate analysis of variables predicting overall survival (Group 1).

CI, confidence interval.

8.4.3 Risk stratification

The variables analysed in univariate and multivariate analyses were incorporated to construct a set of principal histological criteria to stratify AMP based on its prognosis. A low risk category was defined as all the followings were fulfilled: (1) evidence of subpleural sampling by the presence of corresponding anatomical structures, (2) the depth of invasion limited to the submesothelial compartment, allowing superficial invasion into, or abutting the subpleural structures but no further, (3) the degree of nuclear atypia no more than moderate, and (4) the absence of tumour necrosis. Failing to fulfil any of such assigned the case as high risk.

Significant difference in median OS was seen between the low risk and high risk categories (24.4 vs 12.8 months, P=4.0×10^{-6}), the latter comparable to the literature estimates of all-subtypes invasive PM (Figure 8-4A). It was also predictive of the interval between the initial biopsy and subsequent PM diagnosis (3.5 vs 1.8 months, P=0.014) (Figure 8-4B). By restricting the low risk category to epithelioid subtype, which aligned the criteria with the current morphological definition of MIS (the latter can only be diagnosed in those with epithelioid morphology), further improved the prognostic value (33.4 vs 11.9 months, P=8.6×10^{-8}) (Figure 8-4C). The low risk category was associated with 1-year and 2-year survival of 93.1% and 62.2% respectively, and in 40% of patients subsequent PM diagnoses were made at least 12 months apart (Figure 8-4D). This risk category showed substantially longer median OS than low grade epithelioid PM based on 2-tier grading system (19.3 months) (Chapter 4) (Zhang *et al*., 2020a), and those of nuclear grade I based on 3-tier grading system (25-28 months) (Chapter 4) (Zhang *et al*., 2020a) (Kadota *et al*., 2012) (Rosen *et al*., 2018).

Three alternative criteria were subsequently tested (Table 8-9). The first one incorporated the stromal features and assigned those with pleural plaque-like or desmoplasia stroma to the high risk category, regardless of subtype (Alternative criteria 1). This had a similar prognostic power to the epithelioid-restricted principal criteria (33.4 vs 11.7 months, $P=2.4\times10^{-9}$) (Figure 8-5A-B). Interaction was found between non-epithelioid AMP and the high risk stromal features ($P=1.2\times10^{-16}$) (Table 8-10). The second (Alternative criteria 2) and third alternative criteria (Alternative criteria 3) removed the requirement of evaluating the degree of nuclear atypia and served as modifications to the all-subtype and epithelioid-restricted principal criteria respectively. Neither offered non-inferior prognostic power (Figure 8-5-C-F).

Furthermore, the additional utility of adding pre-diagnostic systemic inflammatory markers to the risk stratification system was explored. All laboratory results were obtained within 30 days prior to the diagnostic procedure. In univariate analysis, elevated NLR and CRP were associated with worse median OS (Figure 8-6). Neither markers, however, predicted overall survival independent of the clinical risk categories based on the epithelioid-restricted principal criteria (Table 8-11).

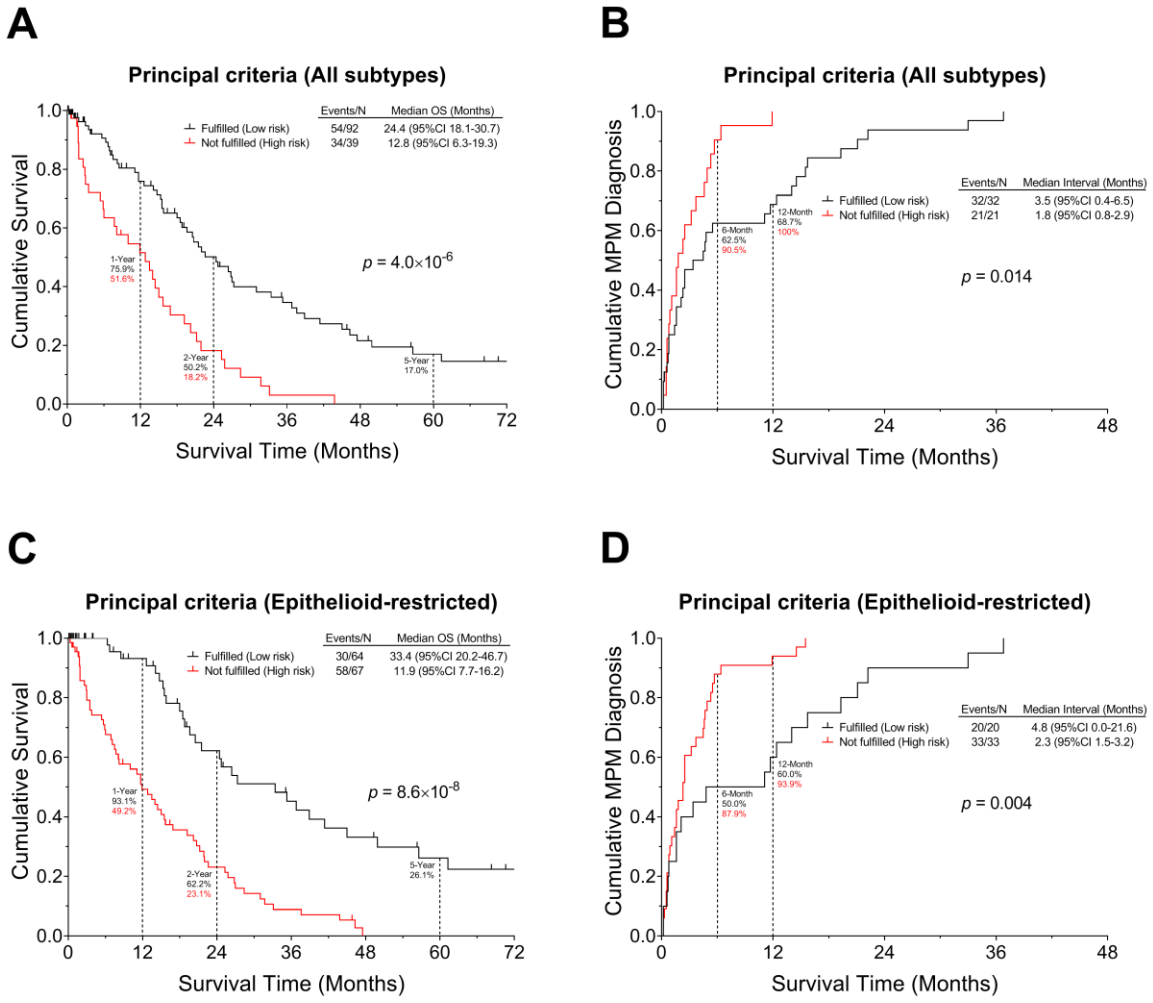


Figure 8-4 Risk stratification of atypical mesothelial proliferation.

(A-B) Principal criteria. **(C-D)** Epithelioid-restricted principal criteria in which epithelioid subtype was necessary to assign to the low risk category.

CI, confidence interval; OS, overall survival.

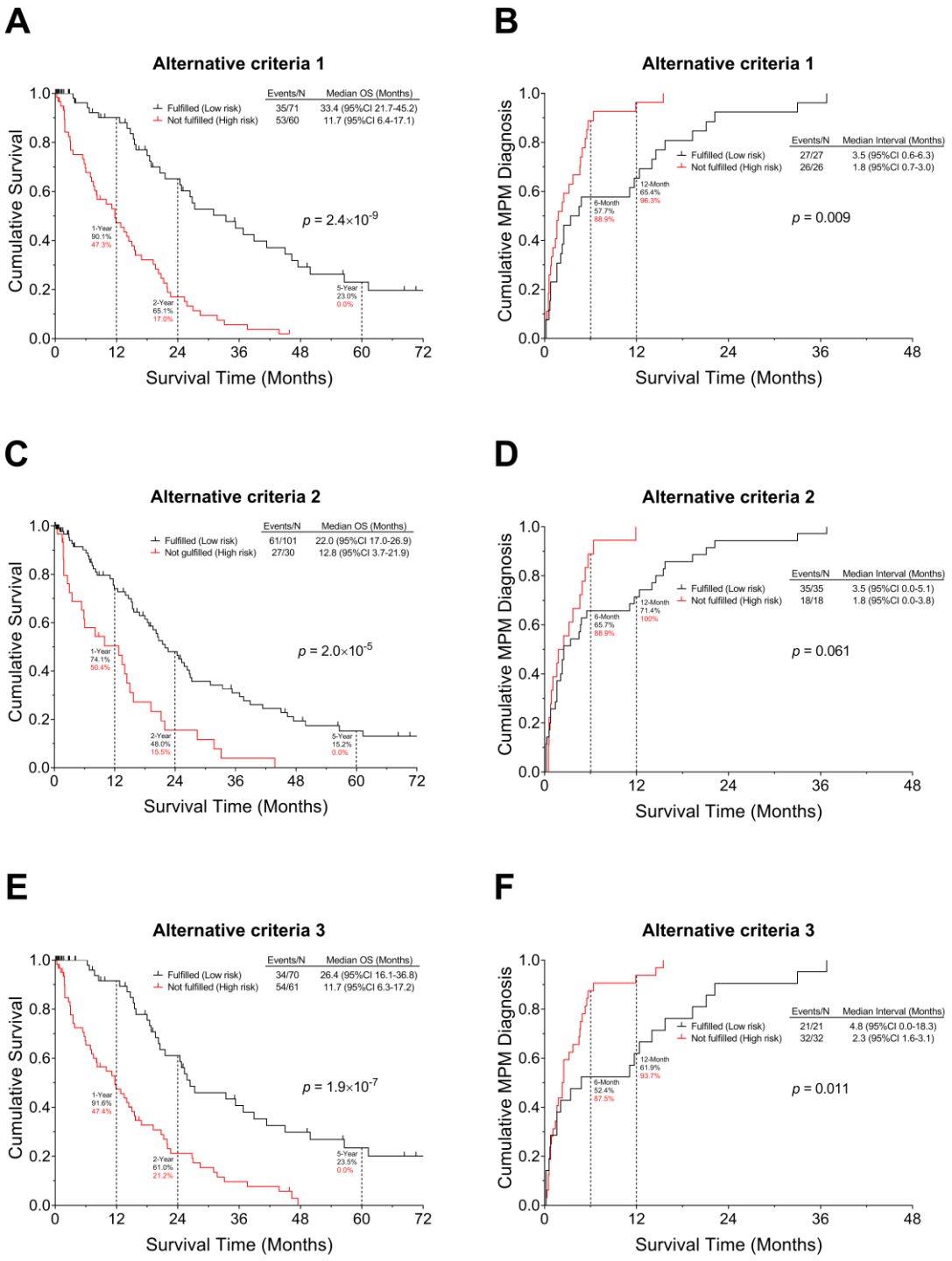


Figure 8-5 Risk stratification of atypical mesothelial proliferation (Alternative criteria).

For each of the alternative criteria, both the prognostic utility **(A, C, E)** and predictive value in relation to the time to subsequent PM diagnosis **(B, D, F)** were tested.

CI, confidence interval; OS, overall survival.

	Principal criteria	Principal criteria (Epithelioid-restricted)	Alternative criteria 1	Alternative criteria 2	Alternative criteria 3
Evidence of adequate sampling	Required	Required	Required	Required	Required
Depth of invasion	Surface Stromal	Surface Stromal	Surface Stromal	Surface Stromal	Surface Stromal
Subtype	Any	Epithelioid	Any	Any	Epithelioid
Cytomorphology	Mild/Moderate	Mild/Moderate	Mild/Moderate	Any	Any
Necrosis	Not allowed	Not allowed	Not allowed	Not allowed	Not allowed
Stroma	Any	Any	No desmoplasia or pleural plaque-like	Any	Any
% fulfilled criteria (Low risk)	70.2%	48.9%	54.2%	77.1%	53.4%
Median OS (months) (Group 1)					
Fulfilled (Low risk)	24.4	33.4	33.4	22.0	26.4
Not fulfilled (High risk)	12.8	11.9	11.7	12.8	11.7
Survival rate (Low risk)					
1-year	75.9%	93.1%	90.1%	74.1%	91.6%
2-year	50.2%	62.2%	65.1%	48.0%	61.0%
5-year	17.0%	26.1%	23.0%	15.2%	23.5%
Median time to PM diagnosis (months) (Group 2)					
Fulfilled (Low risk)	3.5	4.8	3.5	3.5	4.8
Not fulfilled (High risk)	1.8	2.3	1.8	1.8	2.3
Cumulative PM diagnosis (Fulfilled, low risk)					
6-month	62.5%	50.0%	57.7%	65.7%	52.4%
12-month	68.7%	60.0%	65.4%	71.4%	61.9%

Table 8-9 Principal and alternative criteria for risk stratification and associated patient outcomes.

PM, pleural mesothelioma; OS, overall survival.

	Epithelioid (*n*=82)	Biphasic (*n*=22)	Sarcomatoid (*n*=27)	*P*
Myxoid/Cellular fibrosis	79 (96.3%)	16 (72.7%)	4 (14.8%)	1.2×10^{-16}
Pleural plaque-like/desmoplasia	3 (3.7%)	6 (27.3%)	23 (85.2%)	

Table 8-10 Association between subtype of atypical mesothelial proliferation and stromal features.

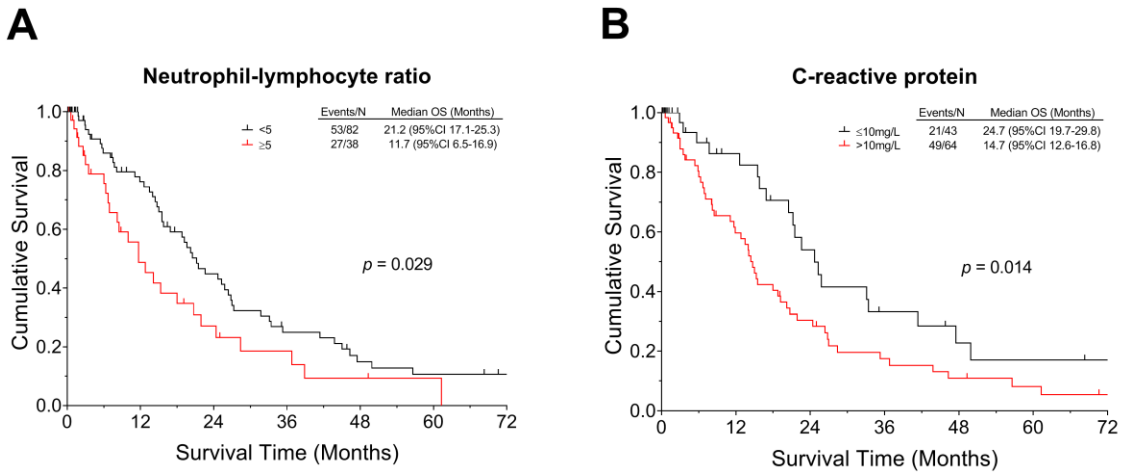


Figure 8-6 Univariate analysis of systemic inflammatory markers predicting overall survival.

All results were obtained within 30 days prior to the diagnostic procedure. Missing values were due to patients not receiving the test(s).

CI, confidence interval; OS, overall survival.

Variable	Hazard Ratio	95% CI	*P*
Principal criteria (Epithelioid-restricted)			
Fulfilled (low risk) vs. not fulfilled (high risk)	0.28	0.17-0.48	3.0×10^{-6}
NLR			
<5 vs. ≥5	1.40	0.79-2.46	0.249
CRP			
≤10 vs. >10mg/L	1.64	0.91-2.94	0.099

Table 8-11 Multivariate analysis of systemic inflammatory markers predicting overall survival.

CI, confidence interval; CRP, serum C-reactive protein; mg/L, milligram per litre; NLR, systemic neutrophil-lymphocyte ratio.

8.4.4 Role of BAP1 and MTAP immunohistochemistry

Of the 31 cases in the prospective cohort, 8 cases were excluded from further analysis as BAP1/MTAP immunohistochemistry were not performed. Among those with dual biomarker status, the majority (14/23, 60.9%) were of low risk AMP. A total of 15 cases (65.2%) showed loss of at least one marker, which was indicative of malignant process and therefore sufficed the criteria for re-classification as PM (Table 8-12). A higher proportion of low risk AMP (6/14, 42.8%) exhibited wildtype BAP1 and MTAP staining patterns than the high risk counterpart (2/9, 22.2%) however the limited number of cases precluded robust statistical analysis.

		MTAP	
		Wildtype	Loss
BAP1	Wildtype	8 (34.8%)	3 (13.0%)
	Loss	6 (26.1%)	6 (26.1%)

Table 8-12 Re-classification of AMP by BAP1/MTAP status.

BAP1, BRCA-associated protein 1; MTAP, methylthioadenosine phosphorylase.

8.4.5 Representative cases

A number of representative cases are described below from both low risk (Case 1, 2, 5) and high risk categories (Case 3, 4), illustrating the clinicopathological features and diagnostic considerations. Cases 3 and 5 were referred from external institutions thereby not included in the study population.

8.4.5.1 Case 1 (Low risk AMP)

This was a 66 year old lady presenting with one year history of recurrent right sided pleural effusion of unknown cause associated with cough and exertional breathlessness, requiring drainage of two litres every three weeks and unsuccessful talc pleurodesis. There was no asbestos exposure, or family history of malignancy. Her past medical history included irritable bowel syndrome but otherwise she had been fit and well. No radiological pleural disease was present at first presentation but pleural thickening, nodularity and septation was seen on a repeat CT scan 12 months after. Initial pleural cytology was negative for malignancy. Three further pleural effusion cytology samples undertaken at RBH showed atypia in two, and negative for malignancy in one, together with a negative pleural biopsy showing talc-related inflammatory changes. Repeated attempts of pleurodesis and indwelling pleural catheter placement did not result in symptomatic improvement.

A VATS pleural biopsy was subsequently taken showing features of AMP (Figure 8-7). There was a surface and superficial stromal proliferation of mild to moderately atypical mesothelial cells, focally with deciduoid cell variant. Nuclear BAP1 staining was lost with focal MTAP loss. Homozygous *CDKN2A* deletion was confirmed using FISH. Pleural effusion cytology taken concomitantly showed atypical mesothelial cells with diffuse BAP1 loss (Figure 8-8).

Following the diagnosis, the patient was referred for but considered ineligible for a major surgical trial for PM, nor chemotherapy due to recurrent infections in the pleural cavity. At 5.2 months post-AMP diagnosis, total pleurectomy and decortication was performed with dual intents to control infection and remove macroscopic disease. The resection specimen showed invasive epithelioid mesothelioma, low grade (nuclear grade II), pathological stage T3 (chest wall invasion) Nx MCR. The patient was alive at 9.7 months post-surgery and 14.9 months following the initial AMP diagnosis.

On retrospective review of all preceding pleural effusion samples, they were of relatively low cellularity with intermixed mildly atypical and bland mesothelial cells. In one sample, taken 9.1 months prior to the AMP diagnosis, there was focal loss of nuclear BAP1 staining limited to no more than a few papillaroid cell clusters (Figure 8-9). The latter was of uncertain clinical significance.

This case was considered low risk AMP, with biomarker profile sufficient for minimally invasive PM.

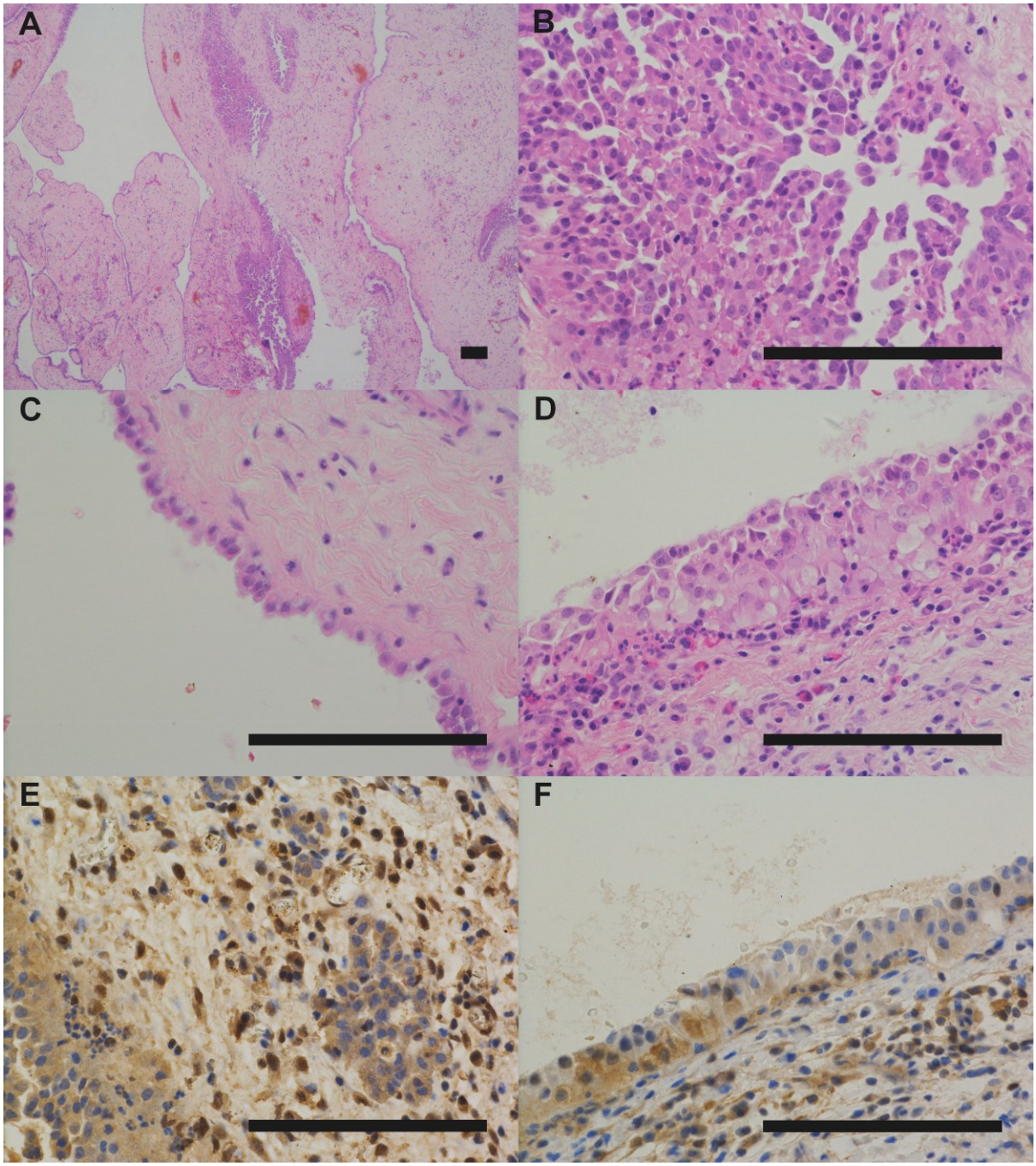


Figure 8-7 Atypical mesothelial proliferation (Case 1, biopsy).

(A) The biopsy was of adequate sampling including subpleural fat (not shown), and there were both a surface and submesothelial mesothelial proliferation. **(B-C)** The surface component was comprised of micropapillary and linear growth patterns. A scattered submesothelial mitotic figure was seen **(C)**. **(D)** Focally the surface component showed

multilayering with deciduoid cell variant. **(E)** The submesothelial component showed loss of nuclear BAP1 staining. **(F)** Focally loss of MTAP staining.

Scale bar = 100μm.

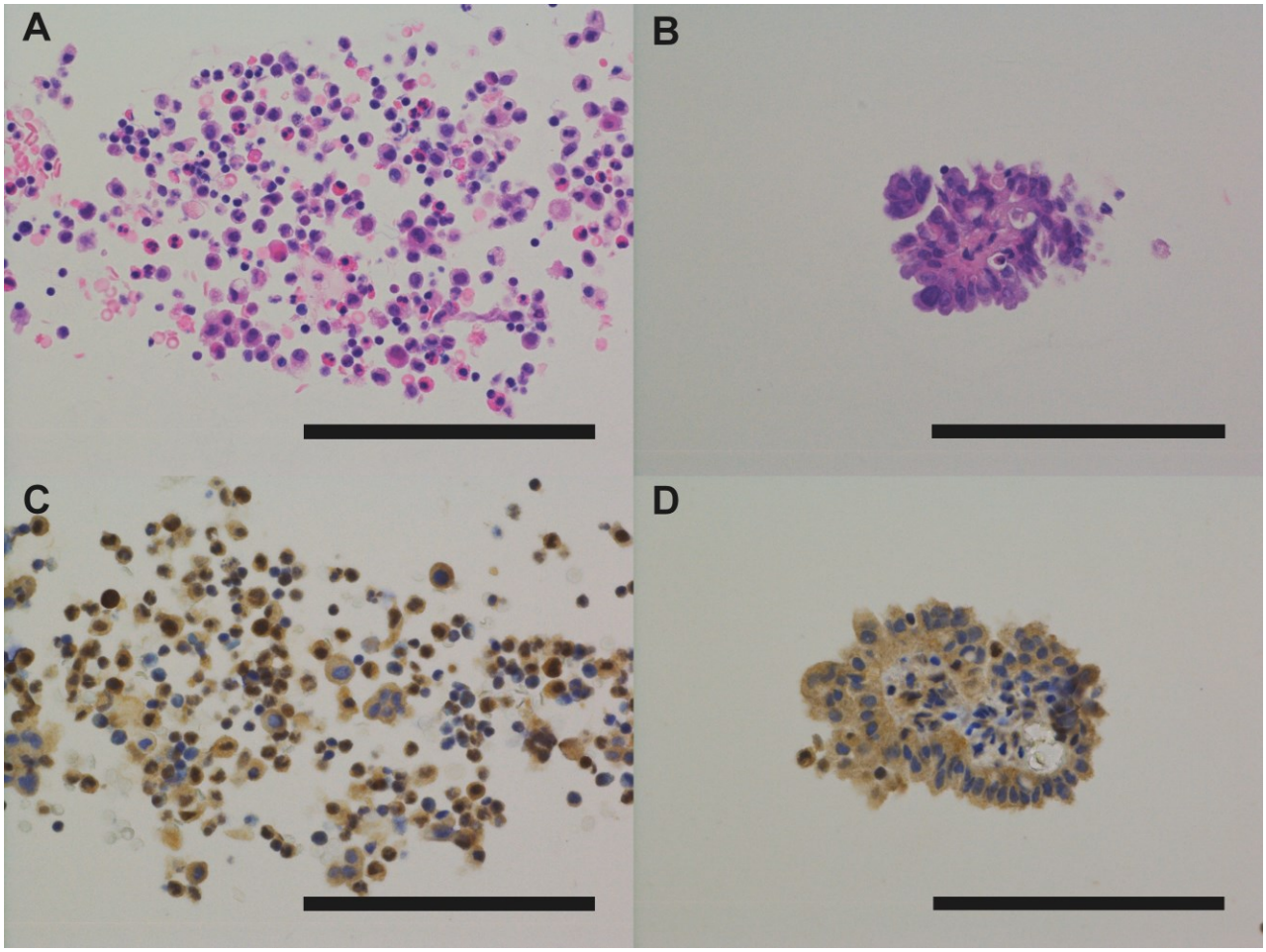


Figure 8-8 Invasive epithelioid mesothelioma (Case 1, pleural effusion cytology).

(A) This was a cellular sample consisted of scattered and papillary groups **(B)** of mildly atypical mesothelial cells set in mixed inflammatory background. **(C-D)** There was diffuse loss of nuclear BAP1 staining.

Scale bar = 100μm.

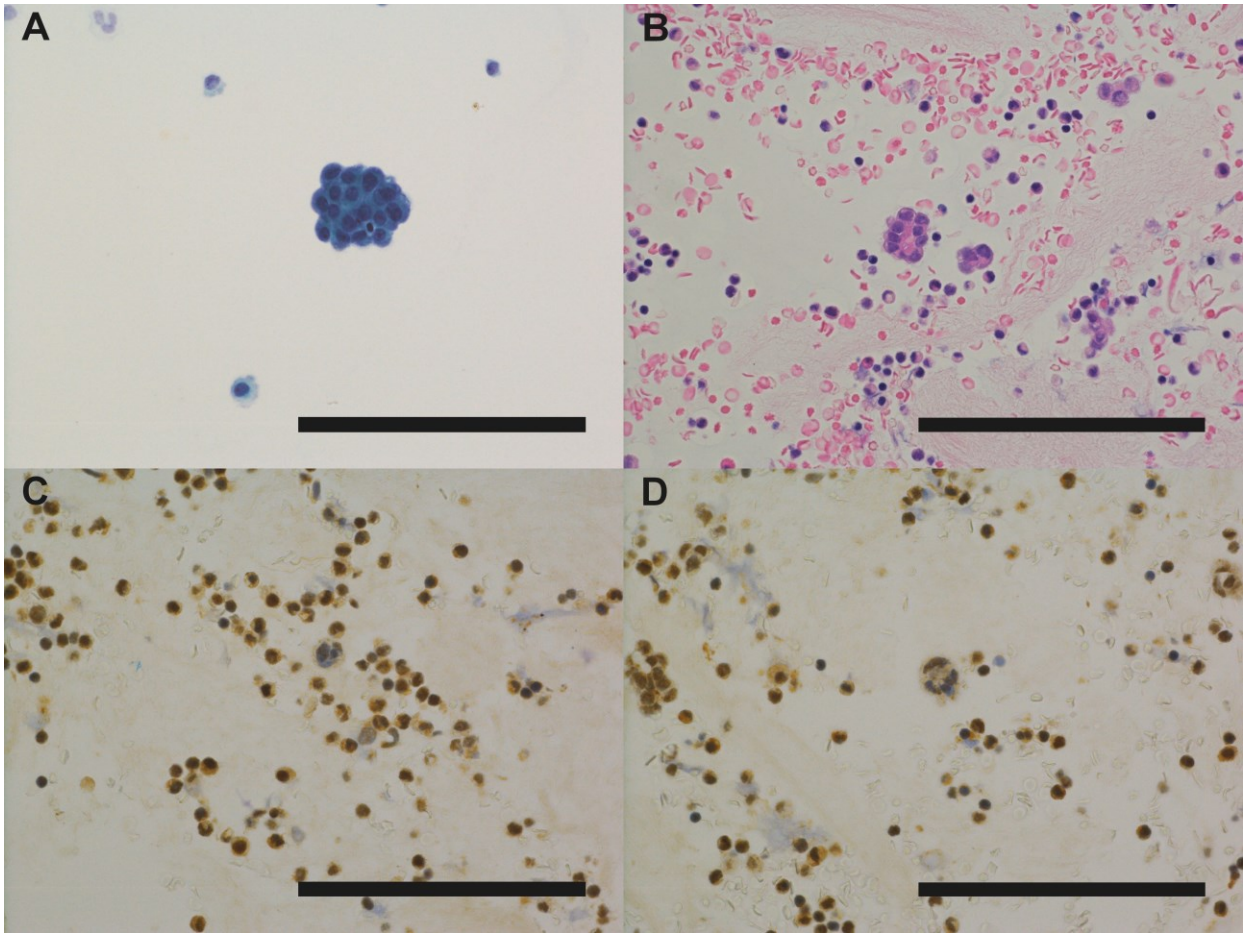


Figure 8-9 Atypical mesothelial proliferation (Case 1, pre-AMP pleural effusion cytology).

(A-B) This was a low cellularity sample consists of predominantly scattered mesothelial cells with very occasional papillaroid groups. **(C-D)** There was focal loss of nuclear BAP1 staining. The patient received a diagnosis of AMP 9.1 months later with diffuse loss of BAP1 staining in the subsequent pleural effusion sample.

Scale bar = 100μm.

8.4.5.2 Case 2 (Low risk AMP)

This was a 68 year old lady who underwent total thymectomy for an early stage type AB thymoma. There was no asbestos exposure but her father had died from mesothelioma with proven occupational exposure. There was no radiological pleural disease or pleural effusion, but intraoperatively thin pleural thickening described as “reticular thin plaque like appearance” was present around the thymic lesion. Histopathology showed sub-millimetre pleural thickening within which there was superficial stromal proliferation of mildly atypical mesothelial cells with trabecular and discohesive growth patterns (Figure 8-10). No mediastinal fat invasion was seen. There was diffuse complete loss of BAP1 staining. This was classified as AMP, and due to the diagnostic uncertainty, no further treatment was offered besides clinical follow-up at an external institution.

At 21.2 months post-AMP diagnosis, a chest wall biopsy confirmed invasive epithelioid mesothelioma, low grade (nuclear grade I), with unequivocal invasion into the chest wall muscles.

This case was considered low risk AMP, with biomarker sufficient for minimally invasive PM.

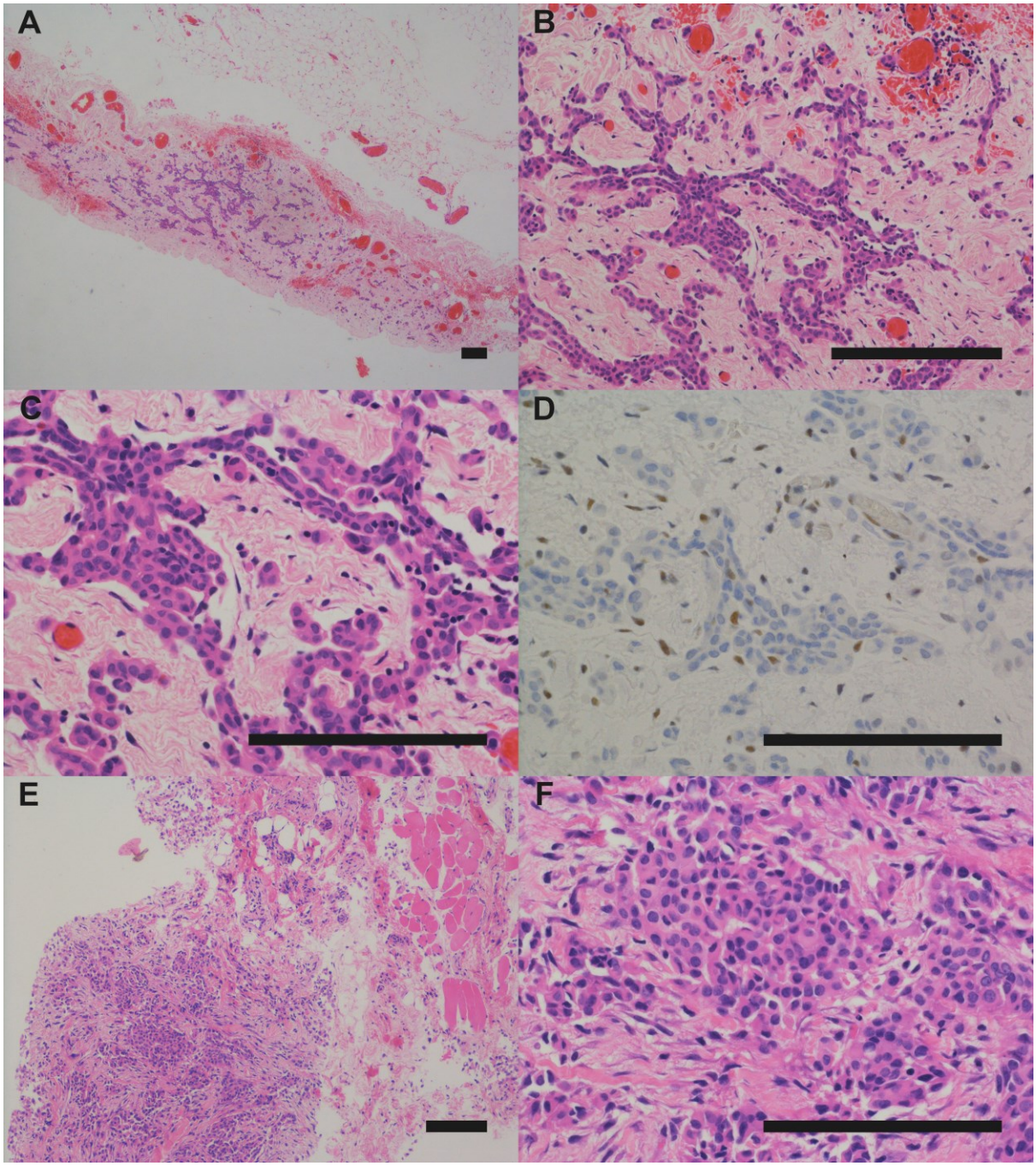


Figure 8-10 Atypical mesothelial proliferation and subsequent PM diagnosis (Case 2).

(A) The atypical mesothelial proliferation was limited to the submesothelial compartment, and no surface proliferation was present. **(B)** The growth pattern was predominantly trabecular, with discohesive secondary pattern. **(C)** The cells show mild nuclear atypia. **(D)** There was diffuse, complete loss of BAP1 staining. **(E)** Subsequent chest wall biopsy showed

unequivocal invasion into the chest wall muscles. **(F)** The malignant cells show similar nuclear features to those in the initial AMP diagnosis.

Scale bar = 100μm.

8.4.5.3 Case 3 (High risk AMP)

This was a 76 year old gentleman presenting with right sided pleural effusion who subsequently underwent pleural biopsy. No information on asbestos exposure or past medical history was available. Histopathology showed fibrotic pleura without evidence of subpleural structure sampling, and a stromal component comprised of mildly atypical mesothelial cells with trabecular growth pattern (Figure 8-11). There was diffuse loss of nuclear BAP1 and loss of MTAP staining. A subsequent pleural biopsy performed 3.8 months later confirmed biphasic mesothelioma, with the epithelioid component exhibiting similar degree of nuclear atypia to the initial AMP diagnosis.

This case was considered high risk AMP, favouring under-sampled invasive PM. The biomarker profile serves as supportive evidence in this setting.

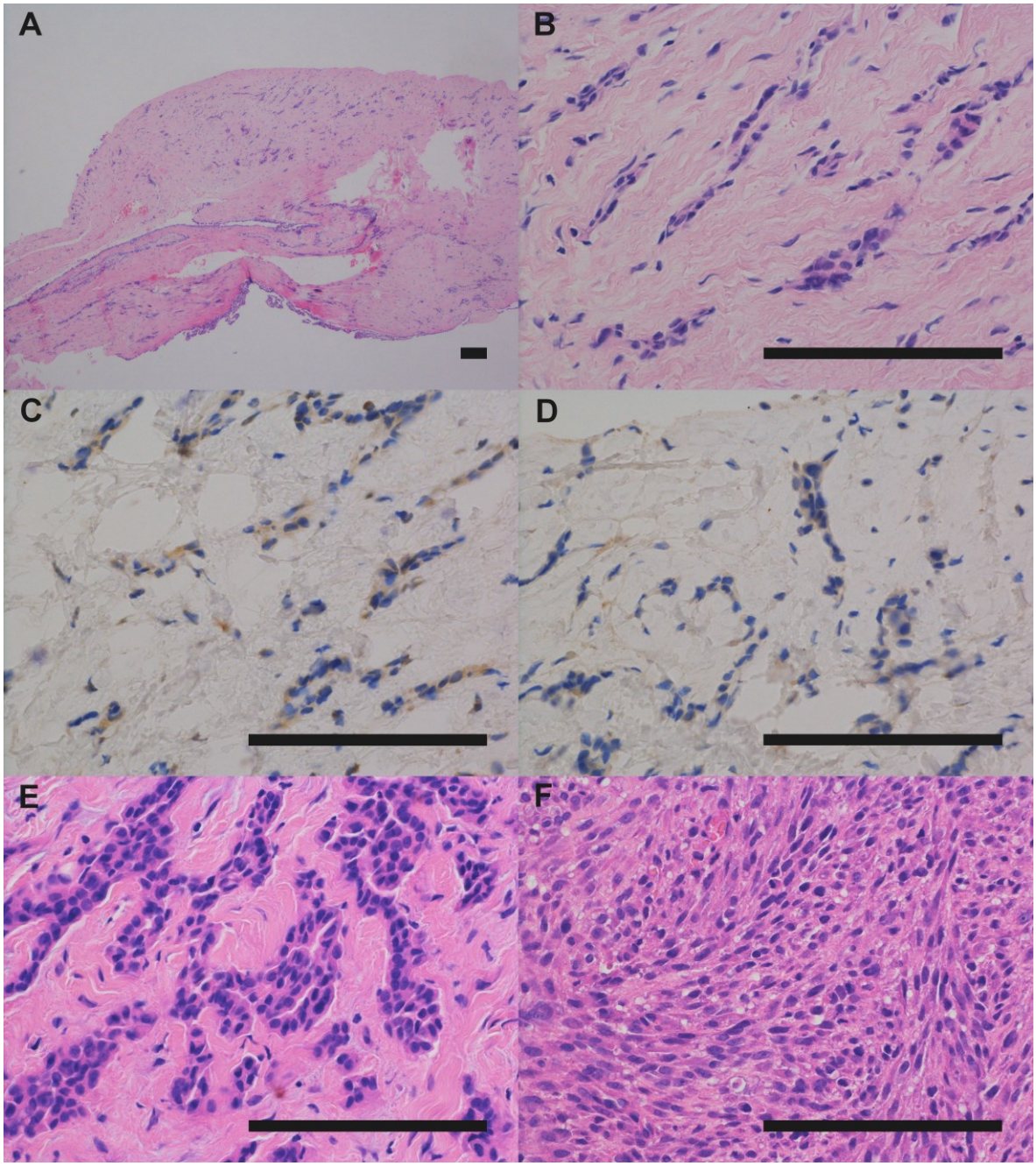


Figure 8-11 Atypical mesothelial proliferation and subsequent PM diagnosis (Case 3).

(A) The pleural biopsy was of limited quantity and extent, with no evidence of subpleural structure sampling. **(B)** The submesothelial stroma contained mildly atypical mesothelial cells with trabecular growth pattern. There was **(C)** diffuse loss of nuclear BAP1 staining and **(D)** loss of MTAP staining. **(E-F)** Subsequent pleural biopsy showed biphasic mesothelioma.

Scale bar = 100μm.

8.4.5.4 Case 4 (High risk AMP)

This was a 67 year old gentleman presenting with 15 month history of right sided pleural effusion, and previous attempts at drainage resulted in rapid re-accumulation. There was proven occupational asbestos exposure. CT showed a large right apical lung mass associated with pleural thickening, suspicious for primary lung cancer, clinical stage T4 N2 (3, 4R, 5) M1a based on TNM classification (8th edition). Intra-operatively there were numerous nodules on both the visceral and parietal pleura.

Histopathology of the pleural biopsy showed moderate to severely atypical mesothelial cells admixed with florid chronic inflammation, fibrin and haemorrhage (Figure 8-12). Subpleural structures were absent and orientation was not possible. The epithelioid component of the proliferation showed cribriform and solid growth pattern, and focally there was features of small cell variant. A spindle cell component exhibiting severe nuclear atypia was also seen. No BAP1/MTAP immunohistochemistry was performed. The patient died 8.1 months after the diagnosis, with no documentation of active oncological treatment.

This case was considered high risk AMP, favouring under-sampled invasive PM.

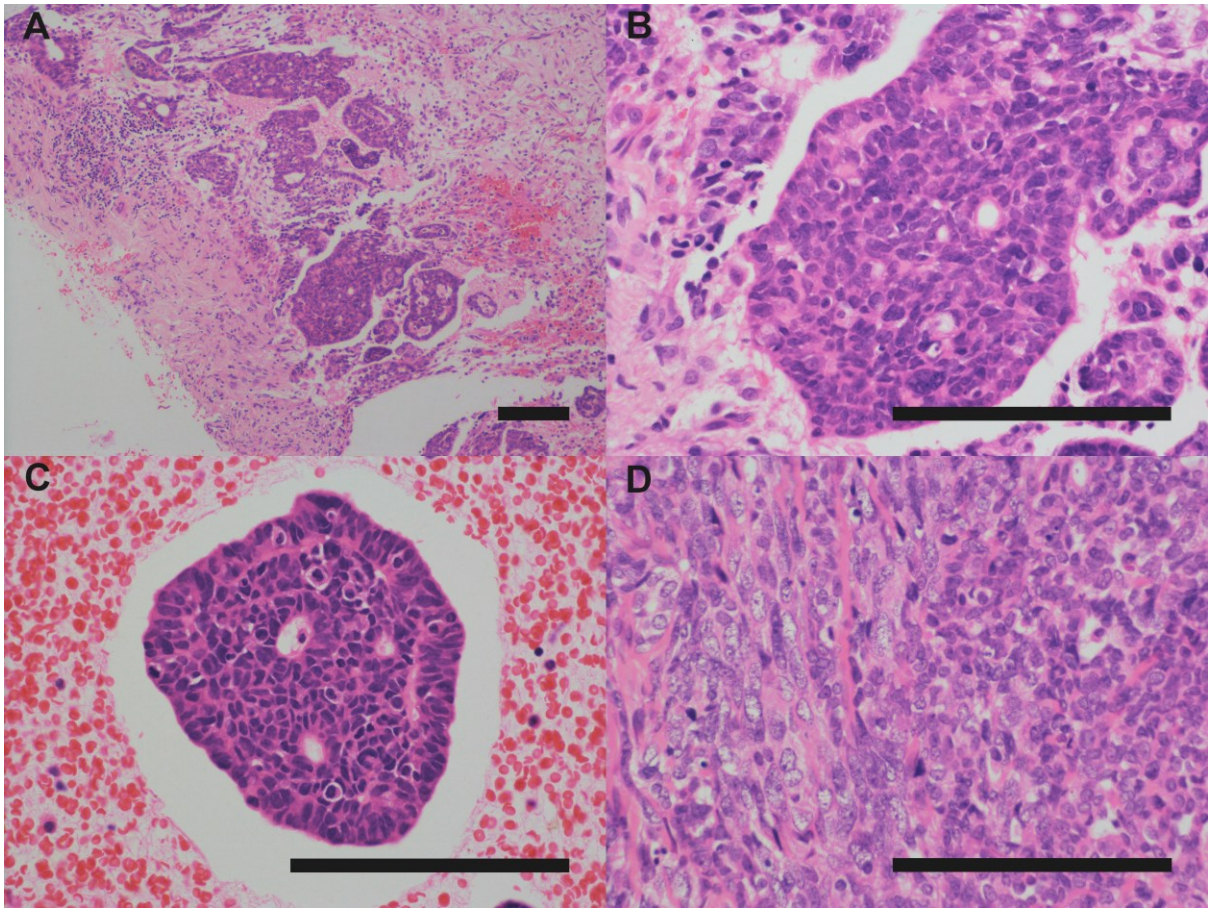


Figure 8-12 Atypical mesothelial proliferation (Case 4).

(A) Atypical mesothelial cells with cribriform and solid growth patterns were seen, admixed with significant chronic inflammation, fibrin and haemorrhage. No subpleural structure was identified. **(B)** The atypical cells show moderate to severe nuclear atypia, and **(C)** small cell variant was seen focally. **(D)** Adjacent to the epithelioid component, there was a minor spindle cell component exhibiting severe nuclear atypia, with pleomorphic nuclei and conspicuous nucleoli.

Scale bar = 100μm.

8.4.5.5 Case 5 (Low risk AMP)

This was an 84 year old gentleman presenting with right pleural effusion associated with right middle lobe collapse. No information on asbestos exposure or past medical history was available. Intra-operatively there were mucosal deposits on the parietal, visceral and diaphragmatic pleura all of which were biopsied.

Histopathology showed adequately sampled pleural biopsies with a surface component consisted of mild to moderately atypical mesothelial cells (Figure 8-13). The growth pattern was predominantly tubulo-papillary but in areas it resembled that of well differentiated papillary mesothelial tumour (WDPMT) with invasive foci (Churg *et al*., 2014) (Galateau-Sallé F *et al*., 2004). Deeper in the submesothelial stroma mildly atypical mesothelial cells with tubulo-papillary growth pattern were present, close to but not infiltrating the subpleural fat. They retained nuclear BAP1 staining but there was focal MTAP loss. Focal MTAP loss was observed in a subset of PM with homozygous *CDKN2A* deletion (Chapter 7, Section 7.4.4), but the exact clinical significance remains to be fully elucidated.

The case was considered low risk AMP, favouring minimally invasive PM. The BAP1/MTAP biomarker status offered limited value based on available evidence. The differential diagnoses include WDPMT with invasive foci, which was highly unlikely given the architectural atypia and presence of multiple macroscopic lesions, and invasive epithelioid PM with WDPMT-like features (Chapter 5) (Zhang *et al*., 2020b). The latter was rare, invariably of low nuclear grade and demonstrated relatively indolent clinical phenotype.

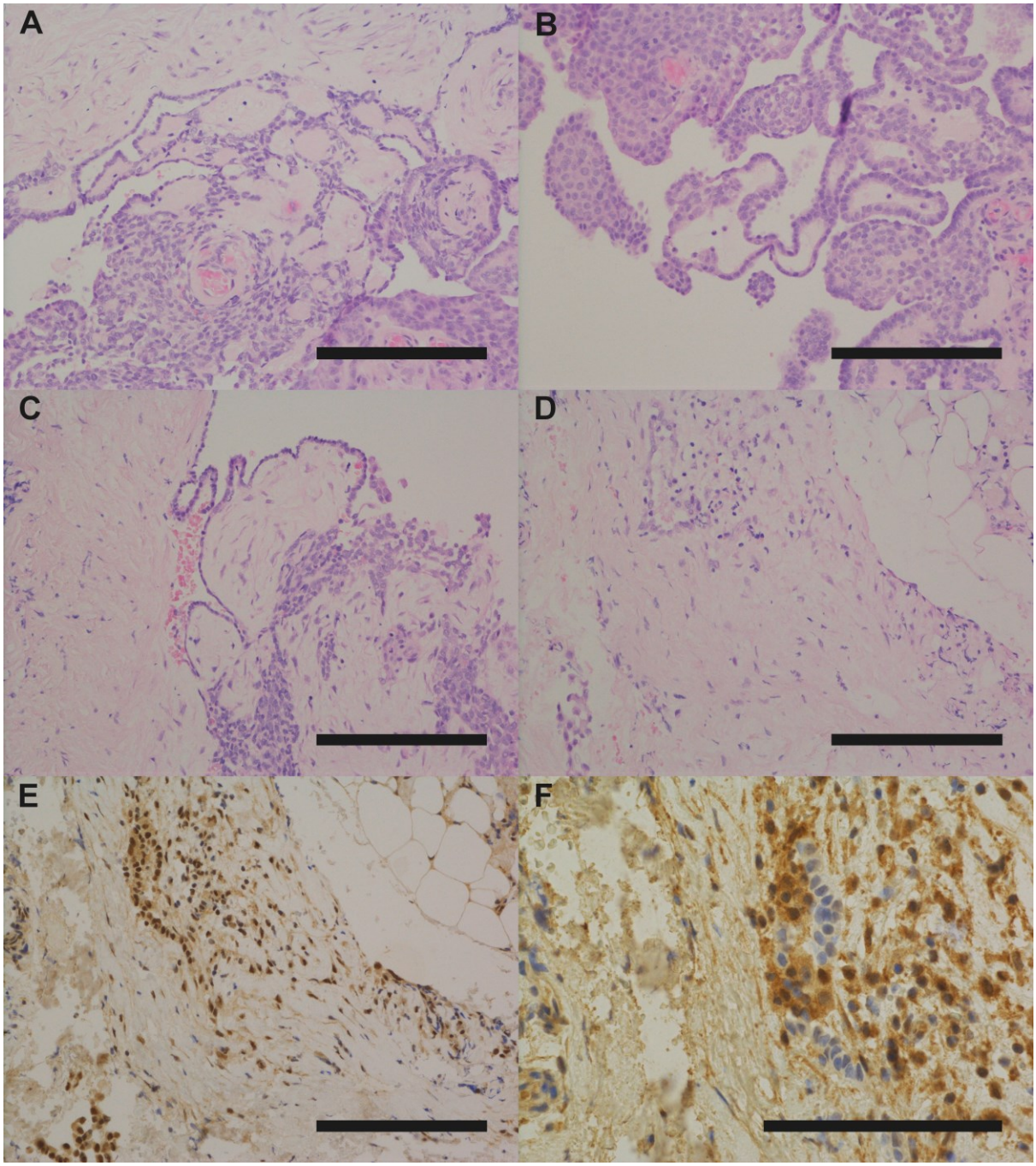


Figure 8-13 Atypical mesothelial proliferation with features resembling WDPMT (Case 5).

(A-B) The surface component showed predominantly tubulo-papillary growth pattern, but focally there were well formed papillae with hyalinised fibrovascular cores, lined by a single layer of cytologically bland mesothelial cells. **(C)** These areas resembling WDPMT merged abruptly with surface mesothelial proliferation showing more complex growth pattern, which was incompatible with the diagnosis of WDPMT. **(D)** Within the submesothelial stroma there

were scattered mesothelial cells with tubulo-papillary growth pattern, and the degree of nuclear atypia was no more than mild. **(E)** The atypical cells retained nuclear BAP1 staining, and **(F)** there was focal loss of MTAP staining.

Scale bar = 100μm.

8.5 Discussion

In this study, a multidisciplinary and systematic evaluation of atypical mesothelial proliferations of the pleura was performed. AMP has been used in diagnostic practice as an umbrella term and represents a highly heterogeneous group of entities with overlapping histological features and clinical phenotypes, encompassing MIS, minimally invasive PM, under-sampled invasive PM of either epithelioid and non-epithelioid subtypes, reactive mesothelial hyperplasia and reactive fibrous pleuritis. This chapter proposes a morphology-based classification system to stratify patients into clinical and statistically significant groups based on prognosis, with the high risk group showing similar outcomes as invasive PM (Table 8-13). Indeed, even low-risk AMP cases had a subsequent diagnosis of PM in 60% of patients, within 12 months after being classified as AMP, indicating the need to identify those with neoplasia as early as possible within the diagnostic pathway

Whilst this has some value in prognostication, the ultimate goal is to make the term AMP redundant and definitively categorised as one of the above groups. Using a limited prospective but ongoing cohort, the present study has shown that the recent introduction of BAP1 and MTAP immunohistochemistry in routine diagnosis of mesothelial diseases carries potential in shifting cases from AMP to a definitive diagnosis of PM.

The patient demographic and clinical characteristics of the population in the present study was similar to the UK PM population (National Mesothelioma Audit, 2018). In the current study the lack of subpleural structure sampling, subtype and presence of severe nuclear atypia in the classification system was incorporated but not stromal features. Although demonstrating utility, the latter is subject to uncertain but likely substantial inter-observer discrepancy. For the former, observations in historical practice were evidenced. Benign mesothelial processes such as reactive mesothelial hyperplasia and reactive fibrous pleuritis can often result in a degree of cytological atypia that overlaps with those seen in PM. The lack of subpleural structure(s) in the tissue materials can be either due to under-sampling by the operator or modality, or replacement of the subpleural plane by pathological processes despite otherwise extensive sampling. There are two possible explanations to the observed adverse prognostic implication of its absence: (1) this is a surrogate of infiltrative growth characteristics hence more advanced disease, and (2) this reflects sampling bias by the operator whom is inclined to sample the areas with the most prominent macroscopic disease, with the latter representing higher disease burden. While the findings in this study reinforce the proposal for more extensive sampling in the diagnostic algorithm, the study data refined the conceptual mandate in the current diagnostic definition of PM, in the way that the evaluation of subpleural structure(s) might no longer be applicable.

The findings from this study are valuable to clinical management and research into PM for several reasons. First, these progressed current knowledge in understanding the natural history of AMP, as at least a proportion of cases in the low risk category which were adequately sampled still proved to be early stage PM. This will allow greater access to surgery and/or clinical trials as patients generally exhibit more favourable functional status with early stage disease and might also harbour a greater extent of epithelioid disease. Second, as high risk AMP carries a similar prognostic outlook to invasive PM, the diagnosis

should trigger further sampling until a diagnosis is made or sampling is viewed as sufficiently adequate to exclude malignancy as far as possible using current sampling modalities, after MDT discussion.

The paucity of well powered studies in the field of AMP rendered direct comparison with published literature difficult. This is further aggravated by variations in diagnostic practice even in mesothelioma specialist centres. Out of the 34 cases from Pillappa *et al*. (Pillappa *et al*., 2018) with an overall censor fraction of 41.1%, 21 were from the pleura. The majority were of epithelioid subtype and 44.1% received subsequent diagnosis of mesothelioma (inclusive of both pleural and peritoneal origins). No median OS was given for the Pillappa *et al.* population but among those with subsequent malignant diagnosis the median OS was 18.2 months. The longer survival exhibited by the relatively limited patient population was suggestive of early stage disease, as described by the original authors.

While well differentiated papillary mesothelial tumour (WDPMT) is a distinct disease entity of benign nature, Galateau-Sallé *et al*. reported a total of 24 cases of WDPMT of which invasive foci were identified in 10 cases (Galateau-Sallé *et al*., 2014). All invasive foci were limited to the submesothelial compartment without unequivocal invasion of subpleural structures. They almost exclusively presented with pleural thickening (9/10, 90%). The 10-year survival of the study population was 30.8%, although with a censor fraction of 70.8% the survival analysis is considered underpowered. In a follow-up multi-institutional study, Churg *et al*. presented 20 cases of WDPMT with invasive foci, 3 of which originated in the pleura and all presented with multiple lesions (Churg *et al*., 2014). They showed extremely indolent clinical behaviour following chemotherapy although the number of cases was too small to reach statistical significance. Although classification in surgical resection specimens or large biopsies should not pose a diagnostic challenge, it would not be unreasonable to hypothesise in small biopsy setting the degree of morphological overlap between WDPMT

with invasive foci, minimally invasive PM and under-sampled invasive PM with WDPMT-like features could lead to misclassification. This should represent an area of future investigations due to the clinical implications.

The major weakness of the current study is its retrospective nature with no systematic follow-up and interval sampling, nor standardisation of sampling protocol. The high degree of subtype concordance in patients receiving subsequent PM diagnosis suggests a substantial proportion of diagnoses are down to under-sampling. This also applies to the low risk AMP category as a majority of patients received a malignant diagnosis within 12 months, indicating an inability to diagnose PM at the time of first biopsy resulting in a delay in diagnosis. In retrospect it is difficult to ascertain the reasons behind re-biopsy in the present study since the intent was not usually documented. Nonetheless diagnostic bias is a distinct possibility, meaning the probability of receiving a second biopsy is high due to symptomatic progression, as in the case of under-sampled invasive PM that was classified as AMP.

The key elements of future work include genomic characterisation of AMP. This involves both conventional methods such as BAP1 and MTAP immunohistochemistry as well as DNA sequencing. In high risk AMP these provide supportive evidence of malignancy in otherwise high confidence diagnosis, while in low risk cases the molecular evidence of malignancy will prove pivotal as low risk AMP often lies within the morphological spectrum of benign mesothelial biological processes. Furthermore, a prospective and systematic evaluation including an interval sampling schedule is warranted. It is crucial to explore the role of multi-site, image-guided and/or anatomical mapping biopsy. The relationship between AMP and MIS warrants further study, and without a robust sampling protocol one cannot obtain the necessary evidence to consolidate, or to nullify, the proposal of a minimally invasive category of PM.

In addition, the utility of pleural effusion-based diagnosis maximising the information value of either or both the cellular and cell free fractions offers an attractive avenue in improving early diagnosis. This could either serve as a reliable and cost-effective adjunct at time of tissue biopsy or a replacement for biopsy at an earlier timepoint. The implementation is anticipated to be challenging and given the biological and physiological complexities of pleural diseases, the scope and influence of pre-analytical variables are unforeseen. The disease burden is likely low, meaning this will require high sensitivity assay platforms with resolution appropriate to the target cell fraction. Finally, given this is an uncommon diagnosis, for pathology departments with substantial pleural diagnostic burden it would be favourable to collate and disseminate local pathological and follow-up data to contribute to the evidence base.

Minimising the use of AMP as a “gray-zone” diagnosis will reduce the burden on clinical practice by obviating the need for repeat biopsies and allowing patients to be considered for surgery, chemotherapy and/or clinical trials at an earlier time point. This will also facilitate research including fundamental science, in understanding the early biological events.

	Mesothelioma *in situ* (MIS)	**Atypical mesothelial proliferation (AMP)**	**Invasive pleural mesothelioma (PM)**
Incidence	Very rare (<50 cases reported)	Uncommon (~10% of all PM)	n/a
Multidisciplinary diagnosis	Essential	Desirable	Desirable*
Pleural effusion	Essential for diagnosis	Usually present	Usually present
Radiological and/or intra-operative pleural disease	Absent	Present	Present
Minimum sampling requirement	100-200mm^2 area	None proposed	3 random anatomical sites
Subtype	Epithelioid	Any	Any
Surface mesothelium	Linear	Linear	Linear
	Small papillary projections or nodules	Complex	Complex
		Absent	Absent
Extent of invasion	None (Surface only)	Submesothelial stroma	Subpleural structure(s)
Molecular signature	*BAP1* mutation (Dacic et al., 2020) Homozygous *CDKN2A* deletion	Unknown†	Diverse
Median time to PM diagnosis	60 months ‡	Low risk: 4.8 months High risk: 2.3 months	n/a
Median OS **	NR	Low risk: 33.4 months High risk: 11.9 months	Epithelioid (Low grade): 19.3 months Epithelioid (High grade): 8.9 months Epithelioid (pleomorphic): 5.4 months Biphasic: 8.8 months Sarcomatoid: 3.9 months

Table 8-13 Comparison between mesothelioma *in situ*, atypical mesothelial proliferation and invasive pleural mesothelioma.

* In exceptional cases multidisciplinary consensus can override histopathological diagnosis, particularly in the setting of inconclusive diagnosis or lack of unequivocal invasion. Such cases mandate multidisciplinary discussion and are expected to be rare. ** For AMP, the survival figures were based on epithelioid-restricted criteria. The presented survival figures for invasive PM were based on local data (1991-2017).

† Loss of nuclear BAP1 staining and homozygous *CDKN2A* deletion were observed in a small number of cases.

‡ A minimum interval of 12 months between the diagnosis of MIS and subsequent PM diagnosis is required for the former.

8.6 Conclusions

Atypical mesothelial proliferation of the pleura is an increasingly historical term, usage of which has changed considerably over the time period of this thesis. The initial intention was to risk stratify AMP cases with a view to improving their management, this work showing that even low-risk cases proved to be PM in 60% of patients. The addition of BAP1 and MTAP has allowed significant reduction in the diagnosis of AMP and allowed more definitive diagnosis on the initial biopsy. Further work is needed to sufficiently evidence the necessity to introduce a clinical and/or pathological minimally invasive stage akin T1mi category for lung cancer (minimally invasive), and the relationship with MIS.

9 General Discussion

In this thesis, the issues of inter- and intra-tumoural heterogeneity in PM, and pursuit of earlier diagnosis, were addressed and evidenced using a number of datasets. Clinical and statistically significant differences in outcomes and key surgical and oncological parameters were observed in patients undergoing multimodality therapy, including radical surgery, which is governed by histological subtype (Chapter 3). Epithelioid PM is the most common subtype and, whilst often regarded as a single disease entity, it can now through the work of this thesis be reliably classified into three subgroups (low grade, high grade, pleomorphic) based on histological features, subject to adequate tissue sampling (Chapter 4). This offers an explanation to variation in prognosis in patients with E-PM, and the overlapping prognostic profile when comparing epithelioid versus non-epithelioid tumours. This subtype also exhibits a diverse spectrum of morphological appearances in relation to architectural growth patterns (Chapter 5) and cytological features (Chapter 6), some of which are unique to the mesothelium while the others are agnostic for tissue lineage. In this thesis, by studying the genotypes of *CDKN2A* and *MTAP* it has been evidenced that the histological classification shows a degree of association with the genomic status but is not in complete agreement (Chapter 7). Finally by employing a similar morphological approach to atypical mesothelial proliferation of the pleura, a novel classification system stratifying patients based on clinical risk has been developed and, within the limitations of the retrospective nature of the study, the hypothesis that at least some of the cases categorised as low risk represented minimally or superficially invasive pleural mesothelioma is rationalised (Chapter 8). This is further supported by the adoption of BAP1/MTAP immunohistochemistry in AMP, where many cases could be upgraded to PM. This allows a definitive diagnosis to be made at an earlier timepoint, both reducing the need for repeat biopsies and permits prompt treatment.

A major challenge in contemporary mesothelioma research is that formal disease classification and the introduction of experimental therapeutic interventions outpaces the progression of the evidence base. Such phenomenon is self-evident in the recent proposals of novel histological entities based on small sample sizes with limited follow-up or independent validation, and the large number of early phase clinical trials without upfront translational portfolios. A good example is the concept of MIS, which is under constant revision, which will again undergo significant changes in terms of eligibility and morphological restraints. It is an extremely rare pathological entity, none have been found in the RBH diagnostic archive over a period of 30 years (Chapter 8, Section 8.3.1). To add to the conundrum, it was recently reported invasive PM with prominent *in situ* component showed prolonged survival compared with invasive PM without (24 months vs 8 months) (Pulford *et al*., 2020). No nuclear grade or molecular data was available, meaning it is practically impossible to ascertain if this denotes another novel entity, or a variant of MIS, or these were merely in fact invasive low grade epithelioid mesothelioma.

The latest WHO classification of thoracic tumours, in relation to the diagnosis and classification of mesothelioma, reflects the scientific endeavours in pathology and fundamental science in the recent years. The classification and its evidence base exerted profound influence on the conceptualisation and the scientific content of this thesis. The changes in formal nomenclature to remove the "*malignant*" prefix to mesothelioma and renaming "*well differentiated papillary mesothelioma (WDPM)*" to well differentiated papillary mesothelial tumour (WDPMT) was due to the recognition of mesothelioma being malignant by nature. The recommendation of adopting nuclear grading system for E-PM represents a step forward in its prognostication, as well as generating further questions in the areas of tumour heterogeneity and sampling requirements - both of which were attempted herein. The inclusion of MIS and a transitional cytological subgroup of mesothelioma were

driven by the integration of conventional histopathology and molecular genetics, despite both are of rare occurrences. In attempt to identify MIS from the RBH patient population, whilst none was found, atypical mesothelial proliferations were of considerable frequency (Chapter 8). Through work conducted in this thesis, it was established that a significant minority of these cases could represent early or minimally invasive disease, and in theory amenable to curative treatment similar to MIS. These newly introduced concepts and entities are likely to trigger the contribution of clinical, pathological and molecular data across the globe, which will in turn improve the next iteration of tumour classification and possibly staging, via a positive cycle.

One might argue in the modern model of academic-clinical partnership, the progression of scientific knowledge inevitably drives transformation of clinical care. This is indeed true in the case of prostatic adenocarcinoma, as has been witnessed over a course of more than half a century since the proposal of grading system by Gleason (Bailar 3rd *et al*., 1966), at which point treatment options were limited. It undertook at least three rounds of major revision, the first in 1974 (Gleason and Mellinger, 1974) when hormonal treatment was being considered in clinical trials, the second in 2005 when improved imaging and biopsy techniques culminated in the introduction of tertiary grades (Epstein *et al*., 2005), and most recently the proposal of grade groups instead of individual grades which offered superior prognostic separation and reproducibility in the context of contemporary treatment regimens (*Epstein et al*., 2016). The Gleason grade withstood the test of time, as it remains a key prognosticator in the era of massive parallel sequencing, with which canonical genomic events including tumour mutational burden, re-arrangement burden and somatic hypermutation or kataegis are associated (Fraser *et al*., 2017). A similar example is the Bloom-Richardson grading system for breast cancer, initially proposed in 1950 (Bloom, 1950), subsequently validated using a larger patient cohort with long-term follow-up (Bloom and Richardson, 1957). It was revised

by Elston and Ellis to improve the operational properties by introducing semi-quantitative scales in the method of assessment (Elston and Ellis, 1991). It predicts patient outcomes independent of clinical variables and multi-gene expression profile (Paik *et al*., 2004), and continues to serve as the gold standard in contemporary diagnostic practice.

The counterargument would state that the conclusions from the work conducted in this thesis suffers from hindsight bias. Diffuse PM has no known cure, nor effective treatment, even in the setting of early disease. Current major guidelines, however, include recommendations for clinical trials, and coupled with changes in classification for example MIS, potentially allow ample opportunity for these newly proposed entities to be part of clinical trials and investigative studies.

Governed by such rhetoric, while this thesis has expanded the evidence base in several key aspects of mesothelioma diagnosis and classification, its main weakness is ingrained in its principal aim and methodology. Heterogeneity in the research of human diseases is an overarching concept, and in many ways poses significant challenges. The results from this thesis were derived from highly cross-sectional data, representing an over-simplified perspective of the disease, spatially limited by the relatively random process of sampling and temporally limited often by tissue sampling at a single time point. Although attempts were made to delineate the spatial heterogeneity, including the description of morphological features and protein expression limited to certain areas of the tumour, the resolution is limited including the lack of investigating the velocity of disease progression. This is an area of PM poorly understood with scanty evidence in the literature, at least in part attributable to the aggressive disease course. Benchmarking exercises involving various subtypes and histological classes are warranted to study the changing tumour and patient characteristics over defined intervals, thus allowing an eloquent description of its natural history, and permits more robust interpretation of clinical trial results as well as patient outcomes.

A multidisciplinary and highly collaborative approach in the study and clinical management of pleural mesothelioma has recently been published, with many recommendations based on expert consensus due to the lack of high grade evidence, presented as a philosophical construct (Nicholson *et al*., 2020). This tackles the issues of segregated and cross-sectional nature of novel data by combining expertise from multiple scientific and clinical specialities in an integrated fashion. Whether this transforms the research landscape of mesothelioma remains to be seen in the coming years. In this thesis, this approach was adopted particularly in the study of atypical mesothelial proliferation, albeit with retrospective data collection. In addition to missing clinical and radiological data, the impact of sampling especially in relation to the number of sites and their anatomical locations could not be assessed fully.

Future research stemming from this thesis can be divided into four domains. First, given the aggressive clinical phenotypes and limited treatment option for PM, the solitary practical function of histological classification is prognostication. Substantial work is required to evaluate the predictive utility of these classification systems, including nuclear grade, in various treatment modalities. The primary and secondary outcomes measures differ by modality, as do the eligible patient populations. A systematic evaluation is thus warranted to ascertain their role(s) with the prospect of improving pre-treatment evaluation and stratification. The focus of research, although not fully supported by evidence, should be upon the initial tissue biopsy as it is conceivable the majority of patients with PM would not endure multiple interval biopsies in their patient journeys. It is also worth exploring the role of digital image analysis and artificial intelligence as they have demonstrated potential in diagnosis, prognostication and identification of morphological features (Courtiol *et al*., 2019) (Galateau-Sallé F *et al*., 2020).

Second, this thesis did not address the role(s) of pleural effusion cytology in improving the diagnosis and treatment stratification of PM. Both the cellular and acellular (cell-free)

fractions represent easily accessible and abundant biological materials, likely harbouring a wealth of clinically and scientifically relevant information. Material needs to be collected and preserved prospectively, for example as cell blocks, in order for this to occur. A frequently quoted figure suggesting poor diagnostic sensitivity of pleural effusion cytology in PM (9/148, 6.1%) was derived from a study predating the use of BAP1/MTAP immunohistochemistry, and did not take into account the clinical and radiological findings influencing the pre-test probability of a positive diagnosis (Arnold *et al*., 2018). Systematic and multi-disciplinary assessment of pleural effusion-biopsy pairs, under a reproducible diagnostic framework akin the Paris system for urine cytology (Barkan *et al*., 2016), will provide pathologists and treating clinicians realistic estimates of the diagnostic confidence. This is of particular value when a tissue biopsy is not indicated either due to patient's functional status or choice. In the context of MIS, the importance of cytology-based diagnosis is likely to be accentuated by the lack of radiological and/or macroscopic targets for biopsy although not replacing the latter (Klebe *et al*., 2021). The acellular fraction of pleural effusion is a vital constituent of the microenvironment, with pro-growth biological activities and unique proteomic profiles (Asciak *et al*. 2021) (Tsim *et al*., 2021) but completely overlooked by pathologists due to specimen processing. The feasibility of detecting human host and prokaryotic DNA has been reported in a number of studies in metastatic adenocarcinoma (Yang *et al*., 2020) (Guo *et al*., 2019) (Hummelink *et al*., 2019) (Kawahara *et al*., 2018) (Lee *et al*., 2018) and mycobacterial infection (Yang *et al*., 2020). The specimen requirement and the impact of pre-analytical variables, however, serve as major hurdles to clinical application and mandate endeavours in fundamental science.

Furthermore, sampling is a central issue to the diagnosis and research of mesothelioma. In this thesis, the proposal of sampling three random sites in the pleura for initial diagnosis was evidenced. Notwithstanding this being a step forward in optimising the sampling protocol for

PM, three key questions remain answered: (1) is a biopsy template or anatomical mapping necessary for accurate diagnosis and prognostication? (2) what is the utility of image-guided biopsy both in the surgical and minimally invasive setting (core biopsy)? (3) do patients with suspected MIS or minimally invasive disease warrant even more extensive sampling than those with radiological and/or macroscopic disease? These questions should be addressed in an adequately powered prospective study with a translational component investigating the biological and genetic aspects of spatial heterogeneity.

Finally, cancer can be regarded as a disease of de-differentiation of the native tissue lineage. The observed morphological distinction and resemblance between PM and normal pleural mesothelium, can be conceived by a heterogeneous and dynamic differentiation programme consisting of (i) preserved or reactivated developmental pathway by which normal mesothelium undertake, (ii) gain of developmental programmes utilised by other lineages, and (iii) creation of *de novo* pathways leading to acquisition of phenotypes not otherwise observed in normal human tissue lineages.

Such lineage reprogramming or plasticity is expected to be explained by a complex myriad of molecular alterations, both at genetic and epigenetic levels. The phenotypes are then in turn selected by extrinsic pressure including tumour microenvironment and treatment. Tumour subpopulations can show a multitude of these characteristics, some being lineage-specific and others lineage-independent, leading to spatial and temporal heterogeneity.

The suggestion that lineage reprogramming plays a key role in this disease is supported by the ubiquitous growth patterns in E-PM, also the high incidence of sarcomatoid transformation (up to 40% of all PM in comparison to <10% of lung cancer), although the latter resides outside the confines of this thesis. It is not yet known how this is governed in PM, nor the cell type(s) involved in the process. Recent work on primary lung

adenocarcinoma, utilising single cell sequencing, revealed highly heterogenous tumour cell subpopulations with distinct lineage characteristics during various stages of tumour development (Laughney *et al*., 2020) (LaFave *et al*., 2020) (Marjanovic *et al*., 2020). Stem-like (Laughney *et al*., 2020) or cells exhibiting "high-plasticity cell state" (Marjanovic *et al*., 2020) characterised transcriptomic and epigenomic programmes show features consistent with primitive lineages (loss of alveolar type II pneumocyte signature) (Laughney *et al*., 2020) (LeFave *et al*., 2020) or a mixed state of differentiation (Marjanovic *et al*., 2020). Whether or not an analogous programme exists for PM, and if so, the cellular and molecular contexts should be a focus of future research.

In conclusion, this thesis interrogated the differential response of PM to therapy, and investigated its inter-tumoural and intra-tumoural heterogeneity, with results showing how this can be organised and refined. Furthermore, it has shown that AMPs are a heterogeneous group of biological processes and that both risk stratification and usage of BAP1/MTAP immunohistochemistry pave the way for earlier diagnosis using a multidisciplinary approach. These findings underpin the biological complexity of a disease with a diverse multi-omic landscape and although much remains to be understood, this thesis contributes to our knowledge of this sophisticated neoplastic ecosystem.

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