PhD Thesis

Half-title: Biological effects of pleural fluid

A study focused on the biological effects of pleural fluid in mesothelioma, and an analysis of the feasibility of a personalised bench-to-bedside pathway for mesothelioma treatment

Rachelle Asciak

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Abstract

Background and aims

Malignant pleural mesothelioma (MPM) carries a poor prognosis and more than half MPM patients do not show disease response to cancer drugs. A more personalised effective approach to MPM treatment is desirable, and the timely development of personalised MPM cell cultures and analysis of drug response/resistance profiles may help guide clinical management of MPM.

MPM or pleural metastases often present with malignant pleural effusion (MPE). There is early pre-clinical evidence of potential biological properties of MPE fluid that may contribute to cancer cell proliferation. Current management of MPE is symptomatic. Should pleural fluid have biological properties, management of MPE may shift towards a more aggressive approach in order to impact on patient survival.

This study aimed to:

a) Analyse a database of MPM patients to explore associations between pleural fluid and survival.
b) Develop patient-derived MPM cell cultures from MPE fluid, with high success rate; develop cell cultures from the same patient at different time points.
c) Analyse the effects of pleural fluid on cancer cells *in vitro*.
d) Provide a description of potential future directions of research.
e) Conduct drug screening assays on MPM cell cultures, to assess feasibility of a bench-to-bedside pathway from MPM MPE fluid to drug resistance/response profiles which would be clinically relevant and useful.

Methodology

a) Data on MPM patients (in 3 UK pleural units) were collected and analysed.

b) MPM cell cultures were established from MPE samples.

c) Cells from established, patient-derived, cancer cell cultures were seeded in pleural fluid, and cell proliferation monitored.

   Primary MPM cell culture was attempted in 100% MPE fluid, with primary MPM cell culture in complete medium as a control.

d) A literature review of animal models studying MPE and MPM, and an experimental model I was involved in the development of, were used to design animal models utilising indwelling pleural catheters to investigate the effects of MPE on pleural tumour growth and on chemotherapy response *in vivo*.

e) DNA sequencing and drug screening were performed on MPM cell cultures.

Discussion and conclusions

Pleurodesis success appears to be associated with improved survival. It is unclear whether prolonged exposure to MPE affects MPM tumour proliferation in humans. This data serves to highlight the need for prospective data and can be used to guide the direction of future study designs.

MPM cell cultures were established from MPE fluid samples with a high success rate, had recognisable malignant features on cytology, included cancer stem cells, and had similar variant profile to MPM tumours. The cell cultures were used to perform quality drug screening assays and DNA sequencing, and the feasibility of a bench to bedside pathway was shown, with the cell cultures as potential tools to refine patient management in MPM.

Cancer cells proliferate strongly in 100% pleural fluid, and to our knowledge this is the first demonstration of this effect, which potentially reflects the intrapleural environment *in vivo*. Although the mechanism is unclear, any form of human pleural fluid (malignant, non-malignant, transudate, exudate) appears to have this effect, and this has potentially significant mechanistic and clinical implications. Moreover, this is the first time that primary cancer cell culture has been performed using pleural fluid alone.

Acknowledgements

This project would not have been possible without the valuable input from many other people involved in this study. I am grateful to the whole team involved, including:

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Publications related to this work

- Pleural fluid has pro-growth biological properties which enable cancer cell proliferation. Asciak R, Kanellakis N, Yao X, Hamid M, et al. *Frontiers in Oncology* 2021.
- The association between pleural fluid exposure and survival in pleural mesothelioma. Asciak R, Kanellakis N, Bibby A, et al. *Chest* 2021
- Patient-derived malignant pleural mesothelioma cell cultures: a tool to advance biomarker-driven treatments. Kanellakis N, Asciak R, Hamid M, Yao X, et al. *Thorax* 2020.
- Novel mouse model of indwelling pleural catheter in mice with malignant pleural effusion. Merrick C, Sherrill T, Kanellakis NI, Asciak R, et al. *European Respiratory Journal, Open Research* 2019.
- Update on Biology and management of mesothelioma. Asciak R, George V, Rahman NM. *European Respiratory Rev*iew 2021.

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Abbreviations

BAP1 = BRCA1 (breast cancer gene 1)-associated protein 1

CT = computed tomography scan

DMEM = Dulbecco's modified eagle medium

DMSO = dimethyl sulphoxide

ECOG PS = Eastern cooperative oncology group performance status

FBS = foetal bovine serum

FDA = U.S. Food and Drug Administration

Hb = Haemoglobin

IPC = indwelling pleural catheter

KRAS = Kirsten Rat Sarcoma virus oncogene

MPE = malignant pleural effusion

MPM = malignant pleural mesothelioma

MSK-IMPACT = Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets

mTOR = mechanistic (or mammalian) target of rapamycin

PBS = phosphate buffer solution

SNV = single nucleotide variant

SV40 = Simian Virus 40

TNF = tumour necrosis factor

UK = United Kingdom

VEGF = vascular endothelial growth factor

Chapter 1: Introduction

Malignant mesothelioma is cancer of the lining of the lung (the pleura), the lining of the abdomen (the peritoneum), or more rarely, the lining of the heart (the pericardium). Malignant pleural mesothelioma (MPM) is the most common type of mesothelioma, with about 97% of cases in the United Kingdom (UK) being MPM, and about 3% being peritoneal mesothelioma. The median age at diagnosis of MPM in the UK is 76 years, and the overall survival is about 10% at 3 years, reflecting the poor prognosis associated with MPM.(*National Mesothelioma Audit 2020 – Mesothelioma UK Charitable Incorporated Organisation (for the Audit Period 2016–18).Royal College of Physicians*)

1.1 Pathogenesis

Asbestos

MPM is mainly caused by the inhalation of asbestos particles, and about 85% of mesothelioma in males is associated with occupational exposure to asbestos, with up to 10% of people with occupational exposure to asbestos developing mesothelioma.(Hillerdal, 1999; Wagner et al., 1960) Mesothelioma was first characterised in 1931, but only linked to asbestos in an epidemiological study in South Africa published in 1960.(Wagner et al., 1960) Apart from mesothelioma, asbestos is also linked to lung cancer, and combined cigarette smoking with asbestos exposure increases lung cancer risk from 10 to almost 100 fold compared to unexposed people.(Røe and Stella, 2015) In contrast, cigarette smoking

does not appear to be associated with increased risk of mesothelioma.(Muscat and Wynder, 1991)

Asbestos has been used since ancient times, for example in pottery, but mass mining only started in the twentieth century when asbestos was used to insulate against heat, fire and corrosion.(Røe and Stella, 2015) Work with brake and clutch linings, construction (especially insulators, pipe-fitters), demolition, dockyards and shipyards workers, carpenters, plumbers and electricians are some of the high-risk occupations.(DeBono et al., 2021) Non-occupational exposures include those through a household member, and living close to an asbestos factory. The use of asbestos was banned in the UK in 1999, and all EU countries, including Malta, were banned from using asbestos in 2005, although compliance with this directive has not been verified in some countries, including Malta. The World Health Organisation recommends that all countries have a National Asbestos Profile (NAP) to aim to eliminate asbestos-related diseases, and although neither Malta nor UK have a NAP that adheres to the NAP format published by WHO, they both have a NAP that is considered to be compatible in content with this format.(Arachi et al., 2021)

There is a long latency period of about 30-40 years between first exposure and development of mesothelioma,(Woolhouse et al., 2018) and therefore new cases of mesothelioma are still diagnosed every year. Furthermore, although asbestos has been banned in several countries, the inhabitants of such countries corresponds to about 16% of the world population, meaning that asbestos continues to be used in a large part of the world, with production now mainly in China, Kazakhstan, Russia.(Baur and Frank, 2021) The incidence of MPM was still increasing worldwide by 2015, although data is lacking on mesothelioma incidence for a large part of the world.(Bianchi and Bianchi, 2014; Odgerel et al., 2017; Røe and Stella, 2015) An epidemiological study on mesothelioma published in

2014 reported that the highest rates of mesothelioma across Europe were found in the UK, the Netherlands, Malta and Belgium. The age-standardised incidence (world population) in men in Malta between 2005-2006 was >3 per 100,000, 2.2 in 2009, and 1.1 in 2012 (and 0.0-0.6 in females), compared to 3.3-3.6 per 100,000 in men in the UK (0.5-0.7 in females) between 2000-2011.(Bianchi and Bianchi, 2014) In the UK, 83% of patients diagnosed with mesothelioma were male, in keeping with the occupational exposure.(*National Mesothelioma Audit 2020 – Mesothelioma UK Charitable Incorporated Organisation (for the Audit Period 2016–18).Royal College of Physicians*)

There is some difficulty with documenting and quantifying the asbestos exposure because some people may not recall it or recall it erroneously, or be motivated by the possibility of being awarded compensatory damages. In addition, different workers, within the same workplace, may be exposed to different quantities and types of asbestos. Non-occupational exposure to asbestos may be present, and there is a high incidence of mesothelioma in some families, where exposure to asbestos may be through the clothing of a member of the family who is an asbestos-worker, although the possibility that there is a genetic predisposition to mesothelioma has also been suggested as discussed later.(Carbone et al., 2002; Huncharek, 1995)

There are two main types of asbestos fibres: amphiboles (straight, long fibres, including amosite (known as brown asbestos), crocidolite (known as blue asbestos), anthophyllite and tremolite), and the more commonly used serpentine (short, curly fibres), mainly chrysotile (white asbestos). Exposure to amphiboles carries a higher risk for mesothelioma than chrysotile.(Gilham et al., 2016; Nicholson, 1991) Biopersistence of the amphiboles has

been documented in both animal and human studies with continuously increasing levels of recoverable lung amphibole fibre levels, whereas chrysotile fibres are able to be partially digested and cleared from the lungs within months.(Churg, 1994) Many patients will have been exposed to both amphibole and chrysotile asbestos.(Carbone et al., 2002)

Inhaled asbestos fibres reach the parietal pleura (the outer layer of pleura) through the alveoli, visceral pleura (inner layer of pleura) and across the pleural space (the space between the parietal and visceral pleural layers), or retrograde through the lymphatic vessels. Chronic inflammation precedes mesothelioma, and is mostly found around stomata and lymphoid patches in the basal parietal pleura, which is the most common site for mesothelioma.(Røe and Stella, 2015) MPM spreads from the parietal to the visceral pleura and invades surrounding tissues and structures; in fact involvement of the visceral pleura denotes more advanced disease (higher stage on the eighth version of the International Association for the Study of Lung Cancer (IASLC) staging system).(Berzenji et al., 2018)

The pathogenesis of mesothelioma is multifactorial. Crocidolite asbestos fibres induce toxicity in human mesothelial cells *in vitro* in a dose-dependent manner.(Bocchetta et al., 2000) This raises the question of how MPM develops if the asbestos fibres kill the mesothelial cells. Crocidolite has been shown to induce macrophage accumulation within pleura and lung, and on contact with crocidolite, the macrophages release TNF-α and human mesothelial cells express TNF-α receptors. The TNF-α – TNF-α receptor interaction activates NF-KB pathway, which allows the human mesothelial cells to divide rather than die.(Yang et al., 2006) If there is sufficient deoxyribonucleic acid (DNA) abnormality caused

by asbestos then the mesothelial cells may develop into mesothelioma. Exposure to asbestos is thought to trigger several processes that ultimately lead to mesothelioma as shown in the figure below (Figure 1.1):

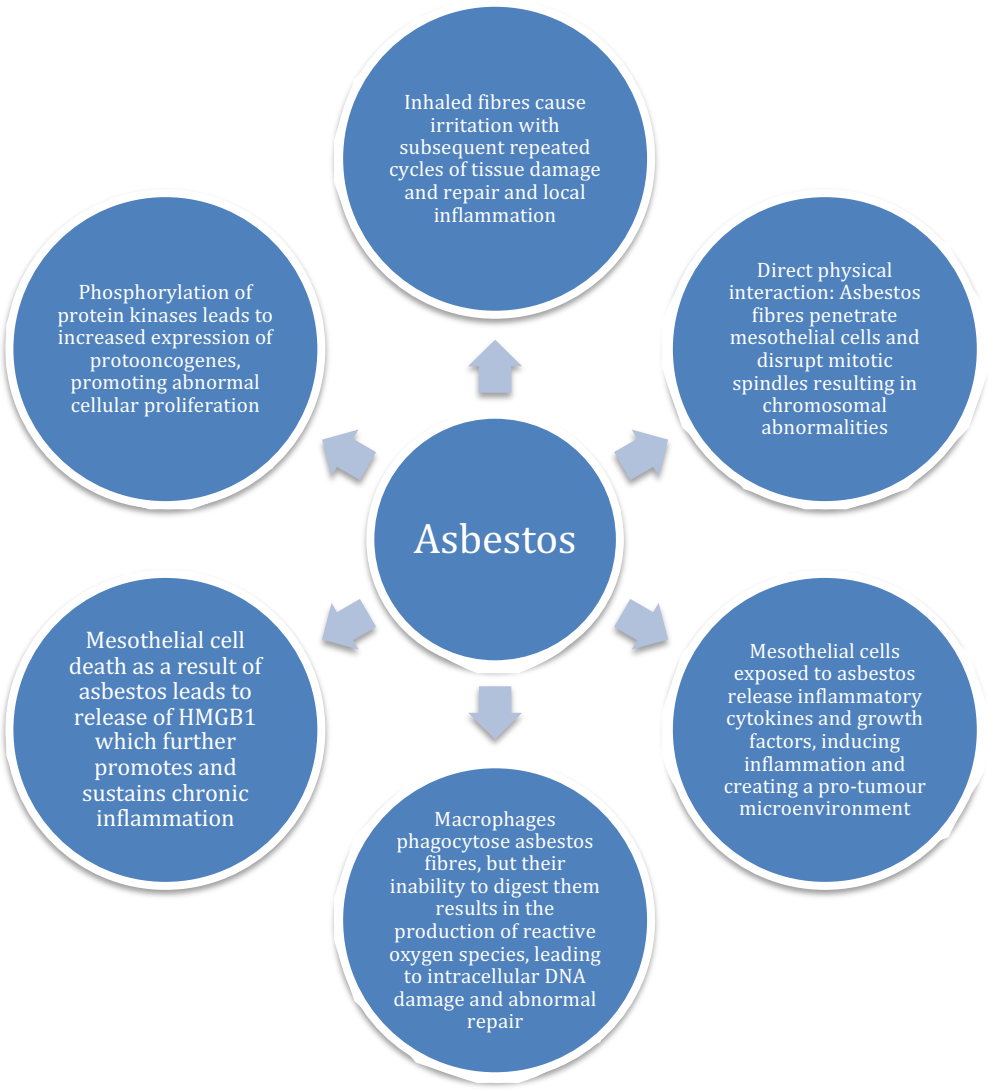


Figure 1.1 shows the multifactorial pathogenesis of mesothelioma development after asbestos exposure *HMGB1 = high mobility group box 1 protein*.(Sekido, 2013, 2008; Vogl et al., 2021; Yang et al., 2010)

However, the majority of workers exposed to asbestos do not develop mesothelioma, and about 20% of cases of mesothelioma are not recognised to be associated with asbestos

exposure.(Jasani and Gibbs, 2012) In addition, mesothelioma develops in adults after a very long latency period, however there are rare cases of mesothelioma that are diagnosed in children,(Brenner et al., 1981) implying that mesothelioma in children either behaves very differently from mesothelioma in adults, or else is not associated with asbestos exposure in the same way. These findings suggest that other factors, apart from asbestos, are involved, and some other possible causes are listed below.

Simian virus 40

Simian Virus 40 (SV40) is a DNA tumour virus, endogenous to the rhesus monkey, that infected humans via contaminated Polio vaccines in the 1950s and 1960s,(Carbone et al., 1999; Kops, 2000) although people who had not received the vaccine were also identified to have SV40 infection indicating the existence of other routes of infection.(Sekido, 2008) Conflicting evidence exists of the potential role of SV40 in mesothelioma.

SV40-like DNA sequences were found in up to 60% of human mesothelioma specimens in France and USA,(Carbone et al., 1994; Galateau-Salle et al., 1998) and confirmed in an independent multi-laboratory USA study (10/12 mesotheliomas found to have SV40 DNA sequences, 83%) and in several other laboratories worldwide.(Rotondo et al., 2019; Testa et al., 1998) In addition, more than 50% of hamsters injected with SV40 developed mesothelioma.(Cicala et al., 1993) Furthermore, human mesothelial cells were found to be more susceptible to SV40 infection and transformation than fibroblasts and epithelial cells, and SV40 and asbestos were found to be co-carcinogenic *in vitro*.(Bocchetta et al., 2000) On the other hand, SV40 was not found in Turkish (*n*=9) or Finnish mesothelioma samples (*n*=49),(De Rienzo et al., 2002; Hirvonen et al., 1999) suggesting potential geographical differences in the presence of the virus, likely related to geographical differences in distribution of administration of SV40-contaminated vaccines.

It has been suggested that in the above studies, the SV40 DNA sequences in mesothelioma represent false positives from contaminating plasmids that result in positive sequencing results,(López-Ríos et al., 2004) however this would not explain the positive results in animal models. Consequently, the role of SV40 in mesothelioma remains controversial, and the International Agency for Research on Cancer did not find enough evidence to classify SV40 as carcinogenic in humans in their report published in 2013.(IARC, 2013)

Radiation

Ionising radiation has been identified as a human carcinogen and risk factor for several cancer types. Cases of development of mesothelioma after radiation exposure have been reported, such as after previous radiation for malignancies, with two epidemiological studies showing increased risk of developing mesothelioma after radiation for testicular cancer with relative risk of 4 (95% CI 2-8.1), and Hodgkin lymphoma with observed divided by expected numbers of cases of mesothelioma (standardised incidence ratio, SIR): 6.6 (95% CI 1.8-16.9) in patients who received radiotherapy, compared to SIR 1.27 (95% CI 0.03-7.1) in patients who did not receive radiotherapy (p<0.05).(Teta et al., 2007; Travis et al., 2005) The risk of mesothelioma is possibly even higher when patients received both radiotherapy and chemotherapy for lymphoma (SIR 5.8 (95% CI 10.2-32.1) and 44.8 (95% CI 23.2-78.3) in patients who received radiotherapy, and radiotherapy plus chemotherapy, for Hodgkin lymphoma respectively).(De Bruin et al., 2009)

Erionite

There is a very high incidence of mesothelioma in Karain and Tuzkoy villages in Turkey, thought to be due to exposure to erionite, a mineral fibre commonly found in the stones of houses.(Baris et al., 1978; Carbone et al., 2002; Rohl et al., 1982) In these villages,

mineralogical and pedigree analysis revealed that there may be an autosomal dominant genetic susceptibility to mesothelioma.(Roushdy-Hammady et al., 2001) There is also evidence from animal models that erionite is a more potent inducer of MPM than asbestos, with 40/40 (100%) of rats injected with intrapleural erionite developing MPM, compared with 19/40 (47.5%) of rats injected with intrapleural asbestos.(Wagner et al., 1985)

Genetic variants in mesothelioma

A specific genetic variant is not common to all mesotheliomas. DNA sequencing of 198 unrelated patients with mesothelioma revealed that at least one germline variant was present in 12%, higher in MPM associated with early onset or family history, and 25% of the variants detected were in *BAP1*, 12.5% in *BRCA2*, and 8.3% in *CDKN2A* (the odds of having a variant: OR 1,658 (95% CI 199-76,224), p <0.001; OR 5 (95% CI 1-14.7), p 0.03; OR 53 (95% CI 6-249), $p <$ 0.001 respectively, compared with the non-cancer control population).(Panou et al., 2018) On the other hand, K-ras activation is rare in mesothelioma suggesting that development of mesothelioma is not dependent on this pathway.(Metcalf et al., 1992) A cross sectional study including 225 patients with mesothelioma reported that *GSST1* null genotype may be protective against mesothelioma,(Franko et al., 2021) although this would need to be further evaluated in larger studies.

1.2 Malignant pleural mesothelioma presentation and diagnosis

MPM most commonly presents with chest pain and shortness of breath, and there is a pleural effusion (fluid collection within the pleural space) at presentation in about 70% of cases.(Rudd, 2010) The normal pleural space contains a small amount of pleural fluid produced by diffusion from the parietal pleura capillaries, and drained via the lymphatic

vessels in the parietal pleura.(Miserocchi, 1997) In malignant disease, accumulation of the pleural fluid (pleural effusion) is thought to result from pleural tumour blocking the lymphatic outflow, as well as intricate interplay between the tumour cells and the host's vasculature and immune response leading to fluid extravasation into the pleural cavity.(Meyer, 1966; Stathopoulos and Kalomenidis, 2012)

The diagnosis of MPM can be challenging because of the variable morphology. Figures 2.2-1.4 show chest x-ray, ultrasound and thoracoscopic views of cases of MPM. The main subtypes of mesothelioma are epithelioid, sarcomatoid, and biphasic, the latter having a combination of features of both epithelioid and sarcomatoid subtypes. Rarer subtypes include desmoplastic and deciduoid. Non-epithelioid subtype generally carries a worse prognosis, with median survival for epithelioid, biphasic and sarcomatoid mesothelioma found to be 19, 12 and 4 months respectively in a database of 1183 patients with mesothelioma,(Meyerhoff et al., 2015) although the pleomorphic subtype of epithelioid mesothelioma has a similar poor prognosis to sarcomatoid mesothelioma.(Galateau-Salle et al., 2016)

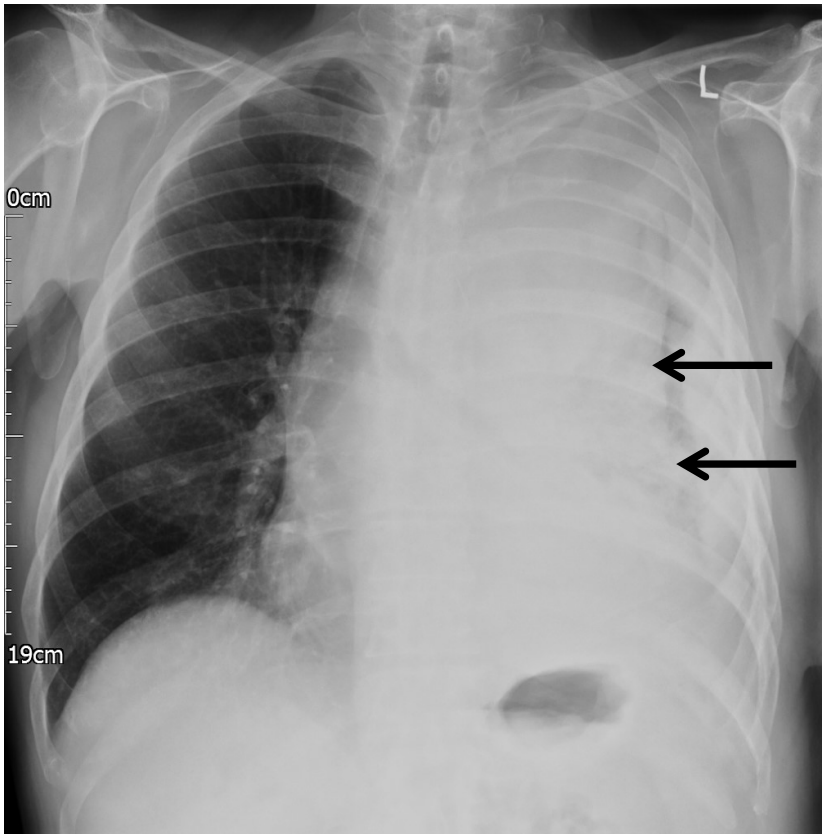


Figure 1.2 Image shows the chest x-ray of a patient diagnosed with malignant pleural mesothelioma. The left side of the patient's chest shows diffuse opacification, with calcified pleural plaques, pleural effusion, and underlying non-expandable lung (arrows show the indented border of the left lung, usually due to thickening of the visceral pleura which restricts further lung expansion.)

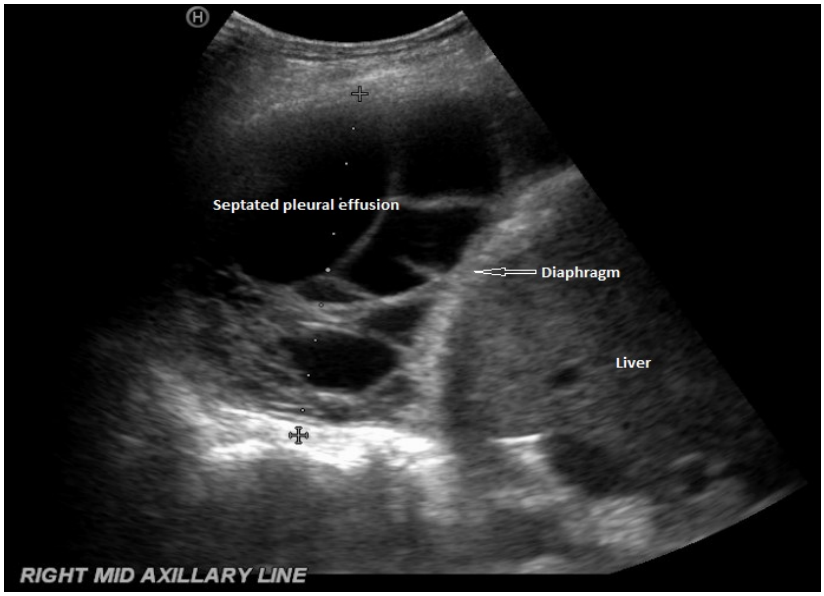


Figure 1.3 Image shows an ultrasound view of the right-sided pleural space. The image shows malignant pleural effusion within the pleural space, with fibrinous strands (septations).

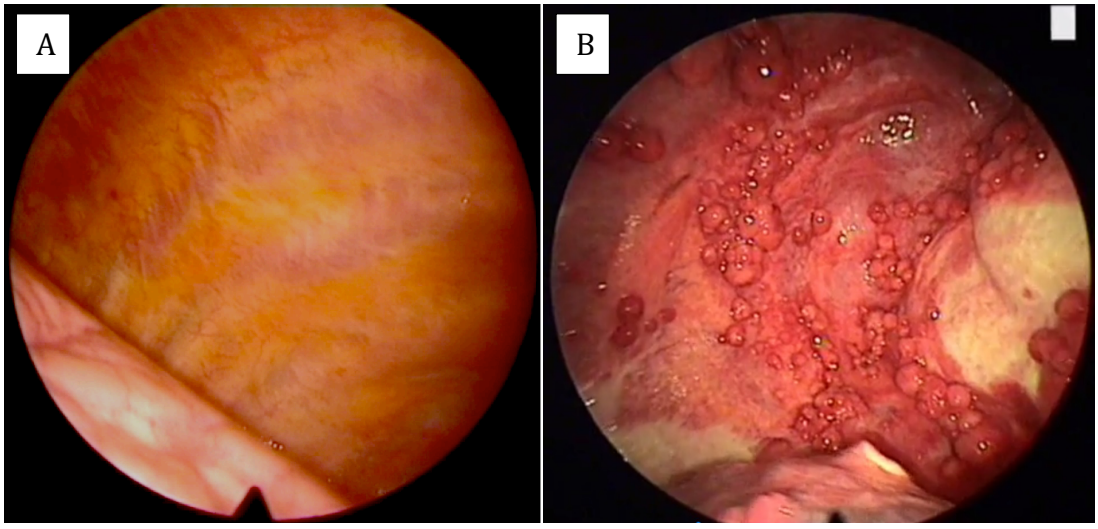


Figure 1.4 Images of the pleura as visualised during thoracoscopy. Thoracoscopy is a procedure in which a small camera is inserted into the pleural space and allows visualisation of the pleura and the taking of pleural biopsies under direct vision. Image A shows the thoracoscopic view of normal parietal pleura, which is smooth, with alternating pink (ribs) and yellow (fat between the ribs) bands. Image B shows the thoracoscopic view of the parietal pleura in a patient diagnosed with MPM. In contrast to normal parietal pleura, here the pleura is thickened, contains pleural plaques (white) and has multiple nodules, which macroscopically would be diagnostic of a malignant process.

1.3 Malignant pleural effusion in malignant pleural mesothelioma

MPE may be large and symptomatic, or it may be small, asymptomatic and not require pleural intervention to drain it to relieve symptoms. In a single centre retrospective study of 556 patients with lung cancer, 40% had MPE, but 50% of these were too small to be aspirated. Despite this, even the latter were associated with poor survival: median survival 12.7 months in patients with no pleural effusion, 7.5 in patients with minimal pleural effusion, 5.5 months in patients with larger MPE.(Porcel et al., 2015) Guidelines recommend observation for asymptomatic malignant pleural effusion (MPE) where the tumour type is already known.(Bibby et al., 2018; Feller-Kopman et al., 2018; Nadal et al., 2021; Roberts et al., 2010) Therefore, only MPEs that are symptomatic are drained, including in cases of mesothelioma. Recurrent symptomatic MPEs are usually treated with

repeated fluid aspiration as necessary if life expectancy is estimated to be less than one month. In other cases, there are two possible options: the insertion of a temporary chest drain into the pleural cavity to drain off the fluid, and then instillation of a talc slurry into the pleural cavity (Figure 1.5) with the aim of inducing inflammation, deposition of fibrin and subsequent adhesion formation between the visceral and parietal pleural layers, sealing off the pleural cavity (pleurodesis); or else the insertion of a tunnelled indwelling pleural catheter (IPC) which allows the fluid to be drained intermittently at the patient's home. Talc instillation via the IPC is emerging as a third possible option for patients (discussed in more detail below). Talc pleurodesis is successful in about 70% of patients,(Dresler et al., 2005) and about 50% of patients with an IPC develop spontaneous pleurodesis after several months.(Van Meter et al., 2011) Currently, standard practice in many pleural units in the UK is to offer patients a choice between these two options, whereas in Malta, patients are usually offered talc as standard first line treatment for recurrent MPEs, with IPCs offered to patients in case of failed talc pleurodesis or if there is underlying non-expandable lung. Since the publication of the results of the IPC_Plus trial, there has been a move towards combining these 2 modalities, and patients may be offered an IPC insertion with talc instilled via the IPC after a period of regular IPC drainage.(Bhatnagar et al., 2018) During the COVID-19 pandemic, there has been a shift towards more IPC insertion, or IPC insertion plus talc via the IPC, rather than chest drain insertion and talc, in an effort to keep patients out of hospital because the latter option involves admitting the patient to hospital for a few days.

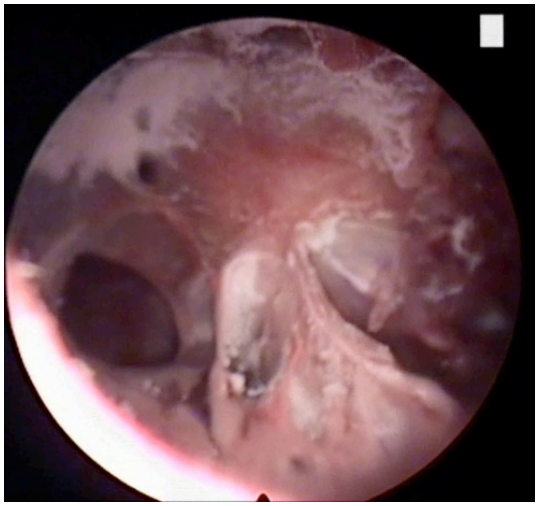


Figure 1.5 Image of the thoracoscopic view of the pleura coated in talc. The image was taken after 4g of sterile graded talc were insufflated into the pleural space.

1.4 Malignant pleural mesothelioma treatment

MPM carries a poor prognosis, with median overall survival of 9.5 months. Treatment with chemotherapy doublet cisplatin (or carboplatin if cisplatin is contraindicated or has adverse effects(Santoro et al., 2008)) and the anti-folate drug pemetrexed (or raltitrexed(van Meerbeeck et al., 2005)) can extend life by about 2-3 months (median survival of 12 *vs* 9 months with cisplatin alone), with a significantly longer median time to progressive disease (6 *vs* 4 months). Where licensed, bevacizumab is recommended to be added to this regime(Kindler et al., 2018; Woolhouse et al., 2018) because cisplatin/pemetrexed/bevacizumab combination was associated with median 19 (95% CI 16-22) month survival *vs* 16 (95% CI 14-18) with cisplatin/pemetrexed alone.(Zalcman et al., 2016)

Chemotherapy is limited to patients who are fit enough to receive it and even then, about half the patients receiving chemotherapy will not respond to it. There is therefore a great need for personalised treatment in MPM in order to deliver treatment that is most likely to be effective, and minimise exposure to unnecessary side effects from treatment that is not likely to be effective.

In summary, there is early preclinical evidence of the biological properties of pleural fluid, potentially facilitating cancer cell proliferation, and leading to chemotherapy resistance. Successful pleurodesis has been observed to be associated with improved survival. This has not been specifically studied in animals and humans to date. If pleural fluid does in fact have these properties, then it may lead to a shift from symptom-guided drainage of MPE to aggressive MPE drainage and prevention of reaccumulation in attempt to decrease cancer proliferation, improve chances of drug response, and aim to impact on patient survival.

There is a need to develop personalised treatment in MPM given the heterogeneity in drug response, genetic abnormalities and patient survival. MPM treatment would ideally aim to target cancer stem cells apart from other cancer cells in order to increase the impact of the therapy. A model that can be used to repeatedly measure evolving genetic composition of MPM and predict drug response and resistance would facilitate this greatly.

There is a burgeoning of research in intrapleural treatment in MPE, with some early promising results. An animal model that allows easy and repeated access to the pleural space would facilitate this research greatly.

1.5 Hypothesis and objectives

The following are the main hypotheses for this study:

- The first hypothesis is that MPE fluid contributes to MPM proliferation and to chemotherapy resistance, and impacts on patient survival.
- The second hypothesis is that a bench-to-bedside pathway is feasible to guide treatment in mesothelioma.

For the first hypothesis, the possibility that MPE fluid has biological properties that contribute to MPM proliferation and impact on patient survival will be evaluated by studying a database of patients with MPM, by performing in vitro experiments. Should there be a signal that pleural fluid indeed has biological properties, then an animal model will be designed to further evaluate this hypothesis.

It would be ideal to personalise MPM treatment to individual patients, increasing the likelihood of treatment response and decreasing the rates of unnecessary drug adverse events. Therefore the second hypothesis is that a pathway, flowing directly from the readily-available pleural fluid from MPM patients (about 70% of MPM patients have a pleural effusion at presentation(Rudd, 2010)), to the laboratory for *in vitro* personalised MPM cell culture, characterisation of the cell cultures, and use of the cell cultures for drug screening and DNA sequencing to obtain information to guide personalised treatment of patients in clinic is realistic and feasible. The cell cultures would need to closely represent the patient's native tumour, the pathway needs to be feasible within a realistic time-frame, and be repeatable in order to re-assess for drug sensitivity and resistance that may change during a patient's

treatment course. If this pathway were found to be feasible, then on a larger scale it may be possible to identify variants associated with drug resistance or response, and enable the feeding back of this information to clinical and oncological teams in a timely manner.

The study was designed with the above hypotheses in mind, and an outline of the aims and questions sought to be answered is shown in Figure 1.6.

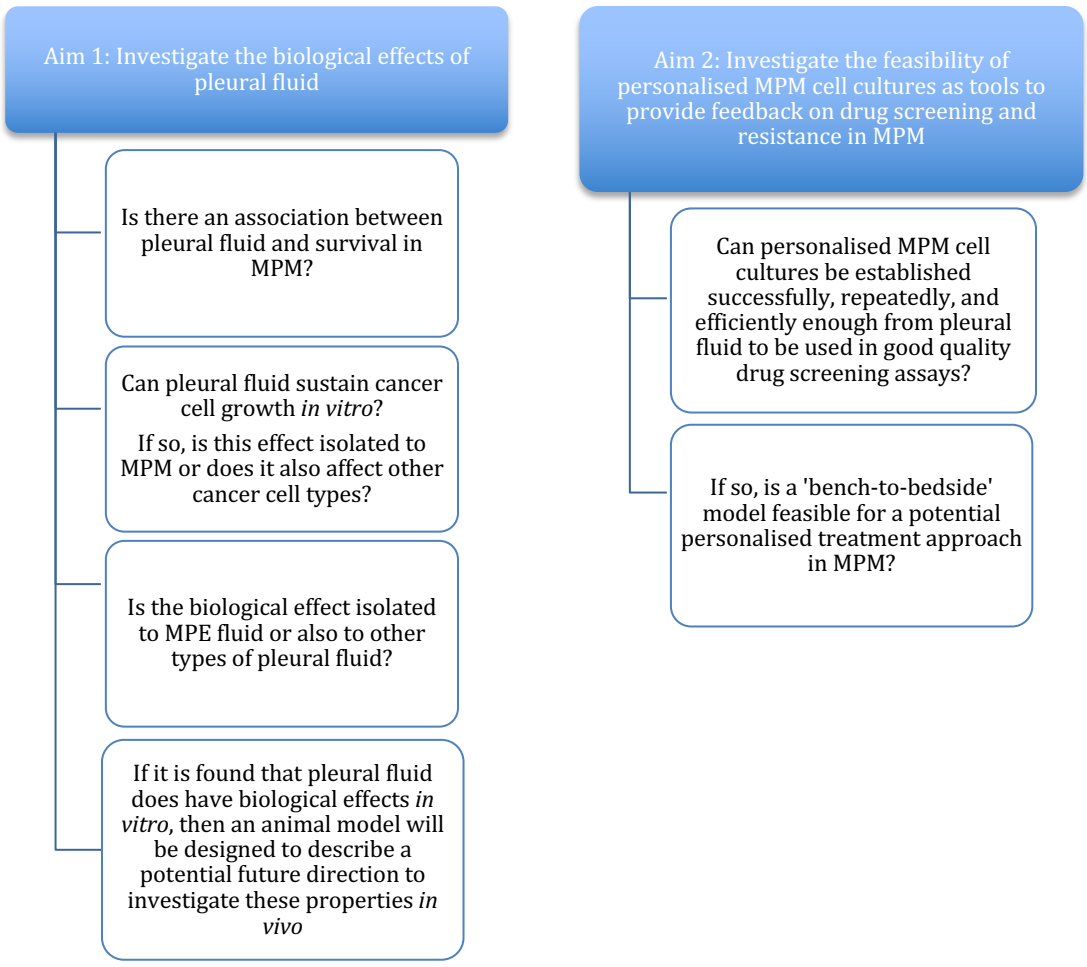


Figure 1.6 An outline of the aims of this project, and the questions that will be explored.

The aims of this study were as follows.

1. **To investigate the association between pleural effusion and survival**

The association between pleural fluid exposure and survival in malignant pleural mesothelioma – a retrospective cohort study

Since MPM is relatively rare, and the factors associated with survival are numerous, the planned database needed to include a large number of patients to enable correction for the factors already identified to be associated with survival. This meant that a prospective study was not possible given the timeframe for this project. In addition, there is as yet no clear signal in the literature that pleural effusion influences survival in MPM, and prior to embarking on a large prospective MPM dataset, an assessment of retrospective data would be valuable. The data would enable a real-life analysis that would accompany the planned *in vitro* and mouse experiment design of this project, albeit within the limits of a retrospective study. The hypothesis was that patients with MPM and longer duration of pleural effusion had worse survival when compared to patients with MPM and shorter duration of pleural effusion. A further hypothesis was that those patients with pleural effusion while receiving chemotherapy had worse treatment-response than patients undergoing chemotherapy who did not have a pleural effusion during treatment. If the data supports this hypothesis, then this study may provide a basis upon which future prospective studies can be based.

The primary objectives were to explore associations between pleural fluid duration and overall survival, and independent association of pleurodesis success and overall survival in MPM. The secondary objectives were to explore associations between pleural fluid

presence (at start of chemotherapy) and chemotherapy response, and associations between pleural fluid presence (at MPM diagnosis) and size (at MPM diagnosis) with overall survival in MPM.

The primary outcome was overall survival, with independent variables of pleural fluid duration and pleurodesis success, defined objectively in a hierarchical structure, universally applied and pre-hoc. The secondary outcomes were chemotherapy response with the independent variable of pleural fluid presence at start of chemotherapy, and overall survival with independent variables of pleural fluid presence and size at MPM diagnosis.

The development of patient-derived malignant pleural mesothelioma cell cultures from pleural fluid: a tool to advance biomarker-driven treatments

The objectives were to develop personalised patient-derived MPM cell cultures from MPE fluid samples, aiming for a relatively high success rate compared with that published in the literature, and furthermore, develop cell cultures from the same patient at different time-points in the patient's disease; and to characterise the cell cultures to confirm presence of malignant features on cytology, stemness as indicated by successful tumoursphere assays, and by DNA sequencing of early and late passage cells in order to confirm that the personalised cell cultures represent cancer cells as close as possible to the patient's native tumour state. These cell cultures would be used for *in vitro* experiments to investigate the biological effects of pleural fluid, as well as for drug screening experiments described later.

Moreover, developing cell cultures from the readily-available pleural fluid of MPM patients with associated MPE, rather than from biopsy tissue, allows the repeated development of

personalised cell cultures, because pleural fluid can be aspirated repeatedly at different time-points in a patient's disease pathway, as long as the pleural effusion persists, when different tumour variants may be present. Biopsy tissue is usually only available at the start of a patient's disease pathway, unless the patient is exposed to repeated biopsy procedures, outside those required clinically, and only if the patient has not achieved pleurodesis which precludes pleural biopsy. For this reason, in this study, MPM cell cultures were developed from pleural fluid samples rather than from solid tumour biopsies, and in addition, cell cultures established from solid tumour samples are more likely to have fibroblast contamination.(Ruiz et al., 2016)

Investigating the biological properties of pleural fluid: in vitro experiments

In order to further explore the potential biological effects of pleural fluid, a series of *in vitro* experiments were designed, using cancer cell cultures established from MPM MPE fluid, and pleural fluid. The objective was to analyse whether pleural fluid alone is adequate for cancer cell proliferation *in vitro*, including primary cell culture as well as established cancer cell cultures.

Studying MPE development and MPE fluid effects in vivo using animal models

The main aim of this part of the study is to provide a description of potential future directions of research. Should the biological effects of pleural fluid be demonstrated in this project, with MPE alone providing an environment with adequate nutritional support for human cancer cell growth *in vitro*, both for primary MPM cell culture and for the continued proliferation of cancer cells from established cell cultures, then this data represents an important first step. The next phase requires direct testing of the

hypothesis that MPE has biological properties in a suitable animal model. Data from the literature review together with an experimental model I was involved in the development of, will be used to describe potential future directions of research to further delineate the hypothesis that pleural fluid, which is rich in protein growth factors, provides nutrients and an optimum milieu to support pleural tumour cells, leading to progressive tumour growth and spread. Furthermore, a second experiment will be described investigating the hypothesis that pleural fluid presence is associated with chemotherapy resistance.

2. **To investigate the feasibility of using personalised patient-derived MPM cell cultures to perform high-throughput drug screening and deoxyribonucleic acid sequencing, as tools for delivering personalised MPM treatment**

The objective was to conduct drug screening assays on the MPM cell cultures in order to explore whether the cell cultures are adequate for good quality drug screening assays, and whether the drug sensitivity / resistance profile would be available in a timely manner to potentially impact on the patient's clinical management. The drug screening assays would use a list of drugs already approved for clinical use in other primary malignancies, aiming to identify drugs that are as effective as, or more effective than, cisplatin/pemetrexed combination (the current most commonly used first line treatment in MPM) *in vitro*.

The ideal would be to develop a feasible bench-to-bedside pathway from MPM MPE fluid to personalised MPM cell culture, to drug screening and DNA sequencing on the culture cells, within a clinically acceptable time-frame, so that the results of the predictions on drug

resistance / response profiles would be clinically relevant and useful (Figure 1.7), and this study represents a first step towards achieving this.

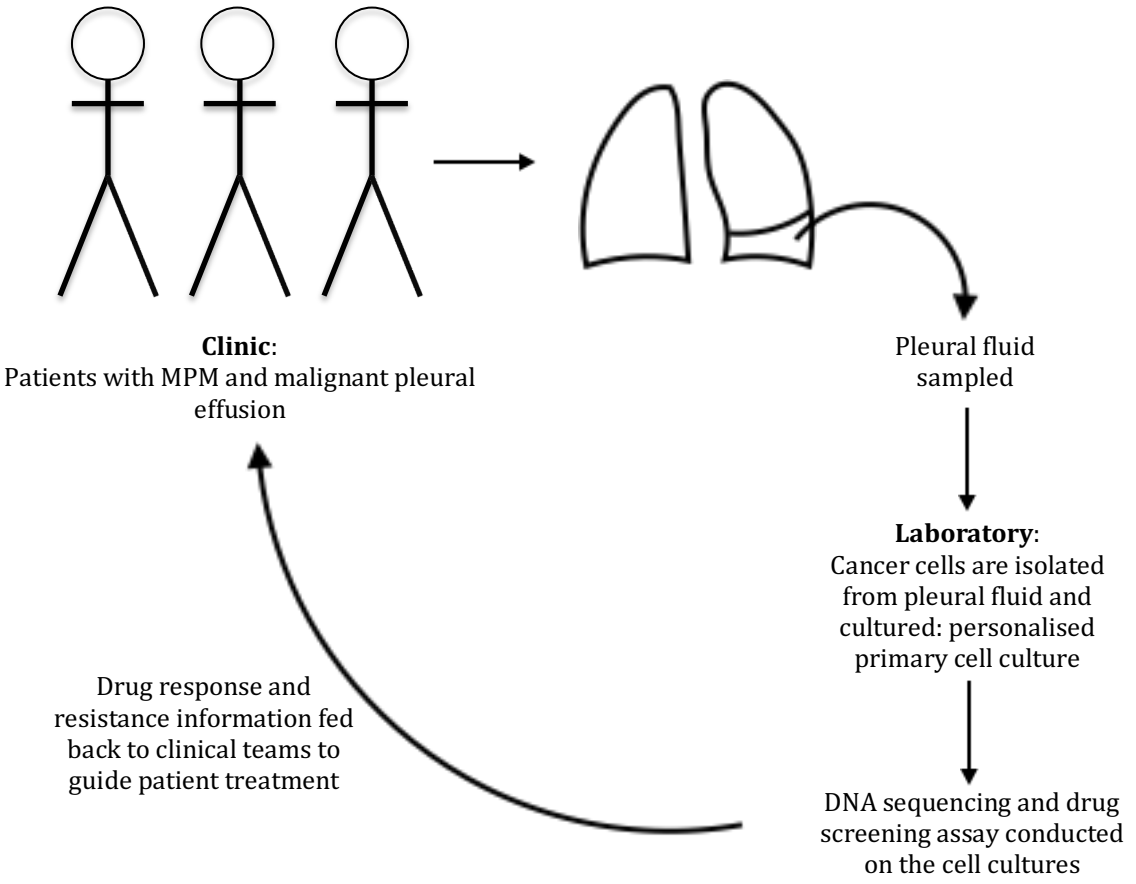


Figure 1.7 shows flow chart of the the proposed bench-to-bedside pathway. This study investigates whether such a pathway would be feasible. Pleural fluid from patients with MPE secondary to MPM is sampled and sent to the laboratory. Cancer cells are cultured from the fluid, and information from drug screening and DNA sequencing results fed back to clinical teams in order to guide MPM treatment. *MPE=malignant pleural effusion, MPM=malignant pleural mesothelioma.*

This project fosters strong collaboration between clinical and laboratory researchers (University of Malta, Oxford University Hospitals NHS Foundation Trust, University of

Oxford, NHS Greater Glasgow and Clyde, North Bristol NHS Trust, Target Discovery Institute at the Nuffield Department of Medicine in Oxford, and Vanderbilt University in the USA). Collaboration is fundamental in mesothelioma research because the cancer is relatively rare, highlighting the importance of collaboration between clinical teams and researchers, to pool experience, resources, and knowledge.

Chapter 2: Methods

2.1 Literature review

For the literature review for this project, an advanced search on PubMed® was carried out using the terms “pleural fluid” and “survival” (1336 results), “pleural effusion” and mesothelioma” (1842 results), “pleural effusion” and “animal models” (323 results) and “mesothelioma” and “cell culture” (286 results). A total of 520 papers/abstracts were reviewed of which 420 were relevant. The literature review was included in the Introduction section of the thesis.

2.2 The association between pleural fluid exposure and survival in malignant pleural mesothelioma – a retrospective cohort study

Figure 2.1 shows the relevance of this section with reference to the aims of this project.

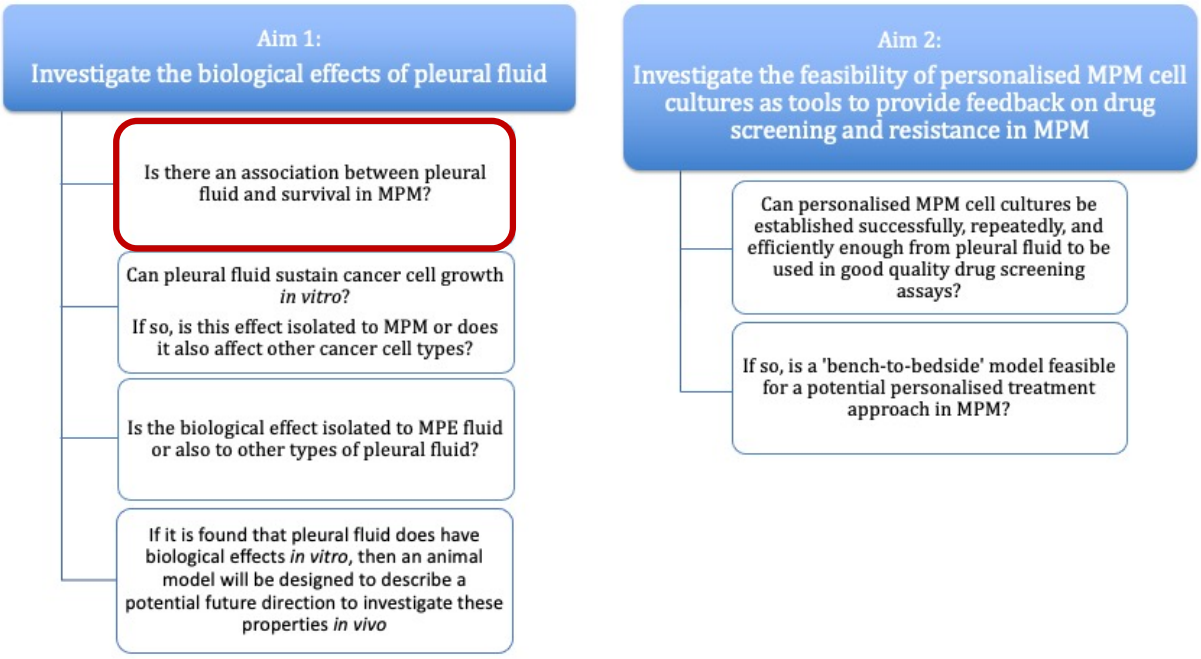


Figure 2.1 Flow chart showing summary of aims and guide to the workflow of the study. In this section, a retrospective database of patients with malignant pleural mesothelioma was analysed in order to explore associations between pleural fluid and survival.

Participants and data extraction

Large numbers of patients were needed, as in addition to the relative rarity of MPM and the need to correct for factors already known to influence survival in MPM, a retrospective dataset needed to factor in cases with missing data which must be excluded from analysis. Such a large dataset was achievable through collaboration between the Pleural Units of three UK health institutions: Oxford University Hospitals NHS Foundation Trust, NHS Greater Glasgow and Clyde, and North Bristol NHS Trust.

Data were collected retrospectively from patients' medical records in the three UK pleural units, on patients documented to have a confirmed diagnosis of MPM (diagnosed by multidisciplinary team meeting, or histocytological proof). Patients were included if they were diagnosed with MPM between 2008-2018 (08/01/2008 to 28/02/2018), since all 3

Pleural Units included in this study had electronic records available from the year 2008 onwards, therefore making it more feasible and accurate to collect all or most of the required data, without relying on hard copies of medical records. Patients who had MPM diagnosed during postmortem examination (*n*=2 in the Oxford cohort) were excluded because it would not have been possible to analyse the effect of pleural effusion on survival beyond MPM diagnosis. Duration of follow up was calculated from diagnosis of MPM to death, and alive patients were censored at time of data collection (25th July, 19th July, 5th November 2018 for Glasgow, Bristol and Oxford data respectively).

Trust and Ethics approval

All data was anonymised at the point research began, and was part of the normal clinical care record with no additional investigations necessary. This research was approved by the Health Research Authority (HRA): IRAS project ID 244245.

Baseline demographics and features, and known predictors of survival in MPM in the literature

Given the large volume of data that needed to be collected on a large number of patients, the collection of unnecessary data was limited by choosing data that was most likely to be useful in the analysis in order to correct the data for factors already known to influence survival. These included factors in a published prognostic model (Brim's model) that was derived after analysis of 482 patients with a confirmed diagnosis of MPM, and weight loss was found to have the greatest influence on survival, with non-epithelioid histological MPM subtype, poor ECOG PS, low haemoglobin (Hb) and low serum albumin levels also being associated with worse outcomes.(Brims et al., 2016) This model had been

prospectively validated in 177 patients with MPM, and used to predict death at 18 months. The data collected in this current study therefore included:

- MPM subtype, as this has been shown to be consistently associated with survival in previous large series;(Brims et al., 2016; Curran et al., 1998; Marinaccio et al., 2003; Meyerhoff et al., 2015; Montanaro et al., 2009; Musk et al., 2011; Neumann et al., 2004; Nojiri et al., 2011; van der Bij et al., 2012; Verma et al., 2018)
- Blood test results (serum albumin and Hb) at MPM diagnosis or within 2 weeks of the diagnosis, since serum albumin and Hb were both included in the Brim's decision tree model as factors influencing survival in MPM;(Brims et al., 2016)
- ECOG PS at diagnosis, since this has been found to be associated with survival in MPM in other large studies;(Brims et al., 2016; Curran et al., 1998; Nojiri et al., 2011)
- Age at diagnosis, since older age was consistently identified as a factor being associated with worse survival in several large studies;(Marinaccio et al., 2003; Milano and Zhang, 2010; Montanaro et al., 2009; Musk et al., 2011; Neumann et al., 2004; Nojiri et al., 2011; van der Bij et al., 2012)
- The presence of other diagnosed malignancies in order to ensure that other end-stage metastatic malignancies that may also influence survival of patients with MPM are noted and the relevance of these analysed in the survival analysis;
- Other treatments received by patients for MPM including radiotherapy, surgery and immunotherapy to ensure that the treatment of MPM across the cohort was similar and that there was no major bias because of a variety of different MPM treatments received;
- Pleural fluid biochemistry results for the Oxford cohort were collected for a sub-analysis to analyse correlation between survival and pleural fluid biochemistry.

The data collected is summarised in table 2.1.

Table 2.1 Summary of the data collected for the analysis. ***ECOG=Eastern cooperative oncology group; MPM=malignant pleural mesothelioma***

Data parameter	**Details**
Details of patient at time of MPM diagnosis	Date of diagnosis Age ECOG performance status Presence of symptoms related to MPM Presence of weight loss
Blood test results at MPM diagnosis	Serum albumin Haemoglobin
MPM histological subtype	Epithelioid, biphasic, or sarcomatoid (including desmoplastic and lymphohistiocytoid)
Survival	In number of days from diagnosis of MPM
Details on pleural effusion	Presence and size at time of MPM diagnosis Presence and size at start of chemotherapy Details of pleural effusion management Details of pleurodesis success Total duration of exposure to pleural fluid
MPM treatment received	Systemic mesothelioma therapy received Response to first line chemotherapy Radiotherapy treatment Surgery for mesothelioma
Any malignancy other than mesothelioma	Whether present or not and primary site of malignancy

Presence and duration of pleural effusion at MPM diagnosis, and survival

Available serial posteroanterior chest x-rays, and thoracic ultrasound scan reports, available from the date of diagnosis of MPM, and including at the start of chemotherapy, were reviewed for the presence and size of pleural effusion. The investigators reviewed both the reports of chest x-ray and ultrasound scans, as written by the reporting radiologist/sonographer, and also at the chest x-ray image itself to confirm presence/lack of pleural effusion. Where pleural effusion was reported on chest x-ray but thoracic ultrasound during the same period was reported to detect no pleural effusion, then this

was documented as 'no pleural effusion' for purposes of this study. This is in recognition of the fact that pleural thickening and small pleural effusion appear similar on chest x-ray.

In addition, data were collected from medical records on date of MPM diagnosis, pleural effusion presence at diagnosis, any measures taken to treat the pleural effusion, the total duration of the pleural effusion, and patient survival from diagnosis of MPM. The total duration of a pleural effusion was calculated as the total number of days a pleural effusion was present overall. For example, if a pleural effusion was drained to dryness 7 days after having been detected on chest x-ray for the first time, the pleural effusion recurred two months later requiring IPC insertion, and IPC was subsequently removed because of IPC-related spontaneous pleurodesis 100 days after IPC insertion, then total pleural effusion duration was calculated as 7 days plus 100 days (107 days in total).

The time exposed to pleural fluid was measured in total number of days from diagnosis of MPM, however due to the inherent bias that patients who die soon after MPM diagnosis would have shorter duration of exposure to pleural effusion recorded, the time exposed to pleural effusion was also calculated as a percentage of the patient's life after MPM diagnosis (total duration of pleural effusion in number of days since MPM diagnosis divided by the survival in number of days since diagnosis of MPM, multiplied by 100). This methodology is similar to Thomas R et al,(Thomas et al., 2017) where total number of days spent in hospital after a procedure were calculated as a percentage of total days in the trial (from procedure to death or to end of follow up period).

If patients developed ascites secondary to mesothelioma, the duration of tumour exposure to ascites was not included in the exposure to MPE fluid analysis, because only pleural tumour exposure to MPE fluid was studied.

Pleurodesis success

Data were collected on whether pleurodesis was achieved at any point after MPM diagnosis or not, regardless of whether pleurodesis was spontaneous, IPC-related, or secondary to chemical or surgical pleurodesis. Data was also collected on pleural interventions required to control fluid accumulation. Pleurodesis success was defined and categorised according to a combination of radiological and clinical parameters:

- Complete pleurodesis - no further MPE documented on radiology (available computed tomography (CT) scans, chest x-ray or ultrasound) at any point after the MPM diagnosis, and no further pleural intervention required (until death / time of data collection);
- Partial pleurodesis - persistent small to moderate (size 1-2, i.e. ≤25% of hemithorax, further described below) MPE but not requiring further pleural intervention for symptom-relief (patients who achieved pleurodesis temporarily but eventually had small-moderate asymptomatic MPE develop again were categorised as 'partial pleurodesis' too);
- No pleurodesis - persistent recurrent MPE requiring further intervention to relieve symptoms (patients who achieved pleurodesis temporarily but then had relapse requiring intervention to drain symptomatic MPE were categorised as 'No pleurodesis' too).

Patients with non-expandable lung would often fall under the latter two categories however this was not specifically looked into. Data on other factors that have been associated with pleurodesis success, including the ability to mount an adequate

inflammatory response (as measured by C-reactive protein),(Mercer et al., 2020) and the degree of pleural tumour bulk, were not available for inclusion in this analysis.

Effect of pleural effusion size on survival

The main aim was to assess whether the presence and duration of pleural effusion influenced survival in MPM, however small pleural effusions may not bathe the tumour sufficiently to influence cancer cell proliferation. Therefore, an assessment of whether the size of the pleural effusion was associated with survival was planned.

Data was collected on the size of the pleural effusion present on chest x-ray. The grading of the pleural effusion size on posteroanterior chest x-ray (Figure 2.2) was as follows:

- 1 - Blunting of costophrenic angle
- 2 - Fluid occupying ≤25% of hemithorax
- 3 - Fluid occupying 26-50% of hemithorax
- 4 - Fluid occupying 51-75% of hemithorax
- 5 - Fluid occupying >75% of hemithorax.

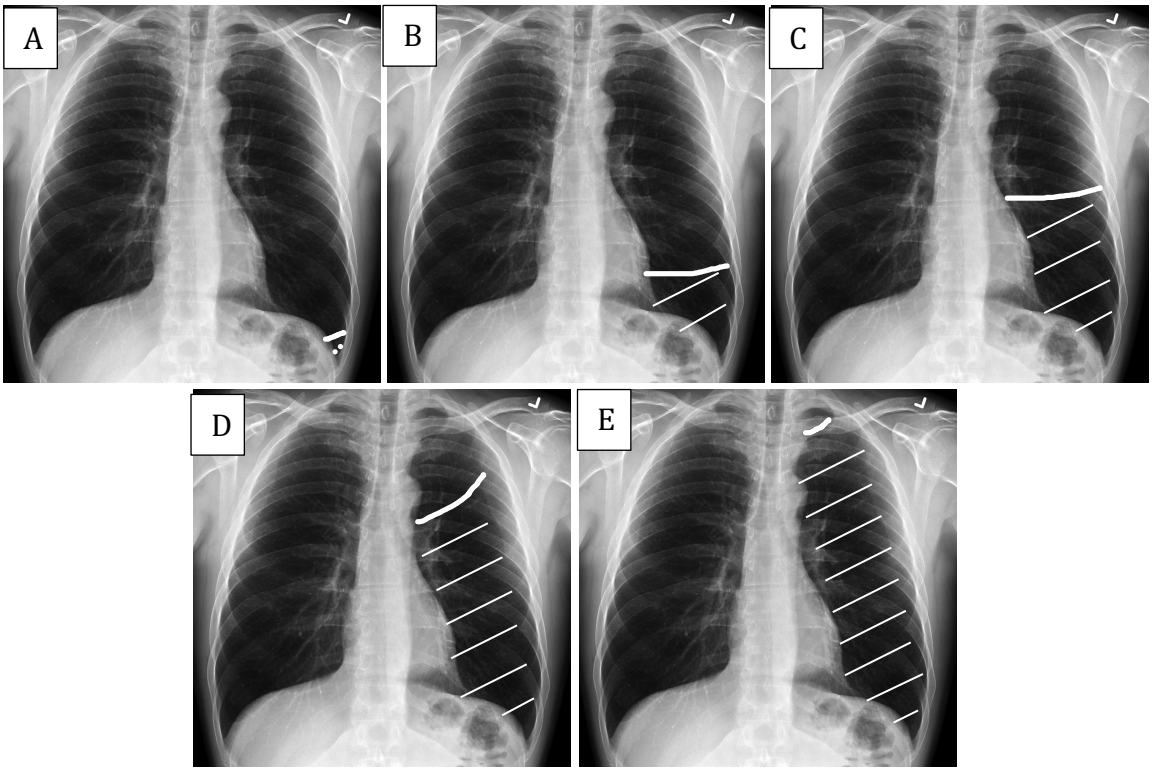


Figure 2.2 shows chest x-rays with white lines to show examples of the pleural effusion sizes (in this case, a left sided pleural effusion) according to the grading system used: image A corresponding to grade 1 (blunting of left sided costophrenic angle), image B to grade 2 (pleural effusion covering 25% or less of the hemithorax), image C to grade 3 (pleural effusion covering 26-50% of the hemithorax), image D to grade 4 (pleural effusion covering 51-75% of the hemithorax) and image E to grade 5 (pleural effusion covering >75% of the hemithorax).

This grading system was originally used in patients with post coronary artery bypass graft pleural effusion in studies published in 1999 and 2002,(Light et al., 2002, 1999) however since then the grading system has been used in other pleural effusions too.(Cartaxo et al., 2011; Livingston et al., 2017; Misra et al., 2007; Porcel et al., 2002) A volume of about 200ml pleural fluid is detected as a meniscus at the costophrenic angle on a posteroanterior chest x-ray, and 500ml pleural fluid would obscure the hemidiaphragm,(Blackmore et al., 1996) correlating with a grade 1 and grade 2 effusion size respectively according to the above classification. As discussed below, determining pleural fluid presence and size from a chest x-ray has its flaws, whereas ultrasound scans detect pleural fluid with up to 100% sensitivity.(Kalokairinou-Motogna et al., 2010; Soni et al., 2015) However chest x-ray

images are more readily available for data collection than ultrasound scan images. Furthermore, for one pleural unit (Oxford), the chest x-ray findings were correlated with the clinic ultrasound notes to confirm presence of fluid.

Effect of pleural effusion presence on chemotherapy response

To compare presence of pleural effusion with chemotherapy response, data were also collected on any cancer treatment received and the dates of treatment, whether patients responded to chemotherapy or not, in addition to whether pleural effusion was present at the start of chemotherapy or not. For the purposes of determining chemotherapy response, data were collected from the radiologist report of the CT scan taken after the end of chemotherapy, and stable disease, partial or complete response (as determined by reporting radiologist) were considered to be chemotherapy-responsive, while progression of disease (as determined by reporting radiologist) was considered to be chemotherapy-unresponsive for the purposes of this study.

Time / treatment bias

Given that the management and diagnosis of MPM may have changed over time, in terms of earlier diagnosis or better availability of treatment, the data were divided into 2 year epochs, according to the year in which the MPM diagnosis was made, to assess for epoch time bias (potential improved survival due to improvements in healthcare over time). There were only 3 patients diagnosed in 2018 (when data collection ceased) therefore these were included in the epoch 2016-2018; while all other epochs were 2 year periods: 2008-2009, 2010-2011, 2012-2013, 2014-2015, 2016-2018.

Statistics

To assess for independent association of pleurodesis success with survival, multivariable regression analysis (Cox) was conducted including pre-determined variables which were potentially associated with survival in MPM according to the literature: weight loss, MPM histological subtype, ECOG PS, serum albumin, serum Hb, age, chemotherapy, as well as pleurodesis success, with survival in number of days from date of MPM diagnosis as the dependent variable. A backward selection model was used, including known predictors of outcome in MPM (which were retained), and then associations of clinical interest and those that demonstrated significance in univariable modelling (at the 0.1 threshold). In addition, Kaplan Meier curves were used to estimate survival according to pleurodesis success.

Pleural effusion duration was also assessed using a Cox model with a time dependent covariate, with the dependent variable as survival in number of days from MPM diagnosis.

Chi square and Fisher's exact test were used to compare patients who received chemotherapy and had a MPE at the start of chemotherapy to those who received chemotherapy but did not have an effusion at the start of chemotherapy, and to analyse chemotherapy response according to the size of the MPE at the start of chemotherapy.

In addition, raw correlations between baseline characteristics and survival were explored. Cox univariable regression was used to compare survival according to presence of weight loss, ECOG PS, serum Hb, and serum albumin at MPM diagnosis, MPM histological subtype, age, MPM treatment (chemotherapy, radiotherapy, immunotherapy, surgery), MPE presence and MPE size at MPM diagnosis, whether complete, partial or no pleurodesis was achieved, and according to whether talc was received or not.

An assessment of the cohort survivor effect was conducted, to analyse survival over time, given that data were collected from the year 2008 onwards and MPM management and MPM services available may have varied over the years since then; an assessment of the effect of the presence of symptoms associated with MPM at time of diagnosis of MPM on survival, because it was postulated that patients with incidental MPM diagnosis are those with earlier stage disease than patients who present with symptomatic disease when the MPM is likely to be more advanced; and an assessment of survival across the different Pleural Units was conducted since patients in each Pleural Unit hail from different areas in the UK representing potential population bias, and their management may vary with different practices across Pleural Units.

GraphPad PRISM version 8.3.0 for macOS (GraphPad Software, San Diego, California USA, www.graphpad.com) and IBM SPSS Statistics for Macintosh version 25.0.0.1 (Armonk, NY: IBM Corp) were used for data analysis, statistics and graphs. A p value of <0.05 was considered to be statistically significant.

2.3 The development of patient-derived malignant pleural mesothelioma cell cultures from pleural fluid: a tool to advance biomarker-driven treatments

Figure 2.3 shows the relevance of this section with reference to the aims of this project.

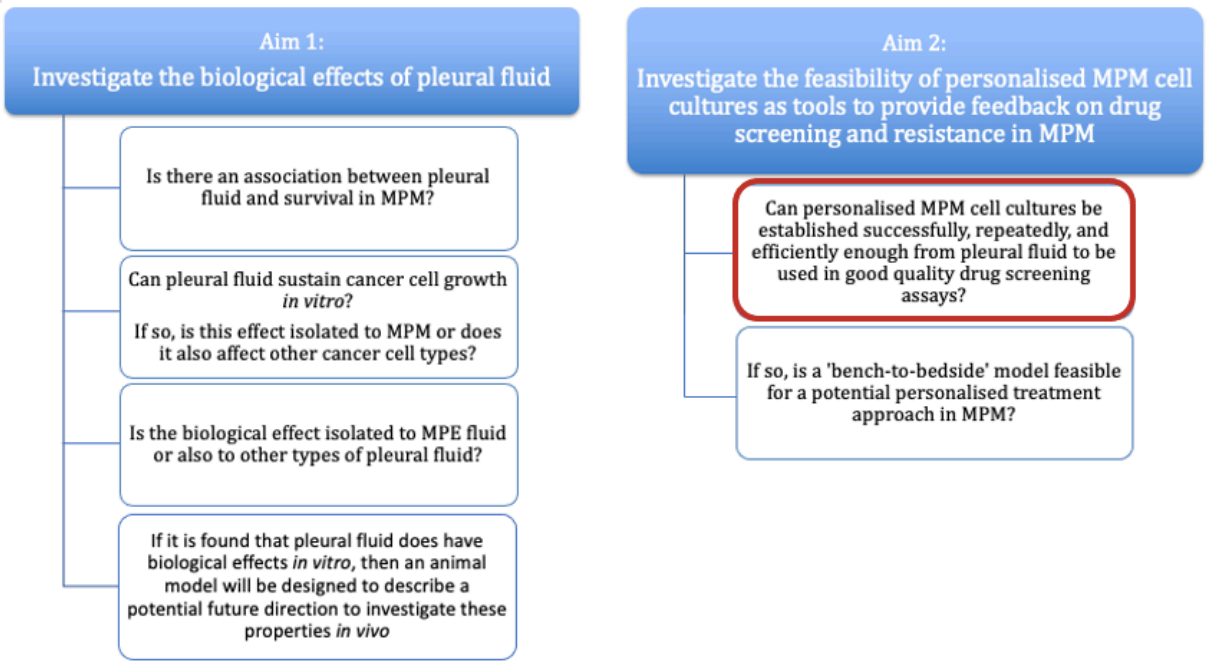


Figure 2.3 Flow chart showing summary of aims and guide to the workflow of the study. In this section, personalised patient-derived malignant pleural mesothelioma cell cultures were developed and studied to determine whether these are adequate for timely high quality drug screening assays and DNA sequencing. The cell cultures were also developed for use in the in vitro pleural fluid experiments described later.

Ethics

Patients included had given consent for inclusion in the Oxford Radcliffe Pleural Biobank (Research ethics committee: South Central Oxford C 09/H0606/5+5) - a project in which patients presenting to hospital for a pleural procedure voluntarily give their consent to donate samples of blood, pleural fluid and pleural biopsies, in order to create a 'bank' of samples available for ethically approved research projects. Ethics approval was obtained to use Oxford Radcliffe Pleural Biobank samples for the purposes of our study (Oxford Radcliffe Biobank Central University Research Ethics Committee (CUREC) number: 15/A251).

Method for cell culture

Pleural fluid samples, 50-60ml in volume (this volume is readily available in patients with MPE undergoing therapeutic fluid drainage procedures, and is an adequate volume for cell culture purposes, with extra volume of leftover pleural fluid for back up samples/further experiments as described below), were collected (March 2017 to February 2019) from patients with MPM and MPE, who underwent pleural procedures as part of their standard medical care.

The cell culture used in this project was an adherent, or monolayer, culture, where cells are grown attached to a solid substrate, as this is appropriate for primary cancer cell culture. Most mammalian cells, including MPM cells, are anchorage-dependent, and can be cultured more easily on a suitably treated surface such as a cell culture plate, as opposed to suspension culture, where cells are grown floating in culture medium. Adherent cultures are appropriate for most cell types including primary cultures, and although the cells may be adapted for suspension culture, this would require agitation of the medium in order to facilitate gas exchange. Adherent cultures do away with the agitation step, and in addition, they allow easy visual inspection under a light microscope, although cell growth and proliferation is restricted by the available surface area. The cells were detached from the solid substrate by using an enzyme, trypsin, or mechanically. For the MPM cell culture, 58cm^2 sterile polystyrene cell culture plates were used. Cell culture 'treated' plates, i.e. where the growth surface has been specially treated by increasing negative charge on the hydrophilic surface, were used for the more sensitive adherent cells in the case of primary cell culture (passage 0 to 5), whereas cell culture plates with a standard growth surface were used for adherent MPM cells beyond the fifth passage.

In order to have adequate cell culture success rate, while allowing standardization enabling comparison with other studies in the literature, the culture medium used in this experiment was Dulbecco's modified Eagle medium (DMEM) (31966047 Gibco, Waltham, MA; high (4500mg/L) glucose, and with phenol red) with 10% foetal bovine serum (FBS) (Sigma-Aldrich® USA origin, MFCD00132239) and 10,000 units penicillin, 10mg streptomycin per ml (P0781 Sigma-Aldrich®, St. Louis, MO) (antibiotics decrease contamination rates, although may alter gene expression and regulation in some cell types;(Ryu et al., 2017) penicillin/streptomycin are the commonly used antibiotics in MPM cell culture(Blum et al., 2015; Chernova et al., 2016)): this is hence referred to as 'complete medium'. MPM cell cultures and cell lines have been reported to grow better in the presence of 5% and 10% FBS,(Pass et al., 1995) than in medium without supplemented FBS. The growth factors in FBS stimulate replication and prevent cellular apoptosis, however, especially in high concentrations, may also affect gene and protein expression and therefore may alter the cell phenotype. Although other studies of MPM cell culture used 5%,(Manning et al., 1991) 15%(Versnel et al., 1989) and 20% FBS,(Kalra et al., 2015) most studies have used 10% FBS,(Chernova et al., 2016; Garay et al., 2013; Hsu et al., 1988; Kobayashi et al., 2008; Martarelli et al., 2006; Orengo et al., 1999; Pasdar et al., 2015; Sato et al., 2011; Suzawa et al., 2016; Usami et al., 2006) and this allows standardisation and therefore comparison between results obtained by different researchers. This is why 10% FBS was used for the MPM cell culture in this present study.

Data was collected on the histopathologist's fluid cytology result reported as part of the patients' clinical care at the time of fluid sampling, and on MPM staging (eighth version of the International Association for the Study of Lung Cancer (IASLC) staging system), as determined by a computed tomography (CT) scan performed around the time (within 6

weeks) of MPE fluid sampling, again as part of the patient's clinical care. Other clinical data collected from the existing medical records included Hb, urea, white blood cell count, platelet count, serum albumin, and pleural fluid biochemistry (glucose, LDH and protein levels), in order to enable description of the dataset.

The method used for cell culture was as follows:

1. The pleural fluid samples from patients diagnosed with MPM were taken immediately to the laboratory for cell culture, and processed within 2-3 hours of sample collection.
2. The fluid was centrifuged at 800 x g for 10 minutes to separate the cells from the supernatant. The supernatant was aspirated and saved for use in experiments requiring MPE fluid from patients with MPM.
3. The sedimented cells were resuspended in 3-5ml of red blood cell lysis solution (Qiagen®) depending on how blood-stained the original sample looked.
4. Cells were kept at room temperature for 5 minutes.
5. The sample was then centrifuged again at 500 x g for 5 minutes. The supernatant was aspirated and discarded, and the pelleted cells washed with phosphate buffer solution (PBS) (Sigma-Aldrich® MFCD00121855) to remove any remaining red blood cell lysis solution.
6. The sample was centrifuged again at 500 x g for 5 minutes. The pelleted cells were resuspended in 1ml complete medium and then transferred to a cell culture treated plate (83.3902.300, Sarstedt, Nümbrecht, Germany) with a further 9ml complete medium in it, and incubated at 37°C and 5% CO_2.
7. The plates were studied regularly under a light microscope, to check if cells were present and proliferating, the level of confluency, to check the cell morphology to confirm healthy status, and to look for signs of bacterial or fungal contamination.

8. The culture medium used contained DMEM with phenol red (phenolsulfonphthalein). Phenol red is a pH indicator, and gives a pink-red colour at physiological pH, but turns orange-yellow when the pH drops, indicating the presence of acidic by-products of the proliferating cells that are consuming the nutrients in the medium. The main acidic by-product in mammalian cells is lactic acid, which can be toxic to cells and accumulation of lactic acid results in a decreased pH in the surrounding medium, which is not optimal for cell growth. Therefore, if the culture medium was noted to gradually change from pink-red to an orange-yellow colour, the medium was aspirated and replaced with 10ml of fresh complete medium.

When cells, under a light microscope, were seen to cover about 70-80% of the available space within the culture dish (this was 70-80% confluency), the cells were passaged, or split. Ideally, mammalian cells in adherent cultures should be passaged when they are in log phase, before reaching the plateau phase at close to 100% confluence, because growth rate slows or stops at this stage and it would then take cells longer to recover when reseeded. This is why passaging of cells when they were beyond 80% confluency was avoided. The technique used to split the cells was similar to that used by other groups,(Manning et al., 1991; Pass et al., 1995) and was as follows:

1. The culture medium was aspirated carefully, taking care not to touch the aspirator tip to the bottom of the dish where the cells were adhered; cells were then washed twice with 1ml of PBS. Serum, calcium and magnesium from the complete medium inhibit trypsin and therefore need to be washed off prior to the addition of trypsin.

2. Trypsin (Sigma-Aldrich® trypsin solution 10X, CAS 9002-07-7, diluted to 1X with PBS without phenol red), a proteolytic enzyme that dissociates adherent culture cells from the

dish)(Huang et al., 2010) was then added (1ml) to the plate, and the plate was incubated at 37°C for 5-6 minutes.

3. The plate was then inspected under a light microscope to ensure that at least 90% of the cells had become detached from the plate (seen to be floating within the trypsin solution). If not, then the plate was either incubated for a further 30 seconds or so before being checked again, or tapped gently to encourage detachment of cells.

4. Complete medium (5ml) was then added to the dish, and a serological pipette was then used to repeatedly aspirate the fluid within the dish to manually detach any remaining attached cells. The dish contents were transferred to a 15ml conical centrifuge tube, and centrifuged at 500 x g for 5 minutes.

5. The supernatant was then discarded, so discarding the trypsin. The pelleted cells were resuspended in complete medium (the fraction of cells to be split was calculated depending on the noted growth rate of the cell culture (speed at which cells reached confluence). For example, for cell cultures that proliferate more rapidly, 5ml of complete medium were used and one-fifth (1ml) were retained for further passaging, whereas in cells known to proliferate more slowly, 3ml complete medium were used and one third (1ml) were retained for further passaging). The 1ml of the cellular solution was transferred to a new dish with 9ml of fresh complete medium, with subsequent incubation at 37°C and 5% CO_2.

6. The cell culture name, date and passage number were clearly labelled on the plate.

The initial cells that were seeded included both cancer cells and other non-cancer cells found in the MPE fluid of patients with MPM. However, non-cancer cells do not have “immortal” properties of cancer cells, and these eventually reach senescence during the process of repeat passaging, and die off (usually by passage 20). The fact that some cells

survived and kept proliferating after serial passages, showing no signs of senescence, was an indication that an immortalised cancer cell culture was being established.

For this project, a cell culture was considered to be established when all cells appeared phenotypically similar, and this was observed to occur usually by passage 20, although it varied greatly from one cell culture to the other. Passage 20 was reached within about 4-6 months from fluid sampling. However, for the purposes of the experiments, earlier passage cell cultures were used in order to minimise genetic variants that inevitably occur with each serial passage, and therefore to have a cell culture as representative of the patient's *in vivo* tumour as possible.

Unsuccessful cell culture was defined as those in which cells were not adherent within 7 days of seeding, those that did not proliferate within 60 days of seeding, and those that became senescent after passaging. Unsuccessful cell cultures were discarded.

Cells (after culture without antibiotics for several days, and when at about 90% confluence) were screened for *Mycoplasma spp.* by polymerase chain reaction every 4 months, using Mycoplasma PCR detection kit (MP0035, Sigma -Aldrich, St. Louis, MO). Mycoplasma contamination is difficult to detect but may impact on reliability and reproducibility of experiment results.(Drexler and Uphoff, 2002)

A proportion of cells was frozen at regular passages (method below) so that the occasional contamination and discarding of a plate was not detrimental to the experiments. Also, continuous culture may lead to genetic instability, therefore some cells were preserved in cryogenic storage so as to have a working stock of cells at lower passages, to allow

examination of the genetic profile at the various passages, enabling identification of genetic alterations that potentially occur with serial passaging.

Freezing of cells

The cells were frozen at high concentration (when a cell culture plate has reached, or has almost reached, confluence), and with a solution containing dimethyl sulphoxide (DMSO) (Sigma-Aldrich® D8418), a cryoprotective agent, decreasing the freezing point of the medium and the risk of ice crystal formation which may damage and kill cells. The freezing medium (90% FBS + 10% DMSO – the most commonly recommended and used freezing medium for mammalian cell culture)(Kielberg V. Cryopreservation of mammalian cells – Protocols. Thermo Scientific. Available online at http://tools.thermofisher.com/content/sfs/brochures/D19575.pdf, accessed 8th July 2019; Cell Culture protocol 7: cryopreservation of cell lines. Merck. Available online at https://www.sigmaaldrich.com/technical-documents/protocols/biology/cryopreservation-of-cell-lines.html, accessed 8th July 2019; Cryopreservation guide. Biological industries. Available online at https://www.bioind.com/media/wysiwyg/product/cryostem/CryopreservationGuide.pdf, accessed 8th July 2019) also contained serum in order to provide a more optimal nutrient-rich environment for cells to increase the chance of them surviving cryopreservation. The method used to freeze the cells is included in the Chapter 6: Appendices, section 6.3.

For long-term storage, i.e. >6-8 months, back up samples of cells were stored in liquid nitrogen. Cells were thawed when required for experiments (method in Chapter 6: Appendices, section 6.3).

Confirming MPM cell culture

There are different cells in pleural fluid, including white blood cells, red blood cells and fibroblasts. In order to confirm that cancer cell cultures had been established, rather than other cell cultures, cell culture cytology and tumoursphere assays were performed.

Cell culture cytology

- The cells of 5 randomly selected cell cultures, at around passage 20, were treated with trypsin and then centrifuged, and the supernatant discarded;
- Cells were diluted to 1,000,000 cells/ml in 0.1% w/v Bovine Serum Albumin/PBS (A9418 Sigma-Aldrich®, St. Louis, MO);
- Cytocentrifugation (A78300003, ThermoFisher Scientific, Waltham, MA; Cytospin™) was carried out at 500 x g for 5 minutes. Three slides were prepared per cell culture at 3 dilutions per cell culture (70 µl, 100 µl and 150 µl containing 70,000, 100,000 and 150,000 cells respectively).
- The slides were prepared and stained with May-Grünwald-Giemsa (MGG) staining according to the following protocol:
  - The cells were fixed with methanol for 2 minutes.
  - The cells were stained with May-Grünwald stain for 6 minutes (1:1, buffer: May-Grünwald stain).
  - The cells were then stained with Giemsa stain for 40 minutes (1.4ml Giemsa stain to 50ml buffer).
  - Slides were washed with PBS and then dried.

The buffer used was sodium hydrogenophosphate anhydrous (2.56g) and potassium dehydrogenophosphate anhydrous (6.63g), diluted to 1 litre in water (Milli-Q®, i.e. water that has been purified and deionised to a high level by a purification system).

- The slides were then shown to a blinded NHS Consultant histopathologist with expertise in MPM, who provided a description of the cells and whether there were features of malignancy.

A table of reagents used and suppliers is included in Chapter 6: Appendices, section 6.4, table 6.4.

Tumoursphere culture

I performed the tumoursphere culture under supervision of a biomedical post-doctorate scientist with experience in tumoursphere assays. Tumoursphere culture was performed in order to characterise the cancer stem cell population within the population of cultured cells. Cell cultures used were at relatively high passage (passage 15-20) to ensure that they consisted of a predominantly cancer cell population. Tumourspheres were cultured in serum-free medium in low attachment plates. The protocol used was also used by Johnson et al,(Johnson et al., 2013) and in summary:

1. Cells from one of the established MPM cell cultures were centrifuged and the pellet resuspended in 1ml tumoursphere medium (20ng/ml Epidermal Growth Factor, 10ng/ml Basic Fibroblast Growth Factor, 5µl/ml insulin, 0.4% bovine serum albumin, 500ml DMEM/F12; 50x B27 supplement was added to the medium just before the experiment)
2. Cells were counted using an automated cell counter and 5000 cells per well (with 200µl of tumoursphere medium) were seeded in a 96-well ultra-low attachment plate. Standard protocols advise 100µl of fluid per well in a 96-well plate to be used for experiments,

however given that in tumoursphere cultures the medium is not changed regularly, 200µl were used instead in order to ensure adequate nutrition was present for the cells.

3. The outer wells were filled with complete medium to correct for evaporation errors.

4. The plates were incubated at 37°C and 5% CO_2 in an IncuCyte® machine (a live cell analysis system where cells are analysed while being kept in an incubator).(https://www.essenbioscience.com/en/products/incucyte/, accessed 13th December 2019)

5. Regular images of the whole wells were taken, and tumoursphere growth and numbers were monitored.

Method for DNA Sequencing

In order to further characterise the cell cultures, and to determine whether cells from passage 20 cell cultures had acquired several new variants during serial passages when compared to passage 0 (i.e. to determine whether passage 20 cells were also representative of the patient's native tumour despite having undergone serial passaging *in vitro*), the genomic sequence of passage 20 cell cultures was compared to that of cells from passage 0 cell cultures. This step also aimed to assess whether the cell cultures developed would yield good quality DNA sequencing results in order to assess the feasibility of the bench-to-bedside pathway described later (i.e. to determine whether patient derived MPM cell cultures were of adequate quality to be used for drug screening assays and DNA sequencing analyses. This would be an important step in the feasibility assessment as the cell cultures need to be adequate for high quality analyses if they are to be considered as possible tools to potentially determine drug resistance / response patterns specific to the patient).

DNA was extracted from three of the MPM cell cultures (the same 3 cell cultures that were used for the drug screening assay described later), both at early (passage 0) and late (passage 20) passage, using a DNA extraction kit (Blood and Cell Culture DNA Mini Kit, Qiagen®). Each cell culture was expanded and DNA was extracted from 3 dishes per cell culture (i.e. 3 dishes at passage 0 and 3 dishes at passage 20), then the extracted DNA for each cell culture was pooled (pooled triplicate samples). The kit allows the genomic DNA to be purified using genomic tips, optimised buffer systems are used to carefully lyse cells, and then genomic DNA binds to Qiagen® resin packed in the genomic tip which is designed to operate by gravity flow for the purification procedure. The method used for DNA extraction followed the kit's standard protocol (summary of protocol in Chapter 6: Appendices, section 6.3).

Whole genome sequencing with comparison of early (passage 0) with late (passage 20) passage variants was performed using Applied Biological Materials, Inc (abm®; Richmond, Canada) as sequencing service provider, and on an Illumina® HiSeq X platform (Illumina, San Diego, CA) – a high throughput sequencing system.(https://www.illumina.com/systems/sequencing-platforms/hiseq-2500.html, accessed 13th December 2019)

Quality control of the DNA samples was assessed by Qubit DNA assay and agarose gel electrophoresis. All samples passed internal QC control. DNA library was constructed using NEBNext DNA Library. Prep Kit was used following manufacturer's recommendations. Quality control of the final library was assessed by qPCR and Agilent bioanalyzer.

The Genome Analysis Tool Kit (GATK) pipeline was used,(McKenna et al., 2010) and this is the most popular DNA sequencing programming framework to facilitate development of analysis tools for whole genome sequencing (WGS) data. Files in Binary alignment map (BAM) format were aligned to the Genome Reference Consortium Human Build 38 (GRCh38), a human genome assembly.

To identify somatic variants (acquired genetic variants that can be passed on to daughter cells during cell division) VarScan version 2.40 was used (a software tool that detects variants in next generation sequencing (NGS) data).(Koboldt et al., 2012) VarScan2 had been found to perform best out of several somatic variant callers tested.(Stead et al., 2013) The data was filtered and visualised using MIG (Multi-image genome viewer).(McGowan et al., 2013)

The DNA sequencing results were analysed as follows:

- The MSK-IMPACT panel of gene variants were compared between early and late passage cell cultures, and new variants identified in the late passage, that were not present in the early passage cell cultures, were analysed further. The panel of genes used in this study is available on the FDA website (https://www.accessdata.fda.gov/cdrh_docs/reviews/den170058.pdf, accessed 7th February 2019)
- In order to identify significant variants, the following variants were excluded: intronic, upstream, downstream, untranslated regions variants, as well as non-frameshift deletions, non-frameshift insertions, and synonymous SNVs. Exonic variants, including non-synonymous SNVs, stop-gain and frameshift variants were analysed further.

For the purposes of comparing early with late passage cell cultures, DNA sequencing results were analysed with a main focus on LR, radial SVM and ClinVar as discussed in the literature review (Introduction chapter).

2.4 Investigating the biological properties of pleural fluid: *in vitro* experiments

Figure 2.4 shows the flow of the study and the relevance of this section with reference to the aims of this project.

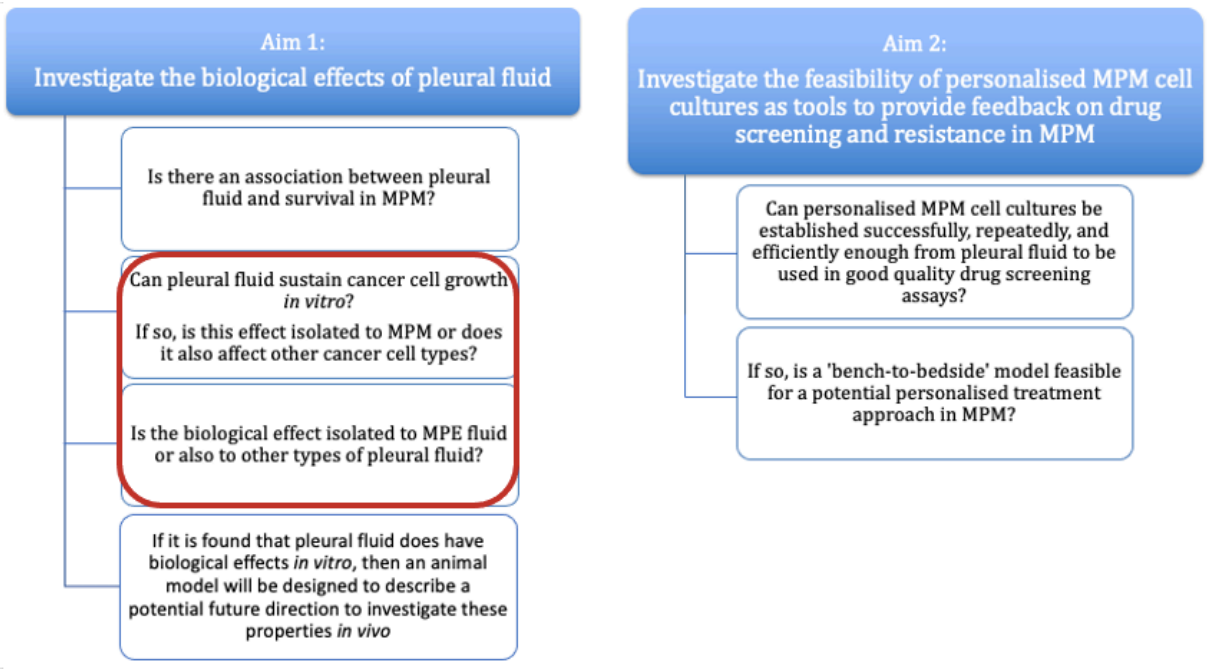


Figure 2.4 Flow chart showing summary of aims and guide to the workflow of the study. This section analysed whether pleural fluid alone was adequate for cancer cell proliferation *in vitro*.

This work was awarded a research grant for laboratory consumables and DNA sequencing expenses (OHSRC – Oxfordshire Health Services Research Committee).

Ethics

MPM cell cultures from pleural fluid samples were used for the experiments, as they are patient-derived cell cultures that closely resemble the native tumour.(Kanellakis et al., 2020) Pleural fluid (50-60ml) was collected from patients with MPM who underwent pleural procedures as part of their standard medical care, and who consented for inclusion in the Oxford Radcliffe Pleural Biobank (Research ethics committee: South Central Oxford C 09/H0606/5+5). This study was approved by the Oxford Radcliffe Biobank Central University Research Ethics Committee (CUREC) number: 15/A251.

Experiment design

The pleural fluid was processed within 2-3 hours of sampling. The method for primary cell culture is described in the cell culture chapter.

The MPM cell culture was used for further experiments when cells were at passages 15-20, in order to ensure that a majority cancer cell population was used, but limiting genomic drifting. In addition, cell cultures were used for experiments when they were 80-90% confluent in the cell culture plate to ensure that they were in the log phase and that there were adequate numbers of cells present for the experiment.

The experiments were designed with cell culture cells seeded in 100% MPE fluid, in order to simulate *in vivo* conditions as closely as possible, as reflective of the environment *in vivo*. The initial pilot experiments were a test of different methods of measuring cell viability as the pleural fluid tended to adopt a gel-like consistency, and this made it difficult to use protocols designed for a fluid medium rather than a more viscous medium. Despite this gel formation, what became clear from the initial experiments was that MPM cells grow and

proliferate within 100% pleural fluid alone, without any addition of full culture medium. If pleural fluid alone is adequate to support MPM cell proliferation in an artificial *in vitro* environment, where cells obtain nutrients solely from the surrounding pleural fluid, then the potential for pleural fluid to result in MPM cells' proliferation in the pleural cavity *in vivo* where conditions for cell growth are much more optimal seems highly likely. This would support the hypothesis that MPE fluid has biological capabilities *in vitro*.

Demonstrating cell proliferation in vitro

An increase in cell proliferation in the presence of pleural fluid needed to be demonstrated in order to show that pleural fluid had biological properties capable of supporting cell proliferation *in vitro*. The tendency of pleural fluid to adopt a viscous gel-like consistency meant that a number of methods needed to be tested to determine the optimal way of demonstrating cell proliferation:

- Visual examination and documentation of confluence via images of the wells

To confirm cell proliferation, the cells seeded in pleural fluid were assessed under a microscope and serial images of the wells were recorded in order to document increasing confluence.

- Luminescence assays

Cellular viability was initially measured using luminescence assays, specifically CellTiter-Glo® luminescent cell viability assay which quantifies the adenosine triphosphate (ATP) present, representing the presence of metabolically active cells. The method used is detailed in Chapter 6: Appendices, section 6.4. The medium/pleural fluid was removed and cells were washed with PBS to remove debris and dead cells which would have affected the background assay. This had been tested by the laboratory team prior to these experiments and so the standard protocol for CellTiter-Glo for experiments with

pleural fluid amended to include removal of the medium prior to addition of the reagent.

Complete medium and human serum were tested as controls, however the results obtained were variable. One possible reason for this is that the pleural fluid tended to adopt a gel-like consistency (Figure 2.5), and when the gel was discarded to enable the addition of CellTiter-Glo® solution to determine cellular viability, cells that were growing within the gel would be discarded too, leading to variable results in cellular viability. Fresh pleural fluid was used as often as possible to reduce any potential effect that freezing may have on the tendency of the pleural fluid to form a gel, and the pleural fluid was removed as carefully as possible in order to minimise loss of live cells.

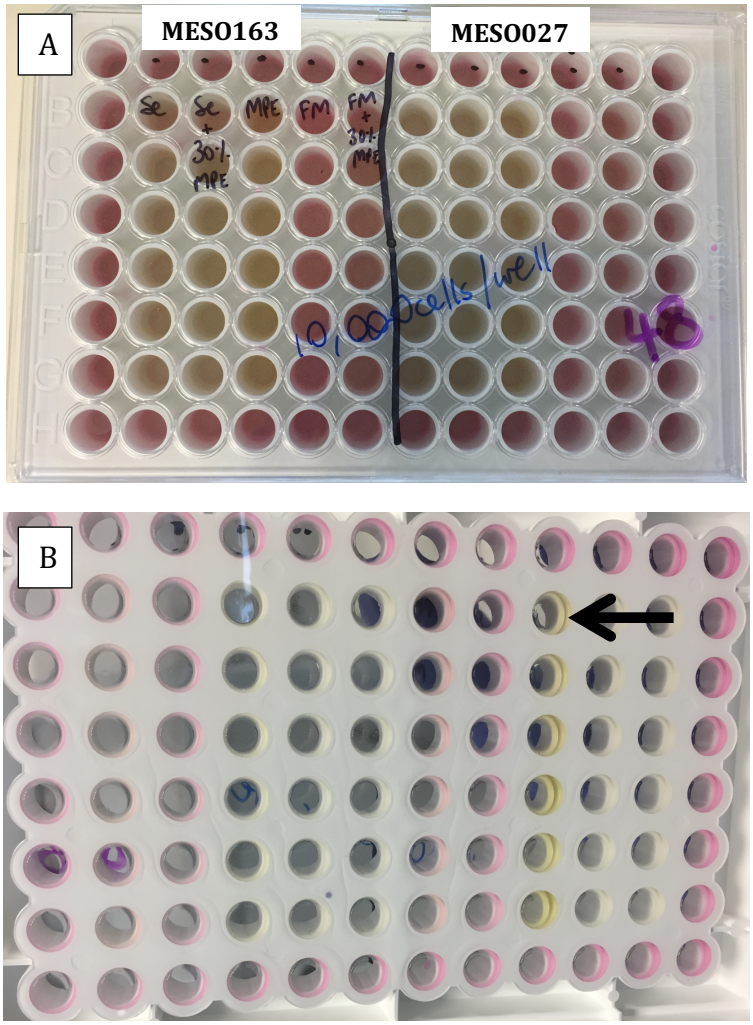


Figure 2.5 shows a white opaque walled 96-well plate. Image A: The cell cultures MESO163 and MESO027 were being tested with 100% serum (columns 2, 7), serum with 30% malignant pleural effusion fluid (MPE) (rows 3, 8), 100% MPE (rows 4, 9), 100% complete medium (rows 5, 10), and complete medium with 30% MPE (rows 6 and 11); 10,000 cells were seeded in each well, but no cells were seeded in rows A and H, and columns 1 and 12, as these were instead filled with 100µl of complete medium in order to limit evaporation from the cell culture wells and to obtain a value for background luminescence. Image B: the same 96-well plate, as viewed from below after fluid was discarded from the wells. The wells that contained 100% MPE fluid can be seen to contain residual gelified MPE (black arrow) within them despite an attempt to empty the wells of fluid. On observing the wells under a light microscope, a decrease in the number of cells present was noted upon removal of the gel, implying that cells were being lost with removal of the viscous pleural fluid, leading to inaccurate and unreliable cell viability results.

- Crystal violet stain

An attempt was made to assess cell viability using crystal violet to stain adherent cells. Dead cells lose adherence and are therefore lost as floating cells when the culture medium is removed, but live (and therefore adherent) cells are left behind and crystal

violet stain binds to the proteins and DNA within the cells, giving an indication of cell viability.(Feoktistova et al., 2016)

Cells were seeded in a 6-well plate in pleural fluid as described above, and the protocol described by Feoktistova et al. was adapted for a 6-well plate. (Feoktistova et al., 2016) In summary, 48 and 72 hours after cells were seeded, the pleural fluid was discarded and 1ml 0.5% crystal violet stain per well was added. Following incubation at room temperature for 20 minutes, the wells were washed twice over with water and allowed to dry.

- Calculation of cell size

Using Fiji (ImageJ) version 2.0(Schindelin et al., 2012) the area of a sample of 3 cells from each image was calculated and a mean of the sizes obtained. This enabled comparison of the size of the cells seeded in different pleural fluid types.

- Calculation of confluence from images of the wells

In order to compare cell viability and to calculate the doubling times for the cells, the confluence percentage was calculated from the images obtained from the Incucyte® using Fiji (ImageJ) version 2.0(Schindelin et al., 2012) by measuring the pixels in the area of the well covered by cells, and expressing it as a percentage of the total area of the well in the image.(Busschots et al., 2015) This method published by Busschots et al generates an area fraction output that can be used as a representative of the approximate confluence, and was found to correlate with cell counts using a haemocytometer (average correlation co-efficient (Spearman correlation test) 0.99+/-0.008 and 0.99 +/-0.01 for two ovarian carcinoma cell lines, with *p* 0.01 for both cell lines), but with the advantage of being a non-invasive test that does not disturb the cell culture.(Busschots et al., 2015) In this present study, this allowed analysis of the confluence without the need to remove pleural fluid from the wells, therefore

minimizing loss of cells. Below is an example of the images generated (Figure 2.6). This method allowed manual setting of the threshold for each image, to ensure that only the area of the well covered by cells was included when calculating percentage confluence, while if the percentage confluence were to have been automatically calculated directly by the Incucyte® machine based on the images obtained, there would have been the risk of overestimating percentage confluence if the surrounding viscous pleural fluid were to be erroneously included as area covered by cells. This data was used to generate growth curves. Doubling times were calculated from the period of most rapid growth because this is representative of the cells during log phase (as opposed to the initial lag or end plateau phase).

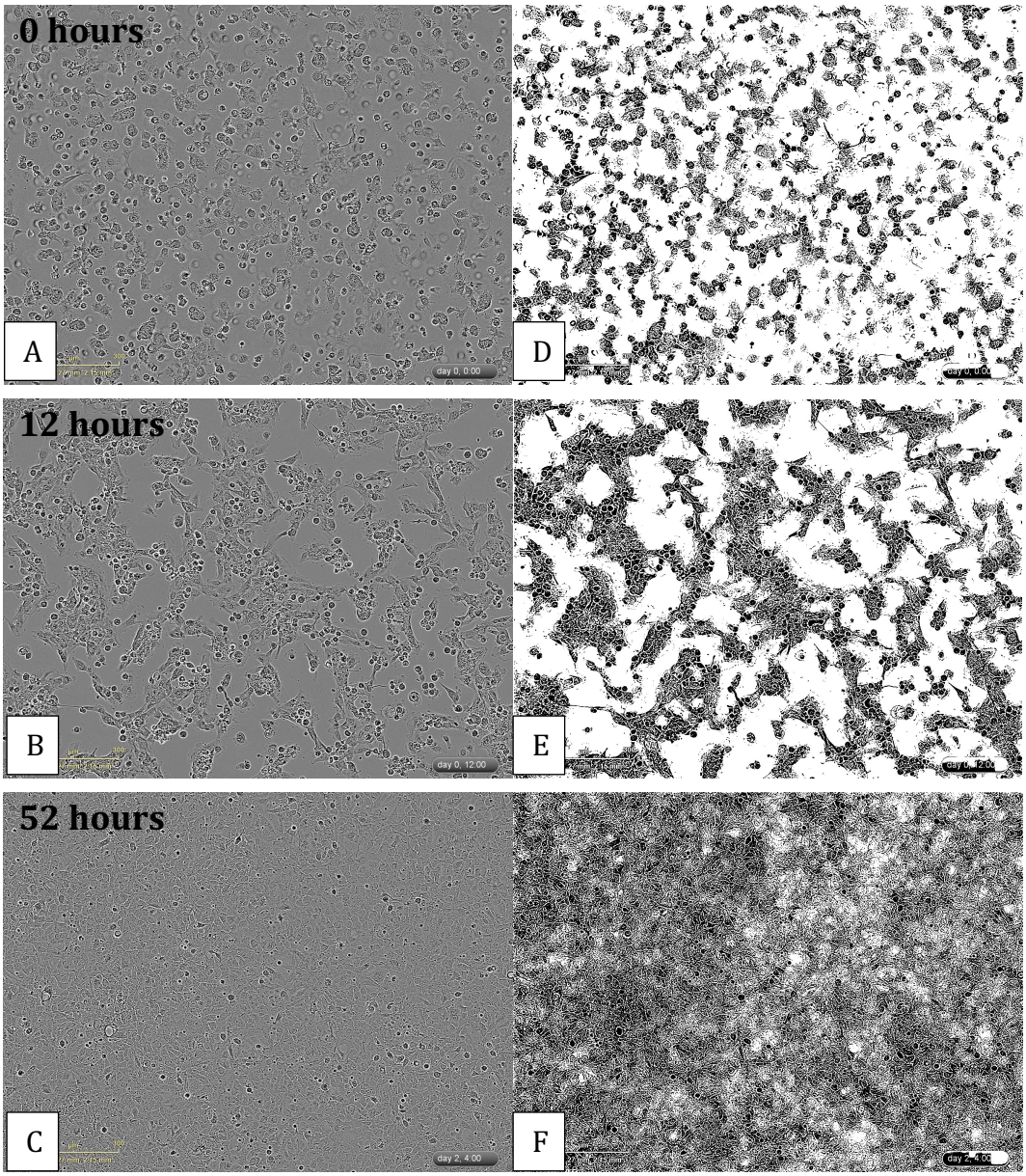


Figure 2.6 shows images of MESO024 cell culture seeded in heart failure transudate pleural fluid. Images A, B, C are the original images taken by Incucyte® at 10x magnification, and images D, E, F are the corresponding images generated by Fiji (ImageJ) version 2.0(Schindelin et al., 2012) with manual adjustment of the threshold. Percentage confluence was calculated as 20.2% at seeding (A, D), 29.3% at 12 hours after seeding (B, E), and 50.8% at 52 hours after seeding (C, F).

Method used for experiments testing the biological effects of MPE

It was clear from initial experiments that MPM cells grow and proliferate within pleural fluid alone, without any addition of complete medium. The following experiments were therefore designed to further investigate this.

Proliferation of cells from established MPM cell cultures in pleural fluid (exudate and transudate MPE fluid, and transudate non-MPE pleural fluid)

- Initial experiment in 6-well plates:

1. MPM cells were seeded in 6-well plates, as per steps 1-6 in the method for 'Seeding cells for experiments' in Chapter 6: Appendices, section 6.4, but in step 2, pelleted cells were instead resuspended in pleural fluid, and similarly, in step 6, cells were made up to the dilution with pleural fluid instead of starvation medium.
2. 2ml of the final solution were transferred to each well in a 6 well plate with 100,000 cells per well.
3. The cells were incubated at 37°C and 5% CO_2.
4. Serial images of the cells within the wells were taken daily, using ZEISS Axiocam 506 mono, to monitor growth and proliferation.
5. The pleural fluid was refreshed every 48 hours (fluid was carefully aspirated from the wells and replaced with fresh pleural fluid, taking care to minimise disruption of cells within the gel-like pleural fluid).

- Subsequent experiment in 96-well plates:

In addition, 20,000 MPM cells from established MPM cell cultures were seeded per well in a 96-well plate, initially in starvation medium for 12 hours, then the starvation medium was changed to 100µl of exudate MPM MPE fluid (biphasic MPM MPE fluid, with total fluid protein 39 g/l, glucose 2.3 mmol/l, LDH 679 IntUnit/l) or transudate MPE fluid (metastatic lung adenocarcinoma MPE (cytology positive) with total fluid protein 8 g/l, glucose 5.7 mmol/l, LDH 190 IntUnit/l). There were at least 6 replicates for each cell culture at each time point. The pleural fluid was refreshed every 24 hours. Cell viability was assessed at 4, 8, 12, 24 and 48 hours using CellTiter-Glo®. A well-characterised, commercially available biphasic MPM cell line CRL-2081(MSTO-211H)™ (derived from human MPE fluid) was purchased from ATCC® and used as a control.

Experiment using MPM MPE fluid, transudate MPE fluid, and non-MPE pleural fluid with Incucyte®

Once it was demonstrated that MPM cells are able to proliferate *in vitro* in 100% MPE fluid alone, four MPM cell cultures (MESO163 (epithelioid), MESO174 (biphasic), MESO024 (biphasic), MESO027 (epithelioid)) were seeded at 20,000 cells per well in a 96-well plate, directly in pleural fluid (100µl of the same exudate MPM MPE fluid, transudate MPE fluid, or transudate non-MPE from a patient with heart failure-related pleural effusion (total fluid protein 25 g/l, glucose 5.7 mmol/l, LDH 97 IntUnit/l)), without a preceding period in starvation medium (minimum of 3 replicates per cell culture). Cells were then incubated at 37°C and 5% CO_2 in an Incucyte® machine, and the machine was set to take regular images of the wells. The percentage confluence was calculated from the images using Fiji (ImageJ) version 2.0(Schindelin et al., 2012) as described above.

Proliferation of cells from established MPM, breast carcinoma and lung adenocarcinoma cell cultures in pleural fluid

The latter experiment (using MPM MPE fluid, transudate MPE fluid, and non-MPE (benign) pleural fluid in Incucyte® machine) was repeated with a breast carcinoma (BRST156) and lung adenocarcinoma (LNG183) cell culture, to explore whether non-MPM cancer cell cultures also proliferate *in vitro* in pleural fluid alone.

Method for primary MPM cell culture in matched MPE fluid, with primary culture in full culture medium as a control

An experiment was designed to assess whether primary MPM cell culture was possible using only matched MPE fluid from the same patient as the culture medium (100% MPE fluid). Larger samples of MPE fluid were saved from MPM patients undergoing large volume MPE fluid drainage as part of their clinical care. The same method as the 'method used for cell culture' described earlier was used, seeding cells in complete medium, but in parallel, half the cells from the same MPE fluid sample were seeded directly in 100% MPE fluid instead of in complete medium. In summary, the method used was as follows.

1. Pleural fluid samples from patients diagnosed with MPM were centrifuged at 800 x g for 10 minutes and the supernatant was aspirated and saved for later use.
2. The sedimented cells were resuspended in 3-5ml of red blood cell lysis solution (Qiagen®) and cells were kept at room temperature for 5 minutes.
3. The sample was then centrifuged again (500 x g for 5 minutes). The supernatant was aspirated and discarded, and the pelleted cells washed with PBS (Sigma-Aldrich® MFCD00121855).
4. The sample was centrifuged one last time (500 x g for 5 minutes). The pelleted cells were resuspended in 1ml of the MPE fluid saved from step 1 (or in 1ml fu complete ll medium

for the control), and then transferred to a cell culture treated-plate with a further 9ml MPE fluid (or in 9ml complete medium for the control) in it, and incubated at 37°C and 5% CO_2.

5. The MPE fluid was refreshed every 48 hours, and the plates were monitored regularly under a light microscope.
6. The cells were allowed to proliferate until >90% confluence. They were then split, and about 70% of cells were transferred to a new culture dish this time in complete medium. An image of the established cell culture was taken.

Statistics

The main aim of these experiments was to investigate whether cancer cell proliferation *in vitro* was possible using pleural fluid alone. An analysis of growth rates was conducted, comparing growth curves obtained with different types of pleural fluid (exudate MPM MPE, transudate MPE and transudate heart failure pleural fluid). This comparison was made using the non-parametric Kruskal-Wallis test for the multiple samples. Kruskal-Wallis test was also used to compare mean size of cells seeded in the different pleural fluid types. GraphPad PRISM 8 was used for the growth curves and the statistics.

2.5 Studying malignant pleural effusion development and malignant pleural effusion fluid effects *in vivo* using animal models

Figure 2.7 shows the flow of the study and the relevance of this section with reference to the aims of this project.

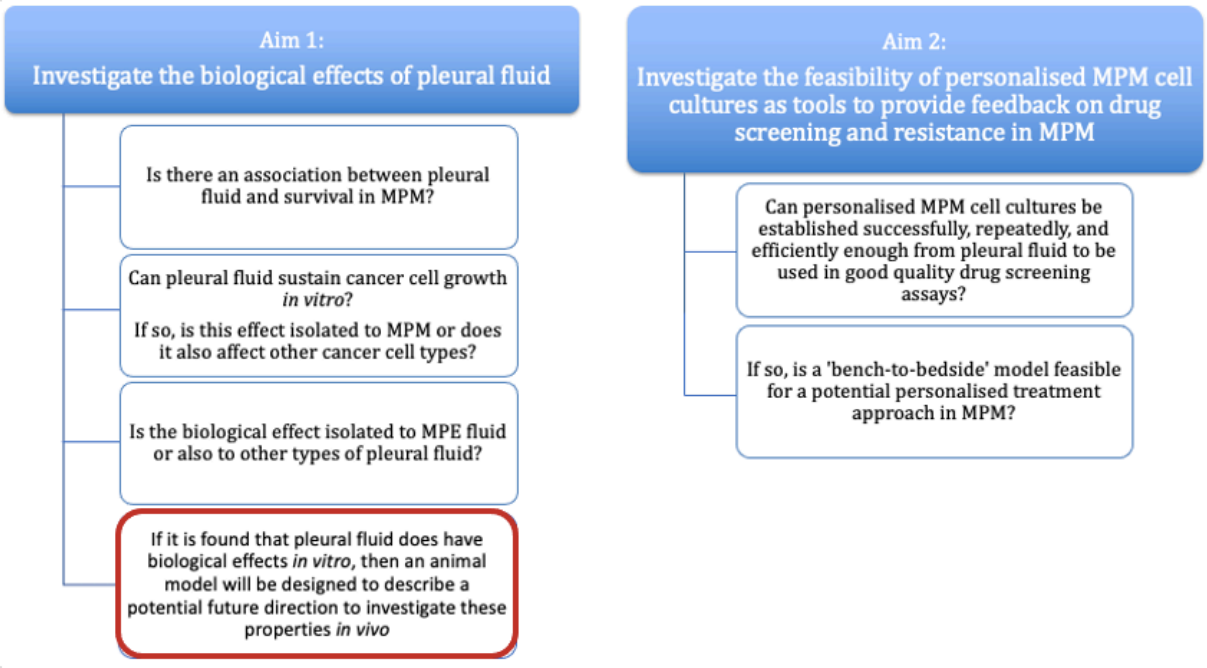


Figure 2.7 Flow chart showing summary of aims and guide to the workflow of the study. In this section, knowledge from a mouse model with indwelling pleural catheters as well as knowledge from the literature was used to design a mouse model to describe potential future direction of research.

A literature review on animal models studying MPE was carried out and the information was used together with a novel model of indwelling pleural catheters in mice, to design mouse model experiments aimed at investigating the biological properties of pleural fluid *in vivo.* The literature review is included in Chapter 1: Introduction and Chapter 3: Results, section 3.4.1, and the animal models design are included in Chapter 5: Future directions, section 5.4.

2.6 High-throughput drug screening and deoxyribonucleic acid sequencing of patient-derived malignant pleural mesothelioma cell cultures

Figure 2.8 shows the flow of the study and the relevance of this section with reference to the aims of this project.

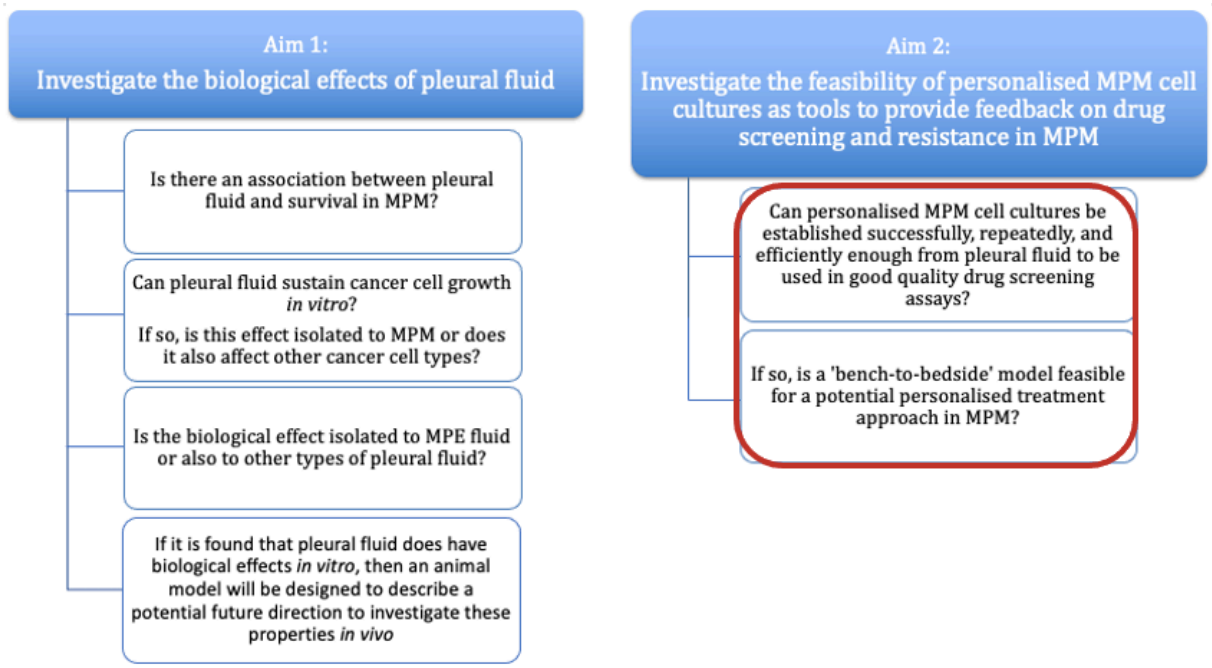


Figure 2.8 Flow chart showing summary of aims and guide to the workflow of the study. In this section, the aim was to see whether it is feasible within the clinical life of a patient, to use personalised cancer cell cultures to assess the patient's own cancer cell vulnerability to treatment, in a reliable and timely manner to help affect treatment outcomes.

DNA sequencing

DNA was extracted, and whole genome sequencing was then performed using abm® as sequencing service provider, as described earlier.

Drug screening

High throughput drug screening was conducted in collaboration with the Cellular High Throughput screening facility at the Target Discovery Institute (TDI), Nuffield Department of Medicine, University of Oxford, UK. A drug library (TDI expanded Oncology drug set, included in Chapter 6: Appendices, section 6.6) of 316 drugs approved for different types of malignancies was used.

For drug screening experiments, cellular viability was measured using AlamarBlue® resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide), a blue weakly fluorescent dye, irreversibly reduced in mitochondria of viable cells to resorufin (pink and fluorescent). The amount of fluorescent resorufin produced, measured by a fluorometer, is proportional to the number of viable cells. Resazurin reduction assays are relatively cheap, and more sensitive than widely adopted and popular tetrazolium assays.(Nakayama et al., 1997; O'Brien et al., 2000) The disadvantages of the resazurin assay are the potential fluorescent interference from the compounds being tested, and the direct toxic effect that the resazurin has on the cells, especially given that the assay requires a period of incubation time, increasing the possibility of these artefacts.(Pace and Burg, 2015) Furthermore, resazurin measures metabolically active cells and therefore does not accurately distinguish between cytotoxic and cytostatic effects, therefore it would be essential to conduct further experiments that directly measure cell death prior to translational application. The incubation time also adds a further plate-handling step than the ATP assay used in the other cell culture experiments in this study, increasing the chance for errors.(Riss et al., 2004) To mitigate this, automated cell seeding and counting was used, the PerkinElmer Janus® automated workstation. Automation reduces human error with manual liquid handling and counting, reduces pipetting and dilution errors, and increases efficiency because thousands of cells can be analysed in a shorter period of time. Automation therefore minimises bias and gives more reproducible results. Despite these limitations, resazurin's low cost and wide availability make it suitable for the goal of assessing feasibility of the use of personalised cell cultures as tools within a translational model that would be efficient and accessible.

The method used for the drug screening assay was as follows:

- The MPM cells were confirmed to be mycoplasma-negative prior to the drug screening experiments.
- Using Janus® G3 automated liquid handling workstation (AJV001, PerkinElmer, Waltham, MA), 3 cell cultures established in our laboratory (MESO163, MESO174 and MESO033; at 2,000, 1,600 and 1,600 cells per well respectively; final volume 100µl per well) were seeded in 384-well plates (3512, Corning, Corning, NY) (Figure 2.4). The number of cells seeded was chosen based on the growth dynamics of each cell culture (prior to the drug screening assay, a growth assay was performed seeding cells from the 3 cell cultures at different concentrations in 384-well plates, and the same resazurin assay planned for the drug screening assay was done to measure cell viability. This allowed an estimate of the number of cells needed to be seeded for the drug screening assay, aiming to achieve approximately 60-70% confluence at 48 hours).
- Six plates per cell culture were used (2 replicates each for three drug concentrations: 100nM, 1uM and 10uM). During this primary screen, the aim was to establish the most efficient concentration, and using multiple cell and drug concentrations increases the chance of obtaining maximal sensitivity. If a hit is discovered during the initial high throughput drug screening assay, then more accurate secondary assays may be planned, but for the purposes of this study, a primary screen was adequate to determine whether the cell cultures were adequate for quality drug screening assays.
- The plates were incubated for 24 hours, and then the drugs were added to the cells and the culture medium. The drugs were dispensed using Echo® 550 liquid handler (Labcyte, San Jose, CA) and then diluted using the Janus® workstation. The negative control

was PBS and the positive control was cisplatin/pemetrexed combination (the most commonly used first line combination chemotherapy for MPM).

- Cellular viability was assessed 48 hours post treatment using the 384 Resazurin protocol. The media were changed to 20µl of resazurin solution (phenol red-free DMEM media (31966047, Gibco, Waltham, MA) supplemented with 10% FBS, and 10ug/ml resazurin (199303, Sigma-Aldrich, St. Louis, MO)) in each well, then incubated for 2 hours at 37°C.
- Fluorescence was then measured using PerkinElmer EnVision™ 2104 multimode plate reader (Perkin Elmer, Waltham, MA).
- Each value obtained in relative fluorescence units (RFU), was divided by the mean of the values of the negative control (PBS) in that plate. A mean value (X) was calculated for the resulting values for replicate 1 and replicate 2 (taking a mean activity level across replicate measurements minimises bias due to variability).
- The *Z-score* was calculated for each averaged value according to the following formula:

Z = (X – population mean) / population standard deviation

The Z factor was also calculated for each dilution of each cell culture (equation in Chapter 6: Appendices, section 6.6).

- DNA sequencing was conducted on these same 3 MPM cell cultures.

Day 0:
Cells were seeded in complete medium: 2000 cells per well for MESO163, 1600 cells per well for MESO033 and MESO174

Day 1:
Cells were treated with chemotherapy drugs diluted with DMSO
(24 hours post seeding)

Day 3:
Cellular viability measured 48 hours post treatment, using Resazurin (resazurin added to cells and 2 hours later cell viability assessed).

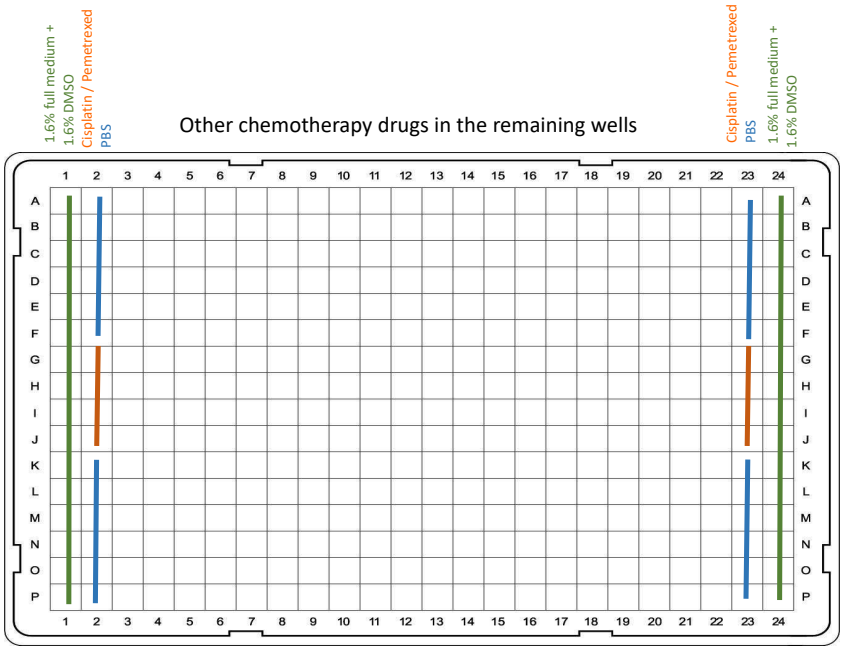


Figure 2.9 shows a schematic representation of the method used for the drug screening assay. Six 384-well plates per cell culture were used (2 replicates, at 100nM, 1uM and 10uM drug dilutions). The outer columns (A1 to P1 and A24 to P24) were cell-free and filled with 1.6% complete medium + 1.6% DMSO (to reduce evaporation errors and because DMSO is toxic to any microbes that may contaminate the plate from the outer wells), wells G2-J2 and G23-J23 were treated with cisplatin / pemetrexed (positive control), wells A2-F2, K2-P2, A23-F23 and K23-P23 were treated with PBS (negative control). *DMEM = Dulbecco's modified eagle medium, PBS = phosphate-buffered saline; DMSO = Dimethyl Sulfoxide; PBS = Phosphate buffered saline*.

Chapter 3: Results

3.1 The association between pleural fluid exposure and survival in malignant pleural mesothelioma – a retrospective cohort study

3.1.1 Literature review

There is some evidence that MPE may not just be a bystander of malignancy and may actually have biological properties leading to cancer growth, and resistance to chemotherapy. An analysis of two datasets of patients (*n*=60 and *n*=259) with MPE undergoing talc pleurodesis found that successful pleurodesis was associated with improved survival.(Hassan et al., 2019) A retrospective study of 240 successful IPC insertions for MPEs reported that the median survival was significantly longer in patients who achieved spontaneous pleurodesis than in those who did not (254 days (95% CI 177-331) and 71 days (95% CI 54-88) respectively).(Tremblay and Michaud, 2006) Patients who achieve spontaneous pleurodesis after IPC insertion are patients in whom the MPE fluid stops forming in the pleural cavity and stops draining via the IPC. Therefore in patients who do not achieve IPC-related spontaneous pleurodesis, the pleural tumours and metastases have increased duration of exposure to MPE fluid. Another, also retrospective, study of 172 patients with mesothelioma undergoing pleural biopsy and talc poudrage at video assisted thoracoscopic surgery (VATS), reported that the presence of pleural fluid on chest x-ray at 3 months post talc pleurodesis attempt, was more strongly correlated with poor survival

(adjusted hazard ratio 2.5 (95% CI 1.7-4.4%)) than other factors such as clinical mesothelioma stage >2 (adjusted hazard ratio 2.4 (95% CI 1.5-4.3%)) and Eastern cooperative oncology group performance status (ECOG PS) >1 (2.2 (95% CI 1.3-4.2%)). The only factor more strongly correlated with survival than pleural fluid recurrence at 3 months was non-epithelioid histology (adjusted hazard ratio 2.8 (95% CI 1.8 to 5.1%)).(Rena et al., 2015) These studies are limited by their retrospective design, and in addition, in the latter study, what was interpreted as increased pleural fluid on the x-ray may have been increased tumour bulk, associated with worse survival, since chest x-ray is a poor diagnostic tool for pleural fluid. Despite this, the results of these studies might imply that successful pleurodesis, and therefore limitation of the pleural tumour's exposure to MPE fluid, may be associated with improved survival.

Potential reasons for the association between successful pleurodesis and survival may include the following:

- Definitive management of recurrent MPE is usually offered to patients with higher PS, and higher PS is associated with improved survival;
- Talc induces an inflammatory response and inflammation secondary to pleurodesis may also enhance immune responses to cancer cells;
- Persistence of pleural fluid as in the case of failed pleurodesis may require further repeated pleural procedures and this may potentially contribute to worsened mortality, although pleural procedures are generally very safe with low risks of complication;
- Pleural fluid may have biological effects that increase cancer cell proliferation, one of the hypotheses of this project, and this theory is discussed in more detail below.

Exfoliated tumour cells floating within the MPE fluid are deprived of the tumour vasculature and the associated nutrients, and have no supportive cell-cell or cell-matrix interactions, however they are still capable not only of surviving, but also of forming secondary tumour foci at other pleural sites. MPE fluid is rich in proteins including growth factors and cytokines, which are pro-inflammatory and angiogenic such as vascular endothelial growth factor (VEGF),(Stathopoulos et al., 2007; Zebrowski et al., 1999) and immunosuppressive such as IL-10,(Chen et al., 1996) while also having low levels of natural killer and suppressor T cells, and low levels of the immunostimulatory cytokine, IL-12.(Chen et al., 1996) A study also showed that tumour-associated macrophages from MPE fluid were not cytotoxic to autologous tumour cells *in vitro*.(Yanagawa et al., 1985) These data suggest a possibility that MPE fluid provides the right microenvironment with adequate nutrients and factors to support tumour cells, while suppressing anti-tumour immune activity, although further research is required to confirm or exclude this.

There is some early pre-clinical evidence of the potential biological properties of MPE fluid. Cheah et al conducted *in vitro* experiments on established MPM cell lines, and reported that MPM cells exposed to 30% MPE fluid from patients with MPM had increased cell viability compared to cells exposed to serum-free medium and to medium with foetal bovine serum (medium is fluid which is manufactured to provide optimal nutrition to cells maintained *in vitro*). The same group also tested the MPM cell lines with standard first line MPM chemotherapy (cisplatin and pemetrexed combination) and found that in the presence of 30% MPE fluid from MPM patients (in culture medium), cells were less likely to die after exposure to chemotherapy than cells that were only exposed to the

chemotherapy and 100% culture medium, concluding that MPE fluid may lead to resistance to chemotherapy in MPM.(Cheah et al., 2017)

MPE may therefore not be a symptom-causing epiphenomenon in MPM alone, but may have biological capabilities supporting MPM cell proliferation, and increasing chemotherapy resistance.(Cheah et al., 2017) This has not been studied in detail in humans to date, and studies in this area are mostly pre-clinical, which do not necessarily reflect the *in vivo* pleural effusion environment. If this theory is proved to be correct, then current management of MPE may be flawed. Such information would shift the current symptomatic management strategy towards complete early drainage of fluid, and aggressive fluid prevention strategies (such as pleurodesis or surgery) to reduce the contribution of pleural fluid on cancer progression, and to improve chemotherapy response.

3.1.2 Results of this study

Baseline demographics and data completeness

A total of 761 patients diagnosed with MPM were included (median age 73 (IQR 67-80, 95% CI 72-74) years; 72% epithelioid, 10% biphasic, 18% sarcomatoid histological subtypes). Table 1 shows the baseline characteristics. There were 9/761 (1.18%) patients with inadequate data on survival.

Table 3.1 showing the summary statistics for the baseline characteristics of the MPM population studied. ***ECOG PS = Eastern Cooperative Oncology Group performance status. *Follow up time = calculated from diagnosis of MPM to date of death or censoring. **Chemotherapy response: responded n=151 (151/248, 60.9%), did not respond n=97, not available n=24. ***Surgery: partial pleurectomy n=1, extrapleural pneumonectomy n=4, pleurectomy decortication n=4, single pleural /lung mass resected n=2.***

Parameter	**Summary statistics**	**Data not available for analysis (*n*, %)**
Age at diagnosis in years (median (IQR, 95% CI))	73 (67-80, 72-74)	0
Status at time of data collection Alive Dead	 69 683	9 (1.2)
Overall survival (days) in patients who died (*n*=683) (median (IQR, 95% CI))	278 (127-505, 253-301)	9 (1.2)
Follow up time (days)* (median (IQR, 95% CI))	301 (139-544, 281-337)	2 (0.3)
Weight loss at diagnosis (*n*, %)	233 (33.9)	74 (9.7)
Haemoglobin at diagnosis (g/l) (median (IQR, 95% CI))	133 (120-147, 132-135)	44 (5.8)
Serum albumin at diagnosis (g/l) (median (IQR, 95% CI))	34 (29-38, 33-34)	54 (7.1)
ECOG PS at diagnosis (*n*, %) 0 1 2 3 4	 174 (27.4) 323 (50.8) 87 (13.7) 45 (7.1) 7 (1.1)	125 (16.4)
Mesothelioma histological subtype (*n*, %) Epithelioid Biphasic Sarcomatoid	 511 (72.1) 70 (9.9) 128 (18.1)	52 (6.8)
Chemotherapy received (*n*, %) Yes No	 272 (36.3)** 477 (63.7)	12 (1.6)
Surgery for mesothelioma (*n*, %)	11 (1.4)***	0
Malignant co-morbidities No Yes	 655 (86.2) 105 (13.8)	1 (0.1)

Primary analysis

Survival according to duration of MPE *after the MPM diagnosis*

Higher percentage of post-MPM diagnosis life exposed to MPE was associated with longer survival (*p* 0.001, HR 1.004, 95% CI for HR 1.002-1.007), when unadjusted for other factors (Chapter 6: Appendices, section 6.1).

A time dependent covariate analysis of MPE exposure and survival was performed, including weight loss, serum albumin at diagnosis, Hb at MPM diagnosis, MPM subtype, ECOG PS, age and whether chemotherapy was received or not. When duration of MPE (in number of days since MPM diagnosis) was analysed as a time-dependent covariate, there was no significant relationship between MPE exposure time and overall survival (HR 1.000, 95% CI for HR 1.000-1.000).

Survival according to pleurodesis success

Successful pleurodesis was strongly associated with survival in univariable analysis. The median survival was 473, 378 and 258 days in patients with complete pleurodesis (*n*=128), partial pleurodesis (*n*=107), and no pleurodesis (*n*=99) respectively (*n*=143 had no effusion throughout their MPM disease, *n*=284 had inadequate data): *p* <0.0001, HR 0.750, 95% CI for HR 0.652-0.864, unadjusted for other factors (Figure 3.1). Patients who received intrapleural talc (*n*=194), whether it was talc slurry through a chest drain or talc poudrage at thoracoscopy, had longer median survival than patients who did not receive any talc intrapleurally (*n*=187) (481 *vs* 369 days, *p* 0.002, HR 0.705, 95% CI for HR 0.567-0.877). Table 6.3 in Chapter 6: Appendices, section 6.2 compares baseline demographics between patients who received talc and those who did not.

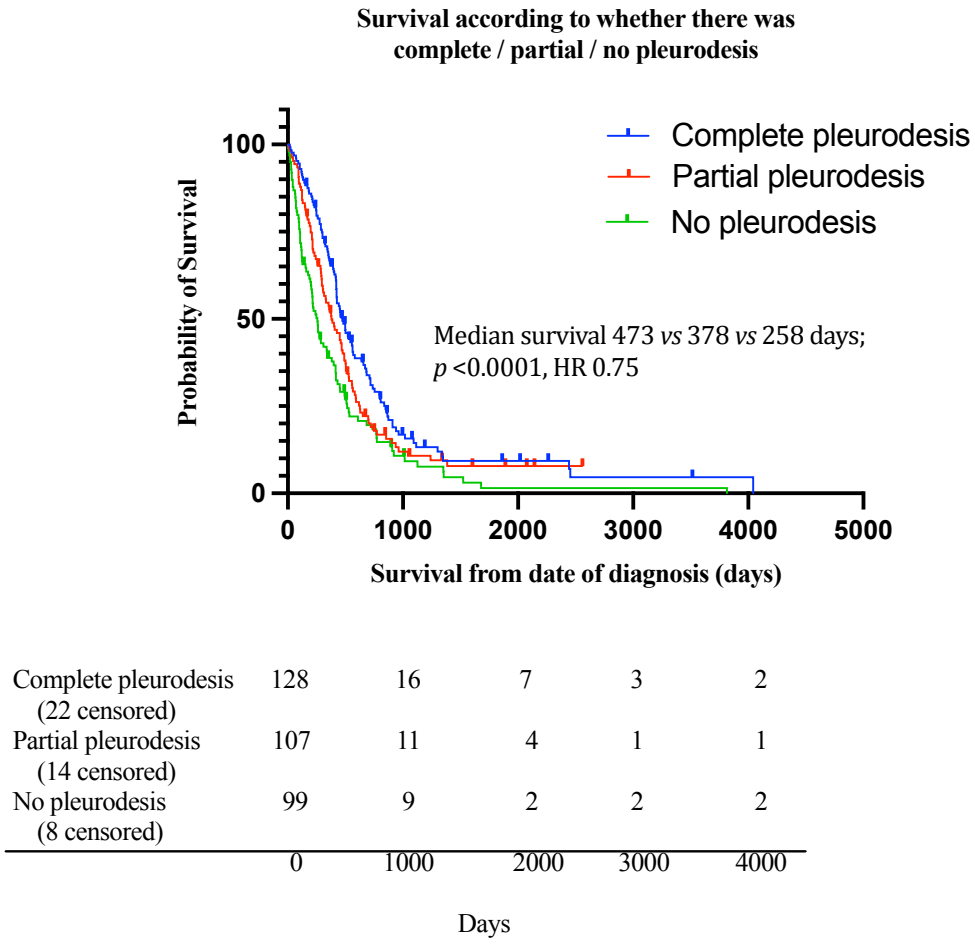


Figure 3.1 shows survival curves according to whether patients achieved complete, partial or no pleurodesis.

Multivariable regression analysis (Cox) was conducted including weight loss at diagnosis, MPM subtype, ECOG PS, Hb at diagnosis, serum albumin at diagnosis, age, and chemotherapy received, as well as pleurodesis success. Pleurodesis success remained significantly associated with longer survival (*p* 0.008) (Table 3.2).

Table 3.2 shows the results of multivariable Cox regression analysis of factors found to be associated with survival in MPM according to the literature, with the addition of pleurodesis success to the model. Cases dropped during this analysis: 1258 (83.6%), highlighting the issue of missing data. ***CI = confidence interval; ECOG PS = Eastern cooperative oncology group performance status; MPM=malignant pleural mesothelioma.***

Variable	Degrees of freedom	*p* value; Hazard Ratio, 95% CI for Hazard Ratio
Weight loss at MPM diagnosis	1	0.09; 0.77, 0.57-1.04
Hb (g/l) at MPM diagnosis	1	0.21; 0.99, 0.99-1.0
Serum albumin (g/l) at MPM diagnosis	1	0.004; 0.96, 0.93-0.99
MPM histological subtype coded as follows in SPSS: **1=epithelioid** **2=biphasic** **3=sarcomatoid**	2	<0.0001
ECOG PS at diagnosis	3	0.02
Chemotherapy received	1	0.08; 1.35, 0.97-1.9
Age at MPM diagnosis	1	0.67; 1.0, 0.99-1.02
Pleurodesis success coded as follows in SPSS®: **0=none** **1=partial pleurodesis** **2=complete pleurodesis**	2	0.008

Secondary analyses

Survival according to the presence and size of MPE at MPM diagnosis

Median survival was 321 *vs* 286 days for patients with and without MPE at MPM diagnosis respectively: *p* 0.237, HR 0.611, 95% CI of HR 0.270-1.384 (inadequate data in *n*=10).

Twenty-one patients had inadequate data on size of effusion or survival, and 740 patients were analysed. Of these, 143 (19.3%) had no effusion (size 0), 257 (34.7%) had size 1-2, 287 (38.8%) had size 3-4, and 53 (7.2%) had size 5 MPE at MPM diagnosis. There was no association between effusion size and median survival (276, 297, 351, and 297 days respectively; sizes 0

vs 1-2: HR 1.117, 95% CI for HR 0.900 – 1.386; 0 *vs* 3-4: HR 1.189, 95% CI for HR 0.959 – 1.474; 0 *vs* 5: HR 1.038, 95% CI for HR 0.7485 – 1.441; 1-2 *vs* 3-4: HR 1.081, 95% CI for HR 0.9038 – 1.292; 1-2 *vs* 5: HR 0.9363, 95% CI for HR 0.6807 – 1.288; 3-4 *vs* 5: HR 0.843, 95% CI for HR 0.608 – 1.170; *p* 0.456).

Chemotherapy response and presence and size of MPE

There were 272 patients documented to have received first line chemotherapy. Forty-five patients did not have adequate data on MPE presence and chemotherapy response, 227 patients were analysed further. When comparing patients who received chemotherapy and had a MPE at the start of chemotherapy (*n*=167, 73.6%) with those who received chemotherapy but did not have a MPE at the start of chemotherapy (*n*=60, 26.4%): 104 (62.3%) and 37 (61.7%) respectively responded to chemotherapy, while 63 and 23 respectively did not (*p* 1.0, two-sided Fisher's exact test).

There was no significant difference in chemotherapy response when analysing the effect of the size of the MPE in patients who had a MPE at the start of chemotherapy (*n*=167): 117 (70.1%) patients had size 1-2 MPE (77 (65.8%) patients responded to chemotherapy), 46 (27.5%) patients had size 3-4 MPE (23 (50%) responded to chemotherapy), and 4 (2.4%) patients had size 5 MPE (4 (100%) responded to chemotherapy); χ^2 6.0, df 2, *p* 0.05 [Chi square] (Figure 3.2).

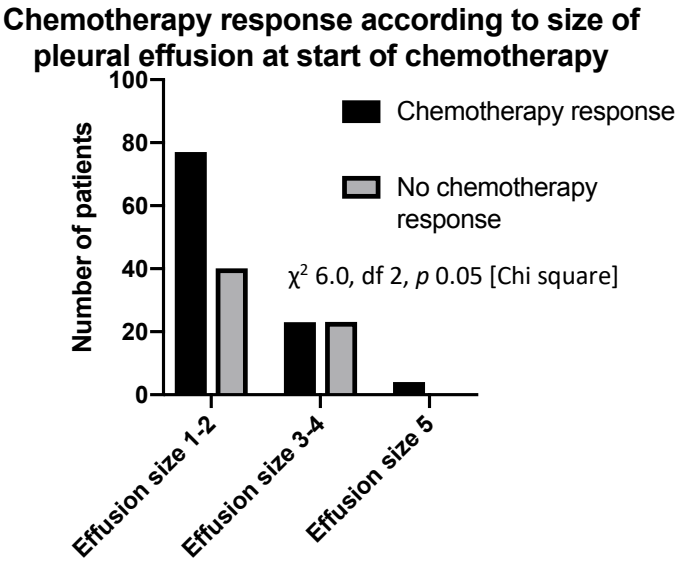


Figure 3.2 shows graph of number of patients who responded to chemotherapy and those who did not, according to the size of pleural effusion at the start of chemotherapy.

Survival association with baseline features

Weight loss, MPM subtype, ECOG PS, serum albumin and Hb at MPM diagnosis, age, and chemotherapy were all significantly associated with survival in MPM (Chapter 6: Appendices, section 6.2, figure 6.6) by univariable analysis, remaining significantly associated with survival (except for Hb and age) by multivariable regression analysis (Cox) (Table 3.3). Therefore, cox regression analysis revealed all the factors in Brims' model(Brims et al., 2016) to remain significant variables (except for Hb).

Table 3.3 shows survival according to factors already identified to influence survival in MPM in the literature, and results of multivariable Cox regression analyses. ***CI = confidence interval; ECOG PS = Eastern cooperative oncology group performance status; MPM=malignant pleural mesothelioma.***

Parameter	Median survival (days)	Statistical analysis results (Cox multivariable regression) (*p* value; Hazard ratio, 95% CI for HR)
Age (years)		
≤60	535	<0.170; 1.009, 0.996-1.021
61-70	386	
71-80	322	
>80	201	
Weight loss		
Yes	247	**0.001**; 1.377, 1.140-1.665
No	342	
ECOG PS		
0-1	366	**<0.0001**; 1.358, 1.204-1.531
2	192	
>2	157	
MPM histological subtype		
Epithelioid	392	**<0.0001**; 1.339, 1.191-1.505
Biphasic/Sarcomatoid	178	
Haemoglobin (g/l)		
121	215	0.135; 0.996, 0.990-1.001
121-153	340	
>153	502	
Serum albumin (g/l)		
≤43	297	**0.002**; 0.974, 0.957-0.990
>43	530	
Chemotherapy received		
Yes	470	**0.003**, 0.789, 0.637-0.976
No	215	

Radiotherapy and immunotherapy were not associated with survival; a small number of patients who underwent surgery survived longer (Chapter 6: Appendices, section 6.1).

Pleural fluid presence or not at the point of MPM diagnosis was not associated with survival (median survival 321 *vs* 286 days respectively: *p* 0.237, HR 0.611, 95% CI of HR 0.270-1.384).

Survival was not associated with presence of other malignant comorbidities, or with time epochs (data not available *n*=10) (Chapter 6: Appendices, section 6.1 and Table 6.1), although patients with symptoms at MPM diagnosis had shorter survival (Chapter 6: Appendices, section 6.1).

Survival varied according to different Pleural units: Glasgow, Bristol and Oxford (median survival was 264, 369, 421 days respectively; data not available on date of death or alive/dead status in *n*=10; Bristol *vs* Oxford: HR 1.022, 95% CI 0.814 – 1.284; Glasgow *vs* Bristol: HR 1.393, 95% CI 1.163 – 1.667; Glasgow *vs* Oxford: HR 1.414, 95% CI 1.184 – 1.690; *p* <0.0003) (Chapter 6: Appendices, sections 6.1 and comparison of baseline characteristics between pleural units is found in Table 6.2).

Subanalysis

Survival according to pleural fluid biochemistry and survival in the Oxford dataset

Data on pleural fluid biochemistry, including glucose, LDH and total protein levels, were available for 110 patients from the Oxford database, and therefore a subanalysis of the effect of the biochemistry of the pleural fluid on survival was carried out on this cohort.

This data was not planned to be included in the Cox regression analysis since it is available for only a small subset of patients.

The results of this subanalysis are shown below (Figure 3.3):

- Survival was 197, 392, and 557 days from diagnosis of MPM in patients with pleural fluid total protein <30g/l (*n*=6), 30-45g/l (*n*=48) and >45g/l (*n*=46) respectively; χ^2 11.7, df 2, *p* 0.003 [Log-rank (Mantel-Cox) test].
- Survival was 294, 489, and 717 days from diagnosis of MPM in patients with pleural fluid glucose 0-3mmol/l (*n*=24), 3.1-6mmol/l (*n*=56), >6mmol/l (*n*=12) respectively; χ^2 7.7, df 2, *p* 0.02 [Log-rank (Mantel-Cox) test].
- Survival was 499 and 216 days from diagnosis of MPM in patients with pleural fluid LDH <600 IU/l (*n*=68) and >600 IU/l (*n*=29), respectively; χ^2 3.7, df 1, *p* 0.6 [Log-rank (Mantel-Cox) test].

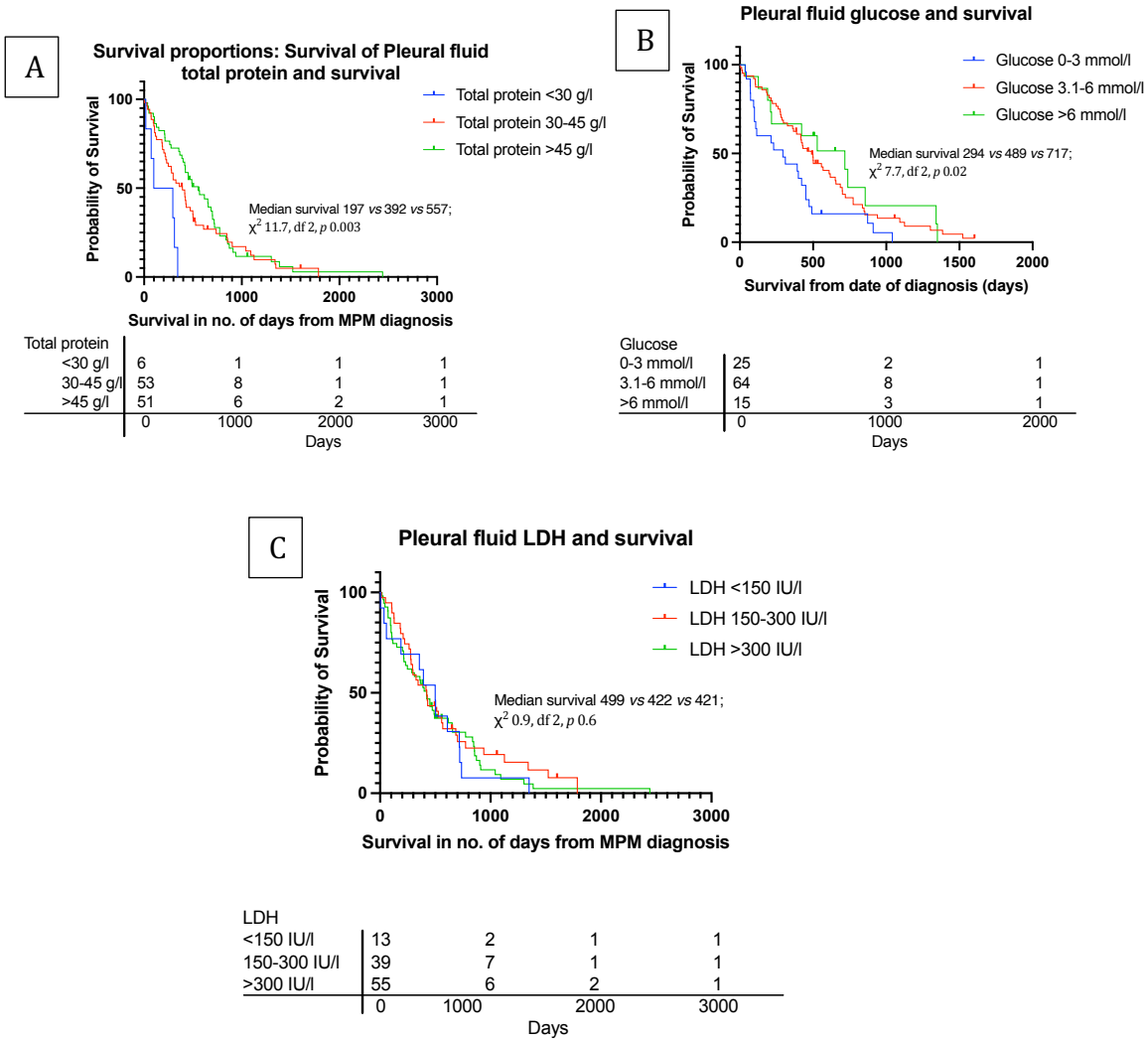


Figure 3.3 shows survival curves according to the pleural fluid total protein (A), glucose (B) and LDH levels (C). ***LDH=lactate dehydrogenase; MPM=malignant pleural mesothelioma.***

3.2 The development of patient-derived malignant pleural mesothelioma cell cultures from pleural fluid: a tool to advance biomarker-driven treatments

3.2.1 Literature review

Treatment for MPM is limited, and chemotherapy is reserved for patients who are fit enough to withstand the potential drug toxicity. Furthermore, the response rate to first line cisplatin/pemetrexed combination was 41% in a clinical trial,(Vogelzang et al., 2003) and even lower (26.3%) when evaluated in a subsequent non-randomised study of 1704

patients.(Santoro et al., 2008) Therefore more than half of the patients who do receive chemotherapy will have risked potential drug toxicity without deriving benefits in terms of disease response. MPM is notoriously heterogeneous, with inter- and intra-tumour heterogeneity at morphological and molecular levels,(Oehl et al., 2018) and its treatment would therefore benefit from a biomarker-driven personalised approach in order to improve treatment response rates, and reduce morbidity and mortality. The various preclinical models that exist are not always representative of human malignancy. MPM cell lines are monoclonal and therefore do not reflect intra-tumour heterogeneity, and the variant load acquired by the several passages of commercial cell lines often means that the cells are not representative of the native *in vivo* tumour and so are not useful for personalised translational studies. A faithful human MPM model to enable feedback of valuable information about disease progression, drug response and resistance would be ideal.

Background

Previously, MPM was induced in animals after inhalation and intrapleural injection of asbestos.(Smith and Hubert, 1974; Stanton and Wrench, 1972; Topov and Kolev, 1987) However although this model is useful to study carcinogenesis and tumour development, it is limited by the lengthy tumour development process due to the latent period of time between exposure to asbestos and tumour development, the lack of reliability of tumour induction (in a similar way that not all humans exposed to asbestos will develop MPM), the cost involved in *ex-vivo* drug screening processes, and the potential variability in species' susceptibility to drugs.(Manning et al., 1991) This limits the model's utility in experiments aiming to discover of new mesothelioma treatments. Therefore, MPM cell cultures

established from the pleural fluid or pleural biopsies of patients with MPM (primary culture) improve the precision and acuity of studies of MPM. This latter model has the advantages of avoiding animal studies and is more time-efficient.

There are a number of available commercial cell lines for use in translational *in vitro* studies, however primary cell cultures and earlier passage cell cultures are considered to be more physiologically relevant when used in *in vitro* models. As discussed, cell lines are monoclonal and do not reflect intra-tumour heterogeneity, and furthermore, commercially available MPM cell lines and cultures have been found to have metabolic upregulation, not seen in primary MPM cell cultures, making the cell lines inappropriate for studies of therapeutic agents that target disruption of the cellular metabolism. In addition, the known MPM genetic instability limits long-term validity of the commercial cell lines.(Chernova et al., 2016)

Thus, developing personalised early passage MPM cell cultures is essential for *in vitro* models to study tumour pathogenesis, and to discover biomarkers and novel therapeutic targets.

Cell culture

Although 2-dimensional cell culture models lack the complexity of the actual tumour, both architecturally and cellularly, they are similar to the tumour native state and provide a superior model for evaluating new agents in early pre-clinical studies,(Chernova et al., 2016) the results of which may then be translated to clinical settings as potential new management approaches or new treatments for patients. However, establishing primary

MPM cell cultures from mesothelioma tissue or MPE fluid is challenging and labour intensive, because the cell isolation and proliferation process takes months of regular care, and utilises several laboratory resources, both for the continued cell culture and for cell storage or freezing.

With cells that are cultured in a cell culture dish, confluence occurs when cells proliferate to occupy all of their available substrate, i.e. when cells are seen under a light microscope to coat all of the available space within the dish. Once the cells reach 70-80% confluence, they are passaged, or split, and transferred to a new cell culture plate with fresh medium, providing more space for continued cell growth and proliferation – also known as the subculture. Normal mesothelial cells have a limited number of times they can divide before they reach senescence (i.e. lose their ability to continue to divide), usually within two months of culture.(Orengo et al., 1999; Pass et al., 1995) On the other hand, cancer cells have developed genetic variants enabling them to evade senescence and become immortalised, and in favourable conditions they will keep dividing indefinitely.(Carter and Shieh, 2015) This is a continuous, or established, cell culture. The time to achieve an established MPM cell culture varies across different studies. A group in Australia established and characterised 5 MPM cell cultures from MPE fluid of patients with MPM, and the cell cultures were considered to be established after 2 months of culture.(Manning et al., 1991) A group from the USA established 9 mesothelioma cell cultures, and the cell cultures were considered to be established after 25 passages or 1 year in culture.(Pass et al., 1995)

Cells in a culture usually have a standard growth pattern, with a lag phase after seeding of the cells, which is a period of slow growth while the cells adapt to the culture environment

and recover from the previous trypsinisation process used to detach the cells from the previous substrate the cells were in. This is followed by a log phase, which is a period of exponential proliferation with cells consuming the nutrients in the culture medium, with each cell population having a different doubling time. When the nutrients are depleted, or confluence is reached, proliferation decreases or ceases, and this is known as the plateau phase.

Cell cultures can be characterised in order to be used for further experiments, and this can be done by assessing the doubling time, documenting the cytological characteristics of the cells, assessing the genetic stability over serial passages, and by performing a tumoursphere assay.

Tumoursphere culture

There is great heterogeneity in MPM tumours with regards to aggressiveness and drug response, and this may be because of the genetic and phenotypic heterogeneity, as well as intra-tumour heterogeneity (temporal and spatial) in clonal composition within different areas of MPM tumours.(Oehl et al., 2018) This heterogeneity emphasises the importance of the need for the development of personalised targeted effective MPM therapies.

One reason for this heterogeneity has been suggested to be the cancer stem cell model. This postulates that tumours are organised hierarchically, with a small subpopulation of tumour cells, called tumour-initiating cells or cancer stem cells, having indefinite self-renewal ability (their propagation maintains tumour initiation ability), and can generate highly proliferative differentiated cells which can repeatedly form the tumour mass.(Reya

et al., 2001; Weiswald et al., 2015) Disseminated cancer cells have been detected distant from the primary tumour site but that never develop into metastatic disease. This may be because the immune system can eliminate some of the disseminated cells effectively, or because most cancer cells lack the ability to generate a new tumour and the theory is that it is only dissemination of cancer stem cells that leads to metastatic disease. If the latter is true, and the cancer stem cell model is correct, then cancer therapies should ideally target cancer stem cells.(Dragu et al., 2015) Cancer stem cells may also lead to drug resistance and tumour recurrence, including in mesothelioma, which is another reason for therapies to target this subpopulation of cancer cells,(Cortes-Dericks et al., 2010) rather than aim to simply shrink a tumour, which would only reflect the treatment's ability to kill other cancer cells, risking leaving the cancer stem cell population intact. Due to lack of distinct morphological features and absence of markers on cell-surfaces, functional assays are used to define and study cancer stem cells.(Weiswald et al., 2015) The experimental definition of cancer stem cells is their ability to form a continuously growing tumour. Serial transplantation of cells in animal models, such as immunocompromised mice, can assess both self-renewal and lineage capacity based on cancer stem cells' unique capacity to form tumours and to re-establish the hierarchical organisation and heterogeneity of the original tumour at each *in vivo* passage.(Visvader and Lindeman, 2008) This however is complicated by the potential for genetic instability within the stem cells present, leading to the generation of diverse cell types, and furthermore, serial *in vivo* assays may take several months.

Therefore, *in vitro* assays have been developed and used to identify cancer stem cells. It is possible to separate cancer stem cells from the rest of the tumour population, and fluorescence-activated cell sorting (FACS) does this by labelling cancer stem cells with

fluorescent-conjugated antibodies and then isolating stem cells based on their surface marker differential expression, fluorescent-based substrates common to cancer stem cells, or based on their cellular proteasome activity.(Lee et al., 2015) However, the availability of fluorescent-conjugated antibodies or substrates and high cost of the cell sorter are limitations of FACS, apart from requiring background knowledge of cell surface markers. Isolation of the cancer stem cells would then require further analysis of the stem cell properties such as tumorigenicity, invasiveness and drug resistance.

A simpler approach would be to use a three-dimensional model – a tumoursphere culture. A three-dimensional *in vitro* model can provide insights not observed in two-dimensional monolayer cell cultures, and can serve as an intermediate between *in vitro* adherent cancer cell cultures and *in vivo* models. These models rely on cancer stem cells' ability to generate tumours, and the capability of cells to form tumourspheres *in vitro* is used as a surrogate marker of cancer stem cells' function. Tumourspheres display all the cancer stem cell characteristics including tumour initiation, invasiveness, anti-cancer drug resistance and capability of contributing to tumour vascularisation.(Lee et al., 2015) Tumoursphere cultures are regarded as one of the criteria for cancer stem cells,(Clarke et al., 2006; Ishiguro et al., 2017) and unlike FACS, do not require background knowledge of the cell surface markers. The tumoursphere culture selectively enriches for cancer stem cell growth, but the spheres also contain more differentiated cells.(Weiswald et al., 2015) Once tumourspheres have been formed *in vitro*, they can then be isolated, expanded, and tested for tumorigenicity in animal models, for cancer stem cell marker expression, and for differentiation.

Tumoursphere culture involves culturing cells onto an ultralow attachment surface in serum-free medium, with supplementation of growth factors such as epidermal growth factor and basic fibroblast growth factor. These conditions enrich cancer stem cell populations because only these cells can survive and proliferate in this environment.(Johnson et al., 2013) To date tumourspheres have been successfully cultured from several cancer varieties,(Lee et al., 2015) including mesothelioma. Adherent cells from established mesothelioma cell lines were used to culture tumourspheres (or mesospheres), and subsequently, adherent cells and mesospheres were injected into immunodeficient mice to assess tumorigenicity. Mesospheres were associated with more efficient tumour initiation than adherent cells. In addition, mesospheres had increased expression of CD24 compared to corresponding adherent cells, and CD24 loss was associated with loss of sphere-forming capacity and lower tumorigenicity.(Pasdar et al., 2015) This provides some evidence of the cancer stem cell theory in mesothelioma, in that stemness (as indicated by CD24 presence, and associated with mesosphere formation capacity) led to increased tumorigenicity in animals. In the same study, serial xenografting of the mesospheres in immunodeficient mice led to increased tumour aggressiveness, as evidenced by decreased lag time to tumour initiation, and in addition, stem cell markers e.g. CD24 gradually increased with serial mesosphere generations, indicating that stemness may increase with each generation.

Three-dimensional cell culture including tumoursphere assays, aims to recapitulate cancer *in vivo* growth conditions, and the isolation of tumourspheres from MPE fluid would allow investigation of the original tumour avoiding more invasive clinical procedures. The identification of mesothelioma stem cells would allow more robust drug screening than that with monolayer cell cultures, and yield more reliable and translatable drug discovery

results, especially since, as discussed, cancer stem cells are associated with drug resistance and lead to tumour relapse, and the identification of drugs that can target cancer stem cells may therefore be key to successful cancer treatment.(Lee et al., 2015) Furthermore, cancer stem cells have been reported to escape immunosurveillance, and enriched cancer stem cells from tumourspheres can potentially allow enhancement of antigen presentation in cancer stem cells and the development of cancer stem cell-targeted immunotherapy.(Lee et al., 2015)

Tumoursphere assays have their limitations. The passage number may affect results because cells in early passage may include transient amplifying cells. These assays are complicated by the fact that tumourspheres form rapidly and this makes it unlikely that they formed from a single cell solely through clonal expansion. In addition, the tumoursphere size may be a reflection of the proliferation rate of other surrounding cells rather than of cancer stem cell traits.(Pastrana et al., 2011) The initial number of cells seeded is very important because tumourspheres need to be distinguished from cell aggregates. Aggregation may be avoided by filtering the cell preparation to obtain a single-cell suspension prior to seeding, and the addition of methylcellulose to the culture medium.(Lee et al., 2015) If aggregates do form, they can usually be easily differentiated from tumourspheres because in tumourspheres cells appear to become fused with one another, and individual cells cannot be identified, whereas individual cells are still identifiable in aggregates.(Johnson et al., 2013) Finally, dormant stem cells may not form tumourspheres because of the lack of as-yet-unknown key components required to activate the stem cells.(Weiswald et al., 2015) Prior to using tumourspheres for drug screening, they should be characterised, especially with regards to tumorigenicity, but also invasiveness and resistance to conventional chemotherapy or radiation.(Lee et al., 2015)

Deoxyribonucleic acid sequencing

Cells that have been cultured for multiple passages can show substantial genetic and physiological characteristics drift, and can confound pharmacogenomics and functional genomic studies.(Horvath et al., 2016) A study comparing early with later passage MPM cell cultures, found that primary cell cultures at low (<10 passages) passage had no differences in metabolic profile, but some high passage (30-50 passages) cell cultures showed additional chromosome structural abnormalities.(Chernova et al., 2016)

Two of the most prominent cancer genome sequencing databases: Sanger Institute's Catalogue of Somatic Mutations in Cancer (COSMIC) and Broad Institute's Cancer Cell Line Encyclopaedia (CCLE) were used to analyse the variant profile of 568 cancer cell lines, and comparisons between COSMIC and CCLE revealed high discrepancies in the variant profiles, with only 57% conformity in missense variants.(Hudson et al., 2014) Furthermore, *BAP1* variants were identified in 22-23% of MPM biopsies by Sanger sequencing,(Bott et al., 2011; Testa et al., 2011) but in as high as 61% of cell cultures from MPM biopsies.(Yoshikawa et al., 2012) In order to further investigate this discrepancy in *BAP1* variants, Sanger sequencing, Multiplex Ligation-Dependent Probe Amplification, cDNA sequencing, copy number analyses, methylation studies of BAP1 promoter and immunohistochemistry were performed on 22 frozen MPM biopsies and 14 (63.6%) were identified to harbour *BAP1* variants or inactivation.(Carbone et al., 2015; Nasu et al., 2015) This discrepancy is thought to be because of poorly sequenced areas of the exome. This implies that Sanger sequencing and targeted next generation sequencing alone may underestimate the frequency of

variants, whilst the whole genome sequencing used in this present study is more comprehensive, and would therefore be more ideal to increase the detection of variants.

The Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) is a highly accurate, highly sensitive and reproducible tumour-profiling assay of variants in solid tumour samples, including variants in oncogenes, tumour suppressor genes, and members of pathways deemed actionable by targeted therapies. It uses next generation sequencing to identify variants in 468 unique genes, and is also capable of detecting low-frequency variants at adequate power.(Cheng et al., 2015) The subsequent use of patient-matched normal controls enabled the compilation of a comprehensive catalogue of tumour-specific variants for every tumour sequenced, producing an unparalleled dataset of matched tumour and normal DNA sequence from advanced cancer patients.(Zehir et al., 2017) The U.S. Food and Drug Administration (FDA) approved MSK-IMPACT in 2017. It includes the genes identified to be commonly mutated in MPM, such as those in the p53/DNA repair pathway (*TP53, SMARCB1, BAP1*) and PI3K-AKT pathway (*PDGFRA, KIT, KDR, HRAS, PIK3CA, STK11, NF2*), with the commonest gene variants in MPM being in *BAP1*, *CDKN2A* and *NF2*.(Carbone et al., 2015; Guo et al., 2015; Hida et al., 2015; Iacono et al., 2015; Zehir et al., 2017) The MSK-IMPACT panel of genes has been used in samples from 40 patients with malignant mesothelioma, and results confirmed that *BAP1* inactivation is the most common alteration, followed by less frequent aberrations including in *NF2* and *CDKN2A*.(Cercek et al., 2015)

A major challenge in whole genome sequencing is distinguishing pathogenic from normal variants. Multiple algorithms were developed that can be used to generate variant function prediction scores (scores that predict the likelihood of a given non-synonymous single

nucleotide variant (SNV) causing deleterious functional change of the protein from common polymorphisms assumed to be mostly tolerable, or functionally neutral), based on different information on the variant, such as its sequence homology, protein structure, and evolutionary conservation. These scores include:

- SIFT (sort intolerated from tolerated),(Ng and Henikoff, 2001) based on protein sequence conservation among homologs
- Polyphen-2 (polymorphism phenotyping version 2),(Adzhubei et al., 2010) based on protein sequence and structure features. Two scores from PolyPhen-2 were trained on different datasets(Dong et al., 2015):
    - Polyphen-2 HDIV (used when evaluating rare alleles at loci potentially involved in complex phenotypes), identifies human deleterious variants by assuming that differences between human protein and its closely-related mammalian homologs are non-damaging
    - Polyphen-2 HVAR (used for diagnostics of Mendelian disease which requires distinguishing variants with drastic effects from human variation), identifies human disease-causing variants by assuming that common human non-synonymous single nucleotide polymorphisms (SNPs) are non-damaging
- MutationTaster,(Schwarz et al., 2010) based on DNA sequence conservation, splice site prediction, mRNA stability prediction and protein feature annotations
- Mutation Assessor,(Reva et al., 2011) based on intra- and inter-species sequence homology of protein families and sub-families
- FATHMM (functional analysis through hidden Markov models),(Shihab et al., 2013) based on sequence homology
- LRT (likelihood ratio test),(Chun and Fay, 2009) a DNA sequence evolutionary model
- VEST3 (variant effect scoring tool, version 3)(Carter et al., 2013).

Conservation scores measure the conservativeness of a given nucleotide site across multiple species where 'conserved' implies evolution is slower than expected, as compared to fast evolving. These include:

- GERP RS (genome evolutionary rate profiling, rejected substitutions),(Davydov et al., 2010) based on DNA sequence conservation
- SiPhy (site specific phylogenetic analysis, using 29 species),(Garber et al., 2009; Lindblad-Toh et al., 2011) based on deduced nucleotide substitution patterns per site
- PhyloP (phylogenetic p values),(Siepel et al., 2006) based on DNA sequence conservation.

FATHMM was found to have the highest discriminative power among independent scores, i.e. found to be the best performing individual deleteriousness prediction method for separating deleterious variants from neutral variants in its quantitative predictions, when compared to other scores including SIFT, PolyPhen-2, LRT, MutationTaster, MutationAssessor, GERP, PhyloP, and SiPhy.(Dong et al., 2015) Ensemble scores combine information from multiple component sources. Two examples of ensemble scores are KGGSeq (a biological knowledge-based mining platform for genomic and genetic studies using sequence data), which uses information from the genetic level, variant-gene level and knowledge level, and CADD (combined annotation-dependent depletion). CADD is a framework that integrates multiple annotations into a single measure for each variant, by contrasting variants that survived natural selection with simulated variants. CADD scores correlate strongly with allelic diversity, functionality, and pathogenicity.(Kircher et al., 2014) KGGSeq was the previously- known best performing ensemble score tested in a study, and also outperformed each of its individual components. However, in the same

study, two novel whole exome ensemble based prediction scores that incorporate multiple deleteriousness prediction scores, Logistic regression (LR) and radial support vector machines (SVM) scores, significantly outperformed both FATHMM and KGGSeq to achieve the highest discriminative power.(Dong et al., 2015)

In addition, ClinVar is an archive of human genetic variants and interpretation of their significance to disease, maintained at the National Institutes of Health. It aggregates information about genomic variation and its relationship to human health, providing information about the clinical impact of a variant. The interpretations of the clinical significance of variants is according to clinical testing laboratories, research laboratories, expert panels and other groups:(Landrum et al., 2018, 2016, 2014)

- CLNSIG is the clinical significance of a haplotype or genotype that includes this variant;
- CLNREVSTAT is the review status, i.e. the level of review supporting the assertion of CLNSIG for the variation.

3.2.2 Results of this study

Personalised MPM cell culture from pleural fluid

Between March 2017 to February 2019, MPM cell culture was attempted in a total of 37 MPM MPE samples (83.8% male, median age 77 years (IQR 14), 81.1% and 18.9% epithelioid and biphasic MPM subtype respectively), and of these, 17 (17/37 45.9%) were established successfully (Table 3.4).

Not surprisingly, of the 17 successfully established MPM cell cultures (i.e. those in which cells were adherent and went on to proliferate, and did not become senescent after passaging), comparison with clinical results in the medical records revealed that

simultaneous pleural fluid cytology results had features consistent with MPM reported in the majority (16/17, 94.1%) of patients: cytology reported as 'suspicious' for MPM *n*=7, 'atypical cells seen' *n*=4, positive for MPM *n*=5; and one sample was cytology negative. Of the 20 samples from which cell cultures were not established successfully, concurrent pleural fluid cytology result was positive for features of MPM *n*=12 (12/14, 85.7%): 'suspicious' for MPM *n*=7, positive for MPM *n*=5; cytology negative (*n*=6), and not available (*n*=2). There was no statistically significant difference between the number of positive and negative pleural fluid cytology results between samples from which cell cultures were established successfully and samples from which cell cultures were not established successfully (*p* 0.09, Fisher's exact test). Figure 3.4 below summarises these results.

There was no statistically significant difference between the number of cases that were early (stages 1 and 2) and late stage (beyond stage 2) between samples from which cell cultures were established successfully and those from which cell cultures were not established successfully (*p* 1.0, Fisher's exact test). Figure 3.5 below summarises these results. The numbers are too small for any definitive conclusions, however, if confirmed on a larger scale, it potentially implies that this model may be applicable to all patients with MPM, regardless of the stage of disease.

Table 3.4 showing the demographic and clinical data for the MPM patients whose pleural fluid samples were used to attempt cell culture. ***F = female, IQR = interquartile range (quartile 1 subtracted from quartile 3), LDH = lactate dehydrogenase, M = male, N = sample size, NA = Not Applicable, MPM = Malignant Pleural Mesothelioma, Mis = Missing data***

	Cell culture successful, *n*=17		**Cell culture unsuccessful, *n*=20**		***p* value (statistical test used)**
	Range	**Median (IQR) / N**	**Range**	**Median (IQR) / N**	
Gender	NA	F=2 M=15	NA	4 16	0.67 (Fisher's exact)
Age (years)	56-87	78 [14]	64-92	77 [14]	0.74 (t test)
MPM Subtype *n*(%)	NA	Epithelioid: 14(82.4) Biphasic: 3(17.6)	NA	Epithelioid: 16(80.0) Biphasic: 4(20.0)	1.0 (Fisher's exact)
Pleural fluid cytology result *n* (%)	NA	Positive/suspicious: 16(94.1) Negative: 1(5.9)	NA	Positive/suspicious: 12(60.0) Negative: 6(30.0) Mis=2(10)	0.09 (Fisher's exact)
MPM staging at time of pleural fluid sampling (early stage= stage I and 2; late stage = >2) *n*(%)	NA	Early: 11(64.7) Late: 4(23.5) Mis=2(11.8)	NA	Early: 15(75.0) Late: 5(25.0)	1.0 (Fisher's exact)
Haemoglobin g/dL	11.1-16.6	14.1 [1.2]	9.5-17.4	13.7 [2.5]	0.29 (t test)
Urea mg/dL	2.6-13.4	4.9 [4.4]	2.4-26.2	5.7 [4.9]	0.71 (Mann Whitney)
White cell count 10^9/L	4.3-14.2	8.9 [4.2]	3.9-14.1	8.5 [4.0]	0.87 (t test)
Platelet 10^9/L	109.0-411.0	265.0 [48.5]	194.0-789.0	293.5 [129.5]	0.39 (Mann Whitney)
Albumin g/dL	27.0-40.0	34.0 [5.0]	23.0-47.0	31.5 [7.5]	0.19 (t test)
Pleural fluid glucose mmol/L	1.5-9.6	5.0 [3.1]	0.6-6.1	4.3 [2.3]	0.19 (t test)
Pleural fluid LDH IU/L	88.0-1934.0	221.0 [487.8]	95-8015	319.5 [486.5]	0.69 (Mann Whitney)
Pleural fluid Protein mg/ml	36.0-52.0	40.0 [4.5]	31.0-54.0	44.5 [11.8]	0.44 (Mann Whitney)

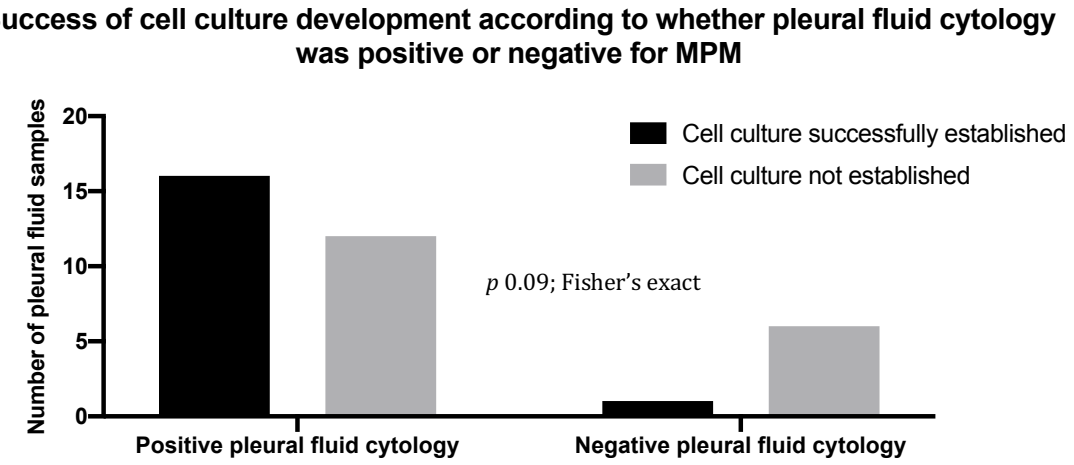


Figure 3.4 shows graph of comparison of the pleural fluid cytology result at the time of pleural fluid sampling, between samples from which cell cultures were established successfully and samples from which cell cultures were not established successfully. Pleural fluid cytology was considered to be positive if the cytology report noted the presence of 'atypical cells', features 'suspicious' of mesothelioma, or 'positive' for mesothelioma.

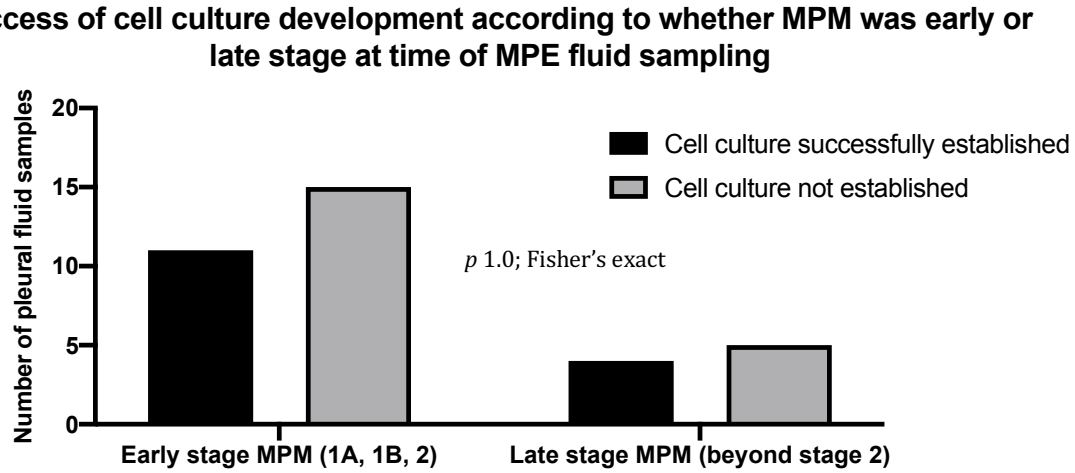


Figure 3.5 shows graph of comparison of the MPM stage (early (stages 1 and 2) and late stage (beyond stage 2) at the time of pleural fluid sampling, between samples from which cell cultures were established successfully and samples from which cell cultures were not established successfully. Data on MPM staging was collected from the computed tomography scans performed at around the time of pleural fluid sampling. *MPE=malignant pleural effusion; MPM = malignant pleural mesothelioma*

It was noted that establishing a MPM cell culture successfully was less likely from heavily blood-stained fluid samples, as also noted in other studies.(Versnel et al., 1989)

All cell cultures were morphologically stable *in vitro*, and displayed diverse growth rates and phenotypes, including cobblestoning and spindle shapes (Figure 3.6), mirroring the inter-individual MPM tumour heterogeneity.

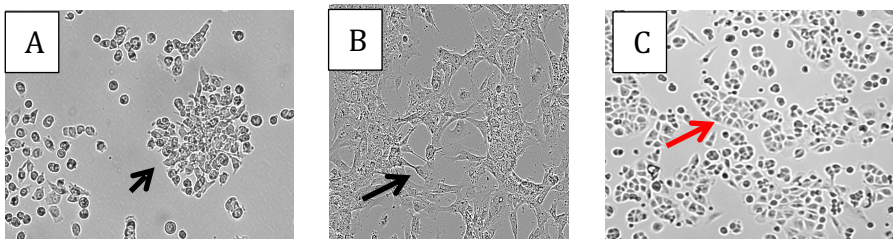


Figure 3.6 shows images at 10x magnification [ZEISS Axiocam 506 mono] of MPM cell cultures MESO174 (biphasic MPM) (A) with colony formation (arrow-head), MESO033 (epithelioid MPM) (B), with spindle-formation (black arrow) and MESO163 (epithelioid MPM) (C) with cobble-stoning (red arrow) patterns.

Cells were able to clone themselves and proliferate, and each cell culture was able to recover and re-grow following cryopreservation and thawing. The images below (Figures 3.7 and 3.8) show cells from established cell cultures proliferating within culture medium.

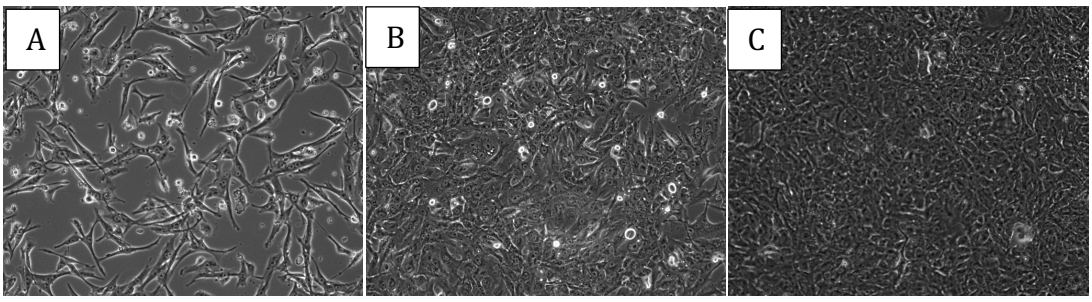


Figure 3.7 shows images of established cell culture MESO174 (biphasic MPM) at 10x magnification [ZEISS Axiocam 506 mono]: 24 (A), 96 (B) and 120 (C) hours after seeding in a cell culture plate, showing progressively increasing confluency.

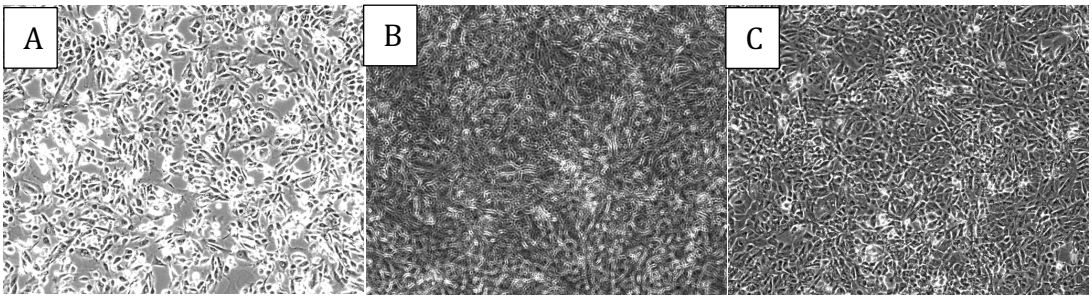


Figure 3.8 shows images of established cell culture MESO027 (epithelioid mesothelioma) at 10x magnification [ZEISS Axiocam 506 mono], 24 (A), and 96 (B) and 120 (C) hours after seeding in a cell culture plate, showing progressively increasing confluency.

The time to develop an established (all cells appearing phenotypically similar) cell culture varied greatly from one cell culture to the other, but was on average about 2 months, and passage 20 was reaching by about 4-6 months from fluid sampling.

Ability to culture cells from same patient at different time points

Importantly, two attempts were made at cell culture from the same patient at different time points. Both attempts were successful, with two MPM cell cultures (both epithelioid MPM (MESO295 and MESO033)) established from the same patient's pleural fluid at different time-points, demonstrating that establishing a personalised cell culture at different time-points is feasible, in order to potentially assess for changing variant status and treatment response/resistance.

Confirming malignant pleural mesothelioma cell culture

Four of the 5 (80%) cell cultures stained with MGG were confirmed to exhibit several hallmarks of frank malignancy. The fifth cell culture had features suspicious for malignancy. Figure 3.9 shows images and a description of the cells.

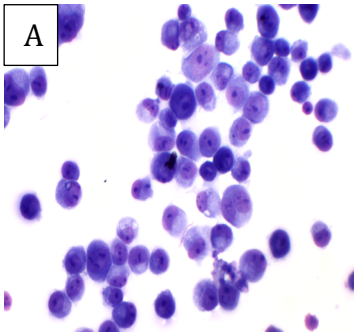


Image A shows MGG of MPM cell culture MESO174 at 10x magnification. Cells show pleomorphic nucleoli, with prominent and often multiple nucleoli compatible with a malignant epithelioid population.
Histopathologist conclusion: frankly malignant.

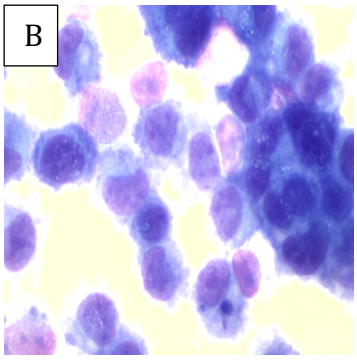


Image B shows MGG of MPM cell culture MESO024 at 40x magnification. Very cellular, quite bland uniform nuclei, small atypical nucleoli, frilly edge with two-tone cytoplasm typical of mesothelial morphology.
Histopathologist conclusion: suspicious for malignancy.

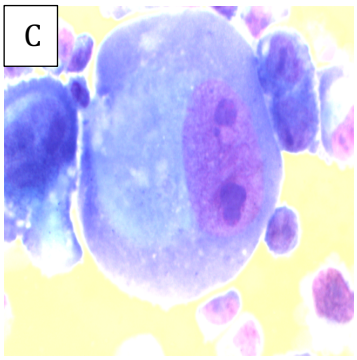


Image C shows MGG of MPM cell culture MESO27 at 40x magnification. The sample showed a pure population of mesothelial-like cells with atypical features and variability within nuclei. The image shows a bizarre cell with large nucleus and very large nucleoli.
Histopathologist conclusion: frankly malignant.

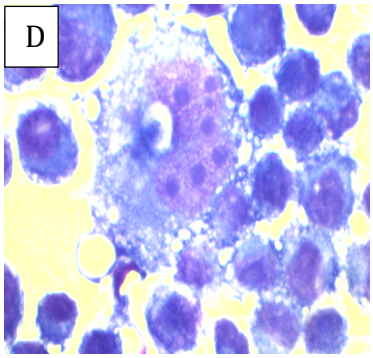


Image D shows MGG of MPM cell culture MESO033 at 40x magnification. The sample showed many small mesothelial-like cells. Image shows cell with bizarre nucleus with seven nucleoli.
Histopathologist conclusion: frankly malignant.

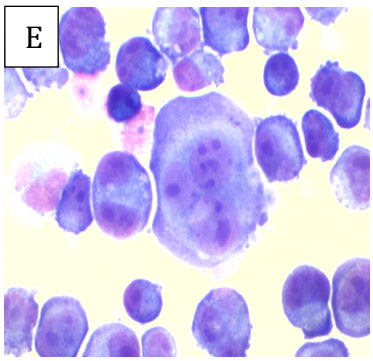


Image E shows MGG of MPM cell culture MESO163 at 40x magnification. Sample showed cells with large and multiple nuclei (binucleate cells). Image shows cells with atypical nucleoli.
Histopathologist conclusion: frankly malignant.

Figure 3.9 shows May-Grünwald-Giemsa stained MPM cell cultures and the corresponding description of the cells by histopathologist. ***MPM = malignant pleural mesothelioma, MGG = May-Grünwald-Giemsa stain***

Tumoursphere culture

Tumoursphere culture was performed in 7 MPM cell cultures, and all (7/7, 100%) the cell cultures successfully formed tumourspheres, indicating the existence of a tumour stem-cell subpopulation. Images were taken on day 6 after seeding the cells. As discussed earlier, tumourspheres can be differentiated from aggregates of cells by the fact that in tumourspheres the cells appear to become fused with each other, as shown in the images below (Figure 3.10), whereas in cell aggregates the individual cells are identifiable.(Johnson et al., 2013)

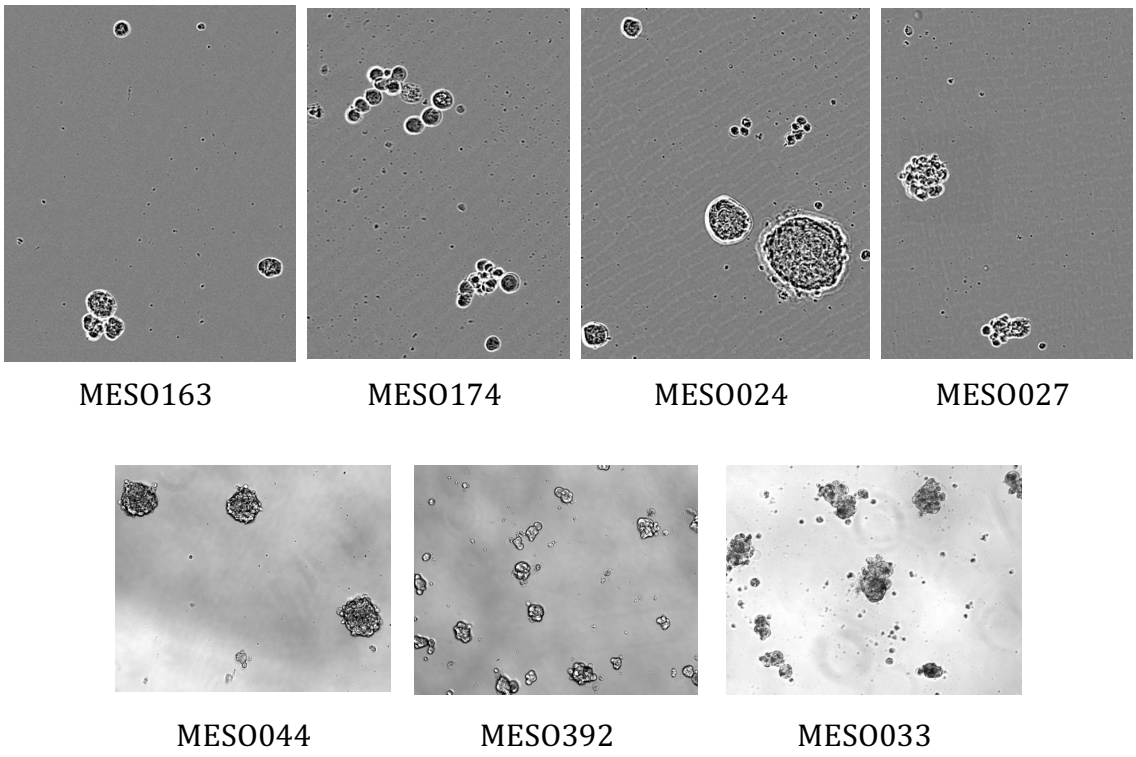


Figure 3.10 show established MPM cell cultures on day 4 after seeding for the tumoursphere assay. The images were taken by IncuCyte® at 10x magnification.

Deoxyribonucleic acid sequencing comparing early *vs* late passage cell cultures

Comparison of early and later passage cell cultures was intended to show that later stage passage and early passage cells did not show significant variability. This was additional characterisation of the cell cultures, but was also important for the later part of the study when the feasibility of a bench-to-bedside pathway was assessed: the personalised cell cultures should not be significantly different from the patient's native tumour if they are to be used as tools to create a drug response/resistance profile for the patient.

Quality assessment

All samples showed high molecular weight band (characterises high quality DNA),(Healey et al., 2014) as shown in Figure 3.11. Although some degradation was seen in the samples, all samples were deemed passable for library preparation.

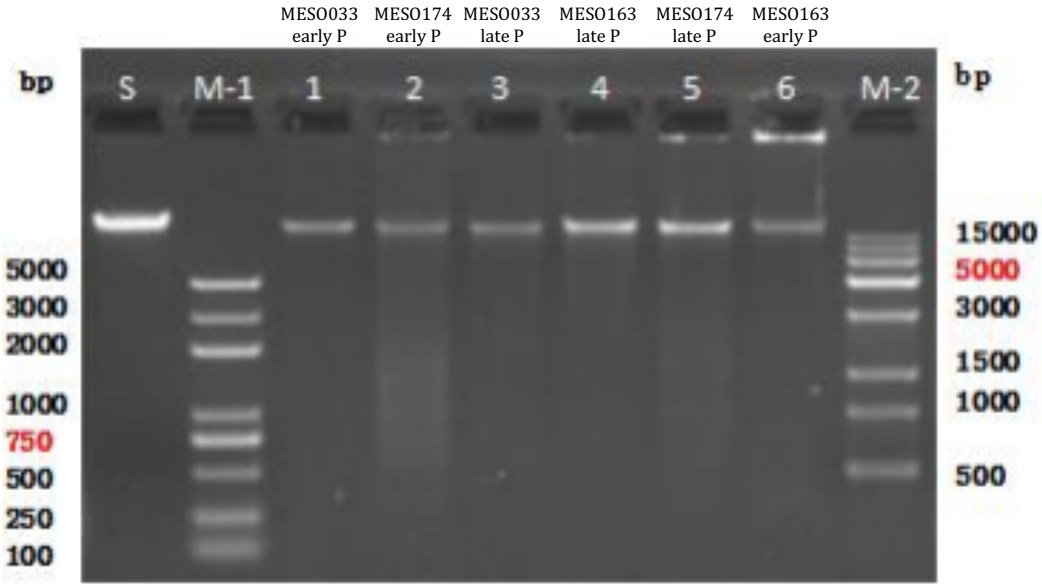


Figure 3.11 showing gel electrophoresis results for the DNA extracted from the MPM cell cultures, where the fluorescence yield reflects the total mass of DNA. The image shows that all cell cultures tested (lanes 1-6) showed high molecular weight band. ***MPM cell cultures: MESO033 (epithelioid MPM), MESO174 (biphasic MPM), MESO163 (epithelioid MPM). M=molecular weight marker; P=passage; S=the positive marker used during DNA sequencing, i.e. a DNA segment of a known size.***

The sequencing data quality was assessed by the Q score. The Q score reflects the probability of incorrect base call: a Q score of 30 (Q30) is equivalent to a base call accuracy of 99.9%, and a Q score of 20 (Q20) is equivalent to base call accuracy of 99%.(Ewing and Green, 1998) Therefore with Q30 sequencing, quality is high enough to confirm that virtually all reads are correct, and is considered to be the benchmark for quality in next-generation sequencing. With HiSeq® systems, nearly all bases have scores >Q30 for paired-end reads (where both ends of fragment are sequenced).(Technical note: Sequencing. Quality scores for next-generation sequencing, Illumina®; available online at https://www.illumina.com/documents/products/technotes/technote_Q-Scores.pdf. Accessed on 6th May 2019) In fact, the Q20 high-quality score for the data ranged from 95.8% to 97.4% and the Q30 score ranged from 90.7% to 94.0%. The optimal GC content (percentage of bases that are guanine-cytosine) for stable bonding of primer with template is 40-60%, and in this study ranged from 40.3% to 41.8% (Table 3.5).

Table 3.5 showing the results of the quality assessment for whole genome sequencing, showing the Q20 and Q30 scores and the GC content for each cell culture sequenced. ***Early = passage 0, late = passage 20, PE = paired-end reads. MPM cell cultures: MESO033 (epithelioid MPM), MESO174 (biphasic MPM), MESO163 (epithelioid MPM).***

Cell culture	PE reads	Q20, %	Q30, %	GC, %	Mapped, %
MESO033, early	311863957	95.98	90.88	41.08	99.87
MESO033, late	339113217	96.39	91.77	40.25	99.86
MESO174, early	334961241	96.57	92.02	40.89	99.87
MESO174, late	334160400	97.44	94.03	40.8	99.7
MESO163, early	365733648	96.58	92.22	41.75	99.79
MESO163, late	350738067	95.81	90.67	40.61	99.85

A summary of detected SNPs, DNA sequence variations where a single nucleotide (adenine, thymine, cytosine, guanine) differs, is shown in Table 3.6.

Table 3.6 showing the single nucleotide polymorphism (SNP) detection. ***CDS=coding sequence; synonymous SNP=a single nucleotide change that does not change the protein sequence; missense SNP=a single nucleotide change that results in a code for a different amino acid; UTR=untranslatable region (in 3' or 5' untranslated regions); ncRNA=non-coding RNA, RNA that is not translated into a protein. MPM cell cultures: MESO033 (epithelioid MPM), MESO174 (biphasic MPM), MESO163 (epithelioid MPM).***

	MESO033, early	**MESO033, late**	**MESO174, early**	**MESO174, late**	**MESO163, early**	**MESO163, late**
CDS	21926	21346	22402	40174	22962	23390
Synonymous SNP	11326	10876	11761	27007	11551	11788
Missense SNP	10121	9976	10129	12486	10802	11019
Stopgain	73	69	83	88	126	126
Stoploss	9	9	13	19	13	13
Unknown	405	425	421	583	478	450
Intronic	1172153	1163733	1217533	1219175	1233704	1235818
UTR3	23589	23337	23816	26265	24545	24679
UTR5	5031	4979	5148	6002	5267	5246
Splicing	64	69	70	84	81	77
ncRNA exonic	8805	8775	9169	9686	9237	9246
ncRNA intronic	137470	136129	140653	141104	141562	141849
ncRNA splicing	51	48	56	54	51	50
Upstream	20623	20408	21006	20991	21756	21654
Downstream	21218	21126	21838	21825	22287	22228
Intergenic	2027027	2011751	2093218	2097878	2157377	2160036
Total	3438720	3412459	3555620	3583931	3639524	3644967

Assessment of new variants in late compared with early passage cell culture

The variants detected by the whole genome sequencing analysis were predominately heterozygous (a variant in only one allele): 55% were heterozygous with one normal variant, 2% heterozygous variants with both variants mutated. The remaining 43% were homozygous (with an identical mutation in both the maternal and paternal alleles) variants (Figure 3.12).

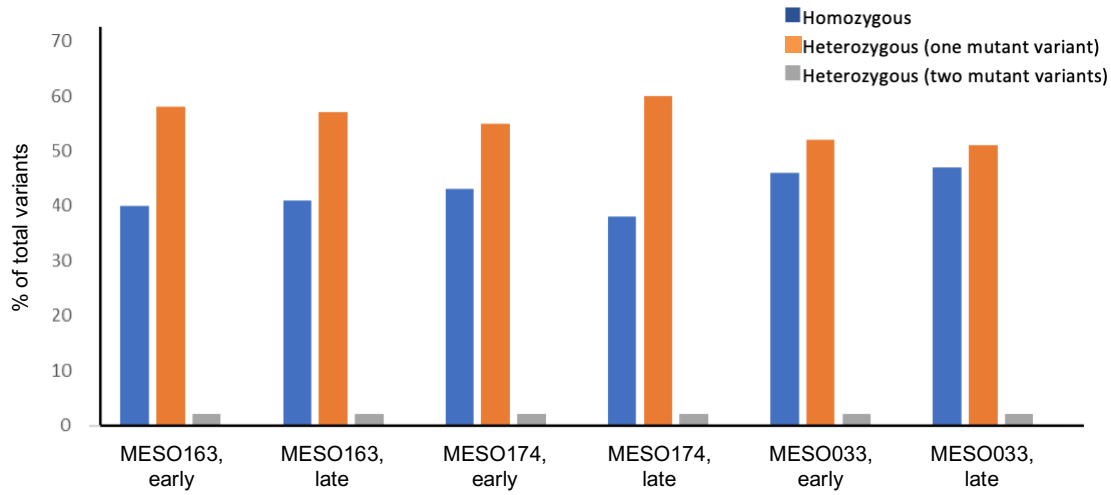


Figure 3.12 shows the results of whole genome sequencing analysis, comparing early with late passage cell cultures, divided according to variants that were heterozygous with one mutant variant, homozygous, and heterozygous where both variants were mutant.

There was a somatic variant rate of about 3 non-synonymous (leading to a change in the encoded amino acid) variants per megabase for all the sequenced cell cultures, similar to that reported in previous studies that have classified MPM as being on the lower end of the somatic burden spectrum among malignancies.(Hmeljak et al., 2018) However, the late passage MESO174 cells exhibited an increased burden of synonymous (do not change the encoded amino acid) variants (figure 3.13). The point variants were predominately (68%) transitions (a point variant leading to a change from adenine to guanine (or vice versa), i.e. purine nucleotides, or a change from cytosine to thymine (or vice versa), i.e. pyrimidine nucleotides) (Figure 3.14).

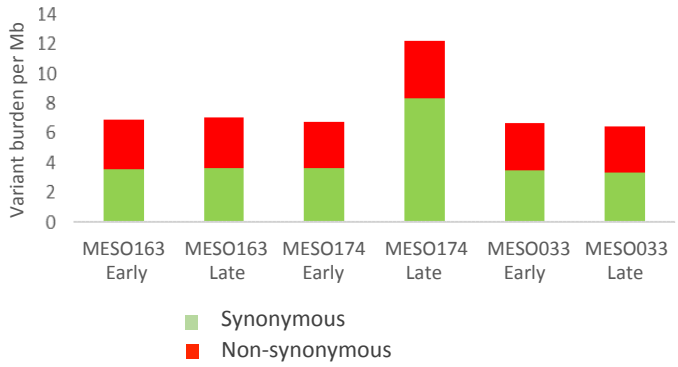


Figure 3.13 Barplot showing the synonymous and non-synonymous variant rates per megabase for the patient-derived MPM cell culture sequencing at two timepoints (early, passage 0; late, passage 20). Late passage MESO174 exhibited increased burden of synonymous mutations (about 8 mutations per megabase) while the other cell cultures showed about 3 variants per megabase for synonymous and non-synonymous variants.

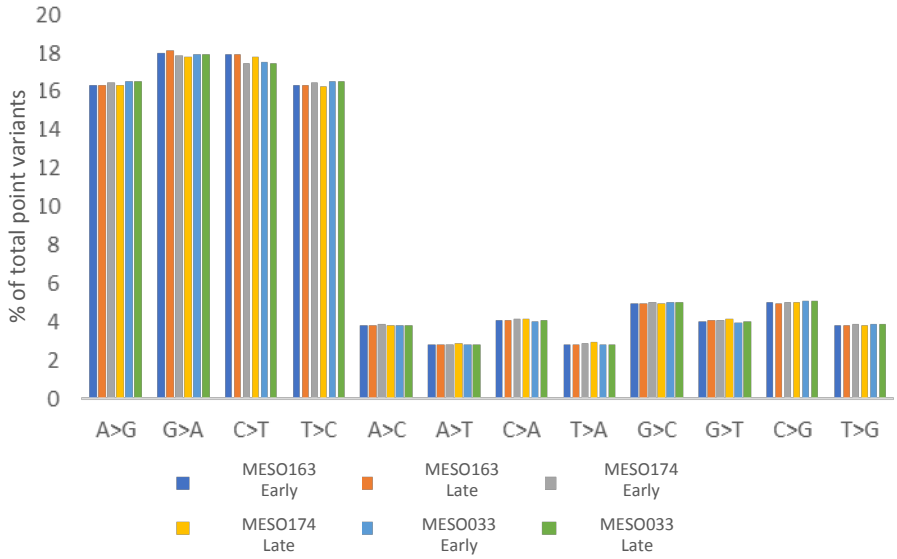


Figure 3.14 Barplots showing the frequency of transition point mutations (A>G, G>A, C>T, T>C) and the less frequent transversion point mutations (A>C, A>T, C>A, T>A, G>C, G>T, C>G, T>G).

There was inter-patient heterogeneity of the variant landscape for the genes known to be MPM-associated tumour suppressor genes: *TRP53*, *NF2*, *BAP1*, *LATS2*, *SETD2* and *CDKN2A* (Figure 3.15).(Hmeljak et al., 2018) This highlights the potential importance of personalised and targeted therapies in MPM.

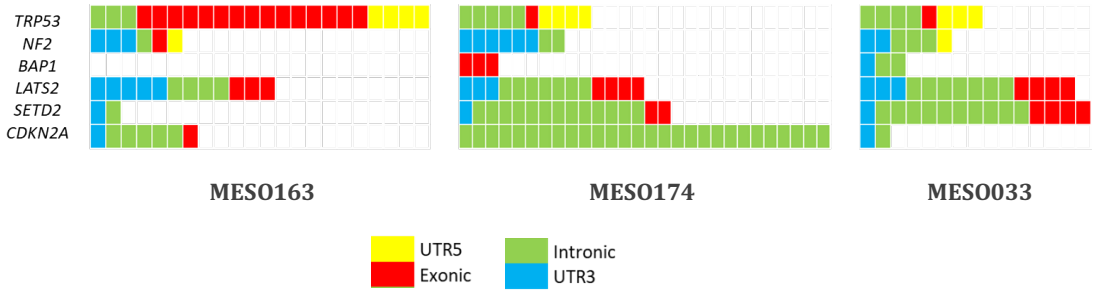


Figure 3.15 Graph showing the functional genomic region for each variant detected in late passage cell cultures for the MPM-related genes: *TRP53, NF2, BAP1, LATS2, SETD2, CDKN2A*. The colours represent different genomic regions. *UTR = untranslatable region.*

The tumour suppressor genes exhibited non-synonymous, stop codon gain, and frameshift insertion variants (Figure 3.16). These findings are in line with previously published data of integrated genomic studies on MPM tumour samples,(Hmeljak et al., 2018) and therefore indicate a resemblance at the molecular level between the patient-derived cell cultures and MPM tumours.

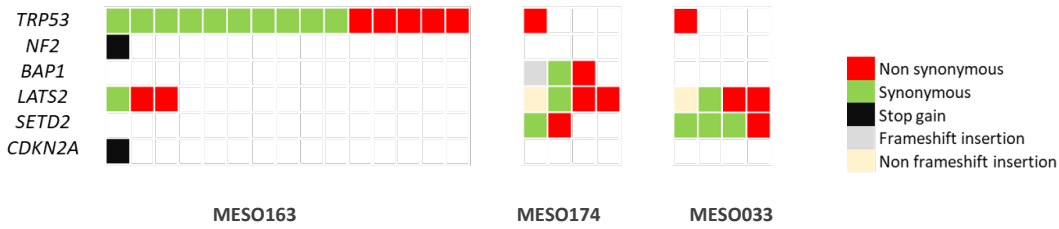


Figure 3.16 Graph showing the exonic variant function for the variants detected in the MPM-related genes: *TRP53, NF2, BAP1, LATS2, SETD2, CDKN2A.* The colours represent different functional consequences of the point mutation.

To identify variants that were due to serial long-term passaging, early and late passage timepoints were compared for each cell culture. As discussed, to focus on the driving variants of cancer-related genes, the FDA-approved panel of gene variants for tumour profiling, MSK-IMPACT (*n*=468) was used.(Cheng et al., 2015) Table 3.7 shows the exonic variants, including non-synonymous SNVs, stop-gain, and frameshift variants, from the MSK-IMPACT panel of gene variants, detected in late passage MPM cell cultures that were not present in the corresponding early passage cell cultures. Variants resulting as 'Tolerable' (T) by both Radial SVM pred and LR pred were excluded from the final assessment. Variants in the human leucocyte antigen (HLA-A and HLA-B) system (*n*=7 in MESO033, *n*=6 in MESO174, *n*=20 in MESO163) were all tolerable by both Radial SVM pred and LR pred.

Table 3.7 shows the variants present exclusively in the late passage cell cultures, predicted to be pathogenic. ***CLNSIG=clinical significance; CLNREVSTAT=review status; D=deleterious; LR=logistic regression; SNV=single nucleotide variant; SVM=support vector machine; T=tolerable.***

MPM cell culture	Chromosome	Gene	Radial SVM score	Radial SVM pred	LR score	LR pred	CLNSIG	CLNREVSTAT
MESO033	3	MLH1	-0.647	T	0.293	T	Pathogenic	Reviewed by expert panel
	20	GNAS	0.786	D	0.831	D	NA	NA
MESO174	3	GATA2	0.525	D	0.853	D	NA	NA
	3	GATA2	0.504	D	0.84	D	NA	NA
	3	GATA2	0.06	D	0.771	D	NA	NA
	3	GATA2	-0.27	T	0.676	D	NA	NA
	3	GATA2	-0.314	T	0.543	D	NA	NA
	3	GATA2	0.347	D	0.782	D	NA	NA
	7	IKZF1	-0.707	T	0.606	D	NA	NA
	12	CDKN1B	-0.329	T	0.573	D	NA	NA
	19	TCF3	-0.236	T	0.728	D	NA	NA
	19	TCF3	0.436	D	0.855	D	NA	NA
	19	KEAP1	0.486	D	0.695	D	NA	NA
	19	JAK3	0.768	D	0.889	D	NA	NA
	19	JAK3	-0.019	T	0.549	D	NA	NA
	20	GNAS	0.228	D	0.675	D	NA	NA
MESO163	1	RRAGC	0.34	D	0.588	D	NA	NA
	5	MAP3K1	0.435	D	0.597	D	NA	NA
	6	ROS1	0.108	D	0.609	D	NA	NA
	14	FOXA1	0.077	D	0.646	D	NA	NA
	14	DICER1	0.723	D	0.816	D	NA	NA
	17	TP53	0.964	D	0.993	D	Conflicting interpretations of pathogenicity	Conflicting interpretations of pathogenicity
	19	KEAP1	-0.261	T	0.512	D	NA	NA

A limited number of non-synonymous and frameshift insertion variants were detected in late passage (passage 20) cell cultures, therefore for experimental assays, cells that were cultured for less than 20 passages were used, as also suggested by Pollard et al.(Pollard et al., 2009)

3.3 Investigating the biological properties of pleural fluid: *in vitro* experiments

3.3.1 Literature review

Background

As discussed earlier, there is some early pre-clinical evidence of the potential biological properties of MPE fluid with Cheah et al. reporting that MPM cells from cancer cell lines exposed to 30% MPM MPE fluid (in culture medium) had increased cell viability when compared to cancer cells exposed to serum-free medium and to medium with foetal calf serum.(Cheah et al., 2017) Although this data provides early evidence of the potential biological properties of pleural fluid, a culture medium of 30% MPE fluid does not necessarily reflect the *in vivo* pleural effusion environment. Furthermore, culture medium that is manufactured specifically to be optimal for *in vitro* cell growth, in terms of nutrients and pH, was used as a control for the experiment. A control that more closely mimics the natural environment of tumour cells in the human body, rather than the artificial specifically formulated culture medium, is more ideal although difficult to achieve *in vitro*. Nevertheless, the study by Cheah et al. is an important first step, fuelling discussion and the designing of further experiments into the biological capabilities of pleural fluid, as if proven to exist, this finding may lead to a shift in management of MPE from only symptom-driven drainage to active therapeutic management even in asymptomatic cases, in an attempt to reduce the chance of disease progression and increase the chance of chemotherapy response.

Although Cheah et al. reported that proliferation of the cancer cells did not differ significantly between cancer cells exposed to matched pleural fluid from the same patient the cell line was derived from and those exposed to unmatched pleural fluid, using matched pleural fluid may potentially reflect what would happen in that same patient in real-life, especially in terms of chemotherapy resistance. In addition, having access to the clinical data of the patient would make it possible to correlate experimental laboratory data with clinical disease progression or regression, and with chemotherapy response or resistance *in vivo*. This is interesting because personalised cancer cell cultures can potentially be used for more than just *in vitro* experiments exploring the properties of pleural fluid, but with the added possibility of using the cancer cell cultures as a tool to guide clinical management of the patient, potentially bridging some of the *in vitro* to *in vivo* gap.

3.3.2 Results of this study

Cells from established MPM cell cultures proliferate *in vitro* in MPE fluid alone

In the initial experiment where MPM cells were seeded in MPM MPE fluid alone in a 6-well plate, cells were observed to proliferate and increase confluence under a microscope. A selection of the serial images of the cells is shown below (Figure 3.17).

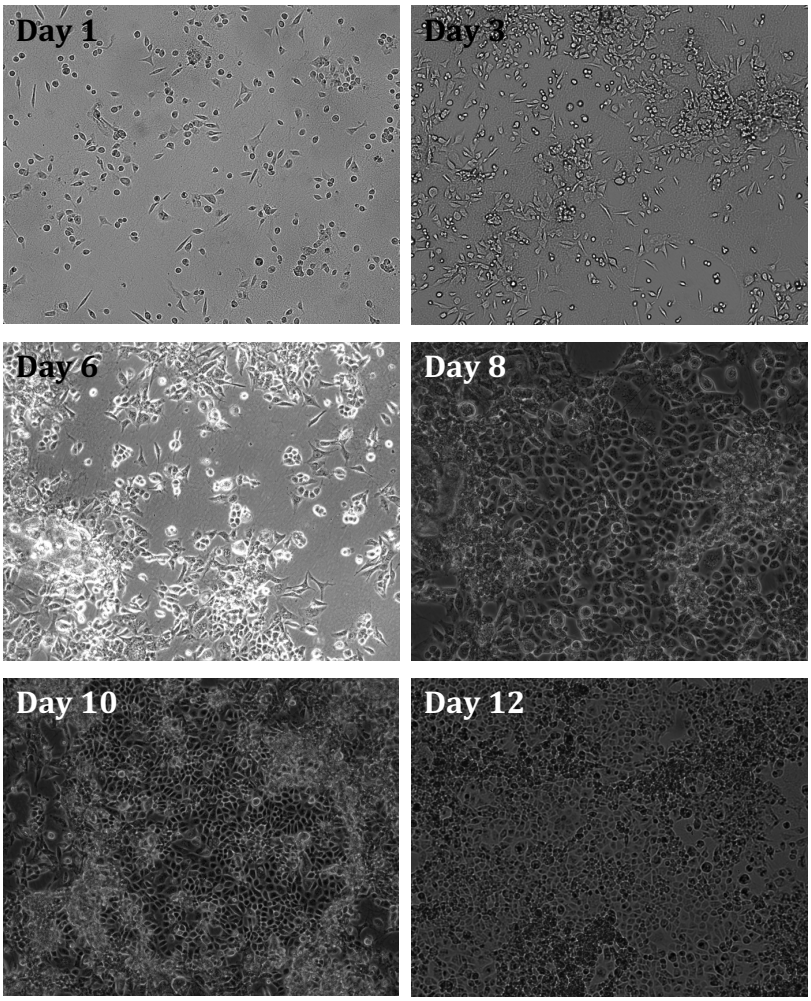


Figure 3.17 shows images taken of MPM cell culture MESO163 seeded in 100% exudate MPM MPE fluid only, at 100,000 cells per well in a 6-well plate. The images were taken on days 1-12 after seeding, and were taken at 10x magnification [ZEISS Axiocam 506 mono]. The cells show increased levels of confluency with time. *MPE=malignant pleural effusion, MPM=malignant pleural mesothelioma.*

During this experiment, the pleural fluid was noted to form a gel contributing to the slightly hazy images seen above. The gel was left within the wells so as to retain any cells that were proliferating within it, and only the fluid itself was aspirated carefully after tilting the plate to one side, as shown in the images below (Figure 3.18), and replaced every 48 hours.

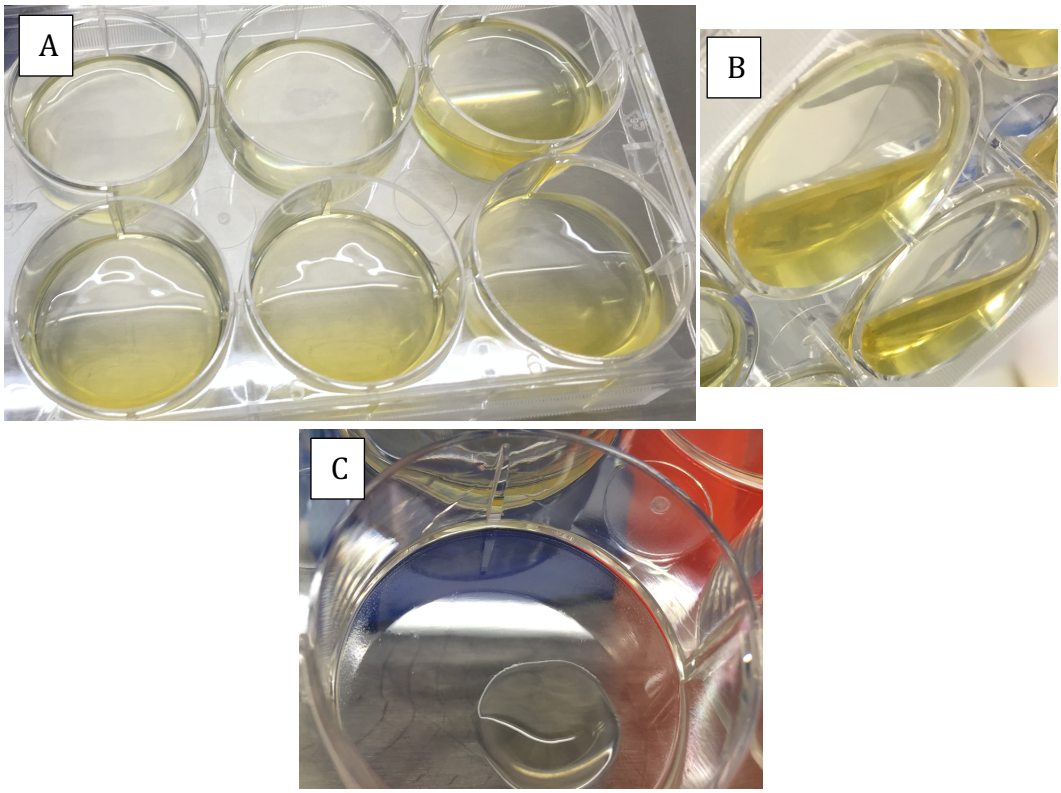


Figure 3.18 shows images show wells within the 6-well plate, containing MPM cells seeded within exudate MPM MPE fluid. Tilting the plate reveals the layer of gelified pleural fluid at the bottom of the wells. Image A: the wells as viewed from above; image B: the wells as viewed from below the plate; image C: a drop of pleural fluid that has adopted a gel-like consistency within the well. ***MPE=malignant pleural effusion, MPM=malignant pleural mesothelioma.***

Crystal violet was used in an attempt to demonstrate the cell proliferation with stains. However, given that the fluid was not all removed because it had adopted a gel-like consistency, and that the remaining pleural fluid contained protein and some cells within in, the stain did not demonstrate the cells clearly against a clear background, as shown in the images below (Figure 3.19).

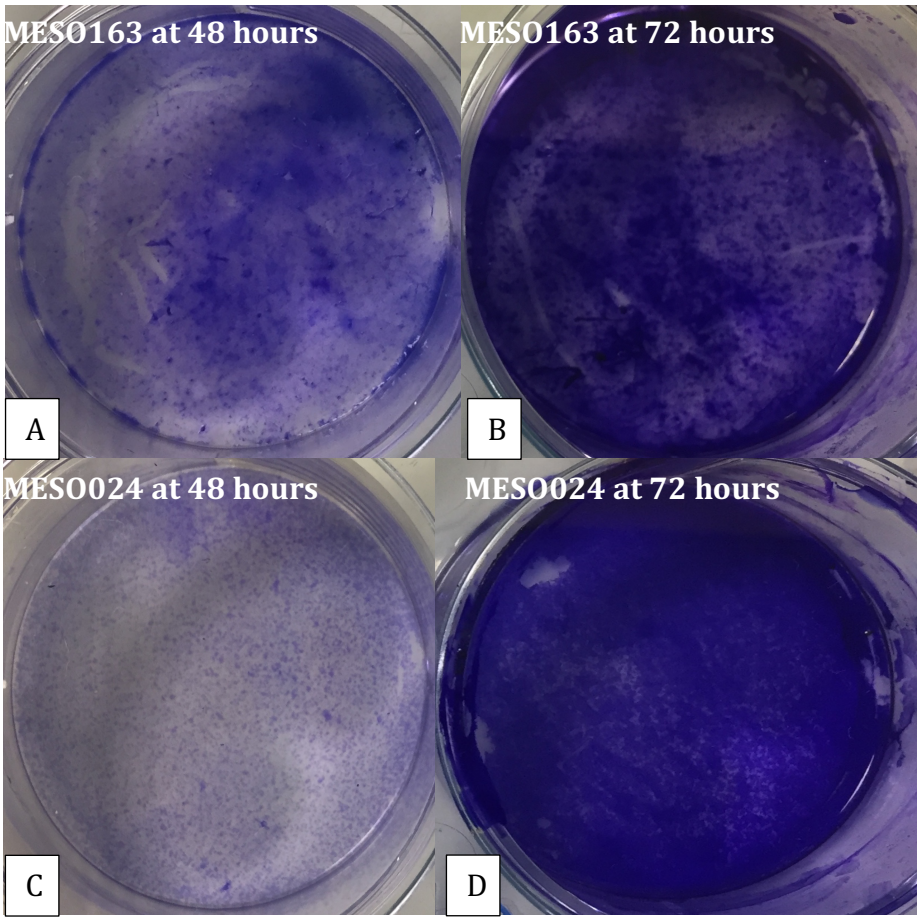


Figure 3.19 shows images show the results of the crystal violet stain of MPM cell culture MESO163 (A and B) and MESO024 (C and D) in a 6-well plate, at 48 (A and C) and 72 (B and D) hours after seeding of the cells in the exudate MPM MPE fluid. MPE=malignant pleural effusion, *MPM=malignant pleural mesothelioma*.

An attempt to denature some of the proteins was made by heat-inactivating the pleural fluid (by heating the fluid to 56°C for 30 minutes) (Protocol for Heat-Inactivation of Serum and Plasma Samples. Montefiori Laboratory Duke University, August 2018. Available online https://www.hiv.lanl.gov/content/nab-reference-strains/html/Protocol-for-Heat-Inactivation-of-Serum-and-Plasma-Samples_Aug-2018.pdf, accessed 3rd July 2019) however no difference was seen in the tendency to form a gel-like consistency after heat-inactivation.

On continued culture of the MPM cells within MPE fluid, the cells were observed to form aggregates and nodules. In addition, the cells were clearly more abundant within the area of the gelified MPE fluid in the well, indicating that the cells were thriving within the gelified MPE fluid and removal of the gel would have discarded several of the MPM cells. This, as well as the aggregates and nodules, are shown in the images below (Figure 3.20).

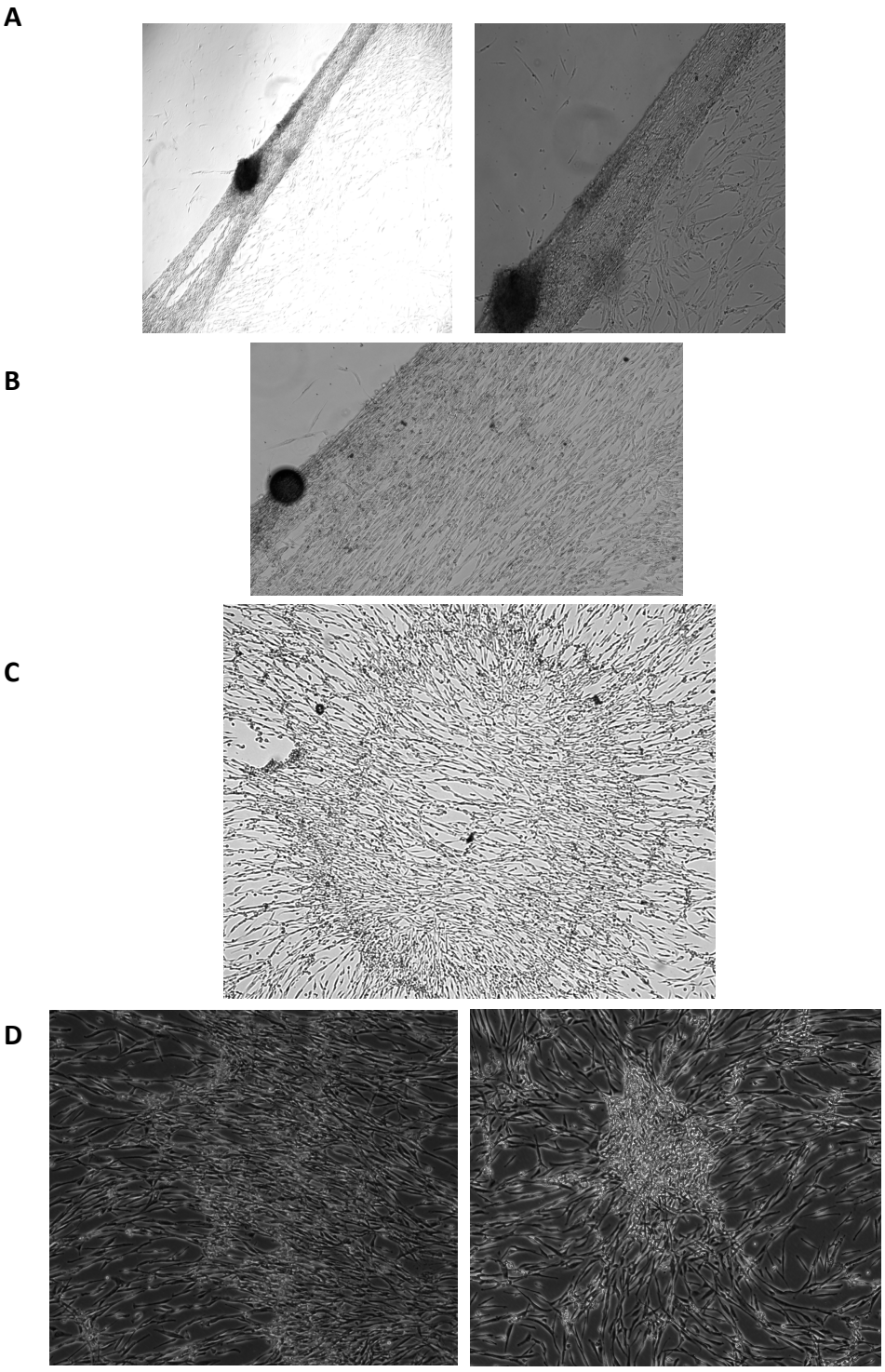


Figure 3.20 shows images of MPM cell culture MESO027 (epithelioid), seeded in exudate MPE fluid at 50,000 cells per well, in a 6-well plate. A **Day 15 after seeding of cells (left: at 5x magnification) (right: at 10x magnification): cells have formed nodule-like aggregates with interconnecting strands.** B **Day 16 after seeding of cells (at 10x magnification): nodule-like aggregate with numerous cells growing within the area where the MPE fluid has formed a gel with a clear demarcation at the edge of the gel beyond which there are fewer cells (top left corner of image).** C **Day 18 after seeding of cells (at x5 magnification) and** D **Day 19 after seeding of cells (at 10x magnification): the cells have aggregated in clusters.**

Cells from malignant pleural mesothelioma cell cultures proliferate *in vitro* in exudate and transudate malignant pleural effusion pleural fluid, as well as in heart failure transudate pleural fluid

Comparison of MPM cells' growth in exudative MPM MPE fluid and transudative MPE fluid showed similar growth rates, as shown in the graphs below (Figure 3.21).

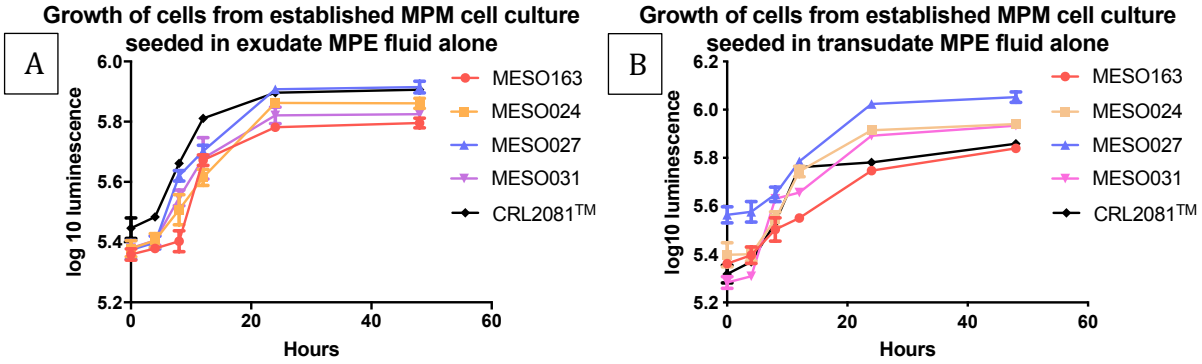


Figure 3.21 shows the growth curves, with mean and 95% confidence intervals shown for each time point, that were obtained after 20,000 MPM cells per well (96-well plate) were seeded in starvation medium for 12 hours, then starvation medium was replaced with exudate MPM MPE fluid (A) and transudate MPE fluid from a patient with lung adenocarcinoma MPE (B). Cell viability was measured at 4, 8, 12, 24 and 48 hours using CellTiter-Glo®. There was no statistically significant difference between the different growth curves in each graph. *Cell cultures: MESO163 (epithelioid MPM), MESO024 (biphasic MPM), MESO027 (epithelioid MPM), MESO031 (epithelioid MPM); CRL2081(MSTO-211H)™ - a well-characterised, commercially available biphasic MPM cell line (derived from human MPE fluid) used as a control.*

The same experiment was repeated, this time also using heart failure (non-MPE) transudate pleural fluid, and cells were incubated in an Incucyte® machine. The percentage confluence was calculated from images of the cells using Fiji (ImageJ) version 2.0.(Schindelin et al., 2012) The resulting growth curves (Figure 3.22) and images of the cells within the wells are shown below (Figures 3.23-3.25). For cell cultures MESO163 and MESO027, there was decreased proliferation with transudate MPE and with non-MPE heart failure transudate respectively, when compared to the proliferation with other types of

pleural fluid tested. However there was no clear decreased proliferation with any one pleural fluid type across all cell cultures tested.

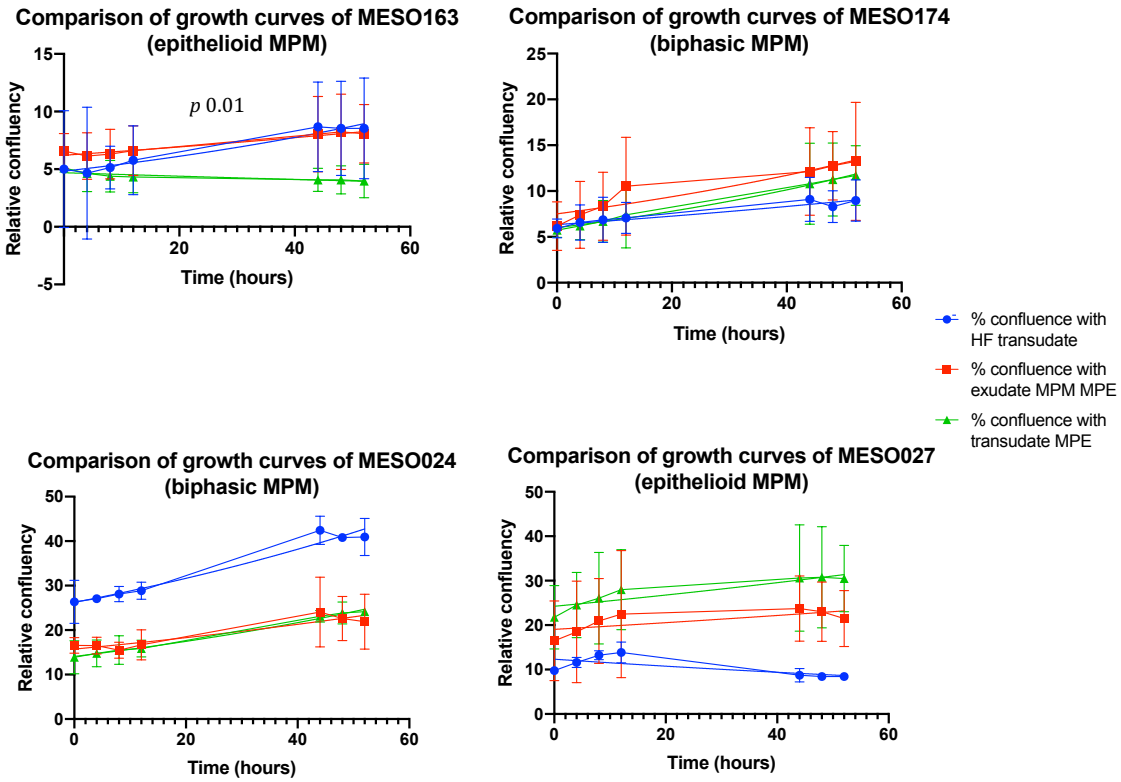


Figure 3.22 shows the growth curves for cells from MPM cell cultures *in vitro*, seeded directly in pleural fluid. The curves show the mean and 95% confidence intervals. There was no statistically significant difference between the growth curves obtained for MESO174, MESO024, MESO027 (*p* 0.1, 0.2, 0.07 respectively). *HF=heart failure, MPE=malignant pleural effusion, MPM=malignant pleural mesothelioma.*

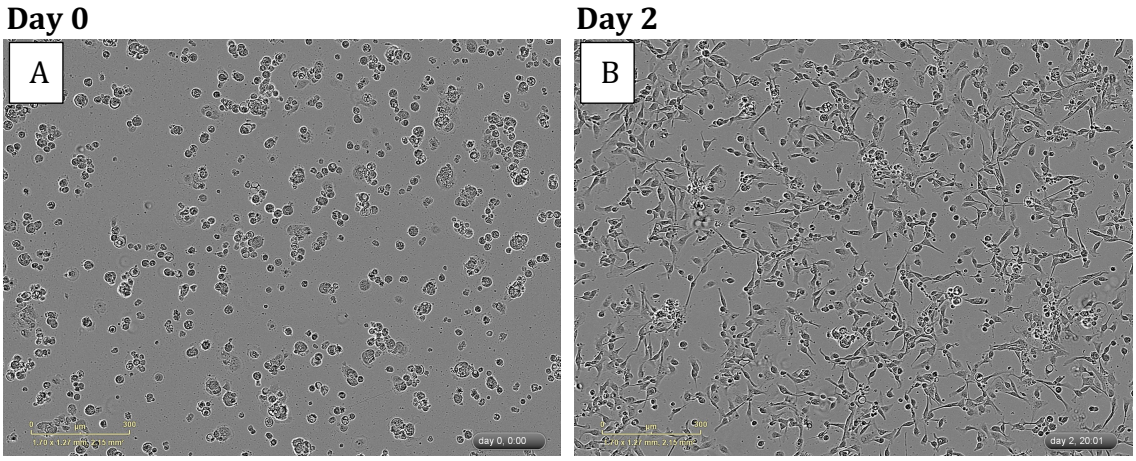


Figure 3.23 shows images of MESO024 (biphasic MPM). Image A: cells after having been seeded in starvation medium for 6 hours and the medium was changed to exudate MPM MPE fluid. Image B: image shows the cells on day 2 after seeding, and there has been proliferation of the cells since they were seeded as shown by the increased confluency. *MPE=malignant pleural effusion, MPM=malignant pleural mesothelioma*.

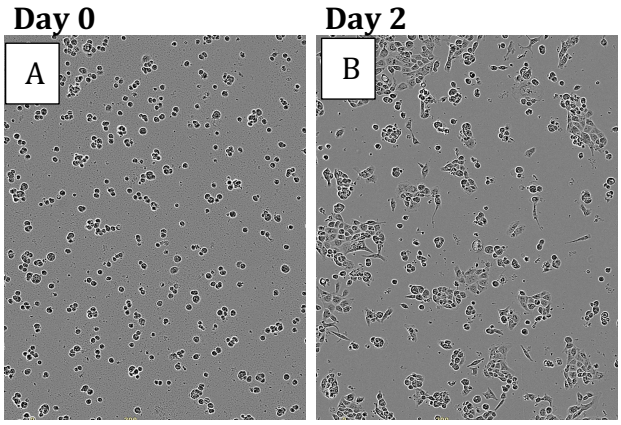


Figure 3.24 shows images of MESO163 (epithelioid MPM) after having been seeded directly in exudate MPM MPE fluid (image A), and the same cell culture 2 days after seeding, showing proliferation of cells (image B). *MPE=malignant pleural effusion, MPM=malignant pleural mesothelioma*.

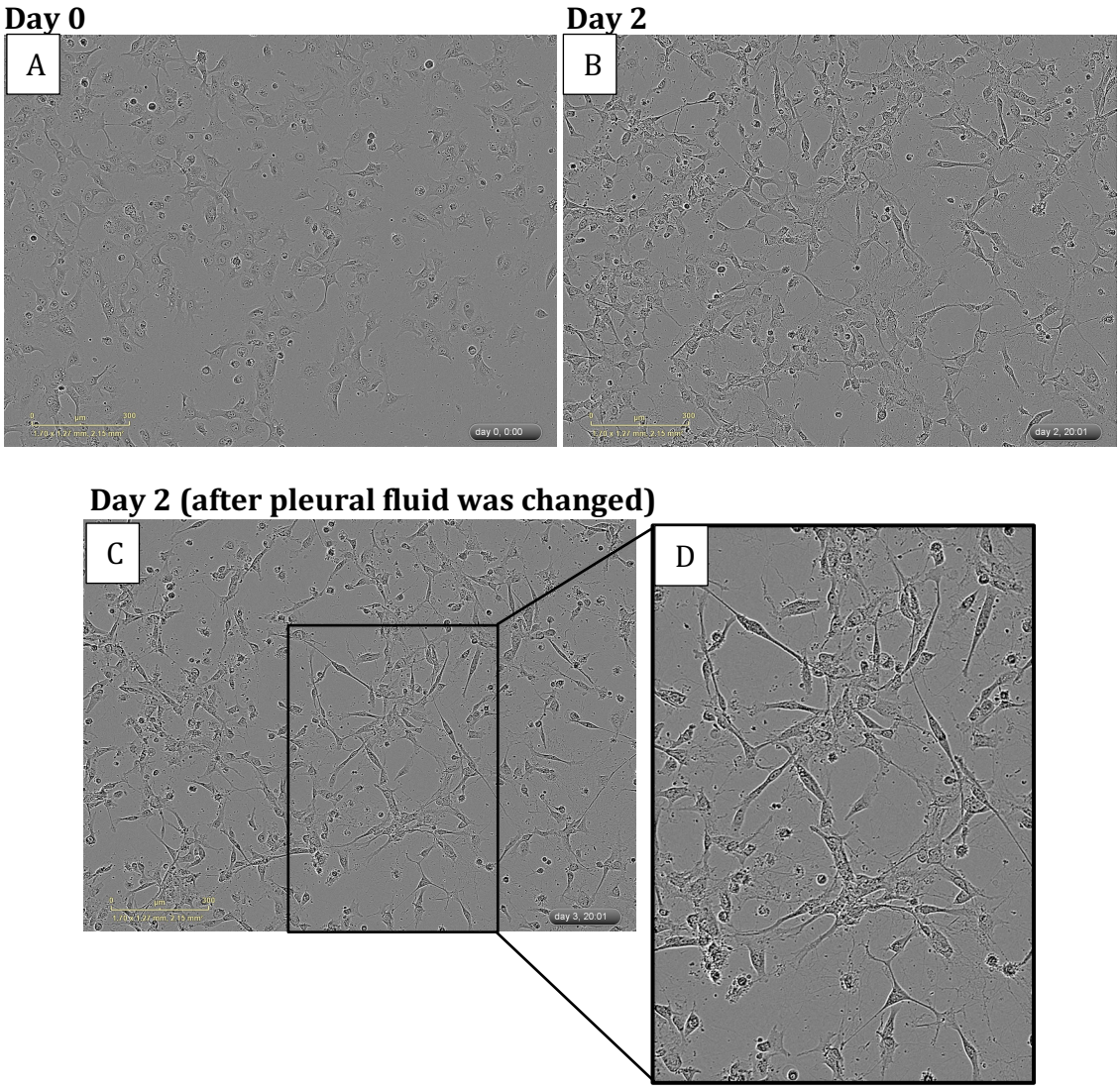


Figure 3.25 shows images of MESO174 (biphasic MPM) after having been seeded directly in exudate MPM MPE fluid (A), and on day 2 after seeding, showing proliferation of the cells (B). Image C shows the same cell culture after the pleural fluid has been changed – the cells appear sharper in this image because in the previous images the pleural fluid had begun to adopt a more gel-like consistency causing the cells to appear to have hazy outlines in the images. Upon changing the pleural fluid, the images had better quality, with the cells appearing to have sharper outlines in the images, however, image C also shows a reduced number of cells when compared to the previous image (image B) indicating that a number of cells were lost with each pleural fluid change. Image D marks the area that has been enlarged in the image on the bottom right, showing the active cells sending out protrusions to migrate or to communicate with each other, revealing that the cells were very much alive and active despite having had only pleural fluid as their culture medium for several consecutive days. *MPE=malignant pleural effusion, MPM=malignant pleural mesothelioma.*

Cells from established breast carcinoma and lung adenocarcinoma cell cultures also proliferate *in vitro* in pleural fluid

The latter experiment was repeated with non-MPM cancer cell cultures in order to explore whether the capability of cancer cell proliferation *in vitro* with 100% pleural fluid is unique to MPM cell culture or not. Both breast and lung adenocarcinoma cells proliferated *in vitro* in 100% pleural fluid alone, and the growth curves are shown in Figure 3.26.

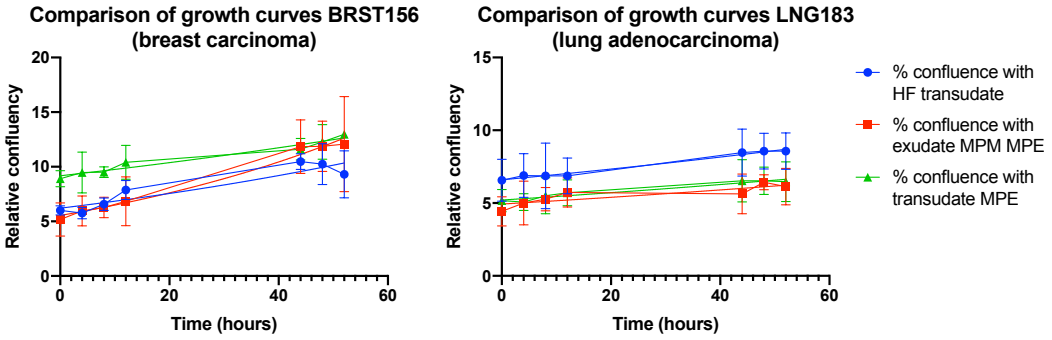


Figure 3.26 shows the growth curves for non-malignant pleural mesothelioma cell cultures *in vitro*, seeded directly in pleural fluid. The curves show the mean and 95% confidence intervals. There was no statistically significant difference between the growth curves obtained for BRST156 and LNG183 (p 0.1 and 0.07 respectively). *HF=heart failure, MPE=malignant pleural effusion.*

Doubling times for cells from cancer cell cultures seeded *in vitro* in pleural fluid

The doubling times for the MPM and non-MPM cancer cell cultures were calculated and are shown in Table 3.8. Cells proliferated even when seeded directly in pleural fluid, i.e. in the absence of a preceding period of time in starvation medium, indicating that pleural fluid alone was adequate for the cells to adhere to the plate and proliferate.

Table 3.8 showing the mean of the fastest doubling times as representative of the doubling time during log phase, for MPM and non-MPM cancer cell cultures, seeded directly in pleural fluid. ***Cell cultures: MESO163 (epithelioid MPM), MESO174 (biphasic MPM), MESO024 (biphasic MPM), MESO027 (epithelioid MPM), BRST156 (breast carcinoma), LNG183 (lung adenocarcinoma). MPE=malignant pleural effusion, MPM=malignant pleural mesothelioma.***

Mean fastest doubling times during exponential growth (log-phase)			
Cell culture	**Cells seeded directly in the pleural fluid**		
	Heart failure transudate pleural fluid	**Transudate MPE fluid**	**Exudate MPM MPE fluid**
MESO163	25.4	-81.8	59.6
MESO174	76	31.1	15.1
MESO024	57.6	58.7	37.1
MESO027	21.1	45.7	22.1
BRST156	17.6	30.6	19.3
LNG183	125.6	95.7	92.2

A comparison was made between the doubling times for each cell culture seeded directly in pleural fluid, and the results are shown in Table 3.9 below. There was no statistically significant increased cell proliferation with one type of pleural fluid than with another across all the cell cultures used in this experiment.

Table 3.9 shows a comparison between the doubling times obtained with the cancer cell cultures in three different types of pleural fluid. ***HF=heart failure, MPE=malignant pleural effusion.***

	Doubling time	*p* values	Kruskal-Wallis statistic
MESO163		**0.01**	6.3
Exudate MPE fluid (E)	59.6	**E *vs* T 0.04**	
Transudate MPE (T)	-81.8	T *vs* HF 0.4	
HF transudate fluid (HF)	25.4	HF *vs* E 0.8	
MESO174		0.1	4.9
Exudate MPE fluid (E)	15.1	E *vs* T 0.2	
Transudate MPE (T)	31.1	T *vs* HF 1.0	
HF transudate fluid (HF)	76	HF *vs* E 0.2	
MESO024		0.2	3.7
Exudate MPE fluid (E)	37.1	E *vs* T 1.0	
Transudate MPE (T)	58.7	T *vs* HF 0.5	
HF transudate fluid (HF)	57.6	HF *vs* E 0.2	
MESO027		0.07	4.6
Exudate MPE fluid (E)	22.1	E *vs* T 0.9	
Transudate MPE (T)	45.7	T *vs* HF 0.1	
HF transudate fluid (HF)	21.1	HF *vs* E 0.9	
BRST156		0.1	4.5
Exudate MPE fluid (E)	19.3	E *vs* T 0.1	
Transudate MPE (T)	30.6	T *vs* HF 0.5	
HF transudate fluid (HF)	17.6	HF *vs* E 1.0	
LNG183		0.07	5.4
Exudate MPE fluid (E)	92.2	E vs T 1.0	
Transudate MPE (T)	95.7	T *vs* HF 0.2	
HF transudate fluid (HF)	125.6	HF *vs* E 0.1	

There was no significant difference in cell morphology noted, and neither was there significant difference in size of cells seeded in the different types of pleural fluid (Figure 3.27).

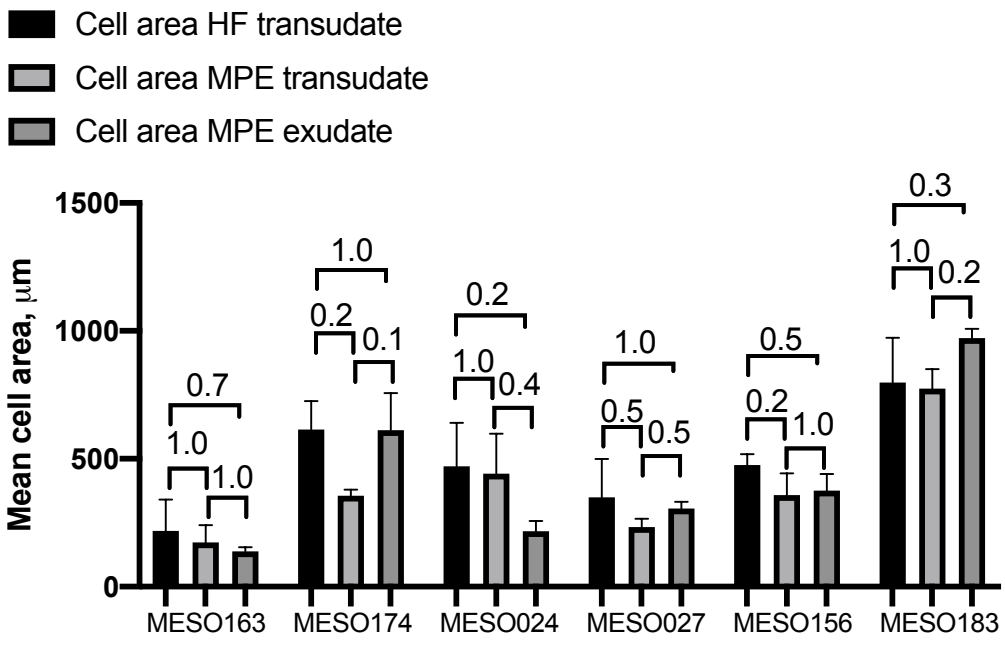


Figure 3.27 shows comparison between mean area of cells seeded in the different pleural fluid types. Values above the square brackets indicate the p values obtained (Kruskal-Wallis test) when comparing each median, and there was no statistically significant difference between size of cells seeded in the different pleural fluid types. HF=heart failure, MPE=malignant pleural effusion

Primary culture of malignant pleural mesothelioma cells can be achieved using malignant pleural effusion fluid alone as culture medium

Primary culture of cancer cells is notoriously difficult, and requires time, skill and laboratory resources. Even under the best conditions, not all attempts at primary cell culture of MPM cells will be successful. Through the above experiments, it was shown that pleural fluid was adequate for cells from established MPM cell cultures to proliferate *in vitro*. In order to

further assess the biological properties of pleural fluid, primary culture of MPM cells in MPE fluid was attempted from 6 MPM MPE fluid samples, with concurrent primary cell culture in standard *in vitro* full culture medium as a control.

In 3 (3/6, 50%) cell cultures, the culture was unsuccessful in both MPE fluid and in full culture medium, in 1/6 (16.7%) cell culture was unsuccessful from the start in complete medium and unsuccessful beyond passage 1 in MPE fluid, and 2 (2/6, 33.3%) attempts were unsuccessful in both MPE fluid and in full culture medium. These results and the associated pleural fluid cytology are summarised in Table 10, together with the associated pleural fluid cytology as reported by the clinical histopathologist as part of the patient's routine clinical care are shown in Table 3.10, and Figure 3.28 shows primary cell culture cells within the plate 43 days after having been seeded.

Table 3.10 shows the outcomes of the MPM primary cell culture in MPE fluid and in complete medium. Cell culture success with MPE fluid was similar to that in complete medium. ***MPE=malignant pleural effusion, MPM=malignant pleural mesothelioma, P=passage.***

Cell culture	MPM subtype	Pleural fluid cytology at time of MPE fluid sampling	Outcome of primary cell culture	
			Cells seeded in complete medium	Cells seeded in MPE fluid
MESO392	Biphasic	Negative	Grew well and frozen at P4 (after >2 months in culture)	Culture dish confluent, cells split and transferred to complete medium on day 43. Went on to becoming an established cell culture beyond P5
MESO051	Epithelioid	Not available	Cells discarded - no attached cells on day 3	Culture dish confluent, cells split and transferred to complete medium on day 38. Cells stopped growing during P1 and were discarded.
MESO397	Epithelioid	Positive	Cells discarded - no attached cells on day 3	Cells discarded - no attached cells on day 3
MESO398	Biphasic	Negative	Cells discarded - no attached cells on day 3	Cells discarded - no attached cells on day 3
MESO402	Epithelioid	Positive	Grew well and frozen at P2 (after >2 months in culture)	Culture dish confluent, cells split and transferred to complete medium on day 39. Went on to becoming an established cell culture beyond P5
MESO064	Epithelioid	Positive	Grew well and frozen at P2 (after >2 months in culture)	Culture dish confluent, cells split and transferred to complete medium on day 40. Went on to becoming an established cell culture beyond P5

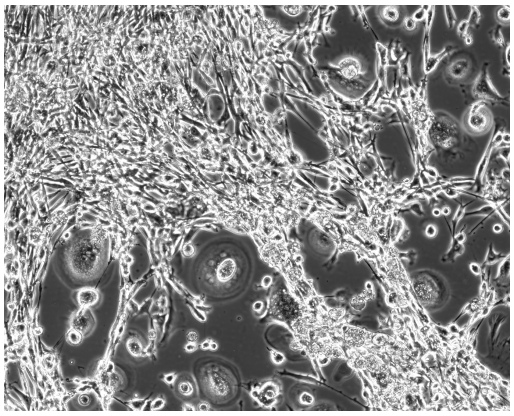


Figure 3.28 shows image of MESO392 taken on day 43 after cells from a fresh sample of MPM MPE fluid were seeded in 100% matched MPE fluid from the same patient. The image shows that the cells have proliferated and adhered to the cell culture plate surface.

3.4 Development of an animal model for malignant pleural effusion in malignant pleural mesothelioma

3.4.1 Literature review

MPE develops in the setting of primary pleural malignancy, as in MPM, or with pleural metastases from a primary malignancy elsewhere within the body. The development of MPE is an important event, signifying decreased quality of life, poor prognosis, and incurability. Although *in vitro* models may provide valuable insights in MPE and allow the development and investigation of human cell cultures, this information is supplementary to the information obtained from *in vivo* models. Tumour cells trigger biological events that eventually lead to MPE formation, and animal models enable the tumour-host interaction within a more clinically relevant microenvironment allowing the full pathobiology of MPE to manifest. On the other hand, animal models are expensive, and unnecessary animal testing is unethical, therefore it is essential to design targeted animal models, the results of which are most likely to translate to clinically relevant outcomes, and with the least possible number of animals, taking into consideration that results in animal models do not always necessarily translate to human disease.

MPE and chemotherapy response

As discussed, Cheah et al. tested MPM cell lines *in vitro* with standard first line MPM chemotherapy (cisplatin and pemetrexed combination) and found that in the presence of 30% MPM MPE fluid, cells were less likely to die after exposure to chemotherapy than cells that were exposed to the chemotherapy drugs and culture medium alone, concluding that

MPE fluid may lead to resistance to chemotherapy in MPM.(Cheah et al., 2017) However this method has its limitations, namely because pleural fluid is similar to serum as both contain growth factors, and cells cultured in greater amounts of serum, e.g. 20-30% (compared to the typically used 10%), show increased proliferation,(Kwon et al., 2016) and therefore this may have been a cause for the decreased cell death with 30% MPE fluid. In addition, the amount of chemotherapy used was not representative of the dose used in clinical practice. In clinical practice the cisplatin to pemetrexed ratio is typically about 1:6.6 (cisplatin 75mg/m^2 and pemetrexed 500mg/m^2 of body surface area, intravenously every 3 weeks),(Pemetrexed s.p.c, available online https://www.ema.europa.eu/en/documents/product-information/alimta-epar-product-information_en.pdf, accessed on 3rd July 2019) while Cheah et al. used a 2:1 ratio: cisplatin 50µg, pemetrexed 25µg, possibly because pemetrexed can be challenging to use *in vitro* since it does not dissolve easily in a solution.

Intrapleural therapy in MPE and MPM

There has been a recent increase in research studies of intrapleural therapy in MPE and MPM, including:

- Intrapleural chemotherapy such as liposome-entrapped cisplatin analogues in MPM, and cisplatin and mitomycin in MPM,(Lu et al., 2005; Rusch et al., 1994)
- Intrapleural gene therapy such as adenovirus-interferon-alpha in MPM,(Sterman et al., 2016)
- Intrapleural immunotherapy such as anti-vascular endothelial growth factor (VEGF) and anti-epidermal growth factor receptor (EGFR) in MPE, and recombinant gamma-interferon in MPM,(Acencio et al., 2017; Boutin et al., 1991)

- Combined intrapleural chemotherapy and immunotherapy, such as pemetrexed and bevacizumab in MPM with MPE.(Chen et al., 2014)

These are only few examples of all the research in intrapleural therapy, and furthermore, there are additional studies of intrapleural therapy in other malignancies apart from MPM. A mouse model with IPC would be valuable to facilitate the design and delivery of intrapleural therapy experiments. Some such examples include:

- In MPM, trials of intrapleural therapy, with cisplatin/pemetrexed combination, mostly included small numbers of patients with patient survival and toxicity results varying widely,(Tsao et al., 2009) therefore further research is necessary in this area, and this can be facilitated by an IPC animal model.
- In MPE secondary to non-small cell lung carcinoma, an intrapleural product of heat-killed *Streptococcus pyogenes*, OK-432, was associated with increased progression-free survival and median survival time when compared to intrapleural bleomycin and cisplatin/etoposide combination, with tolerable side effects in a randomised trial (*n*=102 patients assessed for therapeutic response).(Yoshida et al., 2007) Although the survival differences did not reach statistical significance, OK-432 has been identified as a potential promising therapeutic agent that merits further research, potentially facilitated by the use of an IPC animal model.
- Virus strains with oncolytic potential such as the Vaccinia virus was found to control tumour growth and prevent formation of MPE in a subcutaneous lung adenocarcinoma mouse model.(Weibel et al., 2013) An IPC animal model may help to further explore this virus strain further by studying the effect of it injected intrapleurally in mice with pleural malignancy.

3.4.2 The animal model developed

This work is presented in detail in Chapter 5: Future directions.

3.5 High-throughput drug screening and deoxyribonucleic acid sequencing of patient-derived malignant pleural mesothelioma cell cultures

3.5.1 Literature review

Personalised treatment in MPM

The ideal treatment for MPM would be to have effective personalised treatment, which maximises efficacy but minimises side effects and toxicity. However, this has not yet been achieved, and there have been no clear biomarkers identified that can predict drug response in MPM.

As discussed, MPM carries a poor prognosis, with current recommended first line treatment Cisplatin/pemetrexed/bevacizumab combination being associated with median 19 (95% CI 16-22) month survival *vs* 16 (95% CI 14-18) with cisplatin/pemetrexed alone.(Zalcman et al., 2016) Targeted treatment may be an option in MPM. There is some recent evidence of response to immunotherapy: about 40% of non-epithelioid MPM expresses programmed death ligand-1 (PDL1), and a phase I trial of the anti-PDL1 treatment pembrolizumab showed a 20% response rate when used for MPM treatment, and 52% had stable disease after treatment, with median overall survival of 18 months.(Alley et al., 2017) Two phase 2 trials of Durvalumab, a PD-1 blocker, in combination with first line chemotherapy cisplatin/pemetrexed showed promising results, although phase 3 data is still awaited.(Forde et al., 2020; Nowak et al., 2020) The phase 3 trial, CheckMate-743, compared the combination of anti-PD-1 monoclonal antibody Nivolumab and the human cytotoxic T lymphocyte antigen 4 (CTLA-4) blocking antibody Ipilimumab, with cisplatin/pemetrexed chemotherapy, reporting improved overall survival with the former (median overall survival 18 *vs* 14 months respectively, HR 0.7, *p* 0.002); the

objective response rate was lower with immunotherapy than with chemotherapy (40% *vs* 43%) but with a longer median duration of response rate: 11 months *vs* 6.7 months respectively.(Baas et al., 2021) In October 2020, Nivolumab/Ipilimumab combination treatment was approved by the FDA for first line treatment in non-resectable MPM.(Wright, 2020)

Other treatment modalities for MPM including surgery and radiotherapy have been evaluated, although for surgery in particular, there are only few large randomised trials given the rarity of MPM and the small numbers of patients with MPM who are fit enough to undergo surgery. The evidence that does exist shows that surgery is not consistently associated with improved survival. The British Thoracic Society guidelines do not recommend surgery for MPM outside clinical trials,(Woolhouse et al., 2018) although maximal surgical cytoreduction is recommended by the American Society of Clinical Oncology guidelines in selected patients with early-stage non-sarcomatoid disease, as part of multimodality treatment.(Kindler et al., 2018) MPM typically involves a large pleural area and the delivery of radical radiotherapy doses risks harm to surrounding organs such as lung, liver, and heart. Neither radical radiotherapy used in isolation, nor pre- or post-operative radiotherapy have been shown to improve survival in MPM, and therefore are not recommended in current British MPM treatment guidelines,(Woolhouse et al., 2018) although the American guidelines identify a role for radiation therapy in patients undergoing cytoreductive surgery.(Kindler et al., 2018)

Thus, current agreed first line MPM chemotherapy treatment across guidelines (including European Respiratory Society (ERS), European Society of Thoracic Surgeons (ESTS), European Association for Cardio-Thoracic Surgery (EACTS), European Society for

Radiotherapy and Oncology (ESTRO), British Thoracic Society (BTS) and European Society for Medical Oncology (ESMO) is cisplatin/pemetrexed combination, with the addition of bevacizumab where available and licensed.(Popat et al., 2022; Scherpereel et al., 2020; Woolhouse et al., 2018) However, this treatment is only indicated in select patients who are fit enough (ECOG PS 0-1, and may be considered in PS 2) to withstand the possible toxic side effects associated with these drugs. The bone marrow toxicity side effects of pemetrexed are reduced with folate and vitamin supplements, although other side effects such as nausea, vomiting, fatigue, diarrhoea, dehydration and stomatitis are still more frequent in patients receiving cisplatin/pemetrexed as compared to control groups. Grade 3-4 adverse events (namely hypertension, thrombosis and bleeding) occurred in 71% of patients who received cisplatin/pemetrexed/bevacizumab combination (*vs* 62% of patients receiving cisplatin/pemetrexed alone). Therefore a number of patients will not be fit enough to receive first line chemotherapy, and furthermore, of the select patients who do receive chemotherapy, more than half will risk the associated adverse effects of chemotherapy without deriving benefits in terms of disease response. Even if patients tolerate the full course of chemotherapy, the disease is not cured and median survival is only extended by a few months. Ideally, the response or resistance to cancer treatment would be predicted prior to administration of treatment so that patients who are not likely to respond to treatment may be spared the unnecessary associated side effects, thereby limiting the risk of adverse events to patients who are likely to respond to the treatment.

Deoxyribonucleic acid sequencing

As described earlier, there is high inter- and intra-tumour heterogeneity in MPM, observed at both morphological and molecular levels, and this diversity has therapeutic implications, with varied responses to therapies.(Oehl et al., 2018) Genetic variants have been identified

to correlate with cancer development and survival, as well as with response and resistance to anti-cancer drugs. The tumour suppressor gene (TSG) *TP53* plays a central role in the response to DNA damage and induces cell apoptosis in cells with DNA damage. Variants in *TP53* occur in >50% of all human cancers,(Tian et al., 2013) and in about 5-17% of MPM.(Bott et al., 2011; Bueno et al., 2016; Kato et al., 2016) Mutated *TP53* is associated with significantly decreased survival in MPM compared with wild type *TP53*.(Bueno et al., 2016) Other frequent variants in MPM are increased expression of *EGFR* with overexpression in 64% of 321 tissue samples,(Chia et al., 2021) *CDKN2A/B* TSG loss in up to 60%,(Marshall et al., 2020) and variants in tumour suppressor *NF2* in about 38-50%. These are associated with increased proliferation and invasiveness of mesothelioma. In addition, loss of INK4A/Arf loss in mice with MPM was associated with significantly reduced median survival and highly invasive tumours, suggesting that *INK4A* loss substantially contributes to the poor clinical outcome of MPM.(Jongsma et al., 2008) Variants in TSGs such as *NF2* and *INK4A*, genes involved in apoptosis regulation, may explain at least in part, the resistance of MPM to most conventional drugs because cells are resistant to induction of apoptosis. Conversely, *BAP1* loss is present in about 30-60% of MPM and is associated with improved prognosis.(Guo et al., 2015; Kato et al., 2016; Singhi et al., 2016)

When next generation sequencing was performed on 42 patients with MPM, each had a unique set of genomic aberrations, and no patients had identical genomic portfolios.(Kato et al., 2016) This heterogeneity further emphasises the need for development of personalised effective therapies for mesothelioma. In fact, single arm (non-randomised) phase II trials of targeted treatment with tyrosine kinase inhibitors in MPM, including with sunitinib (*n*=53 MPM patients, 12% disease response, 65% stable, 22% progressive disease), sorafenib (*n*=53 MPM patients, median progression-free survival 5 months) and

imatinib (*n*=25 MPM patients, no objective response), showed only minimal clinical efficacy, perhaps because patients were not selected according to their genomic pattern.(Mathy et al., 2005; Nowak et al., 2012; Papa et al., 2013) DNA sequencing, done in parallel with a drug-screening assay, allows identification of variants and genetic patterns that are associated with a drug response or drug resistance (pharmacogenomics). This might then enable the selection of patients who are more likely to respond to treatment, reduce chances of poor drug response and of adverse reactions, and therefore also decrease treatment costs. Matching variants with targeted treatment has been effective in other cancers, including lung cancer (where patients with anaplastic lymphoma kinase rearrangements responded well to crizotinib).(Kwak et al., 2010) Through next generation sequencing, Kato et al. found a total of 116 aberrations in 42 patients with mesothelioma (pleural and peritoneal), and of these, 112 aberrations were potentially targetable with already existing drugs.(Kato et al., 2016) This same study however was unable to correlate genomic aberrations with clinical outcomes.

The need for personalised treatment is further emphasised by the evidence that several absorption, distribution, metabolism, excretion and toxicology (ADMET) gene-related variations are significantly more common in certain ethnic groups than in others,(Mizzi et al., 2014) and gene variants, including in *TP53*, have been identified as playing a role in drug response in MPM.(Tian et al., 2018)

There is a need to bridge the gap between pharmacogenomics research findings and clinical practice, as to date, translation of the former to the latter has been slow. The advent of whole genome sequencing technology offers a potential solution to bringing personalised drug treatment based on genomic medicine closer to reality. There are however associated

challenges with whole genome sequencing, including a significant cost, and the possible need to train physicians to interpret pharmacogenomics data.

DNA sequencing identifies the exact order of the four nucleotides: guanine, adenine, thymine and cytosine in a DNA strand. RNA is generated by transcription of DNA, therefore reflects active genes. RNA sequencing however does not easily detect genes with relatively low expression levels, and analyses the portion of DNA that has been transcribed into RNA at a given time, so the levels of RNA for various genes will vary over time. Exomes are the protein-coding part of the genome, and whole exome sequencing is associated with a lower cost than whole genome sequencing, together with a lower data storage and analysis requirement. However, several pharmacogenomics effects are caused by amino acid substitutions, and may not necessarily occur within exons. In addition, current capture probes are only able to target already-known exons, and knowledge of all exons is as yet incomplete, therefore unique or rare pharmacogenomic variants in ADMET related genes may go undetected.(Katsila and Patrinos, 2015; Mizzi et al., 2014) Whole genome sequencing solves this issue. In fact, whole genome analysis identified more than 2500 novel variants in the five most well-documented pharmacogenes, of which only 202 resided in exons and proximal regulatory regions.(Mizzi et al., 2014) Moreover, 'next generation sequencing', also known as high throughput sequencing, analyses millions of sequences per run enabling unprecedented opportunities to rapidly analyse whole genomes when compared to traditional DNA sequencing techniques.

Drug screening

The unmet clinical need for improving cancer management has led to the development of drug discovery programmes. Existing compound libraries allow identification of chemical

probes to be used in target validation for drug discovery programmes. Early results from research studies on the gene expression, mRNA, and protein levels associated with a disease, can give rise to the hypothesis that altering a particular protein, gene or pathway (a target) results in improved disease treatment. Once a target is selected, an agent that modulates this target is sought. If an agent is found to be effective in modulating the target to produce a disease response that must be measurable both *in vitro* and *in vivo*, this result must then be validated. The use of multiple validation tests, e.g. both *in vitro* and *in vivo*, increases the confidence in the observed outcome, although even if the agent is confirmed to be effective in producing a disease response, it may not be safe enough to be used in humans at adequate effective doses.(Hughes et al., 2011)

Genes that potentially modulate a particular pathway can be rapidly identified through the process of high-throughput testing where the entire drug library is screened against a cell-based assay. The assay must be reproducible across assay plates, screen days and the duration of the drug discovery programme. The aim of high-throughput screening is to identify drug responses rapidly and accurately.(Hughes et al., 2011)

Drug resistance

MPM cells show high resistance to apoptosis by cytotoxic drugs, and defects in apoptosis are important in tumorigenesis and in drug resistance. For example, TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in most cancer cells but not in most normal cells, however MPM cells seem quite resistant to TRAIL-induced cell death.(Villanova et al., 2008) In addition, there is over-expression of the anti-apoptotic protein FLIP (FADD-like IL-1 beta-converting enzyme inhibitory protein) in MPM cells,(Villanova et al., 2008) and BCL-XL is an anti-apoptotic driver that is overexpressed in MPM.(Xu et al., 2021) Drug resistance can be

fatal for patients,(Brammeld et al., 2017) and early detection of drug resistance together with the identification of drugs that the patient's cancer would respond to is key to successful treatment. Drug resistance profiles exist for other malignancies, such as lung cancer, and some patients who have had chemoresistance assay-directed treatment had significantly longer survival.(Loizzi et al., 2003) In MPM, analysis by extreme drug resistance (EDR) assay of 168 MPM tissue cultures, showed intermediate to high resistance to cisplatin, gemcitabine, or vinorelbine in 27%, 31% and 59% of samples respectively, and resistance to all 3 drugs in 11%.(Mujoomdar et al., 2010) This further highlights the need to identify effective personalised treatment in MPM.

Ensuring and assessing the quality of the drug screening assay

Controls need to be included to monitor the drug screening assay quality, and simple assay protocols minimising the number of wash steps and plate transfers further ensures adequate assay quality.(Hughes et al., 2011) Furthermore, the use of robotics allows more accurate aspiration and transfer of assay reagents and drug, and is more time-efficient than manual techniques.

The Z factor determines the suitability of an assay for use in high-throughput screening,(Kim et al., 2017; Zhang et al., 1999) and the *z*-score represents the difference between an individual value and the population mean in units of the standard deviation (i.e. the number of standard deviations away from the mean) (Chapter 6: Appendices, section 6.6).

Another factor that needs to be taken into consideration to ensure high quality of the assay is the organic solvents, such as dimethylsulfoxide (DMSO), that are often used to store

chemical libraries. At concentrations of >1%, these solvents are toxic to cell-based assays. Therefore, assays need to be designed with this in mind, so as not to be sensitive to the concentrations of solvents used. Furthermore, the compounds within the chemical libraries can become degraded or modified over time when frozen in DMSO solutions, so sourcing fresh compound samples is ideal.(Hughes et al., 2011) Assay false positives and false negatives need to be established, to ensure that these are not unacceptably high.

The doubling time of MPM cell cultures is variable, dependent on the individual cell culture, and the level of confluence. A study reported that maintaining cells of 5 MPM cell cultures at 50% confluence and changing the culture medium every 2 days reduced the mean doubling time from 25-46 hours, to between 18 and >30 hours.(Manning et al., 1991) Rapidly dividing cells are more susceptible to the majority of cytotoxic agents,(Manning et al., 1991) therefore this significant variation may be important when comparing drug screening profiles of the cell cultures. It would be useful to conduct a study into the growth profile of cell cultures prior to drug screening assays, to establish the ideal number of cells to seed in each well for each cell culture.

Drug screening using 2-dimensional cell cultures: limitations and advantages

The difficulty with 2-dimensional monolayer cell cultures is that they do not accurately reproduce the real complexity and 3-dimensional structure in solid tumours. Gene expression of cells in 2-dimensional cultures is different from that found in *in vivo* tumours, and monolayers are unable to mimic all structures and interactions with the surrounding cells and extra-cellular matrix *in vivo*.(Costa et al., 2016) Consequently, chemotherapy resistance may not be fully reflected in a 2-dimensional cell culture. In fact, high-throughput screening of cancer-drug candidates often results in the discarding of about

95% of drugs. The use of more biologically relevant tumour-models can improve translation results and cost-effectiveness. Cells can be assembled into 3-dimensional structures, along with extracellular matrix proteins from the surrounding tumour microenvironment for cellular attachment. A 3-dimensional cell culture has the potential to more accurately represent the treatment effects *in vivo*. Three-dimensional cancer cell cultures can acquire chemotherapy resistance that mimics the chemotherapy resistance observed *in vivo*. One example of a 3-dimensional structure is multicellular tumour spheroids, created by agitation procedures. These exhibit tumour-specific proliferation rates, nutrient / oxygen gradients, and central necrosis. Mesothelioma cell lines that exhibited sensitivity to bortezomib as monolayer cell cultures, acquired resistance to bortezomib as 3-dimensional spheroid cultures.(Barbone et al., 2011) Studies comparing the 2-dimensional to the 3-dimensional cell cultures have yielded varying results, with some showing tumour cells to be less sensitive to anti-cancer therapies in the 3-dimensional structure, and others showing that the anti-cancer therapies were equally or less sensitive in the 2-dimensional cultures.(Lovitt et al., 2014) Apoptotic resistance was observed in 3-dimensional MPM cell lines but not in 2-dimensional cultures, and proteins within the mTOR pathway were identified as having a role in this resistance.(Barbone et al., 2008)

Thus, the main issue with 2-dimensional cell cultures used for drug screening is that they assume that short exposure to a drug *in vitro* is representative of tumour exposure *in vivo*. In addition:

- Cells are often grown in standard incubators with about 20% oxygen partial pressure and this does not represent the steady-state conditions of human organs and tissues (about 1% in the dermis, 14% in arterial blood);

- The plastic cell culture dish does not accurately represent the normal *in vivo* mechanical environment;
- Cell culture screening assays mostly use a single cell type, whereas cells *in vivo* are either in direct contact or communicate over distances with many different cell types;
- Large concentrations of foetal serum and nutrients may promote dedifferentiation of primary cell type into a more foetal-like phenotype, and long-term culture can result in drift in the cells' genetic and physiological characteristics, potentially also leading to loss of expression markers of the original tumour.(Horvath et al., 2016)

However, 2-dimensional cell culture models can provide a means to evaluate new agents in early pre-clinical studies,(Chernova et al., 2016) relatively quickly and cost-effectively. They would be valid for the purposes of a study to assess the feasibility of a bench-to-bedside pathway for MPM patients (see below). Furthermore, their integration with genomic and phenotypic profiling to understand and elucidate new targets may represent the best use of cell culture resources, while still providing useful opportunities to advance drug screening assays.(Horvath et al., 2016)

3.5.2 Results of this study

Ensuring a good quality drug screening assay

The following are the Z factor (robust *Z*-prime or robust *Z'*) results obtained:

- For MESO163 (epithelioid MPM): robust *Z'* 0.5 for 100nM dilution, 0.7 for 1uM dilution, and 0.7 for 10uM dilution (mean 0.6). All indicate an excellent assay since the values are 0.5-1.0.

- For MESO174 (biphasic MPM): robust *Z'* -0.09 for 100nM dilution, -0.3 for 1uM dilution, and *Z'* -0.5 for 10uM dilution (mean -0.2). All indicate that there is too little difference between the positive and negative controls.(Zhang et al., 1999)
- For MESO033 (epithelioid MPM): robust *Z'* 0.5 for 100nM dilution, 0.6 for 1uM dilution, and 0.7 for 10uM dilution (mean 0.6). All indicate an excellent assay.

Two replicate plates were used for each concentration, and the fluorescence measurements for each well in the 2 replicate plates at each dilution were plotted to ensure correlation between the values obtained. The scatterplots below (Figure 3.29) show the Spearman correlation (Spearman correlation was selected because data was not normally distributed) of the results (replicates 1 and 2) obtained at the 3 different drug concentrations used during the drug screening assay (100nM, 1µM, 10µM). There was strong monotonic correlation between the 2 replicates, with mean Spearman r 0.93, 0.93, 0.81 for MESO163, MESO174, and MESO033 respectively, demonstrating robust reproducibility between replicates, therefore a third replicate was deemed unnecessary, and in-keeping with other drug screening assays in the literature using 2 replicates.(Carter et al., 2019; Zinecker et al., 2017)

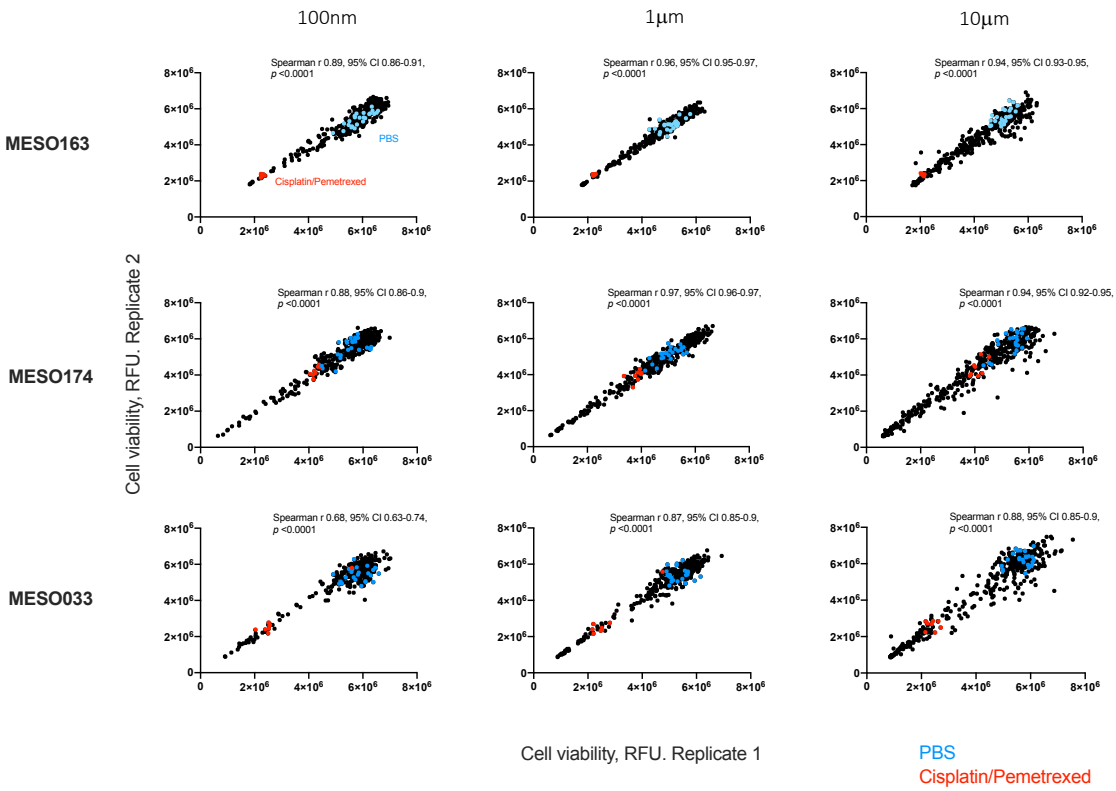


Figure 3.29 shows scatterplots of Spearman correlation analysis of the duplicate samples (replicates 1 and 2) of the high throughput drug screening assays, for cell cultures MESO163 (epithelioid MPM), MESO174 (biphasic MPM), MESO033 (epithelioid MPM), and showing strong monotonic correlation. The figure shows optic density values in Relative fluorescence units (RFU) as measured by the Resazurin assay for all drugs including PBS (phosphate buffered saline, negative control) and Cisplatin/Pemetrexed (first line chemotherapy combination for MPM, positive control).

Correlation, *r*, measures the strength of a relationship between two variables, not the agreement between them; the Bland-Altman plot measures the described agreement between two quantitative measurements.(Bland & Altman, 1999; Giavarina, 2015) Bland Altman plots of the percentage difference between replicate 1 and replicate 2 measurements (Y axis), against the mean of these measurements (X axis) show low levels of bias, indicating that there is little difference between measurements obtained in the replicates (Figure 3.30).

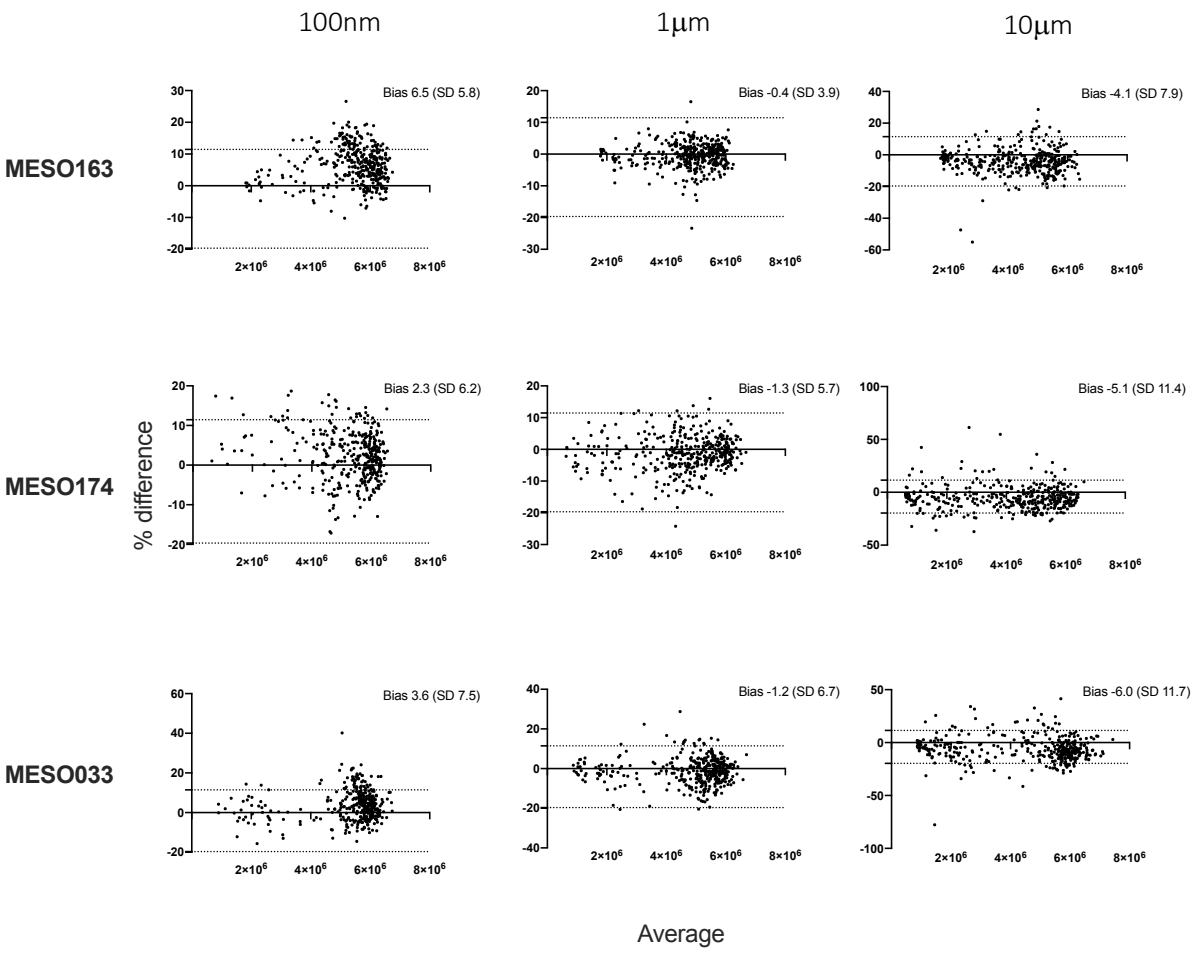


Figure 3.30 showing Bland Altman plots for the duplicate samples (replicates 1 and 2) of the fluorescence measurements (representing cell viability) for each well in the high throughput drug screening assays, for cell cultures MESO163 (epithelioid MPM), MESO174 (biphasic MPM), MESO033 (epithelioid MPM), showing low levels of bias. The broken lines represent the 95% limits of agreement. The difference between the replicates appears to increase with the average cell viability, and this is likely because the drugs on the left side of the graph (resulting in decreased cell viability) are those that were more effective against the cells. A less effective drug (on the right side of the graph) is likely to show greater difference between the two replicates than the more effective drugs that will kill the majority of the cells.

To further investigate the Z factors, and to confirm that the results for the positive control in the high-throughput drug screening assay were correct (given that for cell culture MESO174, there was lack of response to the selected positive control, Cisplatin/Pemetrexed), a serial drug dilution response assay (2-fold dilution, at 10 concentrations: 0mM, 0.006mM, 0.01mM, 0.02mM, 0.05mM, 0.1mM, 0.2mM, 0.4mM, 0.8mM, 1.6mM) for Cisplatin/Pemetrexed was conducted, to establish the drug response curve for each cell culture. The results confirmed that MESO163 was more sensitive to

Cisplatin/Pemetrexed than MESO174 and MESO33 (although the IC50 was similar for MESO163 and MESO033: 0.02nM and 0.03nM respectively). MESO174 was relatively resistant to Cisplatin/Pemetrexed (Figure 3.31). These results again reflect the heterogenous inter-patient response to chemotherapy in MPM.

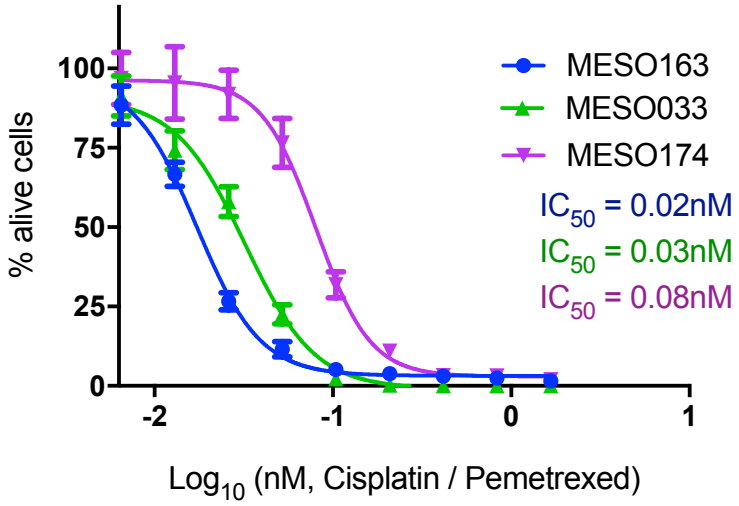


Figure 3.31 shows the drug response curves (mean and SD) of the patient-derived cell cultures MESO163 (epithelioid MPM), MESO033 (biphasic MPM) and MESO174 (epithelioid MPM) for the combination of Cisplatin/Pemetrexed, the current clinical first line chemotherapy regimen for MPM. CellTiter-Glo® luminescent cell viability assay was used.

Results of the drug screening assay

The results obtained from the high-throughput drug screening assay show the inter-patient heterogeneity in the *in vitro* drug response (Figure 3.32), even in those with the same MPM subtype, and reflect the potential importance of developing a personalised treatment strategy.

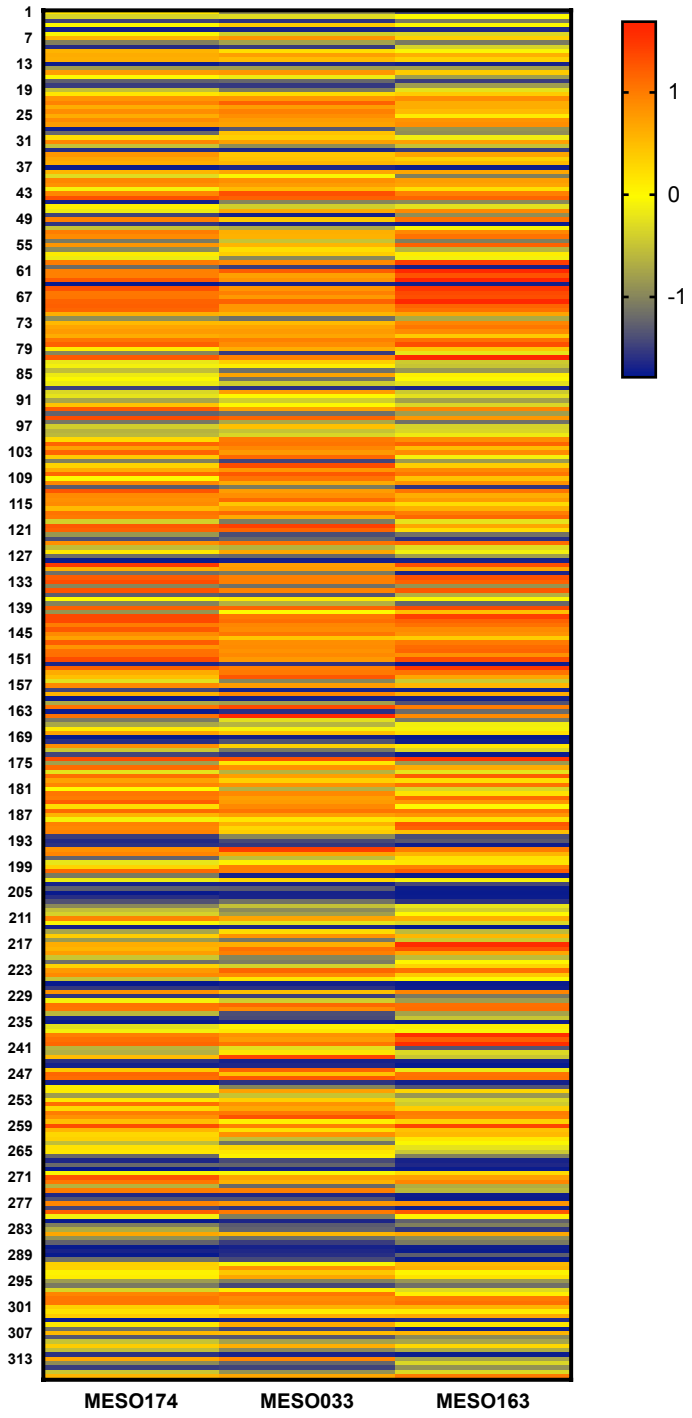
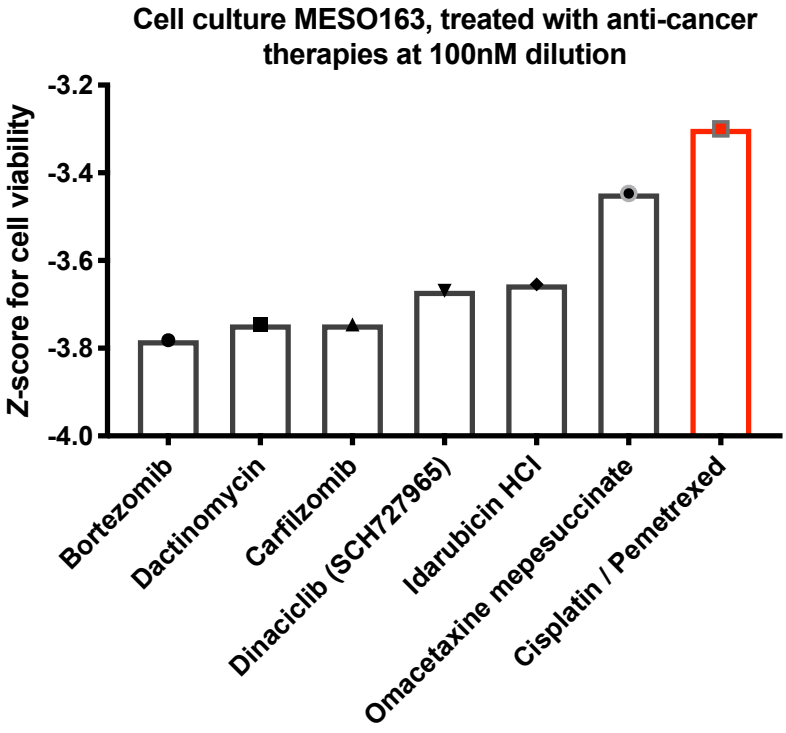


Figure 3.32 shows a heatmap of the drug response at 10μM dilution. Each column represents a primary personalised MPM cell culture (MESO174, biphasic MPM; MESO033, epithelioid MPM; MESO163, epithelioid MPM), and each row represents a tested drug from a drug library of 316 drugs (the numbers correspond to the numbers in the full drug library listed in Chapter 6: Appendices, section 6.6). Cell viability was measured 48 hours post treatment and each drug's response was compared to response to cisplatin/pemetrexed (drug number 1). The colours represent the cell viability *z*-scores across a scale from bright red representing maximal viability (no drug response), while dark blue represents best drug response. This figure shows the inter-patient heterogeneous response to anticancer drugs.

There were several drugs at each dilution that were as effective as, or more effective than, the standard first line MPM treatment used in clinical practice, i.e. cisplatin and pemetrexed combination. The graphs below (Figure 3.33 and 3.34) show the *z*-score obtained for all drugs that were as effective as, or more effective than cisplatin/pemetrexed combination *in vitro*. For all the cell cultures tested at 10uM, and for MESO174, there were several drugs that were more effective than cisplatin/pemetrexed and the large numbers of drugs could not be accommodated in a graph, however the z scores for all the drugs that were as effective or more effective than cisplatin/pemetrexed in the drug screening assay are listed in the tables in Chapter 6: Appendices, section 6.7.



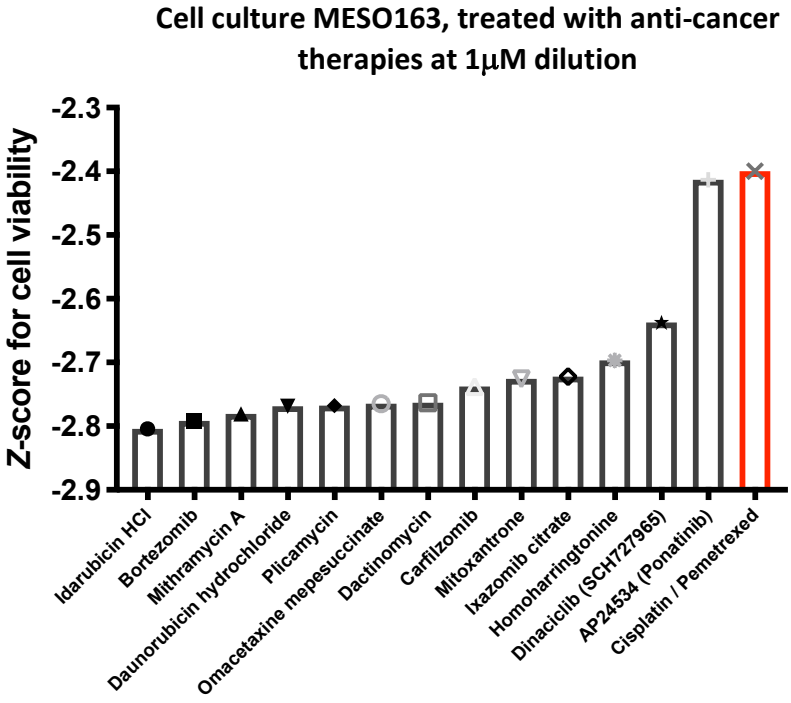


Figure 3.33 shows 2 graphs of cell viability (*z*-score) for cell culture MESO163 treated with the anti-cancer agents in the drug library. The graphs show the *z*-score obtained for all drugs (grey/black marks) that were as effective as, or more effective than cisplatin and pemetrexed combination (red bars represent the mean *z*-score obtained with cisplatin/pemetrexed). Since there were several drugs more effective than Cisplatin/Pemetrexed combination at 10uM dilution, they could not be accommodated in a graph, however the z-scores for all drugs that were more than or as effective as cisplatin/pemetrexed combination in the drug screening assay for all dilutions of drugs with MPM cell culture are listed in the tables in Chapter 6: Appendices, section 6.7.

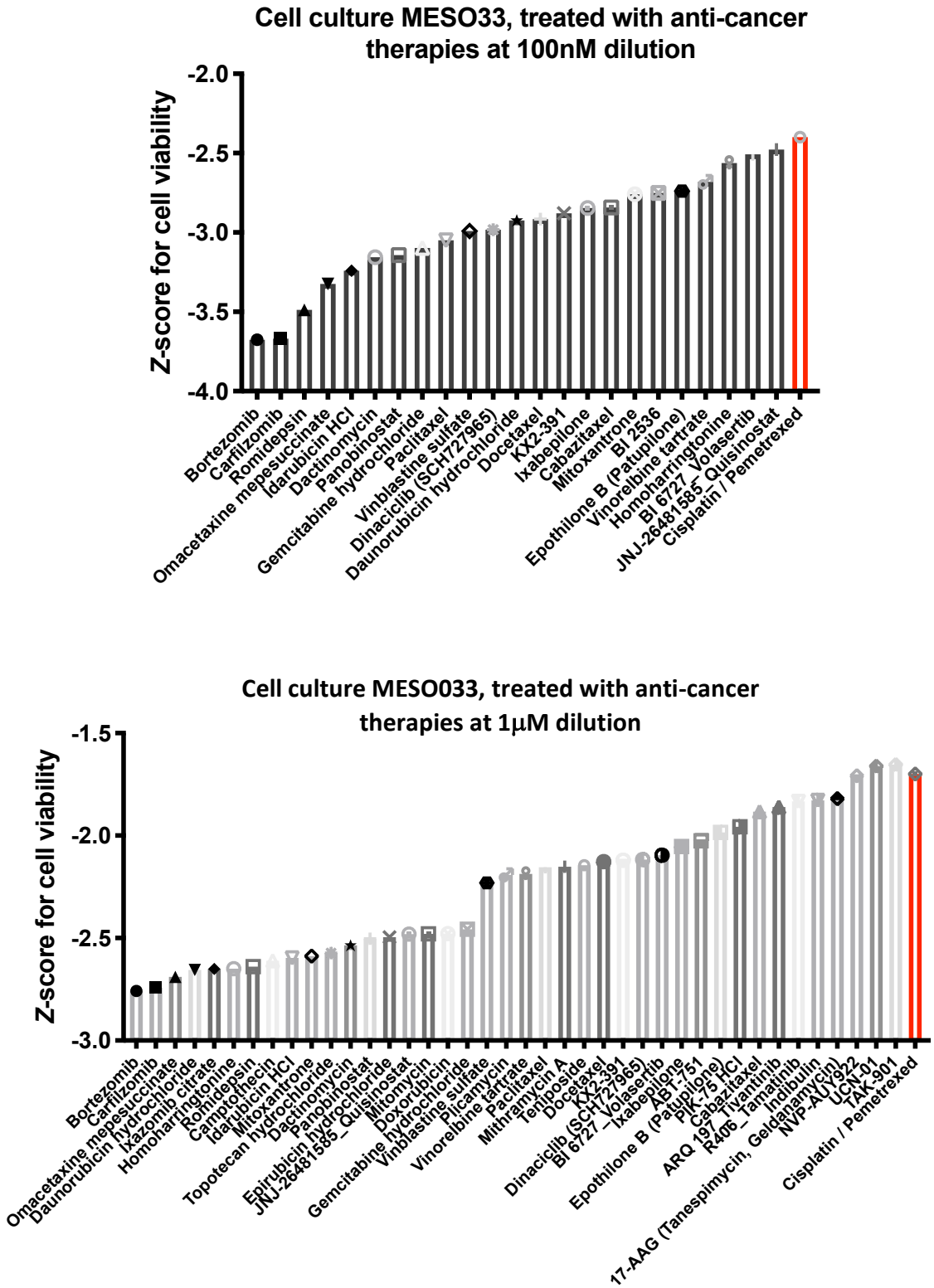


Figure 3.34 shows 2 graphs of cell viability (*z*-score) for cell culture MESO33 treated with anti-cancer agents in the drug library. The graphs show the *z*-score obtained for all drugs (grey/black marks) that were as effective as, or more effective than cisplatin and pemetrexed combination (red bars/marks represent the mean *z*-score obtained with cisplatin/pemetrexed). Since there were several drugs more effective than Cisplatin/Pemetrexed combination at 10uM dilution, they could not be accommodated in a graph, however the z-scores for all drugs that were more than or as effective as cisplatin/pemetrexed combination in the drug screening assay for all dilutions of drugs with MPM cell culture are listed in the tables in Chapter 6: Appendices, section 6.7.

There were 6 drugs that were consistently more cytotoxic than cisplatin and pemetrexed combination across all three cell cultures tested, and at all three dilutions each drug was tested at: bortezomib, carfilzomib, dactinomycin, idarubicin, dinaciclib (SCH727965), and omacetaxine mepesuccinate (previously known as homoharringtonine) (see figure 3.35).

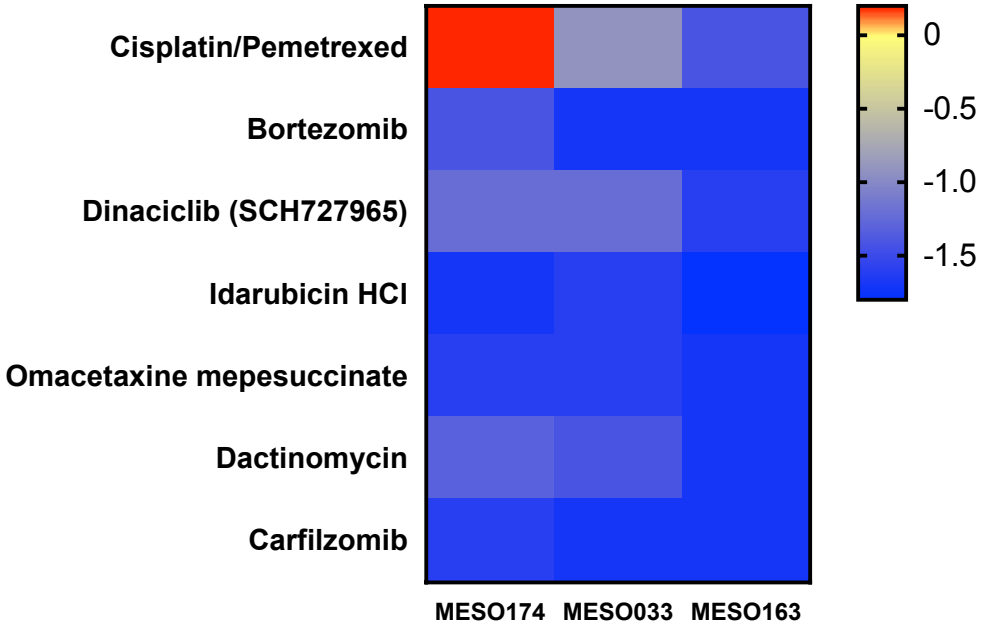


Figure 3.35 shows the heatmap of drug responses for the 6 most effective drugs tested. These drugs were more effective than the positive control, 10uM/1.6uM pemetrexed/cisplatin at all concentrations (100nM, 1uM, 10uM) tested. The drug responses obtained at 10uM dilution are shown, and compared with drug response to Cisplatin/Pemetrexed for each cell culture. The colours represent the cell viability *z*-scores across a scale from bright red representing maximal viability (no drug response), to dark blue representing best drug response.

For cell culture MESO163, these were the only 6 drugs consistently more effective than cisplatin / pemetrexed at each dilution tested, while for MESO033 and MESO174 there were a total of 21 and 44 more effective drugs respectively, as shown in Table 3.11. Proteasome inhibitors, cyclin dependent kinase inhibitors, topoisomerase II inhibitors, protein synthesis inhibitors, and RNA and DNA synthesis inhibitors were among the most effective drugs.

Interestingly, the patient cell culture MESO163 was derived from did receive cisplatin/pemetrexed chemotherapy, and the MPM responded to chemotherapy, as recorded on post treatment CT scan (modified RECIST criteria). On the other hand, the patient MESO033 was derived from also received cisplatin/pemetrexed chemotherapy however progressive MPM was recorded on post treatment CT scan according to the modified RECIST criteria. Although it would not be accurate to interpret *in vitro* results as a definite true *in vivo* representation, in the latter case the lack of response to first line chemotherapy may have potentially been predicted from the drug screening assay given that 21 drugs were found to be more effective than cisplatin/pemetrexed *in vitro*. The patient that cell culture MESO174 was derived from did not receive chemotherapy (Figure 3.36).

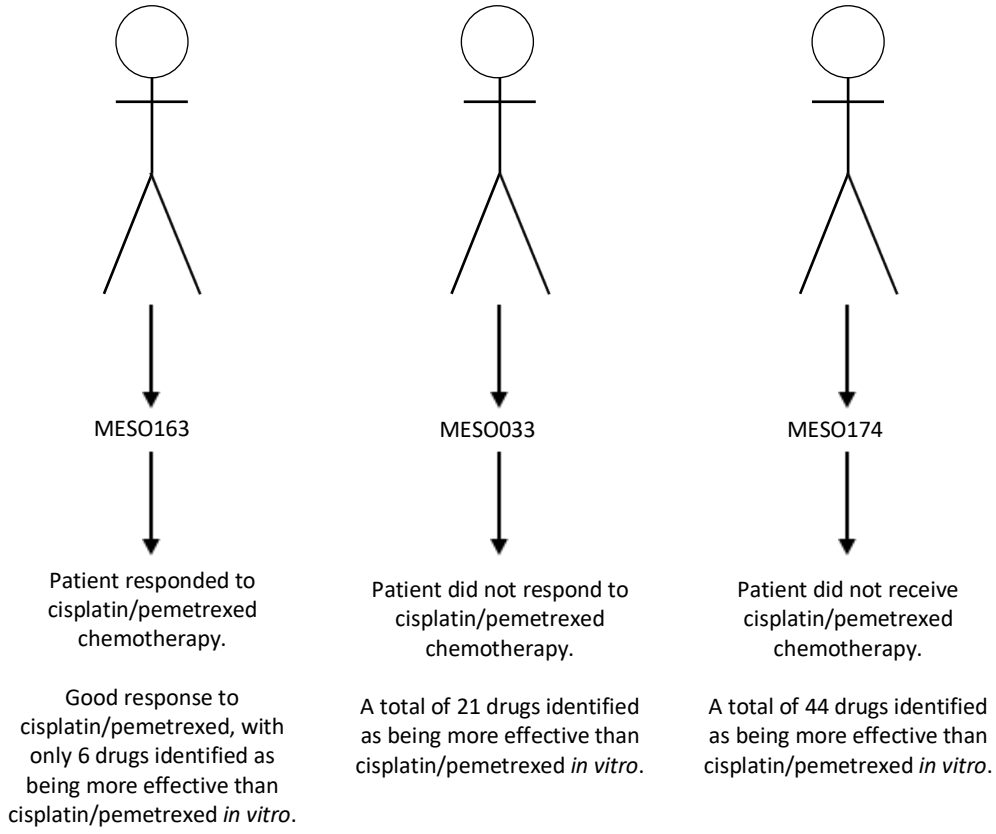


Figure 3.36 shows the cisplatin/pemetrexed response *in vivo*, and the result obtained with cisplatin/pemetrexed *in vitro* drug screening assay on the corresponding personalised cancer cell cultures (MESO163, MESO033, MESO17

Drug tested	Cell culture being tested									Target of drug tested
	MESO163			MESO033			MESO174			
	100nm	1μm	10μm	100nm	1μm	10μm	100nm	1μm	10μm	
Cisplatin / Pemetrexed	**-3.3**	**-2.4**	**-1.4**	**-2.4**	**-1.7**	**-0.9**	**-0.9**	**-0.5**	**0.2**	
Bortezomib	-3.8	-2.8	-1.7	-3.7	-2.8	-1.7	-2.7	-2.2	-1.4	Proteasome inhibitor(Accardi et al., 2015)
Dinaciclib (SCH727965)	-3.7	-2.6	-1.6	-3.0	-2.1	-1.2	-3.0	-2.0	-1.2	Cyclin-dependent kinase inhibitor(Nemunaitis et al., 2013)
Idarubicin HCl	-3.7	-2.8	-1.8	-3.2	-2.6	-1.6	-3.5	-2.4	-1.7	Topoisomerase II inhibitor(Willmore et al., 2002)
Omacetaxine mepesuccinate	-3.4	-2.8	-1.7	-3.3	-2.7	-1.6	-3.2	-2.4	-1.6	Protein translation inhibitor(Wetzler and Segal, 2011)
Dactinomycin	-3.7	-2.8	-1.7	-3.2	-2.5	-1.4	-2.9	-2.2	-1.3	RNA and DNA synthesis inhibitor(Yung et al., 1990)
Carfilzomib	-3.7	-2.7	-1.7	-3.7	-2.7	-1.7	-3.5	-2.4	-1.6	Proteasome inhibitor(Demo et al., 2007)
Romidepsin	-3.0	-2.2	-1.6	-3.5	-2.6	-1.7	-3.8	-2.6	-1.7	Histone deacetylase inhibitor(Tan et al., 2010)
Panobinostat	-2.1	-1.8	-1.2	-3.1	-2.5	-1.6	-3.7	-2.6	-1.7	Histone deacetylase inhibitor(Tan et al., 2010)
Gemcitabine hydrochloride	-1.4	-0.9	-0.2	-3.1	-2.5	-1.5	-1.7	-1.6	-1.0	Nucleoside analog(Plunkett et al., 1996)
Paclitaxel	-1.8	-1.2	0.0	-3.0	-2.2	-1.1	-2.1	-1.5	-0.5	Microtubule stabilizer(Horwitz, 1994)
Vinblastine sulfate	-2.5	-1.7	-0.9	-3.0	-2.2	-1.4	-2.9	-2.2	-1.5	Mitotic inhibitor.Vinca alkaloid microtubule depolymeriser(Jordan et al., 1992)
Daunorubicin hydrochloride	-2.4	-2.8	-1.7	-2.9	-2.7	-1.6	-2.9	-2.3	-1.6	Topoisomerase II inhibitor(Bodley et al., 1989)
Docetaxel	-1.7	-1.3	-0.2	-2.9	-2.1	-1.1	-2.2	-1.5	-0.5	Microtubule stabiliser(Ramaswamy and Puhalla, 2006)
KX2-391	-2.2	-1.5	-0.8	-2.9	-2.1	-1.2	-1.8	-1.7	-1.1	SRC inhibitor(Antonarakis et al., 2013)
Ixabepilone	-1.2	-1.4	-0.7	-2.8	-2.1	-1.2	-1.9	-1.4	-0.6	Microtubule stabilising and mitotic inhibitor(Cobham and Donovan, 2009)
Cabazitaxel	-1.3	-0.5	0.1	-2.8	-1.9	-1.0	-2.0	-1.1	-0.1	Taxane microtubule stabilizer(Abidi, 2013)
Mitoxantrone	-2.6	-2.7	-1.7	-2.8	-2.6	-1.6	-3.2	-2.5	-1.7	Topoisomerase II inhibitor(Bellosillo et al., 1998)
Epothilone B (Patupilone)	-1.8	-0.6	0.0	-2.7	-2.0	-1.1	-2.2	-1.1	0.0	Microtubule stabilising(Cheng et al., 2008)
Vinorelbine tartrate	-2.2	-1.8	-1.0	-2.7	-2.2	-1.3	-2.1	-1.8	-1.3	Vinca alkaloid microtubule depolymeriser,mitotic inhibitor(Gregory and Smith, 2000)
BI 6727_Volasertib	-2.3	-1.3	-1.5	-2.5	-2.1	-1.2	-1.4	-0.8	-0.7	Polo-like kinase-1 inhibitor(Bug et al., 2010)
JNJ-26481585_Quisinostat	-2.2	-1.9	-1.3	-2.5	-2.5	-1.6	-3.4	-2.6	-1.7	Histone deacetylase inhibitor(Venugopal et al., 2013)
UCN-01	-0.7	-0.4	-1.7	-1.4	-1.7	-1.5	-2.7	-2.1	-1.7	3-phosphoinositide-dependent protein kinase-1 inhibitor(Komander et al., 2003)
INK128	-2.0	-2.0	-1.2	-0.3	-0.7	-0.6	-2.4	-1.7	-1.0	Mechanistic target of rapamycin(mTOR) inhibitor(Janes et al., 2013)
Plicamycin	-2.9	-2.8	-1.7	-1.9	-2.2	-1.4	-2.3	-2.1	-1.1	RNA synthesis inhibitor(Nair et al., 2018)
Topotecan hydrochloride	-1.4	-1.6	-1.5	-2.3	-2.6	-1.0	-2.1	-1.9	-1.3	Topoisomerase I inhibitor(Kollmannsberger et al., 1999)
Camptothecin	-1.4	-1.6	-1.5	-2.3	-2.6	-1.2	-1.9	-2.1	-1.2	Topoisomerase I inhibitor(Pommier, 2006)
Mithramycin A	-2.5	-2.8	-1.7	-1.4	-2.2	-1.4	-1.9	-2.1	-1.1	RNA synthesis inhibitor(Miller et al., 1987)
17-AAG (Tanespimycin, Geldanamycin)	-0.9	-0.9	-0.7	-1.4	-1.8	-1.0	-1.8	-1.4	-0.5	Heat shock protein 90 inhibitor(Powers et al., 2013)
Doxorubicin	-1.1	-2.4	-1.7	-1.9	-2.5	-1.5	-1.8	-2.4	-1.4	Topoisomerase II inhibitor(Bodley et al., 1989)
NVP-AUY922 (Luminespib)	-1.8	-1.1	-0.5	-2.2	-1.7	-1.0	-1.8	-1.1	-0.5	Heat shock protein 90 inhibitor(Jensen et al., 2008)
17-DMAG (Alvespimycin)	-1.4	-1.3	-1.4	-1.9	-1.5	-1.0	-1.7	-1.1	-1.5	Heat shock protein 90 inhibitor(Wang et al., 2016)
Epirubicin hydrochloride	-1.0	-2.3	-1.7	-1.8	-2.5	-1.6	-1.7	-2.4	-1.5	Topoisomerase II inhibitor(Järvinen et al., 1998)
GSK2126458	-1.5	-1.8	-1.1	-1.0	-1.3	-0.7	-1.6	-1.8	-1.0	Phosphoinositide 3-Kinase (α/β/δ/γ) inhibitor(Munster et al., 2016)
R406_Tamatinib	-1.8	-1.7	-0.9	-0.1	-1.8	-1.1	-1.5	-1.8	-0.9	Spleen tyrosine kinase inhibitor(Braselmann et al., 2006)
AZD8055	-1.0	-1.6	-1.0	-0.2	-1.1	-0.5	-1.5	-2.0	-1.1	Mechanistic target of rapamycin inhibitor(Willems et al., 2012)
SB 743921	-2.1	-1.4	-1.7	-2.3	-1.6	-1.7	-1.5	-0.8	-1.7	Mitotic inhibitor.Kinesin spindle protein inhibitor(Bongero et al., 2015)
TAK-901	-1.5	-2.2	-1.7	-0.6	-1.7	-1.4	-1.3	-2.5	-1.6	Aurora B kinase inhibitor(Farrell et al., 2013)
Ixazomib citrate	-1.6	-2.7	-1.7	0.1	-2.7	-1.7	-1.3	-1.7	-1.1	Proteasome inhibitor(Xie et al., 2019)
RAF265	-0.1	0.0	-0.2	0.4	0.5	0.0	-1.2	-0.9	-0.2	C-Raf/B-Raf/B-Raf (V600E) and VEGFR2 phosphorylation inhibitor(Williams et al., 2015)
Dasatinib	-1.1	-0.4	-0.1	-0.5	-0.8	-0.3	-1.0	-0.9	-0.6	ABL, SRC and c-KIT inhibitor(Araujo and Logothetis, 2010; Schittenhelm et al., 2006)
MK1775	0.0	-1.0	-0.8	0.3	-0.2	-1.1	-1.0	-1.0	-0.5	Tyrosine kinase (WEE1) inhibitor(Kreahling et al., 2012)
AP24534 (Ponatinib)	-1.9	-2.4	-1.7	-0.3	-0.6	-1.7	-1.0	-1.5	-1.7	ABL, PDGFRα, VEGFR2, FGFR1 and SRC inhibitor(O'Hare et al., 2009)
PIK-75 HCl	0.0	-2.4	-1.7	0.6	-2.0	-1.5	-1.0	-1.8	-1.2	phosphoinositide 3-kinase (PI3K) p110-α inhibitor(Zheng et al., 2011)
Rapamycin (sirolimus)	-1.0	-0.5	0.1	-0.1	-0.3	0.4	-1.0	-0.9	0.1	Mechanistic target of rapamycin inhibitor(Dudkin et al., 2001)

Table 3.11 shows *z*-scores for cell viability obtained for drugs that were consistently as effective as, or more effective than, cisplatin/pemetrexed, and the drug-dilution tested for each cell culture tested. Colour code - Salmon: drugs that were more effective than Cisplatin/Pemetrexed for all MPM cell cultures tested; yellow: drugs more effective in MESO033 and MESO174; blue: drugs that were more effective for MESO174 only. *17-AAG = Tanespimycin,Geldanamycin*

Chapter 4: Discussion

4.1 The association between pleural fluid exposure and survival in malignant pleural mesothelioma – a retrospective cohort study

This is the largest study in the literature to specifically address the association of MPE presence and survival in MPM based on clinical data. Pleurodesis success appears to be associated with improved survival in MPM, however longer MPE duration was not associated with worse survival in MPM albeit within the limits of this retrospective data.

Pleural effusion and survival

An association between time exposed to MPE and survival was not demonstrated with Cox regression and time-dependent analyses. Although *in vitro* data suggest that pleural fluid presence is associated with increased malignant cell growth,(Cheah et al., 2017) this has to date never been assessed using human data. Does this data therefore mean that pleural fluid presence is irrelevant to cancer progression in humans? There are a number of reasons why this conclusion may not be safe, even within this large dataset, as discussed below.

On the one hand, duration of exposure to pleural effusion was not clearly associated with survival, on the other hand, pleurodesis success was associated with improved survival, as also shown in other studies.(Hassan et al., 2019; Korsic et al., 2015) When

analysing the percentage of life exposed to pleural fluid, the total duration of MPE documented to have been present during the post-MPM diagnosis period was assessed, thus e.g. both patients with an IPC *in situ* from MPM diagnosis to death, and patients with a persistent small asymptomatic MPE were classified as having 100% of life post-MPM diagnosis exposed to MPE. When analysing pleurodesis, 'complete pleurodesis' referred to patients with documented complete resolution of MPE at *any* stage of their post-MPM diagnosis life (regardless of the duration of the MPE, i.e. regardless of whether the complete pleurodesis occurred early or late after the MPM diagnosis). This meant that e.g both patients with MPE for 4% of their post-MPM diagnosis life, and those with 80% of their post-MPM diagnosis life could have been categorised as having had 'complete pleurodesis'. Partial pleurodesis referred to patients with persistent (no matter how small) MPE but not requiring further intervention for symptom relief. Therefore, patients may have had a small persistent MPE for 100% of their post MPM diagnosis life, but were categorised as having had 'partial pleurodesis'. 'No pleurodesis' referred to patients with an IPC *in situ* until death, or to those who required further repeated procedures to control the symptomatic MPE and with no documentation of resolution of MPE at any point. Initially, MPE presence/size not correlating with worse survival, and complete pleurodesis correlating with improved survival appear contradictory, however, as illustrated above, two different analyses were conducted:

- The effect of duration of fluid exposure on survival (with its limitations given the unreliability of chest x-ray when assessing effusion presence, as discussed in the limitations below),
- The effect of a patient's ability to achieve pleurodesis on survival.

Therefore a patient who is able to achieve complete or partial pleurodesis at any stage after MPM diagnosis seems to survive longer, perhaps because less advanced MPM

preserves the ability of the visceral and parietal pleura to appose, and allows pleurodesis to be achieved, whereas more advanced bulky MPM tumour prevents the full apposition and adhesion of the visceral and parietal pleura, hence preventing pleurodesis. The analysis of pleurodesis is a more accurate assessment than the percentage of life exposed to pleural fluid, because 'complete pleurodesis' was based on lack of pleural effusion on chest x-ray /ultrasound (thereby removing the bias of fluid *vs* thickened pleura which appear the same on chest x-ray), while 'no pleurodesis' was based on a need for further interventions to drain MPE fluid to relieve symptoms and this was an objective criterion. 'Partial pleurodesis' may still be biased because what was documented to have been a persistent pleural effusion on chest x-ray may well have been pleural thickening. In a review of two datasets of patients with MPE receiving intrapleural talc, successful pleurodesis was associated with improved survival (dataset 1: *n*=51, median survival 16 (95% CI 8.1-23.9) and 5 months (95% CI 2.6-7.5) for successful and failed pleurodesis respectively, *p* 0.007; dataset 2: *n*=259, median survival 11 (95% CI 10.8-11.2) and 6.4 (95% CI 4.9-7.9) for successful and failed pleurodesis respectively, *p* 0.001), maintained after adjusting for the primary tumour type and pleural fluid LDH.(Hassan et al., 2019)

It may be that talc itself has a positive effect on survival, and in fact in this study, patients receiving intrapleural talc had longer survival than patients who did not (*p* 0.003). There is *in vitro* evidence of the apoptotic effect of talc on MPM cells,(Nasreen et al., 2000) and in lung cancer cells,(Lee et al., 2010) while having no effect on normal pleural mesothelial cells' apoptosis. This effect may in part be due to higher levels of endostatin within pleural fluid after talc instillation, converting the pleural space from a highly angiogenic to an angiostatic environment.(Nasreen et al., 2007)

Pleurodesis success was an independent predictor of survival in this dataset, even when all known predictors were accounted for, and it is thus possible that pleurodesis has an effect on survival, the mechanisms of which require exploration. However, it should be noted that talc pleurodesis is also more likely to be considered in patients with higher ECOG PS (usually only considered in patients who have better performance status and life expectancy),(Roberts et al., 2010) thus confounding by indication. In addition, the mode of talc delivery (slurry via chest drain, or poudrage at thoracoscopy, which may well code for different ECOG PS) was unknown, and this may pose a further limitation, although no differences in rates of pleurodesis were found according to mode of talc delivery in a randomised control trial.(Bhatnagar et al., 2020)

Pleural effusion size and survival

It is difficult to draw definite conclusions about the secondary outcomes in this study given that some of the confidence intervals are relatively wide, however the size of a pleural effusion on chest x-ray at MPM diagnosis did not appear to be significantly associated with survival in this cohort. There are conflicting results of the association between MPE size and survival in the literature. In a study of 120 patients with MPE (not specifically MPM-related MPE), massive pleural effusions (defined as those extending from the diaphragm to above the lung hilum on a chest x-ray) were associated with worse survival than in patients with moderate (extending from diaphragm up to lung hilum) effusions (8 *vs* 11 months respectively, p <0.001),(Martínez-Moragón et al., 1998) however another study of 102 patients with MPE found that size of the MPE did not influence the 30 day survival rate.(Amin et al., 2017) This present study included a larger cohort of patients, and accounted for other confounders too, including the factors that are known to be associated

with survival in MPM.(Brims et al., 2016) Thus, the relationship between pleural fluid volume and "stage" or aggression of MPM remains unclear.

Chemotherapy response according to presence and size of pleural effusion at the start of chemotherapy

Similarly, neither presence nor size of pleural effusion at the start of chemotherapy was associated with chemotherapy response. However, this assessment was not an accurate assessment of the total effect of MPE fluid presence on chemotherapy response as the MPE present at the start of chemotherapy may well have been drained soon after, patients may have received intrapleural talc, or had an IPC inserted after the start of chemotherapy, thereby influencing the volume and duration of pleural effusion throughout chemotherapy. In addition, chemotherapy is only considered in patients who are fit enough to receive it, and in MPM it is usually only considered in patients with ECOG PS 0-2, with patients having lower ECOG PS being more likely to receive first line chemotherapy in this study (Chapter 6: Appendices).

Subanalysis of pleural fluid biochemistry and survival

In this current study, higher fluid total protein and glucose levels, and lower fluid LDH were associated with longer survival, although the latter did not reach statistical significance.

In MPE, low glucose has been associated with poor survival,(Amin et al., 2017; Martínez-Moragón et al., 1998) possibly due to greater pleural tumour bulk impairing glucose transfer from the bloodstream to the pleural fluid across the pleura.

Higher pleural fluid LDH levels were associated with worse survival in two studies of patients with MPE (*n*=284 and *n*=120 respectively),(Bielsa et al., 2008; Martínez-Moragón et al., 1998) with a weak association between fluid LDH levels and survival in another (*n*=102).(Amin et al., 2017) In fact, as mentioned above, pleural fluid LDH levels have been incorporated in a prognostic assessment score developed and validated for patients with MPE (LENT score, based on the pleural fluid LDH, ECOG PS, serum Neutrophil to lymphocyte ratio, Tumour type), where pleural fluid LDH levels of >1500 UL are assigned a higher score indicating worse prognosis.(Clive et al., 2014) Higher fluid LDH levels possibly reflect higher degrees of necrosis within the pleural cavity and this may explain its association with worse survival.

Low pleural fluid total protein levels were associated with worse survival in a study of MPE,(Bielsa et al., 2008) possibly reflecting the poor plasma protein levels commonly found in patients in advanced stages of malignancy, although pleural fluid protein level was not related to survival in another MPE study.(Amin et al., 2017)

Baseline characteristics and survival

The previously known factors associated with survival in MPM were also found to be associated with survival in our data, and therefore this cohort is likely to represent real-life data, despite limitations in the dataset. Factors such as weight loss, ECOG PS, and serum albumin, being associated with worse survival is perhaps not surprising given that all these factors tend to be associated with more advanced MPM disease.

Furthermore, patients who presented with pleural effusion had no statistically significant difference in survival when compared to patients who had no pleural effusion at MPM

diagnosis, as also noted by Bibby A, et al.(Bibby et al., 2019) This may be because patients with no pleural effusion tend to present at a later stage (due to lack of breathlessness) when the tumour has invaded the chest wall, therefore both MPM with pleural effusion and MPM without effusion carry a similar poor prognosis. This is in contrast to other primary cancers such as lung cancer, where the presence of MPE indicates more advanced malignancy stage. In fact, MPE is not factored in the assessment of MPM staging.(Pass et al., 2016) As yet, it is unknown why some patients with MPM develop MPE and others do not.

There was no statistically significant difference in survival according to the time period the MPM diagnosis was made in across the years from 2008 to 2018. MPM management has improved over the years, and improved diagnostic methods lead to earlier diagnosis, which should theoretically result in longer survival. The lack of improved survival over the years may be explained by the fact that the treatment of MPM has not changed much over recent years with the same first line chemotherapy being used for over a decade. However new medications such as immunotherapy emerging as possible first line alternatives for MPM may change these outcomes in the future.

Patients with symptoms relating to MPM at diagnosis showed increased survival when compared to patients without symptoms at the time of diagnosis (therefore likely to have had an incidental MPM diagnosis although the number of patients who had no symptoms relating to MPM at diagnosis was very small (*n*=28) when compared to those with symptoms (*n*=576).

Survival of patients with MPM was significantly lower in Glasgow than in Oxford and Bristol. The Scottish index of multiple deprivation (SIMD) takes into consideration multiple factors including income, employment, education, health, access to services, crime and housing. The SIMD analysis across Scotland shows that Glasgow includes >50% of the most deprived areas of Scotland. However, research has shown that despite correction for the socioeconomic profile, Glasgow has higher mortality every year than England and Wales, sometimes referred to as the 'Glasgow Effect', and this mortality excess in Glasgow is likely to be due to a combination of factors, including health behaviours such as smoking and drinking.(Walsh et al., 2016)

4.2 Limitations

This is a retrospective analysis with the associated biases, involving patients referred to three different UK pleural units at different stages of MPM. Apart from the limitation already mentioned above (namely that what is interpreted as a small -moderate pleural effusion on chest x-ray may actually be pleural thickening), the following are other limitations of this study.

If patients died within days of the MPM diagnosis, the tumour exposure to MPE fluid (from diagnosis to death) was very short. This means that short exposure time to pleural fluid may have been falsely correlated with short survival. An attempt to correct for this was made, and the duration of exposure to pleural effusion was calculated as a percentage of the total number of days that a patient survived after MPM diagnosis. However, this method also has its limitations because it was not possible to determine exposure of the MPM tumour to the pleural fluid prior to the date when MPM was diagnosed. Therefore,

exposure of the tumour to MPE fluid was calculated from the date of diagnosis of MPM, despite the fact that several patients would have had pleural effusions long before the actual diagnosis of MPM, especially given that the diagnosis of MPM may be notoriously difficult to make and usually requires pleural biopsy histology to secure the diagnosis. Patients may well have been referred to pleural specialist centres for pleural biopsy long after the pleural effusion was first diagnosed and after several other tests failed to show the true diagnosis. In addition, if patients had successful pleurodesis prior to, or on the date of diagnosis of MPM (such as cases where patients were diagnosed with MPM at thoracoscopy, during which talc was administered with subsequent successful pleurodesis), for the purposes of the analysis, the MPM exposure to MPE fluid was documented to be zero. This means that the exposure of MPM to MPE fluid in such cases was underestimated. In this study, data was collected on thoracocenteses procedures performed as clinically indicated, and not as part of a specific study protocol, and this was inevitable given the retrospective nature of this study.

In addition, when analysing the effect of the presence and size of a pleural effusion on survival, patients with larger or persistent pleural effusions are more likely to have been followed up, and followed up more regularly, therefore their effusion is more likely to have been documented in the medical records, than patients with smaller effusions who may have opted to be followed up by their oncology team or family doctors, and therefore their effusion was less likely to be documented than if they were followed up in a Pleural Unit.

If patients first had unilateral MPE, then developed a contralateral MPE, the total exposure time of the tumour to MPE fluid was calculated for the side the MPM diagnosis was made on, e.g. if MPM was diagnosed on right sided pleural biopsies, then the exposure to the

right sided MPE fluid was included in the fluid exposure time, regardless of whether the patient went on to develop a contralateral MPM MPE throughout the course of their disease.

If patients had only a small to moderate pleural effusion not requiring therapeutic pleural procedures, their presence was also included in the exposure time to MPE fluid, even if the volume was very small and may not have been enough to bathe the pleural tumour adequately.

Ideally all the chest x-rays and ultrasound scans would have been reported by the same independent radiologist / sonographer in order to limit bias.

The cohort included patients diagnosed with MPM between the years 2008 and 2018. In this cohort, survival according to time epochs was analysed, and there was no statistically significant difference, however there may still have been differences in the way MPE is managed (such as increased use of IPC when compared to talc pleurodesis, since chemical pleurodesis was first line of MPE treatment in 2010,(Roberts et al., 2010) and it is only after 2010 that IPC emerged as an acceptable first line alternative to chemical pleurodesis) over the years that may have influenced other outcomes.

Only chemotherapy administered for MPM was considered in this study. At least 3 patients received systemic treatment for other disease (cytarabine/daunorubicin for acute myeloid leukaemia, imatinib for gastrointestinal stromal tumour, and docetaxel for prostate carcinoma) during their MPM disease. It was not possible to determine the effect that other treatment targeting other disease may have had on the MPM progression. With

regards to determining chemotherapy response, MPM can be very indolent, so what was reported to be 'stable' disease following chemotherapy may not necessarily mean chemotherapy responsive since the MPM may have been chemotherapy resistant but slow to progress. Although data on presence and size of the pleural effusion at the start of chemotherapy was available, the details on the duration of pleural effusion throughout the course of chemotherapy was not, and it was not possible to analyse the effect of pleural effusion throughout the course of treatment on treatment-response.

Data on other factors associated with survival in MPE, such as pleural fluid pH,(Heffner et al., 2000) were not included in this analysis, as fluid pH is not part of the standard routine pleural fluid analysis panel and therefore was not available for analysis in this retrospective study.

Finally, due to the retrospective nature of the data, the final Cox regression model dropped 85.9% of cases because of missing values (analysing 14.1% of the initial dataset), highlighting the issue of missing data, including for example, missing ECOG PS data, which is recognized as a factor influencing survival in MPM. This should be only be considered as an exploratory study, given the limitations presented by the large number of missing data especially for the primary outcome of fluid exposure.

4.3 The development of patient-derived malignant pleural mesothelioma cell cultures from pleural fluid: a tool to advance biomarker-driven treatments

This study has shown that MPM cell cultures have been established from relatively small volumes of MPE fluid samples with a higher success rate than that reported in other studies. Samples from the established cell cultures were confirmed to contain cancer cells by a histopathologist with experience in MPM, they were confirmed to contain cancer stem cells by a tumoursphere assay, and passage 20 cells were confirmed to have similar variant profile to passage 0 cells, and thus the cell cultures very closely mimic the *in vivo*, native MPM tumours giving confidence that the cell cultures represent native tumour. Importantly, these experiments show that cell cultures can be established from pleural fluid samples of patients at separate time points; this has the potential to test cancer cells for drug response and resistance at different time-points in a patient's disease.

Establishing MPM cell cultures from pleural fluid

Pleural fluid cytology sensitivity for diagnosing MPM is highly variable, and reported to be 16-73%.(Woolhouse et al., 2018) The higher sensitivities are mostly obtained in specialist centres, probably because of the already high pre-test probability in cases seen in these centres, therefore this may not be applicable to other less specialised centres.(Asciak and Rahman, 2018) In fact, diagnosis of MPM often requires further testing beyond fluid sampling, usually with pleural biopsies being taken for histology. Almost all (16/17) of the samples from which cell cultures were established successfully had features consistent with MPM on the concurrent fluid cytology result, whether these features were defined as atypical cells, suspicious for MPM or positive for MPM in the cytology report. This is expected, as pleural fluid samples that are reported as being negative for MPM on cytology

contain little or no MPM cells, and therefore would have less chance of having a cell culture developed successfully from them, although a study published in 1989 reported that there was no direct relationship between the percentage of tumour cells seen on cytology (on a scale from 'not detected' to 60%) and the success rate for establishing MPM cell cultures,(Versnel et al., 1989) possibly implying that among the larger numbers of cells are several non-viable cells.

Sugaya et al. showed that lung cancer cell lines were more likely to be established successfully at higher stages of the malignancy (11/206 (5.3%) with advanced stage, compared to 4/349 (1.1%) with early stage lung cancer), although most samples used were from resected tumour tissue (*n*=549) rather than pleural fluid (*n*=10) samples.(Sugaya et al., 2002) It has also been reported that long term successful MPM cell culture was more likely in patients with greater MPM tumour burden.(Pass et al., 1995) However, in this study, albeit acknowledging that these were relatively small numbers, successful cell culture was not more likely to be from pleural fluid sampled in late stage (beyond stage 2) MPM.

Previously, MPM cell cultures have been established mostly from MPM pleural biopsy tissue, as opposed to MPE fluid, with cell culture from biopsy tissue reported to be easier.(Pelin-Enlund et al., 1990) MPM cell culture from MPE fluid has been conducted before, however, with lower success rates (4/20 (20%),(Orengo et al., 1999) 3/11 (27%),(Pass et al., 1995) 10/46 (21.7%),(Versnel et al., 1989) and 11/33 (33%, although at least one of these cell cultures did not grow in culture directly from the original MPE fluid sample, with cells being first administered to *BALB/c* nude mice, and the cells from the subsequent xenograft being seeded in culture flasks).(Manning et al., 1991)) One study

reported a 2/4 (50%) success rate, however the sample number was very small.(Hsu et al., 1988) The culture medium and technique used was similar to that of other groups as reported in the literature, however the success rate obtained in this current study, of 17/37 (45.9%) compares very favourably, possibly because of a combination of factors:

- The samples of pleural fluid being processed and cells seeded and incubated within a maximum of 2-3 hours of the fluid being drained from patients - the fluid samples were therefore never refrigerated or frozen prior to being processed reducing the potential for degeneration of the sample;
- Great care was taken to avoid contamination of the samples so that no samples were lost to contamination;
- The laboratory has extensive experience in cell culture;
- Other potential influencing factors on the cell culture success rate may be related to the fluid sampling technique, in that all pleural fluid samples used were taken under strict aseptic conditions with the operator fully scrubbed up, wearing sterile gown and gloves in a theatre environment, potentially reducing the contamination rate of the samples;
- Finally there may also be a difference in the patient population the samples were taken from as compared to the patient population in other studies.

There is also often a lack of adequate relevant clinical information regarding the same patient the cell culture was derived from.(Pass et al., 1995) In this project, all relevant clinical information, including chemotherapy response and presence and size of pleural effusion, was available, and will be used to guide further experiments on the cell cultures. Furthermore, the volume of pleural fluid collected to establish the MPM cell cultures (50-60ml) was lower than that reported in other studies (50-100ml,(Versnel et al., 1989) 100-1000ml,(Kalra et al., 2015) 100-5000ml(Manning et al., 1991)) A study reported using 20ml

MPE fluid samples, however it is unclear how many of the 4 MPM cell cultures established in this study were from MPE fluid samples and how many were from tumour tissue, making it difficult to determine the success rate of establishing cell cultures from 20ml MPE fluid samples.(Usami et al., 2006)

Two MPM cell cultures from pleural fluid of the same patient have been established before, but at the same time point.(Usami et al., 2006) Interestingly, Usami et al. found that these cell cultures, despite being from the same patient at the same time point, were morphologically different, and with different genetic expression,(Usami et al., 2006) highlighting the great heterogeneity that exists in MPM, even within the same tumour. It therefore also highlights the difficulties in finding suitable effective targeted MPM treatment. In this current study, we have managed to establish MPM cell cultures from the same patient but at different time points. This is valuable because it potentially allows the studying of molecular alterations due to MPM progression.

The variability in growth rates exhibited by the various cell cultures noted in this study has previously been noted in the literature: the doubling time in 5 MPM cell lines varied significantly from 18 hours to >30 hours, with an average doubling time of 25-46 hours.(Manning et al., 1991) Clinically, this is evident in the variability seen in MPM progression and patient survival, even in patients with the same subtype and who present at a similar MPM stage. This feature is important in the laboratory as it may affect drug screening tests, since the drug susceptibility may depend on cell cycle effects.(Pass et al., 1995)

Tumoursphere assay

All the 7 MPM cell cultures tested developed mesospheres, and this implies that the cell cultures contain cancer stem cells. This is important because it implies that the cells in the cell culture are indeed cancer cells, mimicking the *in vivo* situation, and furthermore, stem cells can be isolated and used for more robust drug screening assays than those performed on adherent cell culture. As discussed, drug screening assays using tumourspheres rather than adherent cell cultures can test whether drugs target cancer stem cells too rather than just the other cancer cells.

DNA sequencing

The cell cultures were morphologically diverse, with different variants in MPM-related genes, reflecting inter-patient heterogeneity in MPM, even when comparing the same MPM histological subtype. Variants were detected in tumour suppressor genes, as also described in previous studies.(Hmeljak et al., 2018) The small number of additional variants detected in late, when compared to early, passage cells, indicates likely close resemblance to the native tumour even in later passage cells, suggesting that our cell culture method provides a reliable model of the original tumour.

Studies have shown discordance in the genetic profile between the primary tumour and metastatic deposits. A study of 37 lung adenocarcinoma cases found 16% discordance in the EGFR variants between primary tumour and corresponding metastases samples (*N*=37: *n*=21 pleural effusion and pleura, *n*=5 brain, *n*=3 lymph nodes, *n*=2 lung, *n*=2 soft tissue, *n*=2 pericardial effusion and pericardium, *n*=1 adrenal, *n*=1 ovary), and 14% discordance in EGFR variants specifically between primary tumours and pleural metastases (*n*=21) – the pleura being the closest metastases site to lung.(Han et al., 2011) This discordance may be

due to genetic drift or clonal selection that gives rise to the metastatic phenotype, and in addition, some tumours such as non-small cell lung carcinoma, metastasise early and therefore the primary and metastatic tumours evolve independently, leading to variation in the variant profile between primary tumours and corresponding metastases.(Klein, 2009, 2008) This is important because while in this study, harvesting cells from the MPE fluid is likely to represent MPM tumour, because the primary tumour is in close proximity within the pleural cavity, it may not necessarily be true for other non-MPM primary tumours that have metastasised to the pleura. In the latter case, the genetic profile of the harvested metastatic cells from the MPE fluid may be different from that of the primary tumour.

4.4 Limitations

There is growing evidence that cryoprotective agents such as DMSO, may cause variants at a cellular, proteomic, and genomic level,(Baust et al., 2009) and therefore the freezing medium used (10% DMSO and 90% FBS) may be the cause for some of the variants detected in the late passage when compared to the early passage cell cultures. It is unknown whether this effect is reduced with lower concentrations of DMSO, such as 95% FBS with 5% DMSO, however this would have increased the chance of cell death during the thawing process, given that DMSO is used in the freezing medium as a cyroprotective agent. In addition, large concentrations of FBS and nutrients may also promote changes in genetic expression.(Kirby (née Tomkins) et al., 2002; Pandey et al., 2010) Furthermore, trypsin was used as the dissociating agent for adherent cell culture in this study, and it has been reported that cells treated with trypsin during cell culture express different proteins from

cells not treated with trypsin, including proteins that regulate cell apopotosis, cell metabolism and growth.(Huang et al., 2010)

Single cell sequencing of the initial MPM tumour would be required to identify which of the variants detected exclusively in late passages are due to long-term culture, rather than representing a small non-detectable subpopulation in the original MPM tumour.

For the purposes of this study, genetic variants in cancer-related genes were analysed. The genetic variants of regulatory regions were not studied and so mutations resulting in different protein synthesis may have been missed.

As discussed earlier, early passage (such as passage 0) cell cultures consist of a mix of different cell types, and non-cancer cells eventually die off with serial passages, while immortal cancer cells persist in later passages. Comparison of the genomic sequence of passage 0 cells to passage 20 cells may mean comparison of the genomic sequence of a mix of different cell types, with that of a pure cancer cell population. To mitigate for this, the cancer-related genomic variants were assessed (MSK-IMPACT). For the purposes of this study, the DNA sequencing step sought to ensure that cancer cells in passage 20 were still representative of the patient's native tumour cells (i.e. similar to passage 0 cancer cells) and importantly, to ensure that the patient-derived MPM cell cultures were adequate for high quality DNA sequencing and drug screening assay results, which they were. However, if future studies aim to use the patient-derived cell cultures to establish personalised drug response/resistance profiles for patients, then more in-depth genomic sequencing analyses need to be done on a larger scale.

4.5 Investigating the biological properties of pleural fluid: *in vitro* experiments

Cells from cancer cell cultures proliferate *in vitro* in 100% pleural fluid alone

In the subanalysis of the retrospective MPM patient database, higher pleural fluid total protein levels were associated with longer survival. Additionally, MPM cells proliferated similarly in exudate and in transudate MPE fluid *in vitro*, indicating that it is not simply the quantity of proteins within the MPE fluid that gives the fluid the biological capabilities that support cancer cell proliferation *in vitro*. Furthermore, the cancer cells proliferated in heart failure non-MPE transudate pleural fluid, and thus the biological properties of pleural fluid are not solely due to factors secreted by pleural tumours. However, although not a statistically significant difference, across all 6 cancer cell cultures tested (4 MPM, 1 breast carcinoma and 1 lung adenocarcinoma), the slowest growth was recorded with transudate MPE or with heart failure transudate fluid, when compared to exudate MPE fluid. The only difference in doubling time that reached statistical significance was with cell culture MESO163, with a statistically significantly faster doubling time with exudate MPM MPE fluid than with transudate lung adenocarcinoma MPE fluid. Potentially, transudate pleural fluid contains cytokines and nutrients since it is a filtrate of blood via capillaries, and therefore is able to support cancer cell proliferation *in vitro*; however, there have been no good quality studies assessing the components of transudates in this regard.

Cheah et al. reported non-MPM MPE fluid and benign pleural effusion fluid to be associated with increased MPM cell proliferation *in vitro*, by 1.4–2.8 fold and by 1.3–2.2 fold respectively, when compared to serum-free medium as a control.(Cheah et al., 2017) The

difference in this present study is that cells were incubated with 100% pleural fluid as compared to 30% pleural fluid in culture medium, and therefore this study is more representative of the clinical scenario of pleural tumour cells bathed in 100% pleural fluid as would be found in the pleural space.

Importantly, our findings indicate that the biological properties of pleural fluid are not seen solely with MPM cancer cells but also with other non-MPM cancer cells including lung and breast carcinoma. Future studies further exploring this area should therefore focus on other malignancies in addition to MPM.

MPM cells in vitro in MPE fluid have a tendency to form aggregates

Clinically, MPM characteristically forms multiple macroscopic pleural tumour nodules. Similarly, MPM cells in culture, apart from exhibiting piling and sloughing upon reaching confluence which is a common feature of malignant cells,(Manning et al., 1991) were noted to tend to group together as clusters in a colony-like formation on prolonged culture in pleural fluid *in vitro*. This has been observed previously with MPM cells cultured in complete medium at high cell density, while human primary mesothelial cells in similar culture conditions and duration remained in a monolayer.(Kalra et al., 2015; Pass et al., 1995; Tárnoki-Zách et al., 2015) The *in vitro* MPM nodules are described in the literature as being dense, highly cellular, and rich in stress filaments with cell motion being towards the nodules or the interconnecting strands between nodules, as also observed in these current experiments, and the nodules were noted to form also in the absence of cell proliferation, therefore are not clusters of highly proliferative cells.(Tarnoki-Zach et al., 2020; Tárnoki-Zách et al., 2015) The mechanochemical theory of pattern formation is that traction forces exerted by cells on the surrounding medium, result in the transportation of

cells and extracellular matrix (ECM) via strain-oriented ECM filaments that guide cell motility and cell aggregation into clusters. The underlying substrate must be deformable for this to occur, e.g. endothelial cells cultured on gelled basement membrane matrix,(Manoussaki et al., 1996) and the pleural fluid used as a culture medium in these experiments acts as a deformable substrate.

These features observed *in vitro* are important as clinically, cellular mobility is fundamental to tumour progression and contributes to local and distant spread of malignant cells. In addition, cell aggregation into nodules is important because cells within an avascular 3-dimensional nodule have less exposure to systemic drugs, and therefore inhibition of the formation of nodules may potentially help increase drug response in MPM. Myosin II inhibitors such as Y27632 prevent the formation of nodules and decrease MPM cell proliferation *in vitro.*(Tarnoki-Zach et al., 2020) If this *in vitro* evidence is translated to humans, and myosin II inhibitors can prevent formation of nodules and decrease MPM cell proliferation in humans, then they may prevent local spread and metastasis of MPM. By preventing nodule formation, myosin II inhibitors may work synergistically with other systemic anti-cancer drugs enhancing cancer cell response rate to the anti-cancer drugs.(Tarnoki-Zach et al., 2020; Tárnoki-Zách et al., 2015) However, further research is required to explore myosin II inhibitors as a potentially novel MPM treatment.

To my knowledge, this study is the first to demonstrate nodule formation in prolonged cancer cell culture in 100% pleural fluid. Demonstrating that MPM cells form aggregates and nodules in 100% MPE fluid culture may represent a potential new model to test anti-MPM drugs; a model which more closely reflects the *in vivo* scenario of pleural cancer cells

bathed in MPE fluid, especially for experiments seeking to disrupt the aggregate and nodule formation to allow better penetration of drugs.

Primary MPM cell culture in 100% MPE fluid alone has a similar success rate to primary MPM cell culture in full culture medium

In addition to pleural fluid having the ability to support cell proliferation from established cancer cell cultures *in vitro*, 100% MPM MPE fluid has the ability to support the primary cell culture of MPM cells, extracted directly from pleural fluid, without the need for addition of any other nutrients. Furthermore, MPE fluid was able to support cell proliferation in primary MPM cell culture at a similar rate to full culture medium – fluid specifically manufactured to contain optimum nutrition to support cell proliferation.

Primary cancer cell culture can be notoriously difficult and is a delicate process requiring time, extreme care to avoid contamination, and requires numerous laboratory resources and reagents. It is traditionally performed using artificial specially-manufactured cell culture medium, containing all the nutrients required to provide the optimal environment for cell proliferation *in vitro*. Despite these conditions, the reported success rates of primary MPM cell culture in the literature are variable: 4/20 (20%),(Orengo et al., 1999) 3/11 (27%),(Pass et al., 1995) 10/46 (21.7%),(Versnel et al., 1989), 11/33 (33%),(Manning et al., 1991) and one study reporting a 2/4 (50%) success rate, however the sample numbers in this latter study were very small (*n*=4).(Hsu et al., 1988) The success rate of primary MPM cell culture from pleural fluid and using artificial full culture medium in this current project was 17/37 (45.9%). The primary MPM cell culture using MPM MPE fluid instead of full culture medium was 3/6, 50%, and identical to the control (concurrent primary cell culture of the same cells in full culture medium with otherwise identical

conditions). Possible reasons for the relatively higher success rates for MPM cell culture obtained in this project may be because of a combination of factors, including samples of pleural fluid being taken under strict aseptic conditions, potentially reducing the number of samples lost to contamination or infection, the fluid samples being processed and cells seeded and incubated within a maximum of 2 hours of the sample being taken from patients, and the laboratory having extensive experience in cell culture. There may also be a difference in the patient population the samples were taken from as compared to the patient population in other studies, in terms of length of time of MPE or chemotherapy naivety.

Lung cancer primary cell culture from pleural fluid has been performed using 30% sterile-filtered MPE supernatant in the culture medium, aiming to avoid eliminating tumour cell subpopulations that depend on the tumour microenvironment for survival, thus attempting to maintain intratumour heterogeneity,(Basak et al., 2009) with the culture medium being replaced every 5-7 days. The 30% MPE value had been chosen after comparing primary cell culture with various percentages of MPE fluid from 10-100%, and finding that cultures with 10% and 100% MPE showed increased numbers of dead cells, while 30%, 50% and 70% MPE was associated with no apparent differences in culture integrity and numbers of dead cells. The authors report that the cultures with 30% MPE, when compared to parallel cultures with 10% FBS, had increased pleural fluid cell growth rates and this was confirmed in another similar study,(Ruiz et al., 2016) however this may be a reflection of the increased percentage of MPE when compared to FBS, because increasing percentages of FBS may be associated with increased growth rates in some cell cultures.(Ryan, 1979) In the current study, primary MPM cell culture was performed with 100% MPE as culture medium, and the success rates obtained were potentially partly due

to the fact that the pleural fluid was replaced more frequently, every 48 hours, therefore replenishing nutrients. Apart from the proof of concept that MPE fluid is adequate for primary MPM cell culture, the primary cultures in MPE fluid are more representative of the primary tumour in humans because of the maintenance of part of the tumour microenvironment (pleural tumour *in vivo* is bathed in pleural fluid in the setting of MPE), and may therefore potentially allow the studying of intratumour heterogeneity in culture in a setting that more closely resembles the *in vivo* environment.

Primary cell culture in serum-rich media has its advantages as serum provides a source of growth factors and nutrients to support cell growth, leading to faster growth rates. However, there is serum-to-serum variability in terms of performance due to varying nutrients within different serum samples, and the serum may lead to cell differentiation resulting in a cell population different from the native tumour cells.(Price and Gregory, 1982) The serum used to supplement culture medium is usually FBS; finding a suitable alternative is desirable as, apart from the cost implications of using FBS, there are also ethical implications in terms of animal protection with regards to the harvesting and collection of FBS. Pleural fluid is similar to serum, containing growth factors and nutrients that support cell proliferation, and also more closely reflects the *in vivo* environment of pleural cancer cells, and therefore may present a potential alternative to FBS for primary cell culture. However, similar to serum, there is great fluid-to-fluid variability in terms of the biochemical profile, hormone content and growth factor content, therefore may lead to variable cell proliferation rates. Furthermore, experiments with large numbers of samples are required to determine whether primary cell culture in pleural fluid leads to a significantly different cell population from that resulting from primary culture of cells in standard culture medium with 10% FBS.

Pleural fluid has a tendency to form a gel-like substance

A transudative pleural effusion results from an imbalance between the hydrostatic and oncotic pressures in the setting of relatively normal pleura, and an exudative pleural effusion results from an inflammatory process with pleural injury. Exudates contain higher levels of protein and inflammatory markers such as LDH than transudates do.(Light, 1972) Highly viscous MPE has been reported to be associated with MPM, possibly secondary to higher hyaluronic acid levels within the fluid, although it is unclear why this happens.(Cheah et al., 2021) The coagulation system is closely linked to the inflammatory process, orchestrating cell proliferation, migration and synthesis of inflammatory mediators. It is normally activated to promote healing, however chronic activation of the coagulation system can lead to chronic release of procoagulant factors such as tissue factor, cellular activation protein modulation via transformation of fibrinogen into fibrin, and histological changes promoted by cytokines. Disordered fibrin turnover within the pleural space has been reported in some studies, favouring procoagulant activity and fibrin deposition in exudative pleural effusions.(Idell et al., 1991) The highly proteinaceous nature of exudative MPE fluid, with high levels of inflammatory mediators leads to a complex situation with many factors contributing to the fluid properties, and, as yet, it is not clear whether the overall state within the pleural space in MPE is procoagulant or anticoagulant. A study reported increased production of thrombin as reflected by higher levels of fragment 1+2 in exudate pleural fluid (*n*=39: 9 parapneumonic, 15 tuberculosis and 15 MPE), and lower levels of fibrinogen (suggesting high consumption) when compared to transudate pleural fluid (*n*=15). This suggests a procoagulant effect within exudate pleural effusions. However, the same study also found higher levels of fibrin degradation

products and d-dimer, with levels being higher than serum levels, in exudates than in transudates, suggesting that a fibrinolytic process is underway. Moreover, thrombin-antithrombin complex levels were higher in exudates, antithrombin being able to block activated thrombin function, suggesting that a counterbalance to procoagulant effect is present in exudates.(Vaz et al., 2009) This balance between pro- and anti-coagulant within exudative pleural effusions seems to be tipped in favour of anticoagulation given that MPE fluid does not usually spontaneously coagulate within the pleural space, and the failure of MPE fluid to coagulate and form a fibrin clot within the pleural cavity enables its accumulation.

A further possible reason for pleural fluid anticoagulant properties may also be influenced by mast cells within the pleural fluid. In related studies, levels of mast cells within human MPE fluid were higher than in benign pleural effusion fluid. In addition, in mouse models of lung and colon adenocarcinoma, mice lacking mast cells did not develop MPE, while matched mice with mast cells developed MPE and had increased tumour growth. Mast cells secrete multiple inflammatory and vasoactive mediators, including tryptase, which induce pleural vasculature leakiness, and trigger nuclear factor-κβ activation in pleural tumour cells leading to pleural fluid accumulation and tumour growth.(Giannou et al., 2015) Nuclear factor-κβ maintains cell viability by inhibiting apoptosis, in fact nuclear factor-κβ inhibitors led to decreased tumour burden and MPE volume in a mouse model of lung adenocarcinoma.(Stathopoulos et al., 2006) One theory explaining why pleural fluid does not coagulate within the pleural space is that the tryptase secreted by mast cells prevents this because tryptase breaks down fibrinogen (fibrinogen is converted to fibrin by thrombin, leading to clotting and thrombosis, and tryptase therefore prevents this).(Giannou et al., 2015; Prieto-García et al., 2012)

Clinically, although most MPE is fluid in consistency and easily aspirated, in longstanding pleural effusions, the pleural fluid can become viscous and difficult to aspirate or drain with a needle including larger-bore aspiration catheters. In the literature, the reported features of a long-standing pleural effusion are that they tend to be lymphocytic, and there is an increased chance of pseudochylothorax,(Karkhanis and Joshi, 2012) however there is a lack of literature on the commonly encountered viscous, gel-like pleural effusion. Malignant pleural fluid is most commonly an exudate, with high protein, albumin and lactate dehydrogenase (LDH) levels. In a study of 70 exudative pleural effusions, the viscosity of pleural fluid was found to be significantly correlated with pleural fluid protein, albumin and LDH levels.(Yetkin et al., 2007) There is possibly higher protein secretion or leakage into the pleural fluid in more advanced malignancy due to higher inflammation levels, or potentially some of the water is able to be re-absorbed by the pleura infiltrated by malignant disease, but the proteins are not as easily re-absorbed (water is able to cross biological membranes more easily than larger proteins do (Kedem and Katchalsky, 1958)) and therefore the underlying biochemical composition of the fluid eventually changes to one that is richer in protein and as a consequence, has a more viscous consistency.

The tendency for the pleural fluid to form a gel-like substance *in vitro* hindered some aspects of the experiments, as the quality of some of the images of the cells was poor, each aspiration of the viscous pleural fluid from the wells resulted in the discarding of several cells that were thriving within the gel-like substance and not adhered to the base of the plate, and as it was not possible to stain the cells with crystal violet to demonstrate progressive increased confluence because the viscous pleural fluid absorbed the stain and therefore did not provide adequate contrast between the cells themselves and the

surrounding environment. This made accurate assessment of cell proliferation difficult, and 3 separate ways of assessing cell proliferation were used in the experiments in an effort to counterbalance this bias: the use of CellTiter-Glo® luminescent cell viability assay, the calculation of the percentage of cell confluence using Fiji (ImageJ) on images obtained using Incucyte® machine, and observation of the wells (and recording of images of the wells) under a microscope to confirm cell activity and increased proliferation. All three methods demonstrated unequivocally that cancer cells thrive and proliferate in 100% pleural fluid alone *in vitro*. This tendency for the pleural fluid to form a gel-like substance *in vitro* may be because the anticoagulant factors are denatured by the freezing process (pleural fluid was stored frozen prior to being used for these experiments), or perhaps when *in vivo*, the procoagulant properties are continuously being counterbalanced by fresh anticoagulant factors, and this continuous supply is not available *in vitro*.

Potential ways to overcome the difficulties caused by the gel-like pleural fluid in a 2D culture, as well as to better mimic the *in vivo* conditions, include dynamic cultures, such as using plate rockers, growing cells in a vacuum Flexcell® device, or ideally, using 3D cell culture.

4.6 Limitations

Pleural fluid viscosity limited the ability to demonstrate cell proliferation and cellular viability using the traditional ways applicable to cells cultured in clear full culture medium. To overcome this, a combination of methods were used to assess cellular proliferation, including live images, luminescence assay, and using software to calculate relative confluence. A continuous fresh supply of matched MPM MPE fluid to continue the

prolonged MPM primary cell culture *in vitro* was not possible, therefore cells from pleural fluid were extracted and seeded in matched pleural fluid from the same patient, and the fluid was refreshed every 48 hours until the plate reached confluence. At that point, cells were split and seeded in complete medium and cell culture continued as per usual protocol. However, the fact that an established (all cells phenotypically similar) cell culture appeared within 2 months of continued cell culture indicates that at passage 0, pleural fluid had adequate nutrition to sustain the cancer cells *in vitro*.

4.7 Studying malignant pleural effusion development and malignant pleural effusion fluid effects *in vivo* using animal models

This discussion is included in Chapter 5: Future directions, including a discussion about limitations of described animal models.

4.8 High-throughput drug screening and deoxyribonucleic acid sequencing of patient-derived malignant pleural mesothelioma cell cultures

This was a feasibility study assessing if it was possible with high quality results to enable a pathway from readily available MPE fluid to laboratory cell culture of the MPM cells allowing *in vitro* drug screening and DNA sequencing. The aim was achieved and this pathway is feasible; drug screening and DNA sequencing results of patient-derived MPM cell cultures were available with good data integrity within 2 months from the sampling of pleural fluid. This timeframe means that it is possible to allow feedback of drug resistance and response profiles to clinicians treating patients, and this may enable guiding of cancer

treatment, if the *in vivo* response pattern is shown to be correlated with real life clinical response.

DNA sequencing

The identification of predictive markers to identify drug-responders and non-responders before treatment is initiated is important, since treatment response rates correlate with tumour phenotype and overall survival.(Blayney et al., 2012; Ceresoli et al., 2001)

DNA sequencing should help to identify variants that may be targeted with cancer treatment, however in mesothelioma this is particularly challenging. When 10,945 tumour samples (varied primary malignancies) from 10,336 patients were sequenced using the MSK-IMPACT assay, 37% of samples were found to harbour a clinically relevant alteration. However, mesothelioma (*n*=106 mesothelioma samples in total) had the lowest percentage of samples with an actionable variant. Those mesothelioma samples that did have actionable variants had the lowest level of actionability based on the identified variant (the authors assigned a level 3b actionability, i.e. predict for response to investigational agents in clinical trials for a different tumour type).(Zehir et al., 2017) This further highlights the uphill struggle to identify clinically useful therapies for MPM despite the use of advanced techniques. A simplified model using cell cultures with drug screening and DNA sequencing can help increase the number of patients who can access this testing, since it does away with the need for pleural biopsy tissue; in addition, it can be repeated at different time points allowing the potential for identification of genetic modification that occurs during the time course of the disease. This may augment the probability of identifying actionable targets.

Drug screening

The results of the drug screening assay showed 6 drugs to be consistently more effective than cisplatin and pemetrexed combination, i.e. they were more effective at killing cancer cells *in vitro* than the standard first line chemotherapy currently available for mesothelioma. These were bortezomib, carfilzomib, dactinomycin, idarubicin, dinaciclib (SCH727965), and omacetaxine mepesuccinate. As discussed, results from *in vitro* experiments do not always translate to *in vivo* clinical effects in terms of drug response, and furthermore, the toxicity and adverse effect profile *in vivo* needs to be considered. However, with regards to the latter point, all 6 drugs have been approved by the FDA for malignancies other than MPM, and therefore have already undergone testing in humans with profiling of their ADMET characteristics when used for other malignancies.

The mechanisms of action of each of these drugs, and their potential in mesothelioma, are discussed below.

Bortezomib

Bortezomib is the first therapeutic proteasome inhibitor to be used in humans.(Chen et al., 2011) It has been approved for treatment of multiple myeloma, mantle cell lymphoma, other plasma cell disorders and non-Hodgkin's lymphoma.(Tan et al., 2018; "Velcade 3.5mg powder for solution for injection - Summary of Product Characteristics (SmPC) - (eMC)," n.d.) There is early *in vitro* evidence of the efficacy of bortezomib in MPM.(Gordon et al., 2008; Sun et al., 2006; Szulkin et al., 2013) This preclinical evidence spurred the setting up of early clinical trials, however, the results of a phase II trial of bortezomib in unselected

MPM patients were disappointing, with bortezomib monotherapy exhibiting insufficient activity to warrant further investigation in this group of patients.(Fennell et al., 2012) Similarly, a prospective phase II trial of first line treatment with bortezomib combined with cisplatin in 82 mesothelioma patients recruited between 2007-2010, showed the primary endpoint of progression free survival rate at 18 weeks to be 53% which was within the 80% confidence intervals (42-64%).(O'Brien et al., 2013) This implies that mesothelioma may harbour inherent resistance mechanisms.

Carfilzomib

Carfilzomib, like bortezomib, is a proteasome inhibitor. It is approved for use as treatment in multiple myeloma refractory to treatment with bortezomib and immunomodulatory therapy. It was tested in phase I and II trials in advanced solid tumours, and one patient with mesothelioma was included in the phase I trial. The conclusion of the trial was that carfilzomib inhibited proteasome in blood, but had only limited anti-neoplastic effects at the doses tested.(Papadopoulos et al., 2013)

Dactinomycin

Dactinomycin is a polypeptide antibiotic, which intercalates into DNA preventing progression of RNA polymerases and is therefore commonly used as a transcription inhibitor. It was the first antibiotic to be used for cancer treatment, such as gestational trophoblastic neoplasia, Wilm's tumour, and Ewing's sarcoma.(Bensaude, 2011) In mesothelioma cell lines, it has been found to have synergistic activity in combination with recombinant human TNF alpha (rHuTNF alpha) *in vitro*. However when the mesothelioma cell line was transplanted to nude mice as a subcutaneous tumour, there was no significant inhibition of tumour growth with the combination of dactinomycin and rHuTNF alpha, but

there was significant cachexia. This suggests that *in vivo*, the synergy is mainly manifest as side effects, although this study was limited by the fact that nude (immunosuppressed) mice were used, making it difficult to draw conclusions of the potential effect of the drugs in immunocompetent humans.(Bowman et al., 1991) More recently, dactinomycin was found to be synergistic with the protein synthesis inhibitor, RG7787, to kill a variety of cancer cell lines, including human mesothelioma cell lines, and the combination caused tumour regression in mice with pancreatic and stomach cancer cell lines.(Liu et al., 2016)

Idarubicin

Idarubicin is an anthracycline, and like dactinomycin, is derived from *Streptomyces* bacterium. It is used in the treatment of some haematological malignancies such as acute non-lymphocytic leukaemia and acute lymphocytic leukaemia, and in advanced breast carcinoma.(Cersosimo, 1992) Clinical trials of anthracyclines such as doxorubicin and epirubicin, as single agent treatment for mesothelioma have only demonstrated low (0-15%) response rates, although anthracycline use as part of combination regimens has shown reasonable response rates,(Jackman, 2009) with the highest response rates (28.5%) obtained when combined with cisplatin in a systematic review published in 2002.(Berghmans et al., 2002) Combination of anthracyclines with other agents may decrease the cytotoxic dose required. The combination of doxorubicin with acid prepared mesoporous microspheres to deliver the doxorubicin directly to mice mesothelioma tumours, intrapleurally or subcutaneously, enabled delivery of effective cytotoxic doses, lower than the required systemic (and lethal) dose of doxorubicin.(Hillegass et al., 2011) Observational studies report improved survival, when compared to historical controls, in patients with peritoneal mesothelioma who underwent surgery and intraperitoneal doxorubicin.(Yan et al., 2007)

Dinaciclib (SCH727965)

Dinaciclib (SCH727965) is a novel cyclin dependent kinase (CDK) inhibitor, inhibiting CDK2, 5, 1 and 9, and also blocks thymidine DNA incorporation. The loss of cell cycle control is common to all human cancers and is often associated with aberrant activation of CDKs which are critical regulators of cell cycle progression. Dinaciclib has been shown to have the potential to inhibit growth of several human cancer cell lines (mesothelioma was not tested), in both *in vitro* and *in vivo* models.(Parry et al., 2010) Early studies show that dinaciclib has activity against refractory chronic lymphocytic leukaemia,(Flynn et al., 2015) osteosarcoma,(Fu et al., 2011) melanoma,(Abdullah et al., 2011) and relapsed multiple myeloma.(Kumar et al., 2015)

Omacetaxine mepesuccinate

Omacetaxine mepesuccinate (formerly known as homoharringtonine) is a natural alkaloid and protein translation inhibitor, which acts by preventing the initial elongation step in protein synthesis. It has been approved for use in refractory chronic myeloid leukaemia. There is evidence of omacetaxine mepesuccinate's efficacy in solid tumours, and it suppressed the growth of lung adenocarcinoma expressing Kras variant both *in vitro* and in animal models,(Weng et al., 2018) however Kras variant does not seem to play a critical role in the development of mesothelioma in humans or in rats.(Ni et al., 2000)

Mechanisms of drug resistance in MPM

As seen in some of the above examples, *in vitro* drug screening results are not always translatable to clinically useful information. A better understanding of the underlying

mechanisms behind drug resistance in MPM would be useful to identify potential targets for future MPM therapies that overcome these mechanisms. Over the past few years there have been advances in mapping some of the mechanisms associated with resistance to conventional treatment in MPM. When deposited in the lung, the crocidolite asbestos fibres may cause hypoxia and iron chelation at the fibre surface, altering intracellular iron availability. In a study, activation of hypoxia-inducible factor 1-alpha was caused by crocidolite, by iron-chelator, and by hypoxia, and this was associated with increased doxorubicin resistance in human MPM cells.(Riganti et al., 2008) In another study, epigenetic methylation of growth factor-1 induced transcriptional silencing of thymidine phosphorylase in MPM cell lines, leading to the inability of thymidine phosphorylase to convert capecitabine to active drug. This was associated with resistance to capecitabine.(Kosuri et al., 2010)

This was an initial assessment of the feasibility of the bench-to-bedside pathway, using three cell cultures. If drug screening and DNA sequencing are done on a larger scale, it may potentially be possible to provide a "score" for likely susceptibility to first line chemotherapy as well as identifying potential new drug targets.

4.9 Limitations

The following are limitations of personalised cell cultures in the current study.

- Although the success rate of cell culture was higher compared to previous studies, this figure (45%) needs to be increased further aiming to develop personalised cell cultures for

more patients, and the reasons for which some samples fail to generate a cell culture require investigation.

- There is a delay of about two months between seeding cells after obtaining pleural fluid, to establishing a MPM cell culture. In the context of the clinical pathway, where it takes approximately four weeks between sampling, histological/cytological diagnosis, treatment planning, and treatment initiation, this represents a potential delay in therapy if MPM cell cultures are used to direct management. However, if this management is more targeted or associated with better clinical response, then this delay may potentially be acceptable. On the other hand, patients with treatment resistance are likely to have even shorter survival and are the patients who are potentially most likely to benefit from this bench-to-bedside pathway towards personalised treatment, so shortening the pathway to drug resistance/response profiles is ideal. Potential ways to speed up the cell culture process include increasing percentage of serum in the culture medium – 20% FBS has been used for MPM cell culture, however increasing FBS concentration may promote variation in cell phenotype and genetic mutation profile which may produce different drug screening results; cell cultures may also be used for the drug screening assays and DNA sequencing at an earlier passage than used here, however there is a risk of having a larger percentage of non-MPM cells within the cell population, since it is cancer cells that are able to avoid senescence and are able to continue proliferating at serial passages – conducting drug screening assays and DNA sequencing on a cell population that is mixed may alter the drug screening assay results too, and may be less representative of the native MPM tumour.

Finally, this study used serial passages relying on the immortality of cancer cells to enrich for MPM cells. However, other ways of cell enrichment that would reduce the number of passages and reduce the overall time delay include fluorescence-activated cell sorting

(FACS), or magnetic-activated cell sorting (MACS®), to sort non-cancer cells from MPM cells.

- Drug screening and DNA sequencing is a costly process.

- Although the feasibility of this pathway has been shown, *in vitro* drug screening results are not always translatable to human drug response results. The high throughput drug screening assay gives an indication of drug sensitivity of the cancer cells, but does not give an indication of the toxicity of the agents tested. For the purposes of this project, the same drug concentrations (100nM, 1µM and 10µM) were used for the whole drug panel, but the concentrations required to manage MPM *in vivo* might be different and might require tissue concentrations that are not necessarily safe enough for therapeutic administration. The drug screening assay was the primary screen to identify 'hits', and the results will need to be validated by a secondary screen and counter screen to confirm the 'hits'. In addition, MPM is notoriously heterogeneous, morphologically and genetically, and even samples from the same patient may show different genetic profiles. It would therefore be ideal for future studies to study multiple samples from the same patient in an attempt to capture all potential drug targets and to study the potential effect of this heterogeneity in drug resistance/response profiles.

- The positive control used in the drug screening assay was cisplatin/pemetrexed combination. Clinical guidelines advise addition of bevacizumab where available. However, given that bevacizumab is an anti-VEGF agent that targets angiogenesis and not the cancer cells specifically, the MPM cell cultures do not allow testing of the effectiveness of cisplatin/pemetrexed/bevacizumab which has been found to be more effective than cisplatin/pemetrexed alone.(Kindler et al., 2018; Woolhouse et al., 2018)

4.10 How the hypotheses and aims for this study were met

The hypotheses and aims of this study were met as follows:

- The first hypotheses was that MPE fluid contributes to MPM proliferation, and impacts on patient survival. The aim was to investigate the association between pleural effusion and survival.
    - The analysis of the database of MPM patients showed that pleurodesis success was associated with improved survival; on the other hand, the duration of pleural effusion exposure did not show clear association with survival although several limitations of the retrospective study are recognised making it difficult to draw robust conclusions and future studies are therefore required, taking care to address the data issues noted in this study.
    - The *in vitro* experiments showed that pleural fluid, whether cancer or non-cancer related, and whether exudate or transudate, was adequate for cancer cell proliferation *in vitro*, indicating that pleural fluid does have biological properties.
    - Knowledge from a mouse model with indwelling pleural catheter, and data from published literature was used to design an animal model to take this research one step further and study the effects of MPE on MPM progression, and on chemotherapy response.

- The second hypothesis was that a bench-to-bedside pathway for patients with MPM is feasible. The aim was to investigate the feasibility of using personalized patient-derived

MPM cell cultures to perform high-throughput drug screening and DNA sequencing, as tools for delivering personalised MPM treatment.

- MPM cell cultures were established with relatively high success rates, and were confirmed to contain cancer cells including cancer stem cells, and were adequate for good quality drug screening and DNA sequencing. The whole pathway delivered results within about 2 months from pleural fluid sampling. These results met the aim of this study, and show that the pathway is feasible, with some recommendations identified to improve these results further as suggested in the next chapter (Chapter 5: Future directions).

Chapter 5: Future directions

5.1 The association between pleural fluid exposure and survival in malignant pleural mesothelioma – a retrospective cohort study

Despite these limitations, this data serves to highlight the need for prospective data and can be used to guide the direction of future study designs. Clinically, the most important question to answer would be whether MPE should be drained as early and as completely as possible in order to improve morbidity and mortality outcomes for patients with MPM.

It would however also be interesting to know what, if any, effect the MPE fluid has on the tumour itself in humans.

This present data demonstrates the clear need for better quality data in this important area, and to thoroughly answer the above question with robust methodology, I would propose a prospective study. Such a prospective study might involve patients with MPE having an IPC inserted, and then randomisation to either regular MPE fluid drainage, or limited MPE drainage to control symptoms only. Thoracic ultrasound has sensitivity and specificity of up to 100% whereas chest x-ray (posteroanterior, upright) has 82% sensitivity and 88% specificity for pleural effusion detection (using CT scan as reference standard).(Soni et al., 2015) Therefore, the duration and volume of MPE fluid *in situ* could be assessed with regular thoracic ultrasound scans (thereby doing away with the flawed chest x-ray assessment of fluid presence, and avoiding patient-radiation exposure), and

patients followed up until death to assess correlation between MPE and survival. Furthermore, the effect of MPE fluid on the tumour activity and proliferation could be assessed by serial pleural biopsies to check for biological markers indicating tumour activity and proliferation, PET CT scans and repeat CT scans for serial staging, in an attempt to detect any differences between patients with limited exposure to MPE and those with ongoing MPE exposure.

Similarly, chemotherapy response could be assessed in patients whose MPE fluid is drained regularly and in those whose MPE is not, again using thoracic ultrasound to confirm presence and volume, or resolution, of pleural effusion, enabling more accurate assessment of the duration of pleural effusion.

5.2 The development of patient-derived malignant pleural mesothelioma cell cultures from pleural fluid: a tool to advance biomarker-driven treatments

The cell cultures were adequate to be used for further experiments, namely to test the biological effects of pleural fluid, and to perform quality drug screening assays and DNA sequencing in an attempt to establish the feasibility of a pathway towards personalised treatment in MPM. This allows the cell cultures to be used as potential tools to refine patient management in MPM.

Given that the cell culture success rate of 45.9% was high compared to the literature, future studies may adopt this standardised pathway for cell culture including:

- Sampling the pleural fluid under strict sterile conditions in a dedicated theatre for respiratory procedures;
- Transfer the sample to the laboratory immediately to process it, seeding cells *in vitro* within 2-3 hours of sampling of fluid;
- Taking great care to avoid contamination during cell culture;
- Using 10% FBS in the complete culture medium;
- Using the method for cell culture described step-by-step in Chapter 2: Methods, section 2.3.

5.3 Investigating the biological properties of pleural fluid: *in vitro* experiments

The MPM cell cultures established in this project have the capability of forming tumorospheres or mesospheres. Tumorospheres display cancer stem cell characteristics including tumour initiation, invasiveness, cancer drug resistance, and contribute to tumour vascularisation. Serum-free medium keeps the stem cells undifferentiated, in fact a study showed that colon cancer stem cells maintained sphere morphology in stem cell medium, however on removal of growth factors and addition of 10% foetal calf serum, the floating cells became adherent and differentiated, as confirmed by the expression of differentiation markers.(Prasetyanti et al., 2013) If mesospheres from MPM cell cultures in this project were isolated and incubated in pleural fluid, as opposed to the normally used 10% foetal calf serum, it may be possible to study whether pleural fluid has the ability to cause the cells to adhere and differentiate. This would be interesting as if the hypothesis is proved to be correct and pleural fluid has the capability of inducing cancer stem cells to adhere to a substrate and differentiate, then it may be that pleural fluid has more detrimental effects

than just 'feeding' the pleural cancer cells and allowing them to proliferate more readily. The presence of pleural fluid within the pleural cavity may induce cells to adhere to new substrate and form a tumour mass, thereby causing the pleural tumour to spread and contribute to more aggressive and rapidly progressive malignancy.

5.4 Studying MPE development and MPE fluid effects *in vivo* using animal models

This study's data and experiments have shown that achieving pleurodesis is associated with improved survival in humans, and that pleural fluid is adequate for cancer cells to proliferate *in vitro*. This section describes the data from the literature on animal models studying MPM and MPE, and how this data can be applied to design an animal model that would serve as the next step to studying the biological effects of pleural fluid, and the mechanisms behind them.

Background literature data on which the subsequent animal model design is based

Animal models with intravenous, intraperitoneal or subcutaneous (flank models) injection of cancer cells do not fully represent the pleural microanatomy because they lack the pleural tumour microenvironment and interactions between pleural lymphatics and immune system. They are therefore not ideal models to study MPE. The development of knockout mice deficient in genes that are frequently mutated in human MPM has led to mice with MPM and MPE,(Jongsma et al., 2008) however the development of knockout mice is costly and requires specific expertise, and even then, the knockout can affect the development of certain organs, may lead to mouse death,

may lead to a very different phenotype of disease form than in humans, or another gene may compensate for the knocked out gene's function.(Eisener-Dorman et al., 2009)

An animal model that involves cancer cells injected within the pleural space to stimulate MPE production, although also imperfect, would represent a more accurate model to study MPE. The method of delivery of the tumour cells to the pleural space requires minimal surgery and is not associated with increased mortality.(Iliopoulou et al., 2013) Furthermore, the delivery of cancer cells directly to the pleural space simulates human *in vivo* MPE conditions more closely by maintaining the tumour microenvironment within the pleural cavity including:

- Pleural fluid circulation: the parietal pleura produces the pleural fluid, and the fluid is reabsorbed by the lymphatic vessels in the parietal pleura mainly in the lower, more dependent parts of the pleural cavity;(Miserocchi, 1997)
- The communication between the pleural cavity and systemic circulation - the lymphatic system in the pleura allows:
    - Cancer cells to access the pleural space from distant primary tumours;
    - Cells from primary pleural malignancy and lung cancer to access the systemic circulation;(Manac'h et al., 2001; Osaki et al., 2004; Servais et al., 2011)
    - Circulation of immune cells, and this allows their effects on cancer cell growth and proliferation to be assessed.

In a mouse model with tumour cells administered intrapleurally, the formation of MPE is thought to occur in four phases:

- Tumour implantation from day 0 (when tumour cells are seeded intrapleurally) to day 4;
- An inflammatory phase where low volume MPE begins to develop from days 5-8;
- Vascular hyperpermeability as a result of angiogenic factors secreted by tumour foci, leading to MPE fluid accumulation from days 9-12;
- Accelerated growth and spread of the pleural tumour, associated with increased fluid production, leading to mouse morbidity and mortality from days 13 to death.(Iliopoulou et al., 2013)

Animal model design: studying malignant pleural effusion and malignant pleural mesothelioma

The use of mice

An ideal MPE animal model is reproducible, inexpensive, and accurately mimics clinical MPE pathophysiology. Most animal studies of MPE have used mice,(Stathopoulos and Kalomenidis, 2009) although New Zealand white rabbits and immunodeficient rats have been used previously.(Hatton et al., 2002; Ohta et al., 2001) Mouse models are considered to be ideal to study the mechanisms of pleural fluid production in malignancy, and to assess the effect of medication on MPE, for several reasons:

- Their large litter size and small animal size;
- Rapid tumour growth;
- The availability of inbred genetically identical strains;
- Their genome has been entirely sequenced and mice have 90% genome homology with humans;

- The possibility for engineering of gene-deficient and gene-carrier mice, which allows the modelling of genetic disorders, and the assignment of function to genes.(Bockamp et al., 2008; Iliopoulou et al., 2013; Stathopoulos and Kalomenidis, 2009)

On the other hand, models often use cultured cancer cells selected under *in vitro* conditions, in a standard mouse host, and these cannot fully replicate the vast biological heterogeneity in human cancer. The cultured cells may differ fundamentally from the primary tumour, and delivering cancer cells directly to the pleural space is different from the process of metastasis of cells from a primary tumour site to the pleura in humans. Furthermore, great heterogeneity between patients with malignancies, especially MPM, is well known. Therefore the model findings may not be applicable to every patient with MPE and with MPM. Moreover, the introduction of genetic alterations to the mouse may indeed identify gene function, but may also result in severe developmental consequences, which complicate or prevent analysis.(Bockamp et al., 2008) Finally, unlike in humans, where the left mediastinum is separate from the right mediastinum, incomplete separation and therefore communication exists between the two in mice,(Servais et al., 2011) so that the contralateral pleural space cannot be used as a control in mouse experiments. Despite these differences between mouse models and humans, there have been recent advances in MPE animal research that have helped bridge some of the gap that exists between *in vitro* data and clinical data in human MPE.

Immunodeficient vs immunocompetent mice

Many mouse models employ immunodeficient mice to facilitate tumour uptake of allogeneic cancer cells or tissues, such as human lung cancer cells in nude mice,(Martarelli et al., 2006; Yano et al., 2000; Yeh et al., 2006) and in SCID mice.(Boehle et al., 2000; Edakuni et al., 2006) However, as outlined above, there is extensive interplay between tumour and host cells involved in the pathogenesis of MPE. Therefore, MPE animal studies using immunocompetent mice are likely to be more valid than those employing immunocompromised mice, because the host inflammatory response may alter tumour progression, and also because immunosuppression may affect the interplay between tumour and host cells which is crucial for MPE formation.(Stathopoulos and Kalomenidis, 2009) An additional factor to consider is the greater cost of using immunodeficient mice when compared to using immunocompetent mice.(Servais et al., 2011)

Immune-intact Black *C57BL/6* is the most widely used and studied mouse strain, and a reproducible mouse MPE model has been designed and verified in *C57BL/6* mice, with intrapleural injection of syngeneic Lewis lung carcinoma (LLC) cells.(Cui et al., 2009, 2008; Stathopoulos et al., 2006) In two studies, all mice with 1.5 x 10^5 (*n*=40) and 2 x 10^6 LLC cells (*n*=10) injected intrapleurally developed MPE,(Heike et al., 1997; Stathopoulos et al., 2006) making this a reliable model for MPE studies. Furthermore, the MPE was exudative, similar to human MPE.(Stathopoulos et al., 2006) Another study compared different numbers of LLC cells injected intrapleurally: 0.1 x10^5, 0.5 x 10^5, and 1.5 x 10^5 LLC cells in *C57BL/6* mice, with minimal or absence of MPE formation in mice injected with 0.1 x10^5 cells, however worse survival in the mice injected with 1.5 x 10^5 cells (all mice (*n*=40) died by day 19, compared with maximum survival of 25

and 27 days in mice injected with 0.1 x10^5 and 0.5 x 10^5 cells respectively).(Acencio et al., 2015) This highlights the importance of adequate experiment planning depending on the chosen primary endpoint, with experiments aiming to study MPE requiring larger numbers of cells injected intrapleurally to increase the chance of MPE formation, accepting however that the mouse survival may be worsened.

Studying MPM

There have not only been advances in MPE animal model research, but also in MPE animal models specifically studying MPM. Previously, animal MPM models required exposure to asbestos, either inhaled or delivered intrapleurally.(Smith and Hubert, 1974; Stanton and Wrench, 1972; Topov and Kolev, 1987) This required considerable time because of the time lag between exposure to asbestos and development of tumour, and was not a reliable model because of the host heterogeneity with regards to tumour development - not all mice (or indeed humans) exposed to asbestos go on to develop MPM. The crossing of knockout mice deficient in neurofibromatosis type 2 gene, with INK4A/ARF (a locus regarded as one of the most important anti-tumour defenses(Serrano, 2000))-deficient or tumour-related protein 53 (p53)-deficient mice, yields mice with a high incidence of MPM with similar features to human MPM, including development of MPE, despite complete lack of asbestos exposure.(Jongsma et al., 2008)

Previously, MPM mouse models used tumour masses or tumour cell suspensions transferred surgically. More recently, an orthotopic tumour model was developed

involving injection of human MPM cells directly into the pleural cavity of mice, both avoiding surgery and mimicking tumour development in humans.(Servais et al., 2011)

Other MPM animal models utilising the injection of human mesothelioma cells in mice(D'Amato-Brito et al., 2016; Sartore-Bianchi et al., 2007) did not mention the development of MPE therefore their use is limited in MPM MPE research. A group designed a mouse model to study the effect of the mechanistic (or mammalian) target of rapamycin (mTOR) inhibitor temsirolimus in mouse mesothelioma. mTOR is often activated in MPM, and is linked to cell proliferation and survival, as well as migration and angiogenesis, and is associated with a worse prognosis in MPM. AE17 cells (murine mesothelioma cells cultured from ascites and tumours that were formed when *C57BL/6* mice were injected intraperitoneally with crocidolite asbestos) were delivered intrapleurally to *C57BL/6* mice (5×10^5 cells per mouse). The mice developed MPE and temsirolimus treatment was associated with reduced volume of MPE.(Vazakidou et al., 2015) This model represents a valid alternative model for MPM MPE research. Interestingly, another mTOR inhibitor, everolimus, was tested in humans with MPM but disappointingly, there was no overall response rate based on the modified RECIST criteria,(Ignatius Ou et al., 2015) a good example of the difficulties faced in translating *in vitro* and animal model results to clinically useful data.

Method

A novel mouse model with IPC will be described (mouse experiment carried out by collaborators in Vanderbilt, USA) and, together with the knowledge from the literature review, will be used to design a mouse model that describes a potential future direction for research into the biological effects of pleural fluid.

The indwelling pleural catheter mouse model

IPCs have become a mainstay of care and palliation in patients with recurrent symptomatic MPE. They allow regular drainage of MPE, preventing build up of fluid and the associated symptoms, and allow access to the pleural space allowing the administration of drugs, including talc for pleurodesis. A reliable mouse model of MPE utilising IPC, mimicking MPE drainage using an IPC in humans, could serve as a reliable platform for MPE translational research.

Such a model would revolutionise the study of MPE in mice, as repeated thoracocentesis is not possible without animal sacrifice, and an IPC would allow repeated fluid assessment. Furthermore, as intrapleural therapy for malignancy is a burgeoning area of research,(Bibby et al., 2018; Hu et al., 2017; Kim et al., 2004) and IPCs provide direct access to the pleural space, such a model has the potential to enable the efficient testing of novel treatment agents, including the ability to deliver repeated doses to the pleural space, which would not otherwise be possible without animal sacrifice.

The first experimental mouse model utilising IPC in mice with MPE was designed by a research team from Vanderbilt University (Nashville, USA), and Ludwig-Maximilian University (Munich). This study (protocol M1700024-00-AN1) was approved by the Institutional Animal Care and Use Committee.(Merrick et al., 2019) I was involved in the data analysis and interpretation, and the writing of the manuscript. In this thesis I describe an application of this model for the further testing of the hypothesis that pleural fluid has biological properties contributing to pleural tumour growth and chemotherapy response.

LLC cells are derived from a spontaneously developed tumour in the lung of a *C57BL* mouse, the most widely used and studied mouse strain. The LLC cell lines are used in well-established mouse cancer models, and they are immunologically compatible with *C57BL/6* mice. The mice were bred in-house in Vanderbilt (inbreeding leads to genetically identical mice). Furthermore, *C57BL/6* mice are immunocompetent therefore more closely represent the interactions between malignancy and the immune system in humans when compared to experiments with immunosuppressed mice, as discussed earlier.

LLC cells were injected into the pleural space of *C57BL/6* mice (*n*=19). From previous experiments, 7-9 days are required for MPE formation.(Stathopoulos et al., 2006) Therefore, on day 7, the IPC was inserted under general anaesthetic. The experimental design is shown in Figure 5.1.

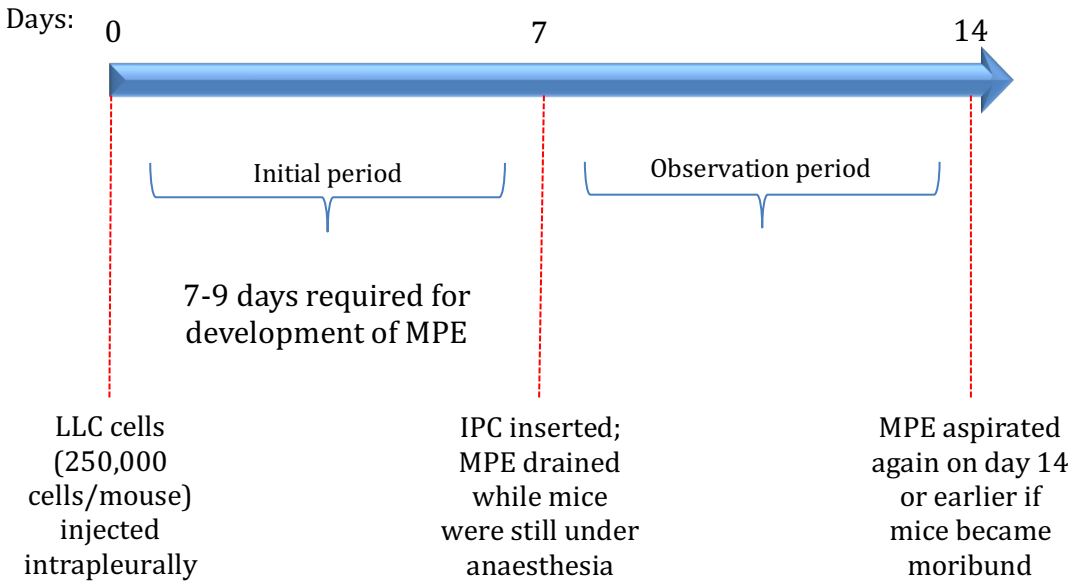


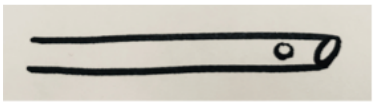
Figure 5.1 shows the experiment design for the MPE mouse model with IPC. ***IPC = indwelling pleural catheter, LLC = Lewis lung carcinoma, MPE = malignant pleural effusion.***

The method used for intrapleural LLC cell delivery and method for IPC insertion is described in the Chapter 6: Appendices, section 6.5, and summarised in the cartoons in Figure 5.2 below. The mouse IPC is shown in Figure 5.3, and a mouse with an IPC in situ is shown in Figure 5.4.

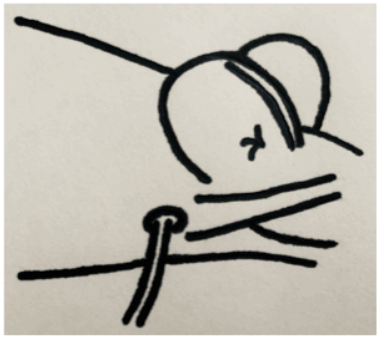
Step 1: LLC cells injected intrapleurally

7 days

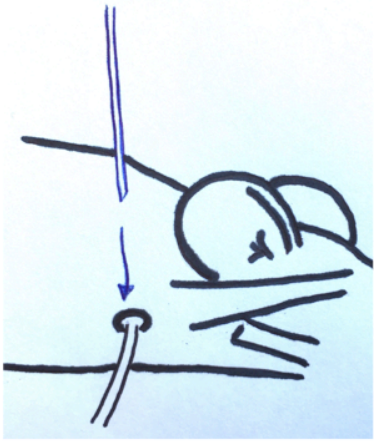
Step 2: puncture through 7th rib space in mid-axillary line



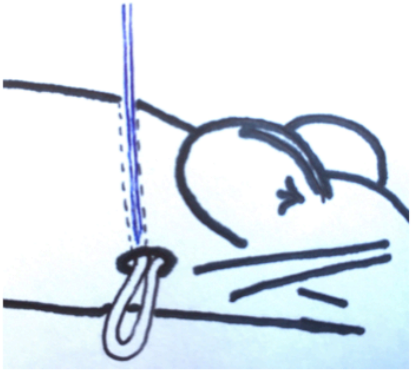
3 holes punctured in distal end of PU-40 tube



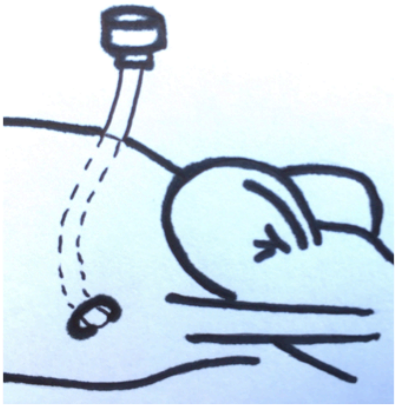
Step 3: PU-40 advanced 1.5cm into the pleural space



Step 4: 16G needle used to puncture the dorsum between scapulae, and advanced through subcutaneous tissue towards catheter insertion site



Step 5: Proximal end of tube passed over needle and out between the scapulae…



…leaving IPC tunnelled through the subcutaneous space

Figure 5.2 Cartoons depicting the method used to insert the IPC in mice.

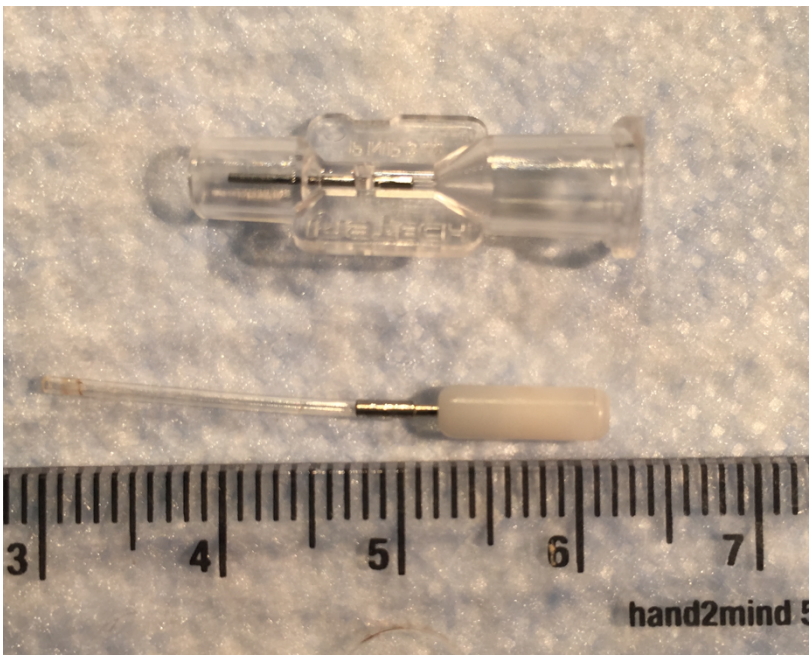


Figure 5.3 shows the mouse pleural catheter. The catheter is composed of polyurethane tubing, syringe accessible port, with syringe adapter.(Merrick et al., 2019)

Figure 5.4 shows the intrapleural catheter tunnelled through subcutaneous tissue with 22G syringe-accessible port on the distal end of catheter. The port is sutured to the skin.(Merrick et al., 2019)

Results of the first IPC mouse model

The mean volume of MPE fluid drained overall (*n*=19) was 188µl (range 0-770µl). Eight mice were excluded from further fluid-drainage analysis because of technical issues outlined in the figure below. The overall MPE drainage from mouse IPC is summarised in Figures 5.5 and 5.6.

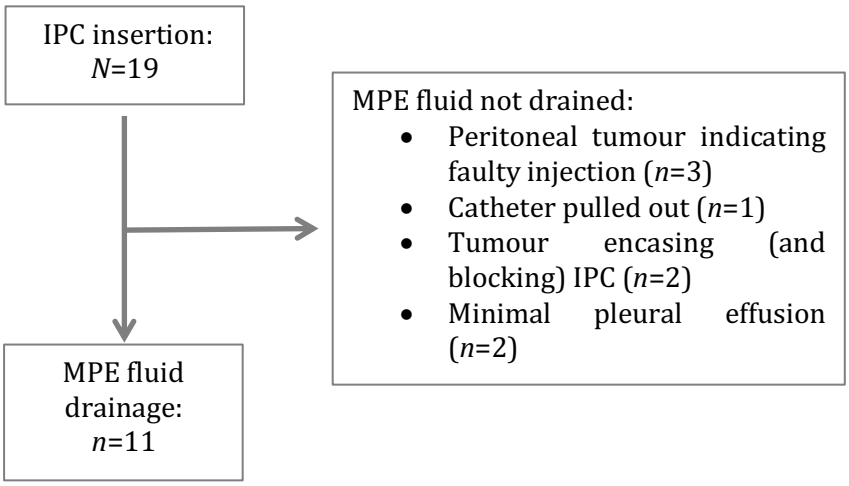


Figure 5.5 shows a flow chart of the reasons why MPE fluid was not aspirated from all the mice who had an IPC insertion. ***IPC = indwelling pleural catheter; MPE = malignant pleural effusion.***

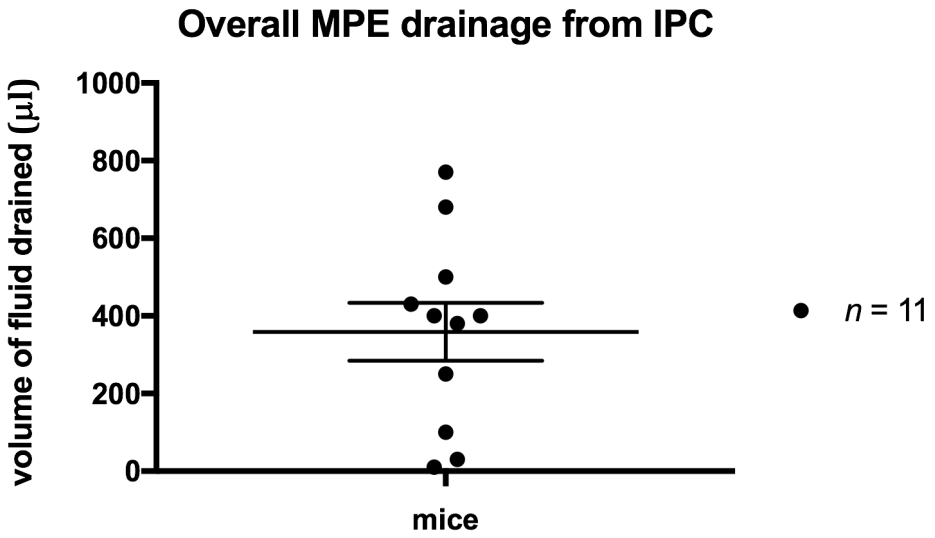


Figure 5.6 shows the scatterplot with the mean (and standard error of mean) volume of pleural fluid drained from mice that had pleural fluid drained (*n*=11). ***IPC = indwelling pleural catheter; MPE = malignant pleural effusion.***

Post-mortem dissection demonstrated bulky pleural tumour with minimal residual MPE (*n*=17) (Figures 5.7 and 5.8). Furthermore, residual effusion with the intrapleural IPC portion enveloped by pleural tumour was found in *n*=2, leading to inability to aspirate fluid via the IPC.

Any potential agent could be injected through the IPC by means of the syringe-accessible port (PinPort, Instech Laboratories, Plymouth Meeting, PA, USA). A volume of 100µl PBS injected intrapleurally was well-tolerated by the mice, indicating that the IPC may also be useful to allow intrapleural drug delivery.(Merrick et al., 2019)

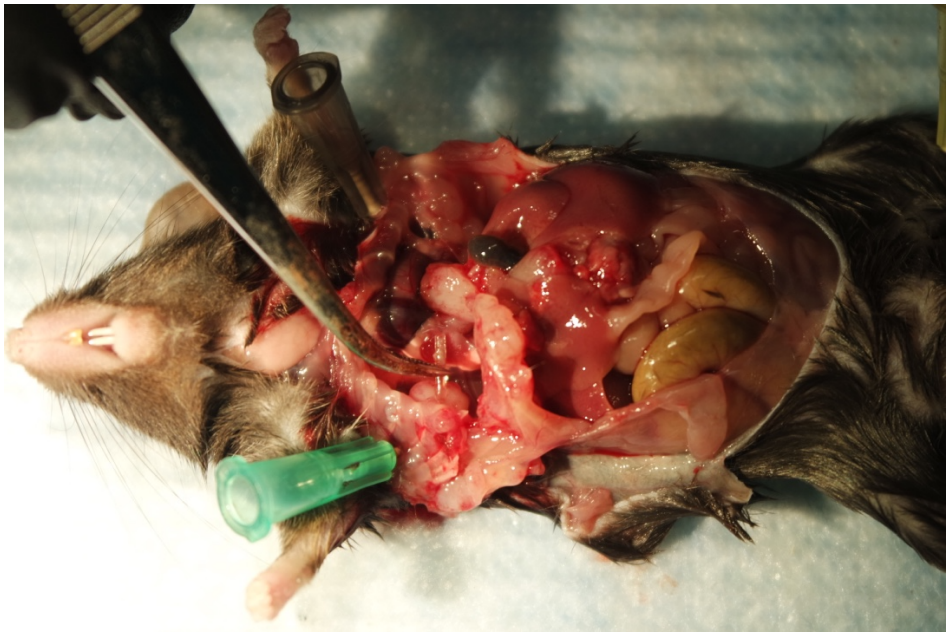


Figure 5.7 shows a postmortem image of mouse with IPC in situ, with the intrapleural tip of the IPC indicated by the tip of the forceps.

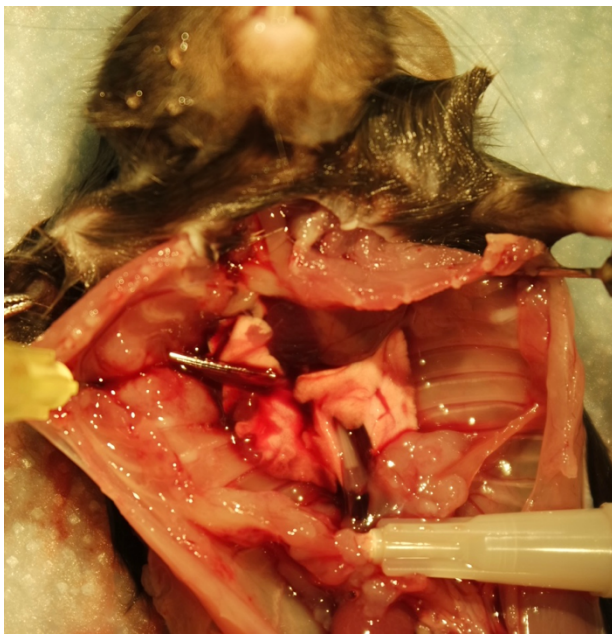


Figure 5.8 shows a postmortem image of mouse with IPC *in situ*, showing the contrast between the right sided pleural cavity in which the LLC cells were injected prior to IPC insertion, with infiltrated thickened parietal pleura macroscopically consistent with a malignant process, and the normal left sided parietal pleura which is smooth, with alternating pink (ribs) and yellow (fat between the ribs) bands. This is similar to the thoracoscopic views of the human pleura infiltrated with malignant disease and normal pleura shown in the introductory chapter (Figure 1.4).

Proposed mouse models to investigate the biological properties of MPE *in vivo*

Experiment 1: studying the biological effects of pleural fluid on pleural tumour bulk

The hypothesis is that MPE fluid contains numerous proteins, immunomediators and inflammatory mediators that contribute to tumour growth and progression. Further to the retrospective analysis and the *in vitro* experiments results described earlier on in this thesis, here a potential future direction to further investigate this hypothesis is described.

Primary and secondary outcomes

The primary outcome of the experiment will be the total tumour burden (by weight of tumour, determined at post-mortem examination). Mouse survival in number of days would not be an ideal primary outcome because mice injected with adequate numbers of mesothelioma cells to reliably form MPE, would not be expected to survive much longer than 14 days in general, so determining differences within groups with such a short overall life expectancy would be difficult and not meaningful in terms of the human disease state.

The secondary outcomes include assessment of pleurodesis on post-mortem thorax histology to determine whether any factors can be identified that are associated with IPC-related pleurodesis.

Proposed method

Selection of mesothelioma cells and mouse strain

MPE formation is crucial because the focus of the proposed experiment is the effect of MPE on pleural tumour burden. Other mesothelioma cell cultures in other mice have not necessarily been associated with MPE production. In fact, there was no mention of MPE formation in studies which used human MPM cells injected intrapleurally in nude Xid mice, human MPM cells injected intrapleurally to athymic nude mice, and AK7 murine mesothelioma cells injected intrapleurally to *C57BL/6* mice.(D'Amato-Brito et al., 2016; Leclercq et al., 2011; Sartore-Bianchi et al., 2007)

A reliable mouse mesothelioma model with MPE has been developed using 5×10^5 AE17 murine mesothelioma cells per mouse, injected intrapleurally in immunocompetent *C57BL/6* mice,(Vazakidou et al., 2015) therefore, the proposed model uses AE17 cells injected intrapleurally in *C57BL/6* mice. Additional advantages of using *C57BL/6* mice are that this mouse strain is widely studied, and AE17 murine mesothelioma cells were derived from *C57BL/6* mice, making them compatible with the same mouse strain.

AE17 cells will be injected intrapleurally in *C57BL/6* mice, at 5×10^5 cells per mouse. This will be combined with the IPC mouse model described above, in order to study the biological effects of MPE in MPM.

Calculation of number of mice needed

Accurate power calculations are not possible because this experiment has not yet been done before. An estimate was therefore obtained as follows. Power calculations were performed to calculate the number of animals needed, by performing a priori two-tailed power analyses using G*Power statistical power analysis tool.(Faul et al., 2007) Since the primary outcome of the experiment is the total tumour burden as measured by the post-mortem weight of tumour, the statistical test of choice was 'ANOVA: fixed effects, omnibus, one-way', with three groups (group 1 with fluid drained completely and IPC left open for drainage, group 2 with fluid drained daily and replaced with saline, and group 3 with fluid drained daily and re-introduced back into the pleural cavity, as discussed below), and assuming large effect sizes (the minimum difference between groups considered to be clinically significant) of 0.8, α-errors (type 1 error probability) 0.05, and power number (type 2 error probability) of 0.8 (80% chance of

rejecting null hypothesis, assuming the projected population effect size is correct, i.e. β-errors less than 0.2; http://www.gpower.hhu.de). This results in a total sample size of 21, i.e. 7 mice per experiment group (critical F 3.55, numerator df 2, denominator df 18, actual power 0.86). However, in the mouse model with IPCs, there were 6 mice out of 19 (32%) who had issues with completing the experiment (3 had peritoneal tumour indicating peritoneal injection of cancer cells rather than pleural, 1 IPC fall out, and 2 had minimal MPE). Therefore to correct for the 32% attrition, the final sample size should be 21/0.68, i.e. 31 total sample size, or about 10 mice per group.(Charan and Kantharia, 2013)

The results of the initial experiment will then inform all further experiments as to the total number needed. In addition, it is not yet known whether tumour burden will reflect treatment outcomes, however lack of tumour growth in humans is a strong surrogate for treatment success, and hence it would be reasonable to assume the same is true for mice.

The control for the experiment

It has been noted that the presence of the pleural fluid itself impacts on mouse survival. In a study of mice with LLC cells injected intrapleurally, mice who developed pleural effusion had decreased activity and responsiveness, and increased shortness of breath, in addition to reduced survival than those mice who did not have a pleural effusion.(Ma et al., 2012) As the pleural effusion develops, the mice have gradual weight loss and decrease in physical activity, and in more advanced stages appear tachypnoeic and cachectic.(Iliopoulou et al., 2013) Therefore a simple comparison of mice with MPE and

mice with the MPE drained would lead to biased results since mice with MPE are more likely to become moribund from the volume of fluid itself. Therefore, a control needed to be included.

Serum is similar to pleural fluid in terms of viscosity and consistency, therefore the possibility of serum from the same strain of mice, *C57BL/6*, was considered as a control: the MPE fluid would be drained in some mice and replaced by equal volumes of serum, thereby eliminating any survival advantage the mice may have purely secondary to effusion volume control. However, serum also contains similar proteins to those in MPE fluid. As discussed in earlier chapters, MPM cell lines have been reported to grow better in the presence of 5% and 10% foetal bovine serum,(Pass et al., 1995) than in medium without supplemented FBS, and my laboratory experiments showed that 100% human serum was adequate for cells to grow *in vitro* (Figure 5.9), including heat-inactivated human serum (whereby the complement proteins would be inactivated). Serum was therefore not thought to represent a valid control to test whether or not pleural fluid presence within the pleural cavity is associated with worse outcomes.

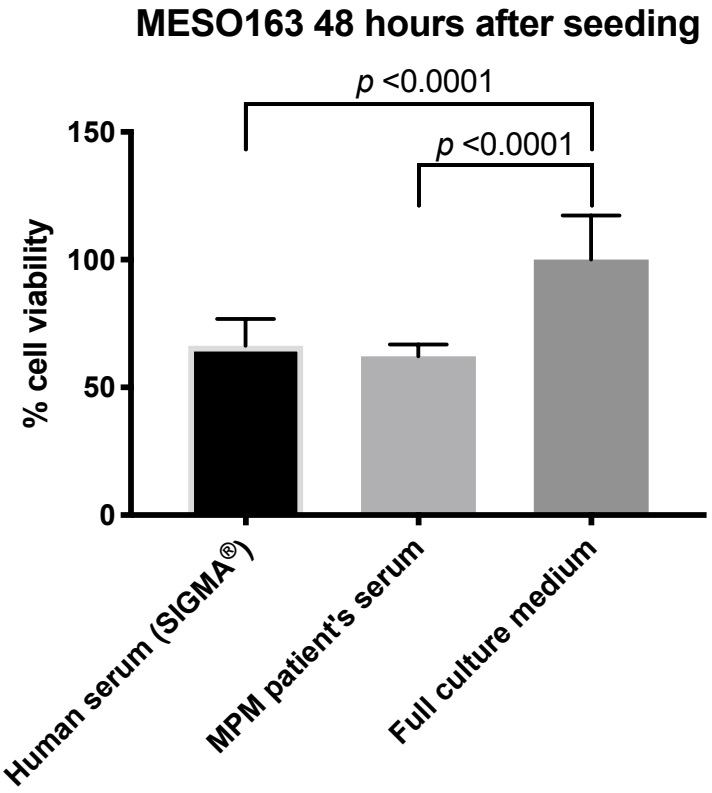


Figure 5.9 shows a graph of the percentage MPM cell viability (MESO163, epithelioid MPM) after cells were incubated with commercial human serum (SIGMA®), with serum from patients with MPM diagnosis, and with full culture medium for 48 hours *in vitro* (*n*=18 replicates). Results show that human serum alone is adequate for MPM cell growth *in vitro* and therefore serum does not represent an adequate control for the planned mice experiments. *MPM = malignant pleural mesothelioma.*

An ideal control would be an inert fluid that is instilled intrapleurally, that is not reabsorbed quickly the way saline is, but just exerts a volume effect intrapleurally in order to correct as much as possible for the morbidity associated with the pleural fluid volume. Such a fluid is not easy to find, therefore an iterative learning process will attempt to determine the ideal control. To start with, saline (0.9% sodium chloride) was selected as the most ideal control to start off with as it is relatively inert, although accepting that some of saline would be quickly absorbed and the fluid volume bias will not be completely eliminated. To mitigate some of this effect, the saline will be replaced on a daily basis.

Determining the volume of pleural fluid tolerated by mice

A pilot study is planned, to analyse the threshold of intrapleural fluid volume tolerated by mice without influencing their survival. *C57BL/6* mice will have IPCs inserted, and the pleural fluid drained to dryness, with varying volumes of fluid re-instilled back into the pleural cavity. If inadequate volumes of pleural fluid are present (from earlier experiments, it was noted that not all mice produce large volumes of pleural fluid, similar to the case in humans where not all patients with MPM produce large volumes of pleural effusion) then serum can be instilled into the pleural space. The aim would be to find the threshold of intrapleural fluid volume beyond which the volume would influence the survival of the mice.

Experimental design

The subsequent experiment is designed to have three groups of mice:

1. The first group would have the pleural effusion drained to dryness (IPC attached to a catheter bag and left open to freely drain any fluid as it is produced, thereby preventing fluid build-up within the pleural cavity – representing patients with the pleural effusion drained completely;
2. The second group will have fluid drained completely daily, and a volume of saline equivalent to the maximum volume of intrapleural fluid tolerated by mice, as identified by the pilot study, instilled intrapleurally. This group will be the control.
3. The third group will have fluid drained completely daily, and a volume of pleural fluid equivalent to the maximum volume of intrapleural fluid tolerated by mice, as

identified by the pilot study, re-instilled intrapleurally, representing patients in whom MPE fluid is left within the pleural cavity.

Mice will be monitored until death to document survival, and sacrificed if they become moribund in order to avoid unnecessary animal suffering. Postmortem examination of tumour weight, and thorax histology will enable assessment of the primary and secondary endpoints. The histological assay may follow the same protocol as that published by Acencio et al:(Acencio et al., 2015) 10% formalin instilled down the trachea to keep the lung inflated, and then organs placed in 10% formalin for a minimum of 24 hours, after which the pleural cavity will be exposed and the tissues fixed in formalin for a day, and 70% ethanol for 3 days. The slides can then be prepared and analysed.

The proposed experiment design is depicted in the following diagram (Figure 5.10).

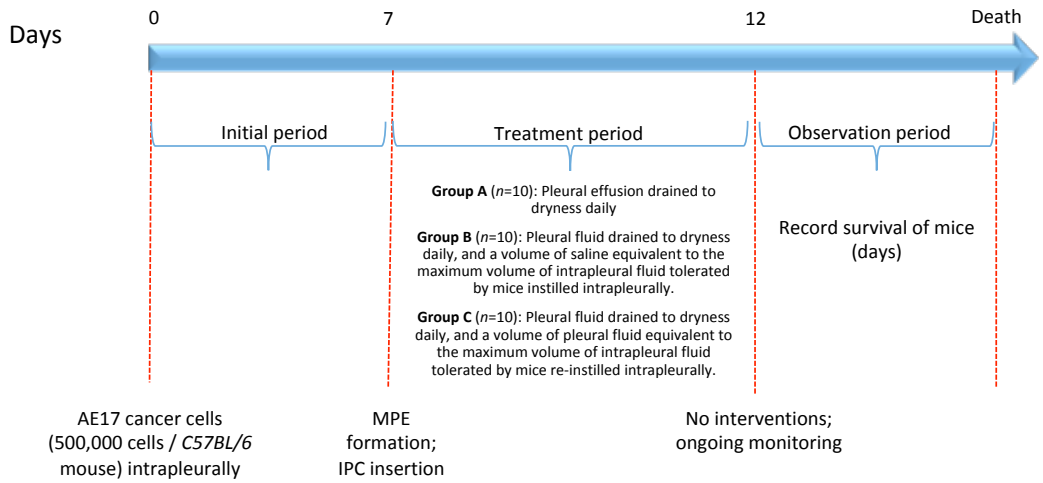


Figure 5.10 shows the proposed experimental design of the mouse model with IPC to investigate the biological effects of MPE *in vivo*. The maximum volume of intrapleural fluid tolerated by mice will be identified through a pilot study. *IPC = indwelling pleural catheter, MPE = malignant pleural effusion.*

Experiment 2: studying the biological effects of pleural fluid on chemotherapy response and resistance

The IPC mouse model potentially allows the investigation of the effect of MPE on chemotherapy response in mice with MPM. The mouse IPC will allow drainage of MPE fluid in some mice, allowing comparison between survival and chemotherapy response in mice with MPE and those in whom the MPE will be drained.

Proposed method

- AE17 cells will be injected intrapleurally in *C57BL/6* mice, at 5×10^5 cells per mouse, as this has already been reported to reliably produce MPE.
- IPCs will be inserted on day 7 as above.
- Half the mice will have the fluid drained completely via the IPC, daily in order to keep the intrapleural space as dry as possible. The other half of the mice will have the fluid drained and then re-injected into the pleural space at the maximum volume that does not significantly affect the mouse morbidity (as identified by the pilot study described above).
- Chemotherapy will be administered to the mice (100mg/kg pemetrexed intrapleurally on days 7-11 and 14-18, and 10mg/kg cisplatin intrapleurally on day 7: the doses tested and identified to be optimal in mice in other studies,(Anraku et al., 2010; Teicher et al., 2000) and administered intrapleurally as this has previously been identified to be less toxic (route yielded a significantly lower C_{max}, whereas area under the curve (reflects the mouse exposure to the drug) was similar in both groups) than when administered intravenously.(Greillier et al., 2009)

- Mice will be monitored until death to document survival, and sacrificed if they become moribund in order to avoid unnecessary animal suffering. The pleural tumour weight and histology can be investigated postmortem to assess whether there are differences between the chemotherapy response in tumours exposed to pleural effusion and those with limited exposure to effusion.

The proposed experiment design is depicted in the following diagram (Figure 5.11).

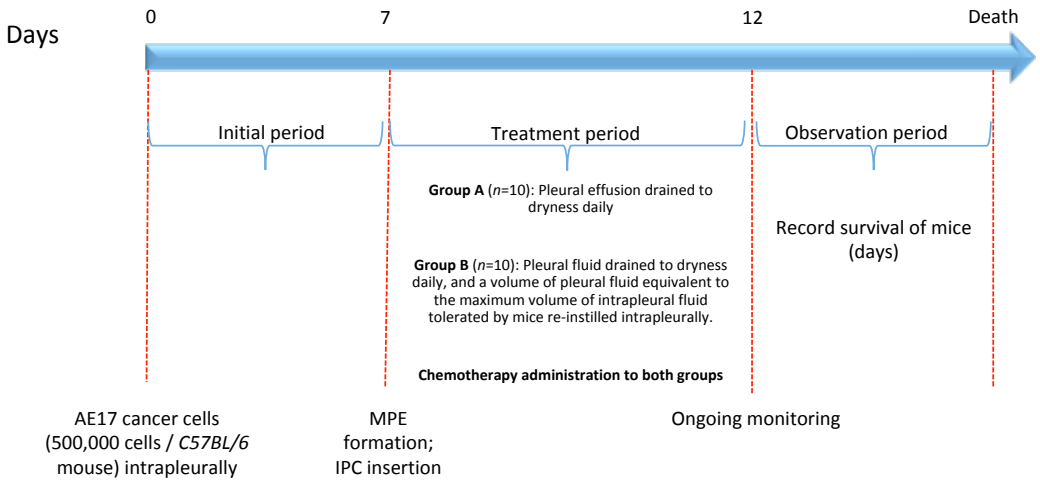


Figure 5.11 shows proposed experimental design of the mouse model with IPC to investigate the effect of MPE on chemotherapy response / resistance *in vivo*. The maximum volume of intrapleural fluid tolerated by mice will be identified through a pilot study. *IPC = indwelling pleural catheter, MPE = malignant pleural effusion.*

Collaborators in Vanderbilt, USA, who have significant expertise and experience in mouse experiments and insertion of IPC in mice, can potentially conduct these proposed experiments, ensuring the highest quality of the eventual results. The proposed

experimental mouse models will enable *in vivo* studies of the biological effects of MPE in MPM.

Although this model may shed light on the presence or absence of pleural fluid biological properties, there remain several unanswered questions regarding MPE, and the IPC mouse model design may potentially help address some of these questions. One such question is whether MPE should be drained as completely and early as possible in humans because of the potential risk of developing non-expandable lung with long-term MPE as the disease progresses.(Feller-Kopman et al., 2018) This would be better studied using randomised controlled trials in humans, comparing patients whose asymptomatic MPE is drained, with those whose asymptomatic MPE is left *in situ*, as per guideline recommendations, and assessing whether the latter group had increased chance of developing non-expandable lung throughout the course of their disease.

Discussion

The novel mouse model seeks to mimic MPE drainage via IPC in humans. It enables the testing of the effect of MPE in MPM and in other cancers and allows recurrent sampling and drainage of pleural fluid which would otherwise necessitate animal sacrifice. It potentially allows the studying of the effect of an IPC in humans because the post-mortem study of the mouse thorax with IPC may allow identification of changes within the pleural space at the site of IPC catheter, and may help to identify factors associated with pleurodesis.

Furthermore, the IPC mouse model enables the testing of the use and effectiveness of intrapleural therapies, including allowing the delivery of multiple doses, in the treatment of MPE.(Merrick et al., 2019) Intrapleural injection of MPM therapy has been postulated to enhance its anti-tumour activity because it has direct contact with the pleural tumour, and high local concentrations via the IPC can be delivered with less systemic toxicity than with systemic chemotherapy.

Limitations

Further studies using this model should take into account the potential small number of mice that may not develop adequate volumes of MPE (*n*=2/19), although this is a natural event seen in other mouse MPE models and indeed also seen in humans, as some patients with MPM will develop MPE while others will not.

The mice might not produce enough pleural fluid, which may pose a problem especially in the group of mice whose pleural fluid is to be drained and then re-instilled at a volume equivalent to the maximum volume of intrapleural fluid tolerated by mice, as identified by the pilot study. One possible solution to this may be the use of an equivalent volume of mouse serum instilled via the IPC into the pleural cavity, instead of pleural fluid, given that pleural fluid and serum should have similar characteristics in terms of biochemical profiles and growth factors.

This experiment studying the effect of pleural fluid on chemotherapy response is limited by the fact that AE17 murine MPM cells may behave differently to human MPM cells, especially with regards to therapeutic response, so results cannot automatically be

extrapolated to be applicable to humans. However, if mice with MPE have worse chemotherapy response when compared to matched mice without MPE, this would be a good starting point to trigger further research in this field.

Other limitations of this model include limitations of all animal studies of MPE. MPE animal models cannot adequately and accurately mimic the great biological heterogeneity present in human malignancy, and especially the great heterogeneity found in MPM, therefore experiment findings may not be applicable to every patient with MPM MPE. Ideally biomarkers would be identified and targeted, including biomarkers predicting patients who will develop MPE in MPM, or patients who will have worse survival if exposed to MPE, or who will / will not have successful pleurodesis. Furthermore, cells from cancer cell cultures are highly selected cells in *in vitro* conditions, and would have therefore undergone substantial genetic and molecular profile modification. As discussed, cancer cell culture DNA sequencing may help identify any major alterations, but the cell cultures will never be exactly identical to the patient's primary tumour. Finally, animal experiments may yield very promising results, but this does not always translate to promising results in humans, as seen with zoledronic acid, where anti-MPE effects and improved survival were noted in mice,(Stathopoulos et al., 2008) but results in a human RCT were disappointing.(Clive et al., 2015)

5.5 High-throughput drug screening and DNA sequencing of patient-derived MPM cell cultures

The MPM cell cultures have the potential to be used as tools for further experiments that may aid new MPM treatment discovery.

- Utilising the patient-derived MPM cell cultures for immunotherapy testing

Cancer immunotherapy holds the potential to be more effective than chemotherapy and with fewer side effects. T cell immunotherapy is an emerging treatment modality in cancer however is not part of standard MPM treatment. The feasibility of the MPM cell cultures panel to act as an *ex vivo* model suitable for the development and assessment of T cell immunotherapy treatment was studied together with collaborators in the immunology unit at the laboratory, and using the patient derived MPM cell cultures.(Kanellakis et al., 2020)

- The use of tumourspheres instead of 2-dimensional cell cultures

The aim of this study was to show feasibility of a bench to bedside pathway to potentially feedback information about likely drug response/resistance to clinical teams in an attempt to guide anti-cancer treatment. The use of 2-dimensional cell cultures was shown to be feasible. As discussed, 3-dimensional cell cultures, such as the use of tumourspheres, are thought to be more representative of a tumour *in vivo* and may therefore give more translatable drug screening results than 2-dimensional cell cultures. As discussed, tumoursphere assays using the patient-derived cell cultures in this study were successful, and therefore the next planned step is to perform drug screening assays on tumourspheres from the patient-derived cell cultures.

- Potential for patient-derived cell cultures, drug screening and DNA sequencing analysis to allow identification of variants associated with drug response/resistance *in vitro*

- On a larger scale, the patient-derived cell cultures offer an *ex vivo* platform suitable for cross-examination of genomic, transcriptomic, and proteomic data with high-throughput drug screening data. A better understanding of the variants associated with response or resistance to drugs *in vitro*, may enable the identification of areas of interest for further investigation and the distinction between driving and passenger variants, and therefore potentially allow the identification of biomarkers to guide personalised treatment.

- Determining what Z score is likely to be associated with good clinical response will help the physician in choosing the drug with the best benefit-risk ratio.

However this requires considerable cost, and the identification of effective drugs *in vitro* is only the first step in a long process towards the identification of suitable drugs *in vivo*. The results obtained would first need to be confirmed with further assays. However, the use of a library of drugs already approved for use in other malignancies means that the ADMET profile and pharmacodynamics properties of the drugs used are already known and established.

Chapter 6: Bibliography

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Chapter 7: Appendices

6.1 The association between pleural fluid exposure and survival in malignant pleural mesothelioma – a retrospective cohort study

Percentage of post MPM diagnosis life exposed to pleural effusion was correlated with survival

The percentage of post MPM diagnosis life exposed to pleural effusion was correlated with survival (*n*=307 with complete data): Pearson *r* -0.16, 95% CI -0,27 to -0.05, R^2 0.03, *p* 0.002, with the negative *r* values implying that less exposure correlates with longer survival. It was noted that the data was mainly clustered at 0 or 100%, implying that most patients either have pleural effusion throughout their post-MPM diagnosis life, or else successfully achieve pleurodesis very soon after MPM diagnosis, with only very few patients in between these two values, as shown in Figure 6.1. The percentage of life post-MPM-diagnosis exposed to pleural effusion was correlated with survival for each Trust involved in this study, and the results are shown in Figure 6.2.

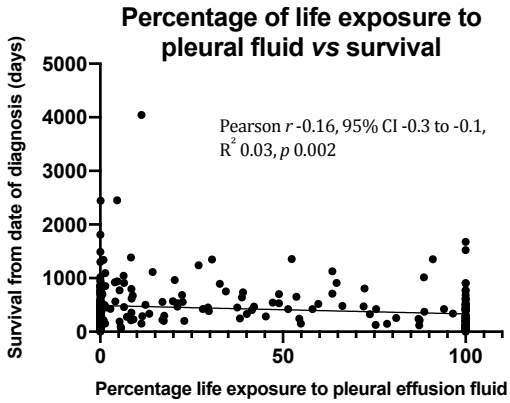

Figure 6.1 shows scatter plot of correlation between percentage of life exposed to pleural effusion fluid *vs* survival.

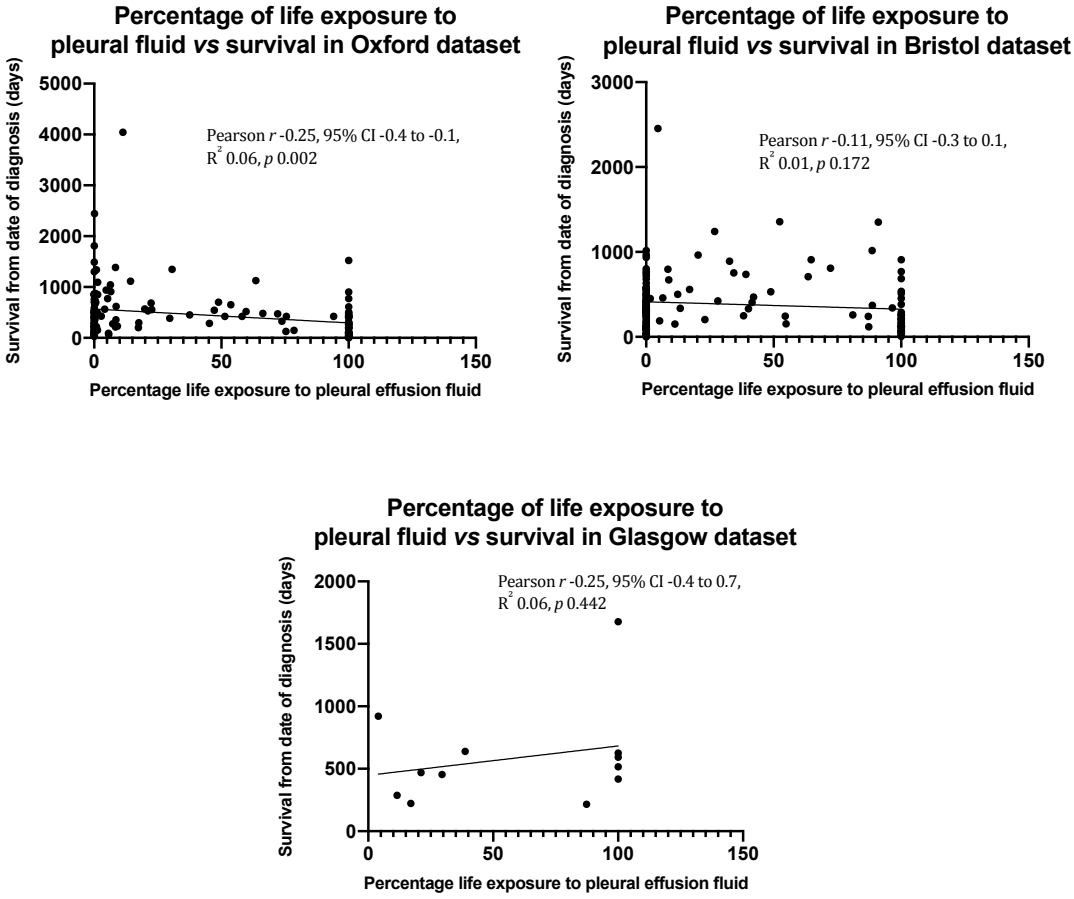

Figure 6.2 shows scatter plots of correlation between percentage of post-MPM-diagnosis life exposed to pleural effusion *vs* survival for the Oxford, Bristol and Glasgow datasets, analysed separately.

Survival according to other treatments for MPM

In order to ensure that there was no major difference in treatments being given for MPM across the cohort of patients, data was collected on surgery, radiotherapy and immunotherapy received by patients for their MPM disease.

There was no significant difference in survival between patients who received radiotherapy and those who did not (*n*=214 and 121 respectively (inadequate data *n*=426), median survival 412 and 306 days, χ^2 0.86, df 1, *p* 0.035, Log-rank (Mantel-Cox) test), neither between patients who received immunotherapy and those who did not (*n*=15 and 181 respectively (inadequate data in 565), median survival 625 and 369 days, χ^2 3.0, df 1, *p* 0.08, Log-rank (Mantel-Cox) test). When comparing patients who received surgery for MPM (other than the initial diagnostic video-assisted thoracoscopic surgery (VATS) for pleural biopsies to diagnose MPM) and those who did not, there was improved survival in patients who had surgery (*n*=13, median survival 939 days) compared to those who did not have surgery (*n*=182, median survival 359 days) (inadequate data *n*=566): χ^2 6.5, df 1, *p* 0.006, Log-rank (Mantel-Cox) test). Figure 6.3 shows the effect of radiotherapy, immunotherapy and surgery on survival in this cohort of patients.

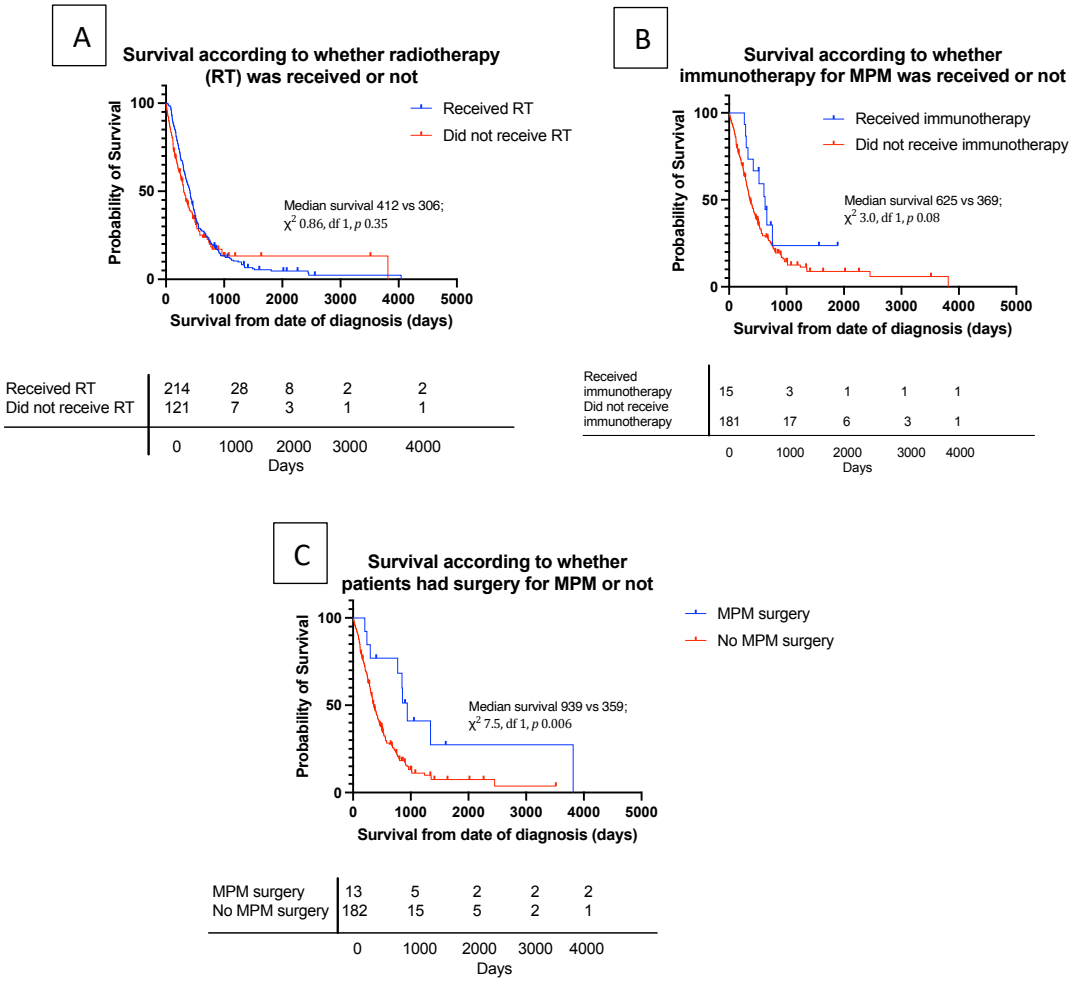

Figure 6.3 shows survival curves of patients who received radiotherapy (A), immunotherapy* (B) and surgery (C) for MPM.** ***MPM=malignant pleural mesothelioma. *n=15 received immunotherapy: nintedanib n=7, pembrolizumab n=4, bevacizumab n=1, IMM/101 n=2, VS6063 FAK kinase inhibitor n=1; **n=13 had surgery for MPM: extra-pleural pneumonectomy n=4, pleurectomy decortication n=4, decortication n=2, partial pleurectomy n=1, other n=2 (resection of cystic pleural lesion histology of which was biphasic MPM, resection of pleural mass histology of which was epithelioid MPM).***

Survival according to time epochs

Median survival was 340 (*n*=126), 264 (*n*=144), 322 (*n*=161), 355 (*n*=175), and 287 (*n*=145) days for patients diagnosed between 2008-2009, 2010-2011, 2012-2013, 2014-2015, 2016-2018 respectively; χ^2 4.3, df 4, *p* 0.36; Log-rank (Mantel-Cox) test (Figure 6.4).

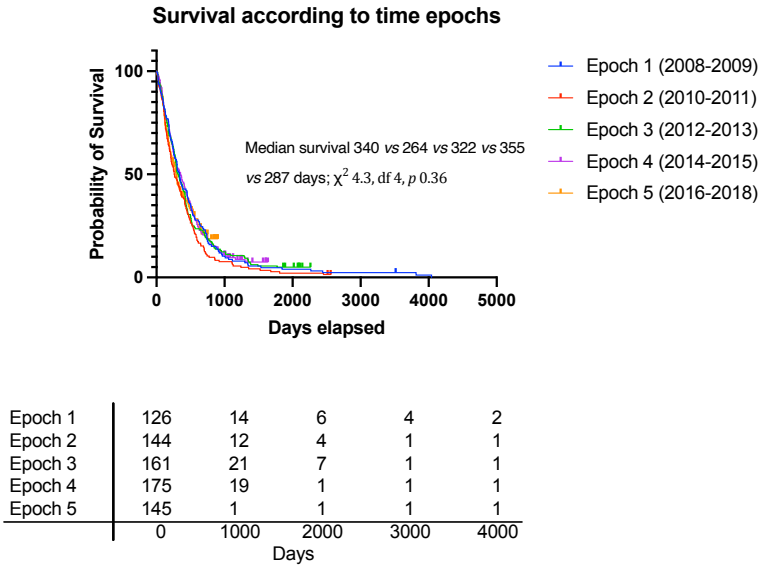

Figure 6.4 shows survival curves of patients diagnosed with MPM according to different time epochs.

Survival according to whether symptoms were present or not at MPM diagnosis

Survival was compared between patients who presented with symptoms relating to MPM (*n*=639), such as cough, chest pain, and shortness of breath, and those who had no symptoms at diagnosis (therefore the diagnosis was likely to be incidental) (*n*=34) (inadequate data *n*=88): median survival 328 days *vs* 465 days respectively, ratio 0.71, 95% CI of ratio 0.48 to 1.03; χ^2 7.9, df 1, *p* 0.005 [Log-rank (Mantel-Cox) test] (inadequate data in *n*=157) (Figure 6.5).

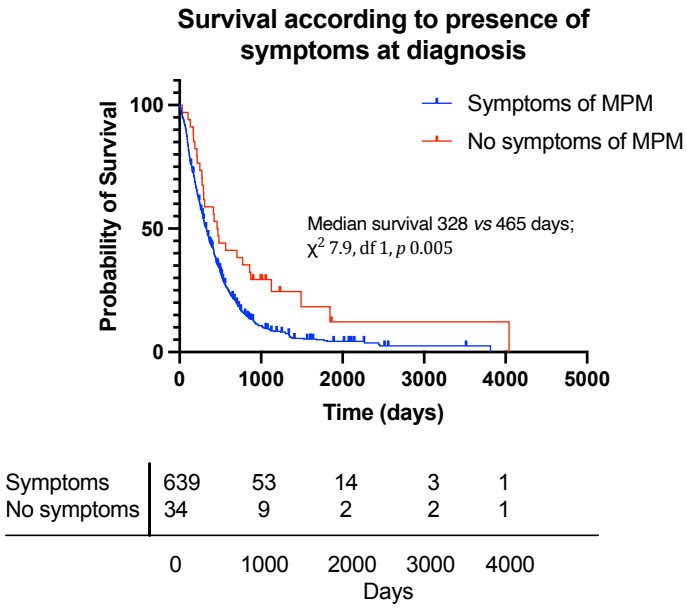

Figure 6.5 shows survival curves according to whether patients presented with symptoms relating to MPM or not (i.e. likely incidental finding in the latter group).

Cox regression analysis revealed all the factors in Brim's model(Brims et al., 2016) to remain significant variables, and epoch time periods remain non-statistically significant variables, however symptoms of MPM at diagnosis did not reach statistical significance (*p* 0.2) when corrected for the other variables. Removal of epoch time periods from the equation did not change the statistical significance of the other factors.

Comparison of patient characteristics between Pleural Units

With regards to weight loss at diagnosis, when comparing individual Pleural Units to each other, there was no significant difference between Bristol and Oxford NHS Pleural Units (*p* 0.3), but significant differences between Glasgow and Bristol Pleural Units (*p* 0.0001), and Glasgow and Oxford Pleural Units (*p* <0.0001) [Fisher's exact test]. There was no statistically significant difference between median Hb at MPM diagnosis between Glasgow and Oxford Pleural Unit (*p* 0.4), but a statistically significant difference between Glasgow and Bristol

Pleural Units (*p* 0.07), and between Bristol and Oxford Pleural Units (*p* 0.004). There was no statistically significant difference between median serum albumin at diagnosis between Glasgow and Bristol Pleural Units (*p* 0.7), but a statistically significant difference between Bristol and Oxford Pleural Units (*p* <0.0001), and between Glasgow and Oxford Pleural Units (*p* <0.0001).

Overall survival was lowest in Glasgow, and highest in Oxford, however patients from Oxford and Bristol were statistically significantly more likely to have weight loss at diagnosis, while patients in Glasgow and Bristol were more likely to have lower serum albumin levels, and Hb levels were lower in patients from Glasgow and Oxford than in those from Bristol.

When analysing the factors known to influence survival in MPM, together with the patient's Pleural Unit in a multiple linear regression model, with survival as the dependent variable, weight loss at diagnosis (*p* 0.03), and the Pleural Unit (*p* 0.03) were statistically significant variables, although serum albumin at diagnosis, MPM subtype, and PS were more statistically significant variables (*p* 0.005, <0.001, <0.001 respectively).

Chemotherapy and ECOG PS

Chemotherapy is only considered in patients who are fit enough to receive it, and in MPM it is usually only considered in patients with ECOG PS 0-2. Therefore patients receiving chemotherapy are likely to also be patients with better ECOG PS. As expected, whether chemotherapy was received or not was correlated with ECOG PS, with patients having lower ECOG PS being more likely to receive first line chemotherapy: Pearson *r* -0.36, 95% CI -0.43 to -0.29, *p* <0.0001.

6.2 Supplementary figures and tables for retrospective analysis of patients with malignant pleural mesothelioma

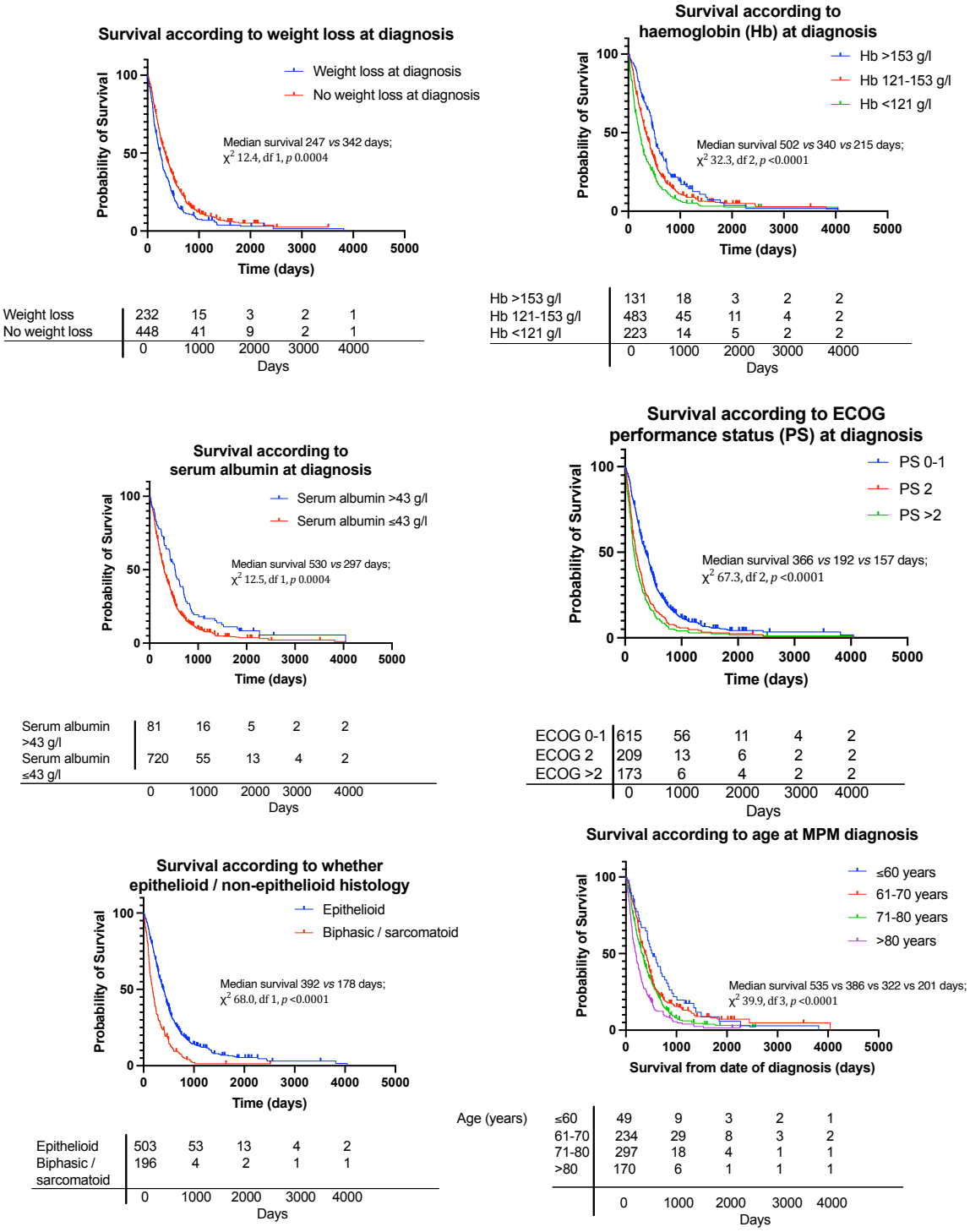

Figure 6.6 shows survival curves for the individual factors (univariable analysis) in an MPM decision tree model,(Brims et al., 2016) and age, found to be associated with survival in MPM, when analysed in this cohort of patients.

Table 6.1 shows comparison of survival between patients with malignant comorbidities and those without. ***Prostate n=21, skin n=17 (melanoma n=7, basal cell carcinoma n=6, squamous cell carcinoma n=3, unknown type n=1), gastrointestinal tract n=16 (colorectal n=13, gastrointestinal stromal tumour n=1, appendiceal n=1, somatostatinoma n=1), haematological n=11 (Chronic lymphoid leukaemia n=3, lymphoma n=5, acute myeloid leukaemia n=1, myeloma n=1, monoclonal gammopathy of unknown significance n=1), breast n=10, non-small cell lung carcinoma n=7, bladder n=7, laryngeal / tongue n=3, testicular n=3, cervical / endometrial n=3, renal cell carcinoma n=2, multiple n=4 (colorectal and prostate n=1, colorectal and bladder n=1, prostate and renal cell carcinoma n=1, prostate and bladder n=1).***

Malignant comorbidities	Median survival (days)	Statistical analysis results
Present* (*n*=104)	306	χ^2 0.76, df 1, *p* 0.38 (Log-rank (Mantel-Cox))
Absent (*n*=646)	303	

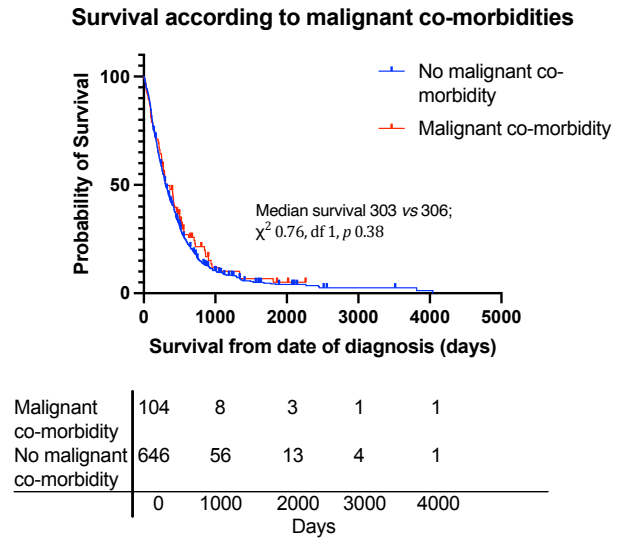

Figure 6.7 shows comparison of survival curves according to whether malignant co-morbidities were present or not.

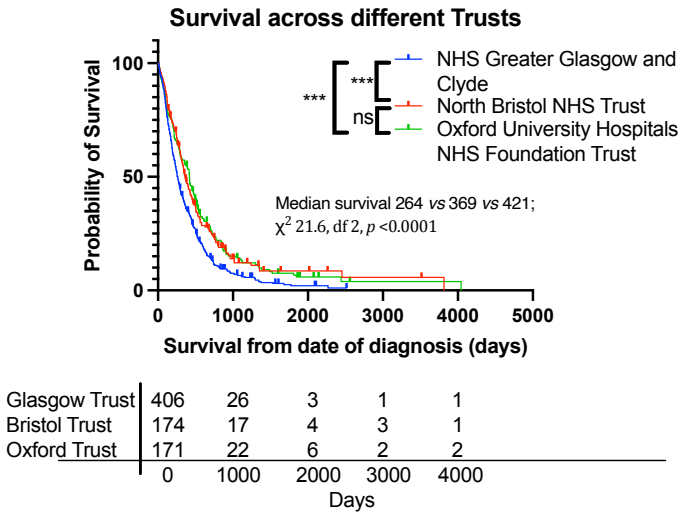

Figure 6.8 shows survival curves of patients diagnosed with MPM in three different Pleural Units in the UK. ****Statistically significant difference, ns = non-statistically significant difference.*

Table 6.2 shows comparison of the factors included in the Brims model, across Pleural Units included in the dataset. ***CI = confidence interval; ECOG PS = Eastern cooperative oncology group performance status; IQR = interquartile range; MPM = malignant pleural mesothelioma.***

Parameter	Trust			Data not available (*n*, for Oxford, Bristol, Glasgow)	Statistical analysis results
	Oxford	**Bristol**	**Glasgow**		
Weight loss at MPM diagnosis	*n*, (%): 68 (54)	73 (47)	92 (23)	53, 21, 0	χ^2 180.1, df 4, ***p* <0.0001**; χ^2 test
ECOG PS at MPM diagnosis ECOG PS 0-1 ECOG PS 2 ECOG PS 3-4	*n*, (%): 112 (84) 15(11) 7(5)	 139 (79) 23 (13) 13 (7)	 246 (75) 49 (15) 32 (10)	45, 1, 79	χ^2 4.5, df 4, *p* 0.3; χ^2 test
MPM histological subtype Epithelioid Biphasic Sarcomatoid	*n*, (%): 110 (68) 23 (14) 30 (18)	 126 (75) 14 (8) 27 (16)	 275 (73) 33 (9) 71 (19)	16, 9, 27	χ^2 5.1, df 4, *p* 0.3; χ^2 test
Haemoglobin (g/l) at MPM diagnosis	Median (IQR, 95% CI): 131 (22, 127 to 134)	138 (28.5,133 to 142)	133 (28, 130 to 136)	16, 0, 28	***p* 0.005**, Kruskal-Wallis statistic 10.7 (Kruskal-Wallis test)
Serum albumin (g/l) at MPM diagnosis	Median (IQR, 95% CI): 36 (10, 35 to 39)	34 (7, 33 to 35)	32 (8.8, 31 to 33)	22, 3, 30	***p* <0.0001**, Kruskal-Wallis statistic 53.4 (Kruskal-Wallis test)

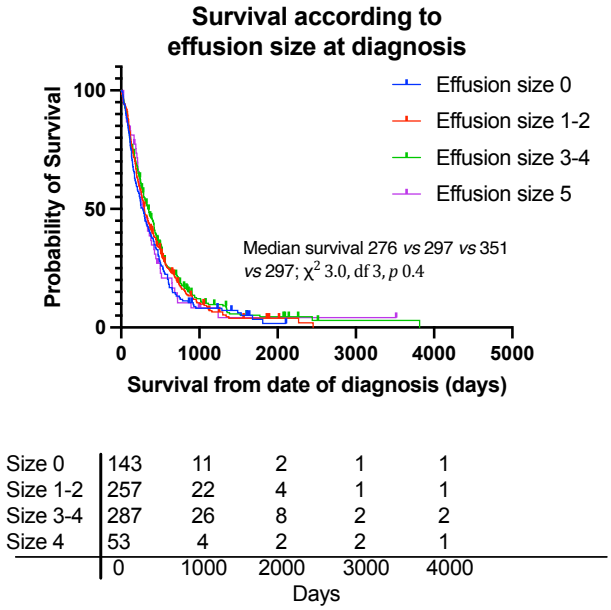

Figure 6.9 shows survival curves showing comparison of survival according to the size of the pleural effusion present at MPM diagnosis.

Table 6.3 shows comparison of the baseline factors for patients who received intrapleural talc and those who did not (data on whether talc was given or not was not available in *n*=380). Patients receiving talc were more likely to have higher serum albumin levels at MPM diagnosis, more likely to have epithelioid MPM subtype, and more likely to be older in this cohort. The higher serum albumin may reflect better nutritional status and general health, and the higher chance of having epithelioid subtype may be a reflection of improved prognosis (biphasic and sarcomatoid subtypes are associated with worse prognosis in MPM), which may be factors that influence a clinical decision to give intrapleural talc. However, again, the high number of missing data is an important factor to bear in mind and it is therefore difficult to draw robust conclusions from this analysis. *MPM=malignant pleural mesothelioma; NA = data not available.*

	Received intrapleural talc (*n*=194)	**Did not receive intrapleural talc (*n*=187)**	**Statistical analysis result**
Performance status			
0	60	54	
1	81	98	*p* 0.22 (χ^2)
2	27	17	
3	7	9	
NA	19	9	
Weight loss at MPM diagnosis			
Y	66	110	*p* 0.32 (χ^2)
N	88	66	
Mean haemoglobin (g/l) at MPM diagnosis	137.4	133.9	*p* 0.07 (t-test)
Mean serum albumin (g/l) at MPM diagnosis	35.9	32.3	***p* <0.00001** (t-test)
MPM subtype			
Epithelioid	149	130	***p* 0.032** (χ^2)
Biphasic	22	17	
Sarcomatoid	17	33	
Mean age at MPM diagnosis (years)	73.3	71.4	***p* 0.025** (t-test)

6.3 The development of patient-derived malignant pleural mesothelioma cell cultures from pleural fluid: a tool to advance biomarker-driven treatments

The method used to freeze the cells

1. Cells were prepared according to the first part of the protocol for splitting a cell culture, i.e. washed twice with PBS, incubated with trypsin, centrifuged, and the supernatant was aspirated without disturbing the cell pellet.
2. The cells were then resuspended in 1ml of freezing medium (90% FBS + 10% DMSO).
3. The suspension was then transferred to a labelled cryogenic vial, and frozen at -80°C.

Thawing of cells

The thawing procedure is stressful to frozen cells. DMSO is toxic to the cells, and frozen cells' deterioration begins above -50°C. Therefore, when thawing the cells, they were exposed to thawed DMSO for the shortest period of time possible, to ensure that the highest possible proportion of cells survive the procedure.

1. The cells were thawed by rubbing the vial in gloved hands for 1-2 minutes.
2. The thawed cells were immediately transferred to a 15ml conical centrifuge tube with 5ml complete medium, and centrifuged at 500 x g for 5 minutes.
3. The supernatant was aspirated, so removing any residual DMSO.
4. The pelleted cells were resuspended in 1ml complete medium and transferred to a cell culture plate containing 9ml of complete medium.
5. The plate was observed under the light microscope to ensure that cells were present, and then the plate was incubated at 37°C and 5% CO_2.

Method used for DNA extraction

The method used for DNA extraction followed the kit's standard protocol:

1. Cell culture samples were first centrifuged at 1500 x g for 10 minutes.
2. The supernatant was discarded and the cells were washed twice in PBS.
3. The cells were then resuspended in PBS to a concentration of 10^7 cells/ml.
4. Buffer C1 (cell lysis buffer: 1.28 M sucrose, 40 mM Tris (tris(hydroxymethyl)aminomethane) chloride, pH 7.5; 20 mM magnesium chloride; 4% Triton X-100) was added to cell suspension, to lyse cells but preserve the nuclei.
5. Lysed cells were centrifuged at 1300 x g for 15 minutes, and supernatant discarded.
6. The cells were resuspended in Buffer C1. The mixture was centrifuged again at 1300 x g for 15 minutes to remove all residual cell debris from the nuclear pellet.
7. Cells were resuspended in Buffer G2 (General Lysis Buffer: 800 mM guanidine hydrochloride; 30 mM Tris chloride, pH 8.0; 30 mM EDTA, pH 8.0; 5% Tween 20; 0.5% Triton X-100). This lyses the nuclei and denatures proteins by vortexing at maximum speed for about 20 seconds to resuspend nuclei. The kit used yields genomic DNA that ranges in size from 20–150 kb, with an average length of 50–100 kb. Vortexing the lysate may reduce the size of the genomic DNA slightly to 20–130 kb, but can help to avoid poor flow rates associated with clogging.
8. Proteinase K was added, and the sample was incubated at 50°C for about 45 minutes until lysate became clear.
9. The sample was then loaded onto the genomic tip and centrifuged. The DNA binds to the column while other cell constituents pass through.
10. The sample was washed to remove any remaining contaminants.
11. Pure high-molecular-weight DNA was eluted and precipitated with isopropanol.

6.4 Investigating the biological properties of pleural fluid: *in vitro* experiments

Method used for CellTiter-Glo® luminescence assay

The reagent contains detergent to lyse the cells, ATPase inhibitors to stabilise ATP that is released from the lysed cells, luciferin as a substrate, and the stable form of luciferase (an oxidative enzyme that can generate light). The ATP and luciferin act as substrates for luciferase to produce bioluminescence. The ATP assay is fast, sensitive and less prone to artifacts than other viability assays,(Riss et al., 2004) and CellTiter-Glo® simply involves adding the reagent to the cells, minimising the number of plate-handling steps required. The method used was as follows:

1. The plates were checked under a light microscope to ensure that there were viable adherent cells present within the wells.
2. If the cells had been seeded in starvation medium, the culture medium was aspirated, the cells in each well were washed with 100µl of PBS, and 100µl of the fluid to be tested (mostly pleural fluid in these experiments) were transferred using a multichannel pipette to each of the wells with cells. The plate was incubated at 37°C and 5% CO_2 until the required time point of the experiment.
3. Upon reaching the desired time-point, any fluid was discarded from the wells.
4. Cells were washed with PBS.
5. 50µl of CellTiter-Glo® solution were transferred to each well (this reacts with ATP in live cells, in so-doing it kills and detaches the cells)
6. The plate was mixed gently to induce cell lysis in the adherent cells.
7. The plate was incubated at room temperature for 30 minutes (the Promega CellTiter-Glo® instructions for use of product suggest letting the plate stabilise for 10 minutes to

allow the luminescence signal to stabilise, however for these experiments, a period of 30 minutes was allowed for the luminescence signal to stabilise as more consistent results were noted this way).

8. TECAN safire^2 multimode multiplate reader, and Magellan^{TM} software were used to measure luminescence, and an example of the luminescence results obtained for a 96-well plate is shown below. The values generated by the Magellan^{TM} software were in relative luminometer units (RLU) (Figure 7.10).
9. The Log10 of the luminescence results obtained were plotted against time to obtain growth curves.

Figure 6.10 shows an example of the luminescence measurements (in relative luminometer units (RLU)) generated when the plate is read using Magellan^{TM} software and the TECAN safire^2 multi-mode multiplate reader.

Seeding cells for experiments

1. When cell culture plates reached 80-90% confluence, the cells were prepared according to the first part of the protocol for splitting a cell culture, i.e. washed twice with PBS, incubated with trypsin, centrifuged, and the supernatant aspirated.
2. The pelleted cells were resuspended in 1ml of complete medium.
3. Then 80µl PBS, 10µl Trypan blue 0.4% solution (Sigma-Aldrich® T8154) and 10µl of the resuspended cells were transferred to a small vial.
4. Subsequently, 10µl of the resuspended cell solution in Trypan blue were transferred to each side of a haemocytometer.
5. Cells on either side of the chamber (within the central grid) were counted under a light microscope and a mean of the 2 readings obtained.
6. The volume of fluid needed to make up the required dilution to transfer a required number of cells per well for the experiment was calculated.

For example:

- If 53 and 45 cells were counted on either side of the Neubauer chamber (mean = 49 cells), the following Neubauer formula was used:

  Concentration = number of cells x 10,000 / number of squares

  where number of squares is the number of squares that cells were counted in, and in these experiments this was 1;

  i.e. for a 1:10 dilution, the mean number of cells in each chamber should be multiplied by 10,000 to identify the number of cells present within 10µl of the solution.

The value was then multiplied again by 10 to calculate the number of cells within a 1ml volume: i.e. $49x10^4$ x10, i.e. 4,900,000 cells in 1ml i.e. $4.9x10^6$ cells in 1ml of cell suspension.

- If the experiment required 50,000 cells per well, and a total of 72 wells were planned to be used in the experiment, then the calculation was based on the volume required for 90 wells in order to ensure that there was sufficient volume of resuspended cells available for the experiment, taking into consideration losses through pipetting and transferring of cells:

  1 well = 50,000 cells, therefore 90 wells = 90 x 50,000 = 4,500,000 (i.e. $4.5x10^6$) cells needed in total for the experiment.

- In the above example, with $4.9x10^6$ cells in 1ml of cell suspension, 4,500,000 cells (i.e. 4.5 x 10^6) were required for the experiment. The volume of resuspended cells needed to have the required number of cells, was calculated:

  $4.5\ x10^6 / 4.9x10^6 = 0.92ml$

  i.e. 0.92ml of resuspended cells were required for the experiment in order to have $4.5x10^6$ cells.

- If a 96-well plate was being used, the experiments were performed with 100µl of solution in each well (the standard volume used in a 96-well plate) so a total of 9000µl or 9ml of fluid was required for 90 wells.

- The required volume of resuspended cells was deducted from the total fluid volume required for the experiment: 9ml – 0.92ml = 8.08ml.

- Therefore 0.92ml of resuspended cells were added to 8.08ml of pleural fluid in order to make up a total of 9ml fluid for the experiment.

7. Finally, 100µl of the final solution were transferred to wells on a 96-well plate, excluding columns 1 and 12 and rows A and H which were filled instead with 100µl of complete medium in order to reduce evaporation errors (fluid may evaporate during incubation, especially from the outer wells, causing varying results in the cell viability assay).
8. The cells were incubated at 37°C and 5% CO_2.

Table of reagents used:

Table 6.4 Table listing the reagents used in the laboratory and the suppliers.

Reagent	Supplier
Dulbecco's modified Eagle medium	Gibco®
Foetal bovine serum	Sigma-Aldrich®
Bovine Serum Albumin	Sigma-Aldrich®
Penicillin, Streptomycin	Sigma-Aldrich®
Red blood cell lysis solution	Qiagen®
Phosphate buffer solution	Sigma-Aldrich®
Trypsin	Sigma-Aldrich®
Dimethyl sulphoxide	Sigma-Aldrich®
Resazurin	Sigma-Aldrich®

6.5 Studying malignant pleural effusion development and malignant pleural effusion fluid effects *in vivo* using animal models

The method used for intrapleural LLC cell delivery

The method for intrapleural LLC cell delivery was similar to that reported by Servais et al,(Servais et al., 2011) and was as follows:

1. The mice were first anaesthetised with isoflurane, with oxygen at 3 litres per minute flow rate, by calibrated vaporiser.
2. Once anaesthetised, the animal fur on the right thoracic flank posterior to forelimb was shaved using electric clippers.
3. The shaved mouse was then placed in a nose cone for ongoing anaesthesia, and mice were placed on a warm circulating water blanket throughout the procedure to prevent hypothermia.
4. With the mouse positioned in the left lateral decubitus position, the right thorax was sterilised with iodine.
5. The subcutaneous skin was then incised with surgical scissors and retracted, and further dissection was performed to reveal the bony thorax and parietal pleura - the pleura was kept intact.
6. Under direct visualisation, a 1cc syringe with 21G needle was used to inject a cell suspension of 250,000 cells in 100µl PBS into the thoracic space.
7. The subcutaneous tissues were secured into anatomic position with topical tissue adhesive.
8. Mice were removed from sedation and monitored until fully recovered.

The method used for IPC insertion

On day 7 after intrapleural LLC cell injection, the IPC was inserted.

1. Three full-thickness punctures were made in the distal end of a polyurethane tube (PU-40) (Instech®) using a 22G needle (to allow enhanced catheter flow, similar to IPCs in humans with multiple fenestrations in the intrapleural portion of the catheter).
2. The mice were anaesthetised with isoflurane and again shaved in the same manner as for intrapleural LLC cell injection, and the mice were then placed in a nose cone for

ongoing anaesthesia, in left lateral decubitus position, on a warm circulating water blanket.

3. The skin was sterilised with iodine and the subcutaneous tissue on the right flank was dissected to expose the thorax.
4. A small puncture was made through the seventh rib space in the mid-axillary line using a 16G blunt tipped needle.
5. Immediately following puncture, the needle was removed and the PU-40 was advanced approximately 1.5cm into the pleural space. Simultaneously, a sharp tipped 16G needle was punctured through the fur on the animal dorsum, midway between scapulae, and advanced from point of insertion, through subcutaneous tissue toward the catheter insertion site in the right flank.
6. The proximal end of the PU-40 tubing was passed over the needle and out between the scapulae.
7. The needle was then removed, leaving the IPC tunnelled through the subcutaneous space.
8. The proximal end of the catheter was anchored at the insertion site with application of GLUture (World Precision Instruments, Hitchin, UK) and the distal end was sutured in place between scapulae with ethilon 5-0 monofilament suture.
9. Finally, the area of dissection was closed with approximation of tissue and application of topical tissue adhesive.
10. The IPC was capped with a 22G syringe accessible port, which was placed on the proximal end of the catheter prior to pleural insertion in order to prevent tension pneumothorax (Figure 3).
11. Pleural fluid was then aspirated while the mice were still anaesthetised.
12. On day 14, mice were sacrificed in a CO_2 inhalational chamber (or earlier if mice were moribund) and final pleural fluid aspiration was performed. After final aspiration, mice were dissected and the pleural space was exposed.

6.6 High-throughput drug screening and deoxyribonucleic acid sequencing of patient-derived malignant pleural mesothelioma cell cultures

Z factor and z-score

The Z factor (Kim et al., 2017; Zhang et al., 1999) is calculated as:

$Z\ factor = 1 - 3(SD_p + SD_n)/(mean_p - mean_n)$

where SD is standard deviation, *p* is positive controls (10uM/1.6uM pemetrexed/ cisplatin, as per standard first line chemotherapy for mesothelioma) and *n* is negative controls (PBS). The Z factor is usually used to measure the assay quality and has a range of 0 to 1. Appropriately robust screening assays have a Z factor of more than 0.4-0.6.

The *z*-score is calculated according to the following formula:

$z\text{-score} = (X - \text{population mean}) / \text{population standard deviation}$

where *X* is the value obtained for fluorescence divided by the mean of the negative control (in this case, PBS). The *z*-score is used to normalise the data and incorporates information on the variation in sample measurements. It is however also sensitive to outliers.

The drug library

The drug library (Target Discovery Institute (TDI) Expanded Oncology Drug Set), including a total of 316 drugs (apart from Cisplatin/Pemetrexed), used in the drug screening assay:

1	Cisplatin/Pemetrexed	32	CHIR-258 (Dovitinib)
2	Galiellalactone	33	Mitomycin
3	SR1 HCl	34	Nelarabine
4	VER 155008	35	Carboplatin
5	BAY 73-4506_Regorafenib	36	(R)-Flurbiprofen (Tarenflurbil)
6	RDEA119_Refametinib	37	Mitoxantrone
7	Brivanib	38	Allopurinol
8	GSK2126458	39	Thioguanine
9	MGCD0103_Mocetinostat	40	Dacarbazine
10	BIBF 1120_Nintedanib	41	Altretamine
11	FK 506_Tacrolimus	42	Ridaforolimus
12	RD162	43	YM155
13	4-hydroxytamoxifen	44	BIRB 796 (Doramapimod)
14	AVN944	45	PHA-739358 (Danusertib)
15	PF-04708671	46	VX-11e
16	AV-951 (Tivozanib)	47	PCI-32765_Ibrutinib
17	Camptothecin	48	ABT-263 (Navitoclax)
18	AZD1480	49	CPI-613
19	Docetaxel	50	BI 2536
20	MLN8237_Alisertib	51	GSK 269962
21	Finasteride	52	MDV3100_Enzaluamide
22	Clomifene citrate	53	Prednisone
23	Idelalisib	54	AZD8055
24	Tetramisole HCl	55	Enzastaurin
25	Raloxifene	56	OSI-027
26	1-methyl-D-tryptophan, 95%	57	Rapamycin (sirolimus)
27	Carmustine	58	MK-4827, HCl salt
28	CUDC-101	59	HA-1077 (Fasudil)
29	Cladribine	60	PIK-75 HCl
30	Tubastatin A HCl	61	Flutamide
31	Exemestane	62	Busulfan

63	Cyclophosphamide	95	Bexarotene
64	Quinacrine HCl	96	PF-04691502
65	Ifosfamide	97	Lapatinib, di-p-toluenesulfonate salt
66	Aspirin (Acetylsalicylic Acid)	98	AC220_Quizartinib
67	Anastrozole	99	Dasatinib
68	Iniparib (BSI-201, IND-71677)	100	PLX4032_Vemurafenib
69	Mitotane	101	Pilocarpine
70	TGX-221	102	Pemetrexed
71	Oxaliplatin	103	Letrozole
72	ABT-751	104	Arsenic(III) oxide
73	Methotrexate	105	Clofarabine
74	PF4800567 hydrochloride	106	Masitinib
75	Pralatrexate	107	Fludarabine phosphate
76	Tandutinib	108	Bicalutamide
77	Mercaptopurine	109	Erlotinib hydrochloride
78	Procarbazine hydrochloride	110	Rofecoxib (Vioxx)
79	Floxuridine	111	Topotecan hydrochloride
80	Gemcitabine hydrochloride	112	FG-4592
81	Uracil mustard	113	Pemetrexed, Disodium salt, Hetahydrate
82	PX-866_Sonolisib	114	GSK 650394
83	I-BET151 (GSK1210151A)	115	Mechlorethamine hydrochloride
84	MK1775	116	XAV-939
85	Motesanib Diphosphate (AMG-706)	117	Methoxsalen
86	Epothilone B (Patupilone)	118	Pomalidomide
87	LY2603618_Rabusertib	119	Cytarabine hydrochloride
88	AT9283	120	Megestrol acetate
89	ABT-869_Linifanib	121	Tretinoin
90	RAF265	122	LY 333531 mesylate_Ruboxistaurin
91	AT 101	123	Lestaurtinib
92	FK-866 HCl_Daporinad	124	Plerixafor
93	Estramustine sodium phosphate	125	LY2784544_Gandotinib
94	AZD4547	126	Everolimus

127	KX2-391
128	XL880 (Foretinib)
129	LY2157299
130	MGCD-265
131	PF 431396
132	Bimatoprost
133	Goserelin acetate
134	Dacomitinib (monohydrate) (PF-00299804)
135	Cisplatin aq
136	BMS-754807
137	Axitinib
138	INK128
139	Nilutamide
140	NVP-LDE225 (Diphosphate salt)
141	Metformin hydrochloride aq
142	Pravastatin
143	Zoledronic acid
144	Valproic acid
145	Enzalutamide
146	Olaparib
147	Lomustine
148	CYT-387_Momelotinib
149	Thalidomide
150	Roscovitine_Selicilib
151	Dexrazoxane
152	Amonafide
153	Lenalidomide
154	Lomeguatrib
155	Celecoxib
156	Indibulin
157	Lenvatinib
158	Bortezomib
159	Pazopanib hydrochloride
160	Crizotinib
161	Vandetanib
162	Sotrastaurin
163	Panobinostat
164	ABT-199
165	AZD2014
166	PKC412_Midostaurin
167	AS703026_Pimasertib
168	LY2228820 (CP868569)
169	JNJ 26854165 (Serdemetan)
170	TAK-901
171	AZ 3146
172	2-methoxyestradiol (Panzem)
173	Doxorubicin
174	EMD1214063
175	NVP-BEZ235_Dactolisib
176	GSK2636771
177	Irinotecan
178	INCB018424 (free base, Ruxolitinib)
179	AZD6244 (Selumetinib)
180	Atorvastatin Ca
181	S-trityl-L-cysteine, 40 mM
182	Prednisolone
183	Aminolevulinic acid hydrochloride
184	Chloroquine diphosphate
185	Azacitidine
186	Vismodegib
187	Trifluridine
188	OSI-906_Linsitinib
189	Pentostatin

190	Tasocitinib_Tofacitinib	222	Deferoxamine mesylate
191	Pipobroman	223	Tamoxifen citrate
192	17-DMAG (Alvespimycin)	224	Methylprednisolone
193	Sunitinib	225	Temsirolimus
194	Stattic	226	AP24534 (Ponatinib)
195	Imatinib	227	Epirubicin hydrochloride
196	Vatalanib	228	CAL-101
197	Erismodegib	229	Teniposide
198	Fluorouracil	230	Bosutinib
199	Vemurafenib	231	Fulvestrant
200	Temozolomide	232	ABT-888 (Veliparib)
201	Ixazomib citrate	233	Triethylenemelamine
202	AG-014699_Rucaparib	234	Belinostat (PXD101)
203	Canertinib	235	Osimertinib
204	Tubacin	236	ZSTK474
205	PF-2341066 (Crizotinib)	237	Bleomycin sulfate
206	Homoharringtonine	238	Amifostine
207	Obatoclax Mesylate (GX15-070)	239	Imiquimod
208	Nutlin-3	240	Streptozocin
209	BIIB021	241	Lapatinib
210	TAK-733	242	PF 477736
211	PF670462	243	Lasofoxifene
212	DCC-2036_Rebastinib	244	Carfilzomib
213	Idarubicin HCl	245	Crenolanib
214	XL184_Cabozantinib	246	Toremifene citrate
215	Simvastatin	247	Vincristine Sulfate (Oncovin)
216	MLN4924	248	AT-406
217	CI-994_Tacedinaline	249	UCN-01
218	Dabrafenib Mesylate	250	(5Z)-7-Oxozeaenol
219	Abitrexate/Methotrexate	251	CHR 2797_Tosedostat
220	NVP-AUY922	252	GDC-0980
221	Vorinostat	253	PD-0332991

254	MK-2206
255	Anagrelide
256	BMS-911543
257	Nilotinib
258	GSK1120212_Trametinib
259	Clafen (Cyclophosphamide, Endoxan)
260	ARRY-162_MEK-162
261	Gefitinib
262	Thio-TEPA
263	Paclitaxel
264	Melphalan
265	Melphalan hydrochloride
266	Tipifarnib (Zarnestra)
267	Cobimetinib
268	Dinaciclib (SCH727965)
269	Ceritinib
270	GDC-0941_Pictilisib
271	Abiraterone
272	Decitabine (Dacogen)
273	Ixabepilone
274	Entinostat
275	Daunorubicin hydrochloride
276	Dactinomycin
277	Chlorambucil
278	Valrubicin
279	Bendamustine hydrochloride
280	Cabazitaxel
281	AZD 7762 hydrochloride
282	BIBW2992 (Tovok)_Afatinib
283	BI 6727_Volasertib
284	MK-0752
285	R406_Tamatinib
256	BKM-120_Buparlisib
287	SB 743921
288	Auranofin
289	JNJ-26481585_Quisinostat
290	Mithramycin A
291	AZD1152-HQPA
292	Perifosine aq/PBS
293	GDC-0068
294	Pazopanib
295	ARQ 197_Tivantinib
296	Sorafenib
297	NVP-BGJ398
298	Dexamethasone (Decadron)
299	XL-147
300	Capecitabine
301	Hydroxyurea
302	Palbociclib
303	Aminoglutethimide
304	Romidepsin
305	PF-3845
306	Plicamycin
307	Varespladib
308	Vinorelbine tartrate
309	PAC-1
310	Alectinib
311	17-AAG (Tanespimycin, Geldanamycin)
312	Omacetaxine mepesuccinate
313	Prima-1 Met
314	Etoposide
315	Vinblastine sulfate
316	Irinotecan hydrochloride
317	Uridine triacetate

6.7 Supplementary tables for drug screening assay

Table 6.5 showing z-scores for drugs that were more than or as effective as cisplatin/pemetrexed combination in the drug screening assay (10uM dilution) of drugs with MPM cell culture MESO163

Small Compound name	Z-score MESO163 with 10uM dilution
Idarubicin HCl	-1.78580533
JNJ 26854165 (Serdemetan)	-1.748971307
Crenolanib	-1.740287181
PF-2341066 (Crizotinib)	-1.727749725
AP24534 (Ponatinib)	-1.722833247
Doxorubicin	-1.722784256
Ceritinib	-1.715075747
Omacetaxine mepesuccinate	-1.714735707
Homoharringtonine	-1.713515599
Auranofin	-1.710204657
Tubacin	-1.709135661
BI 2536	-1.706419392
Amonafide	-1.705355241
Dactinomycin	-1.702492163
Mithramycin A	-1.702328545
Ixazomib citrate	-1.702030184
Osimertinib	-1.701301158
Stattic	-1.700126064
Epirubicin hydrochloride	-1.699522591
Plicamycin	-1.696493371
SB 743921	-1.696035095
Daunorubicin hydrochloride	-1.695755138

Mitoxantrone	-1.695546036
TAK-901	-1.695448236
Bortezomib	-1.693011302
Valrubicin	-1.690984364
Quinacrine HCl	-1.68782252
UCN-01	-1.686424489
Carfilzomib	-1.684673575
Crizotinib	-1.674578374
PIK-75 HCl	-1.673135301
XL880 (Foretinib)	-1.668079888
4-hydroxytamoxifen	-1.66741199
AT9283	-1.657003689
Cobimetinib	-1.654581833
PF 431396	-1.644510458
Dinaciclib (SCH727965)	-1.63105788
BAY 73-4506_Regorafenib	-1.619709174
Romidepsin	-1.605453796
Lestaurtinib	-1.57805882
Obatoclax Mesylate (GX15-070)	-1.553891135
Topotecan hydrochloride	-1.538443978
BI 6727_Volasertib	-1.522065074
Mitomycin	-1.490971529
Camptothecin	-1.485719466
Canertinib	-1.416270304
Cisplatin / Pemetrexed	**-1.4**

Table 6.6 showing the z-scores for all drugs that were more than or as effective as cisplatin/pemetrexed combination in the drug screening assay using 10uM dilution of drugs with MPM cell culture MESO033

Small Compound name	Z-score MESO033 with 10uM dilution
XL880 (Foretinib)	-1.683394314
JNJ 26854165 (Serdemetan)	-1.681775282
Crizotinib	-1.676138917
AP24534 (Ponatinib)	-1.673746538
PF-2341066 (Crizotinib)	-1.672893257
Ceritinib	-1.671322295
Osimertinib	-1.67070079
Bortezomib	-1.669384267
SB 743921	-1.668264214
Carfilzomib	-1.666321218
Ixazomib citrate	-1.66464841
ABT-263 (Navitoclax)	-1.660454124
BI 2536	-1.659483739
Quinacrine HCl	-1.657844079
Canertinib	-1.656294526
Romidepsin	-1.653859577
Homoharringtonine	-1.651930274
Omacetaxine mepesuccinate	-1.649554264
BAY 73-4506_Regorafenib	-1.646634259
Idarubicin HCl	-1.644980203
AZD 7762 hydrochloride	-1.643407682
Mitomycin	-1.64318786
Mitoxantrone	-1.638695327
Crenolanib	-1.630948057

Daunorubicin hydrochloride	-1.625608695
4-hydroxytamoxifen	-1.624471869
Auranofin	-1.621907284
JNJ-26481585_Quisinostat	-1.588912698
Panobinostat	-1.57882264
AT9283	-1.578183537
Epirubicin hydrochloride	-1.558347978
SR1 HCl	-1.542254057
Valrubicin	-1.541414161
UCN-01	-1.541110947
Amonafide	-1.540229625
Doxorubicin	-1.520783107
AZD1480	-1.511687957
Stattic	-1.506949351
BKM-120_Buparlisib	-1.487058409
MGCD0103_Mocetinostat	-1.479835839
Gemcitabine hydrochloride	-1.477429432
PIK-75 HCl	-1.474867248
Teniposide	-1.455655467
Vinblastine sulfate	-1.43717566
Plicamycin	-1.43628292
Clofarabine	-1.432686066
Sunitinib	-1.404116874
Sorafenib	-1.402816648
Mithramycin A	-1.393823292
Cobimetinib	-1.390996981
Belinostat (PXD101)	-1.386181858
LY 333531 mesylate_Ruboxistaurin	-1.366434315

Triethylenemelamine	-1.365845599
TAK-901	-1.354133332
Dactinomycin	-1.351604661
BMS-754807	-1.331570282
Vinorelbine tartrate	-1.321028401
CUDC-101	-1.303487499
Tubacin	-1.289144409
BIBW2992 (Tovok)_Afatinib	-1.273285871
BI 6727_Volasertib	-1.226402284
Dinaciclib (SCH727965)	-1.21583241
Camptothecin	-1.200502438
Etoposide	-1.19947011
Lestaurtinib	-1.1940929
KX2-391	-1.193066248
Ixabepilone	-1.169704823
ABT-751	-1.144079898
MK1775	-1.136568109
AZD4547	-1.132932938
Dacomitinib (monohydrate) (PF-00299804)	-1.131304774
R406_Tamatinib	-1.121491622
Obatoclax Mesylate (GX15-070)	-1.120857083
Epothilone B (Patupilone)	-1.113882205
Paclitaxel	-1.109751734
MLN4924	-1.087599978
2-methoxyestradiol (Panzem)	-1.073331502
Docetaxel	-1.060018918
PF 431396	-1.055600677

Cytarabine hydrochloride	-1.040736373
Vorinostat	-1.039271813
Topotecan hydrochloride	-1.036457864
Cabazitaxel	-1.015955352
ARQ 197_Tivantinib	-1.001458249
PHA-739358 (Danusertib)	-0.98946745
(5Z)-7-Oxozeaenol	-0.97571387
NVP-AUY922	-0.975435432
Indibulin	-0.972811933
17-DMAG (Alvespimycin)	-0.972369253
17-AAG (Tanespimycin, Geldanamycin)	-0.952851385
MK-4827, HCl salt	-0.951285316
BIIB021	-0.936310135
CHIR-258 (Dovitinib)	-0.908053841
Cisplatin / Pemetrexed	**-0.9**

Table 6.7 showing the z-scores for all drugs that were more than or as effective as cisplatin/pemetrexed combination in the drug screening assay using 100nM dilution of drugs with MPM cell culture MESO174

Small Compound name	**Z-score MESO174 at 100nM dilution**
Romidepsin	-3.798242585
Panobinostat	-3.69337413
Carfilzomib	-3.534848474
Idarubicin HCl	-3.514290483
JNJ-26481585_Quisinostat	-3.372467473
Mitoxantrone	-3.249922052
Omacetaxine mepesuccinate	-3.180405028
Dinaciclib (SCH727965)	-2.986162699
Homoharringtonine	-2.981469226
Vinblastine sulfate	-2.940280115
Dactinomycin	-2.91364401
Daunorubicin hydrochloride	-2.878585157
Bortezomib	-2.710310879
UCN-01	-2.691918893
INK128	-2.36400719
Plicamycin	-2.344966244
Epothilone B (Patupilone)	-2.185523056
Docetaxel	-2.168017576
Vinorelbine tartrate	-2.141201132
Paclitaxel	-2.110113043
Topotecan hydrochloride	-2.06627161
Cabazitaxel	-1.979428997
Ixabepilone	-1.892993879
Camptothecin	-1.862990648
Mithramycin A	-1.854222808

KX2-391	-1.849888507
17-AAG (Tanespimycin, Geldanamycin)	-1.789775686
Doxorubicin	-1.76820464
NVP-AUY922	-1.764008966
Gemcitabine hydrochloride	-1.729956085
17-DMAG (Alvespimycin)	-1.726144023
Epirubicin hydrochloride	-1.709515164
GSK2126458	-1.584360511
R406_Tamatinib	-1.544526011
AZD8055	-1.532042062
SB 743921	-1.467054965
BI 6727_Volasertib	-1.388710486
TAK-901	-1.308856767
Ixazomib citrate	-1.301478078
RAF265	-1.218794161
Dasatinib	-1.045043446
MK1775	-1.041610904
AP24534 (Ponatinib)	-1.038311576
PIK-75 HCl	-1.01966679
Rapamycin (sirolimus)	-1.019250675
Lestaurtinib	-0.917335734
Cisplatin / Pemetrexed	**-0.9**

Table 6.8 showing the z-scores for all drugs that were more than or as effective as cisplatin/pemetrexed combination in the drug screening assay using 1uM dilution of drugs with MPM cell culture MESO174

Small Compound name	Z-score MESO174 at 1uM dilution
Romidepsin	-2.637298416
JNJ-26481585_Quisinostat	-2.619548409
Panobinostat	-2.619068096
TAK-901	-2.475356704
Mitoxantrone	-2.466182049
Epirubicin hydrochloride	-2.431029543
Omacetaxine mepesuccinate	-2.426421389
Carfilzomib	-2.425432839
Idarubicin HCl	-2.401591391
Homoharringtonine	-2.369980904
Doxorubicin	-2.369574094
Daunorubicin hydrochloride	-2.300046074
Dactinomycin	-2.168602197
Bortezomib	-2.161535555
Vinblastine sulfate	-2.15737557
Plicamycin	-2.130006778
UCN-01	-2.127871019
Camptothecin	-2.077854341
Mithramycin A	-2.065104632
AZD8055	-2.005968724
Dinaciclib (SCH727965)	-2.000010716
Topotecan hydrochloride	-1.904657698
GSK2126458	-1.837621161
Obatoclax Mesylate (GX15-070)	-1.827497112
PIK-75 HCl	-1.826240595

Clofarabine	-1.826203921
Mitomycin	-1.820014433
Vinorelbine tartrate	-1.816050097
Cladribine	-1.813806503
R406_Tamatinib	-1.772012848
Ixazomib citrate	-1.749520937
KX2-391	-1.716835487
INK128	-1.702418048
Gemcitabine hydrochloride	-1.642877526
Teniposide	-1.611456747
AZD2014	-1.594408061
Docetaxel	-1.544653568
AP24534 (Ponatinib)	-1.524374366
AT9283	-1.521352126
AZD 7762 hydrochloride	-1.515307891
Paclitaxel	-1.461383771
17-AAG (Tanespimycin, Geldanamycin)	-1.447988014
PF-04691502	-1.447420904
PF 477736	-1.427575371
Ixabepilone	-1.416217108
AVN944	-1.401687178
ARQ 197_Tivantinib	-1.372993975
ABT-751	-1.289933308
PHA-739358 (Danusertib)	-1.271354695
Sorafenib	-1.15747372
MLN4924	-1.122941761
Epothilone B (Patupilone)	-1.110081003
NVP-AUY922	-1.075738919

17-DMAG (Alvespimycin)	-1.066751529
Cabazitaxel	-1.050801392
MK1775	-1.011125805
XL880 (Foretinib)	-1.00660615
BIBW2992 (Tovok)_Afatinib	-0.942784874
Dasatinib	-0.906966292
Rapamycin (sirolimus)	-0.900702464
MLN8237_Alisertib	-0.892490238
RAF265	-0.891877219
BI 2536	-0.871798548
CUDC-101	-0.846839248
CHIR-258 (Dovitinib)	-0.845328593
BI 6727_Volasertib	-0.832004419
PKC412_Midostaurin	-0.821666935
SB 743921	-0.820224266
AZD1480	-0.769222814
BIIB021	-0.730688048
Indibulin	-0.683529106
BKM-120_Buparlisib	-0.677980236
OSI-027	-0.635413405
NVP-BEZ235_Dactolisib	-0.635250804
GDC-0941_Pictilisib	-0.611515258
PF-2341066 (Crizotinib)	-0.595803968
BAY 73-4506_Regorafenib	-0.5865036
MGCD0103_Mocetinostat	-0.580806915
Etoposide	-0.575068602
Cisplatin / Pemetrexed	**-0.5**

Table 6.9 showing the z-scores for all drugs that were more than or as effective as cisplatin/pemetrexed combination in the drug screening assay using 10uM dilution of drugs with MPM cell culture MESO174

Small Compound name	Z-score MESO174 at 10uM dilution
JNJ 26854165 (Serdemetan)	-1.73786121
Crizotinib	-1.735382641
PF-2341066 (Crizotinib)	-1.733724273
SB 743921	-1.732081704
Ceritinib	-1.731231074
AP24534 (Ponatinib)	-1.730936566
XL880 (Foretinib)	-1.729398232
Romidepsin	-1.729306749
AZD 7762 hydrochloride	-1.726671061
JNJ-26481585_Quisinostat	-1.72410317
Quinacrine HCl	-1.724010099
Mitoxantrone	-1.722612937
Osimertinib	-1.71738278
Panobinostat	-1.710182164
4-hydroxytamoxifen	-1.707534551
CUDC-101	-1.693700072
PHA-739358 (Danusertib)	-1.684419596
Idarubicin HCl	-1.681665994
BAY 73-4506_Regorafenib	-1.681503841
UCN-01	-1.676381066
Belinostat (PXD101)	-1.670910472
Crenolanib	-1.669622885
Canertinib	-1.668408679
Auranofin	-1.650485742
Cobimetinib	-1.632259731
Amonafide	-1.630110926
Daunorubicin hydrochloride	-1.624817368

Sunitinib	-1.611179434
Mitomycin	-1.601033837
MGCD0103_Mocetinostat	-1.596834543
TAK-901	-1.586841085
Homoharringtonine	-1.585951368
Carfilzomib	-1.565039947
Stattic	-1.561813735
Omacetaxine mepesuccinate	-1.556041436
Teniposide	-1.530748158
BI 2536	-1.528675948
Epirubicin hydrochloride	-1.507797087
AZD1480	-1.481946983
Vinblastine sulfate	-1.478604679
17-DMAG (Alvespimycin)	-1.47646199
AT9283	-1.46736573
SR1 HCl	-1.44978961
ABT-263 (Navitoclax)	-1.448388285
Bortezomib	-1.428031907
Doxorubicin	-1.426321702
Valrubicin	-1.422051826
Lestaurtinib	-1.370959335
Obatoclax Mesylate (GX15-070)	-1.346015638
Dactinomycin	-1.344529437
Vinorelbine tartrate	-1.314034661
Tubacin	-1.264963488
Topotecan hydrochloride	-1.262469137
BMS-754807	-1.26154946
Camptothecin	-1.244687309
BKM-120_Buparlisib	-1.231468229
AZD4547	-1.224355201

Erismodegib	-1.211057201
Cladribine	-1.200045867
Tipifarnib (Zarnestra)	-1.19559963
Dinaciclib (SCH727965)	-1.188096721
PIK-75 HCl	-1.159124664
KX2-391	-1.146036792
Vorinostat	-1.137289467
PF 431396	-1.137177413
Plicamycin	-1.125657532
Mithramycin A	-1.123974446
PF-04691502	-1.091740129
AZD2014	-1.072524587
Sorafenib	-1.067156127
Ixazomib citrate	-1.054764141
AZD8055	-1.054723728
Clofarabine	-1.019046935
BIBW2992 (Tovok)_Afatinib	-1.018518482
GSK2126458	-1.006692535
INK128	-0.985956681
Dacomitinib (monohydrate) (PF-00299804)	-0.974452749
Gemcitabine hydrochloride	-0.973245656
Nutlin-3	-0.944073675
OSI-027	-0.924510229
Etoposide	-0.921986481
ABT-751	-0.897249354
R406_Tamatinib	-0.873500878
NVP-LDE225 (Diphosphate salt)	-0.863100141
LY 333531 mesylate_Ruboxistaurin	-0.83054246
AVN944	-0.820654529
MLN4924	-0.818998158

GDC-0980	-0.799485868
ARQ 197_Tivantinib	-0.788252727
NVP-BEZ235_Dactolisib	-0.779461251
AT 101	-0.73794257
XL184_Cabozantinib	-0.734126167
PKC412_Midostaurin	-0.71669354
Vandetanib	-0.690170075
BI 6727_Volasertib	-0.664410244
PF 477736	-0.645482475
Ixabepilone	-0.623054556
AC220_Quizartinib	-0.618345686
Lapatinib	-0.584396249
Triethylenemelamine	-0.577719668
Dasatinib	-0.57235671
Paclitaxel	-0.531003827
MK1775	-0.52119759
LY2784544_Gandotinib	-0.517177475
17-AAG (Tanespimycin, Geldanamycin)	-0.515227506
Docetaxel	-0.510983673
BIIB021	-0.509708414
CHIR-258 (Dovitinib)	-0.50443871
GSK 269962	-0.494537638
Temsirolimus	-0.479236878
PAC-1	-0.477195767
Irinotecan hydrochloride	-0.46062923
NVP-AUY922	-0.458121842
2-methoxyestradiol (Panzem)	-0.443555574
Lapatinib, di-p-toluenesulfonate salt	-0.382632392
Cytarabine hydrochloride	-0.377993999
Galiellalactone	-0.345874263

TAK-733	-0.343797551
NVP-BGJ398	-0.334775681
ABT-869_Linifanib	-0.311901231
Thioguanine	-0.27001058
Indibulin	-0.239840251
ZSTK474	-0.225605343
Irinotecan	-0.220336898
RAF265	-0.20552476
Fluorouracil	-0.186063125
PX-866_Sonolisib	-0.179706841
LY2603618_Rabusertib	-0.148829267
Motesanib Diphosphate (AMG-706)	-0.14853852
PCI-32765_Ibrutinib	-0.148456954
MK-4827, HCl salt	-0.143142443
AG-014699_Rucaparib	-0.140678719
I-BET151 (GSK1210151A)	-0.103242543
Cabazitaxel	-0.102859744
Bleomycin sulfate	-0.094136452
Ridaforolimus	-0.086357365
Bosutinib	-0.079530189
OSI-906_Linsitinib	-0.075925727
DCC-2036_Rebastinib	-0.051912436
RDEA119_Refametinib	-0.048414046
GDC-0941_Pictilisib	-0.031453108
Epothilone B (Patupilone)	-0.029100455
AV-951 (Tivozanib)	-0.019026249
VER 155008	-0.018982438
Axitinib	-0.012311431
Erlotinib hydrochloride	-0.010661027
S-trityl-L-cysteine, 40 mM	-0.005484219

GDC-0068	0.071710379
VX-11e	0.08671099
(5Z)-7-Oxozeaenol	0.100563404
Floxuridine	0.101068377
Pazopanib	0.117766719
Rapamycin (sirolimus)	0.122380952
CHR 2797_Tosedostat	0.128535032
AS703026_Pimasertib	0.13898124
PF-3845	0.159057427
PD-0332991	0.166777264
Everolimus	0.168215597
Melphalan hydrochloride	0.178230361
MLN8237_Alisertib	0.179413456
Palbociclib	0.198532688
Cisplatin / Pemetrexed	**0.2**