# Personalizing treatment for malignant pleural mesothelioma

Quispel-Janssen, J.M.M.F.

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Josine MMF Quispel-Janssen

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# Personalizing Treatment for Malignant Pleural Mesothelioma

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## Table of contents

CHAPTER 1

# Introduction and Outline of this Thesis

## Mesothelioma



Mesothelioma is a tumor arising from mesothelial cells lining the pleura, pericardium or peritoneum. It usually spreads locally and causes thickening of this lining, accumulation of fluid, or both, leading to symptoms of pain and dyspnea when situated in the pleural cavity, and obstipation and pain when the peritoneum is affected. If untreated, most patients die within 2 years from start of symptoms.

## Asbestos

Inhaled asbestos fibers are recognized as the main causative factor for developing mesothelioma. Asbestos is a term used to describe a group of 6 different mineral fibers that occur naturally throughout the world. Two subgroups can be distinguished based on their structure: the serpentine group and the amphiboles. Serpentine refers to a green, snakelike feature seen in this type of mineral. Chrysotile (white asbestos) is a serpentine mineral and the most commonly used type of asbestos. Amongst the amphibole minerals are amosite (brown asbestos), crocidolite (blue asbestos), tremolite, actinolite and anthophyllite [1]. The fireproofing properties of asbestos were already known in prehistoric times as can be concluded from archeological findings of clay pots containing asbestos fibers to make the pots fire resistant [2]. The ancient Greeks and Romans used asbestos in cloths for various purposes. Famous examples are the wicks used by the Vestal Virgins to maintain an eternal fire burning in the temple of the goddess Vesta. Mining and weaving of the fibers was done by slaves who were known to die early. Plinius Maior, a Roman historian and philosopher described the use of a thin membrane from a goats' bladder to prevent inhalation of asbestos fibers during mining [3]. Asbestos became increasingly popular at the time of the Industrial Revolution since its resistance to heat, electricity and chemicals and its plasticity made it an ideal material to insulate the steam engines and machines that were developed at that time. To meet the need for asbestos, commercial mines were established in Canada, Russia, Scotland, England, Germany and Italy. Mining in Australia, Finland, South Africa and Zimbabwe started a few decades later. In 1899 the Austrian born Ludwig Hatschek developed a technique to add asbestos to cement and called the patented product Eternit which acquired many applications in construction [4]. The harmful effects of asbestos were already suspected in the late nineteenth century as can be concluded from a 1902 report of Lady Anderson, an English Inspector of Factories who included asbestos on a list of dusts that were known to cause harm to man [5]. Scientific proof of its injurious effects became available with publications on asbestosis, a condition first described in 1924 by the British pathologist Cooke as '*Fibrosis of the lungs due to the inhalation of asbest dust*' [6-8]. In 1949 asbestosis was recognized as an occupational disease by the Dutch government [9]; a status the British government already decided to in 1931. This recognition was necessary for a

patient to be considered for a disability allowance. The notion that asbestos had carcinogenic properties and could induce lung cancer was first published in 1938 [10]. Epidemiologic argumentations for this idea were provided by Doll in 1955 [11]. A decade later Gross published his animal experiments in which he intratracheally administered asbestos to rats and found a high percentage of lung carcinomas, a malignancy very uncommon to rats [12]. From 1960 on, it became clear that asbestos could induce not only lung cancer but also mesothelioma, a very rare disease [13-15]. The Dutch doctor Stumphius dedicated his thesis to the health risks of asbestos and analysed the employees of a shipyard and a machine factory on the island of Walcheren that had evident asbestos exposure. He found asbestos bodies in sputum and biopsies of almost all employees and an unusually high prevalence of mesothelioma.

## Epidemiology

In his thesis in 1969 Stumphius warned that due to the widespread use of asbestos, mesothelioma could become a serious health threat, and asked for preventive measures [16]. It was only in 1993 that the Dutch Government banned all use of asbestos products. In 1969 90 cases of mesothelioma were registered in the Netherlands. Since then the incidence has increased more than six times. One would expect the numbers of new cases to drop since no new asbestos products are being used from 1993 onwards. But due to the extensive use in the seventies and the long latency period of 30-50 years, a peak in incidence is expected. This peak is predicted between 2015 and 2021 [17, 18]. However, since 2010, there seems to be a plateau in the Netherlands of around 550 new cases a year [19]. Globally, the mesothelioma incidence varies widely. Rates are highest in successively the United Kingdom, Australia and the Netherlands [20]. Many reasons exist for this global variation. The first reason is obviously the extent of asbestos used: countries with a high grade of industrialization consumed more asbestos. Many of these countries now have prohibited use of all types of asbestos. However, around 140 nations worldwide – mostly low-income countries- still have little or no regulation on asbestos [21]. Secondly, the reliability of the diagnosis may vary. Mesothelioma is notoriously difficult to diagnose. To improve the quality of the diagnostic process, several countries established national panels of expert pathologists that review all suspected cases of mesothelioma. The Dutch Mesothelioma Panel (Nederlands Mesotheliomen Panel (NMP)) started its work in 1969. Another factor that may explain the global variation in incidence, is the diversity in life expectancy throughout the world. The average age at mesothelioma diagnosis is 69. In Russia for example, men die at an average age of 64.7 years and may not live long enough to develop this disease [22]. Apart from asbestos, there exist many (around 390) other mineral fibers that do not fall under asbestos regulations but that are associated with mesothelioma [23]. Erionite for example, occurring in gravel that was used to pave roads in North Dakota in

the United States, is less widely used than asbestos but more potent in causing cancer [24]. Our current patients are likely to have been exposed to asbestos by working in construction, shipbuilding, or the automobile-industry (brake linings), but exposure may have occurred in as many as 70 branches of industry in the Netherlands [25]. These professions explain the male predominance of this disease.



## Treatment

In cancer therapy in general, surgery is the best treatment option to achieve curation. In mesothelioma however, radical resections are extremely difficult due to the widespread distribution of the cancer in the pleural cavity. It is disputable whether treatment for mesothelioma can be curative, but if so, it needs to include chemotherapy and possibly also radiotherapy. Extrapleural pneumonectomy (EPP) -complete resection of the involved lung and pleura- has a high morbidity and non-neglectable mortality and unfortunately, the disease often recurs. Many research papers that advocate surgery describe case series of highly selected patients with a long survival, but the impact of these articles is moderate due to selection bias [26]. A recent comparison between treatment schedules including surgery and schedules without surgery using propensity matching scores, demonstrated improved survival with surgery-including multimodality treatment [27]. However, the best method to assess the value of surgery is through randomization as was done in the Mesothelioma And Radical Surgery (MARS) trial [28, 29]. The conclusion of the authors that EPP offered no benefit and could even harm patients, induced a lot of criticism but did lead to development of new trials with lung-sparing surgical procedures such as extended pleurectomy/ decortication (EPD) [30]. The potential benefit of EPD in combination with chemotherapy is currently evaluated in the MARS2 trial and the EORTC1205 trial. What is evident from surgical trials is that most patients with mesothelioma are not eligible for surgery whatsoever due to poor performance status or disease extent. In the Netherlands, chemotherapy consisting of a platin and pemetrexed combination is considered the standard of care, based on a trial published in 2003 by Vogelzang et al [31]. Surgery-including multimodality treatment is only performed in the context of clinical trials. In many other European countries and the United States however, surgery of mesothelioma is more common.

## Personalized therapy

The general trend in oncology is to move from ‘one size fits all’ to personalized treatment. A personalized approach asks for biomarkers that allow selection of an appropriate drug for a certain patient. With the research described in this thesis, we aim to personalize mesothelioma therapy by combining clinical studies with translational research and

preclinical models. An overview of recently tested systemic treatments with a focus on predictive biomarkers is given in chapter 2 (*Emerging Therapies for Malignant Pleural Mesothelioma*).

## Preclinical models

Conducting clinical trials in a small and frail patient population such as the mesothelioma population is challenging. Difficulty in staging and response evaluation further complicate this. Staging in mesothelioma was mainly based on surgical assessment of disease extent. Since only a small proportion of all patients undergo a surgical procedure, reliability of staging is limited. To improve this, the International Association for the Study of Lung Cancer (IASLC) has constructed a database that resulted in the 8th edition of the TNM classification for MPM published in 2016 [32-35]. In spite of these improvements, staging -and with this stratification of patients in clinical trials- remains a huge challenge. Furthermore, radiologic assessment is notoriously difficult in MPM resulting in large interobserver variation in response evaluation. Assessment of tumor volume may improve this but has not found its way to clinical practice yet [36]. Adequate preclinical selection of compounds is therefore essential to optimally use the limited patient- and medical resources for clinical trials. It is key to develop preclinical models that most accurately resemble the original tumor. Chapter 3 gives an overview of existing preclinical models (*A Catalogue of Treatments and Technologies for Malignant Pleural Mesothelioma*). Mouse models are developed by elimination of INK4/ARF that lead to rapid development of mesothelioma tumors [37]. However, most mice develop sarcomatoid tumors while in humans, epithelioid histology predominates. Therefore, we aimed to develop a model that better represents the human tumor type and simultaneously reflects the genetic diversity of the population. Chapter 4 describes our newly developed culture model of primary tumor cells derived from pleural fluid of patients with mesothelioma, the drug sensitivity assays performed with this model and the correlation with expression profiles and clinical responses (*Chemical Profiling of Primary Mesothelioma Cultures defines Subtypes with Different Expression Profiles and Clinical Responses*).

## Pharmacogenomic profiling

In non-small cell lung cancer (NSCLC) the discovery of genetic aberrations such as EGFR mutations, has had major implications for treatment. At the start of this thesis, the genetic landscape of mesothelioma was largely unknown. Our aim was to explore this landscape in cooperation with the Welcome Trust Sanger Institute and search for genetic alterations that are potentially targetable. This was done by combining data from whole exome sequencing and drug sensitivity screens performed with a large panel of mesothelioma cell lines

including several primary tumor cell lines derived from our patients. Chapter 5 describes the results of this effort (*Comprehensive Pharmacogenomic Profiling of Malignant Pleural Mesothelioma Identifies a Subgroup sensitive to FGFR inhibition*).



## Immunotherapy

The durable properties that make asbestos attractable for industrial applications are the same properties that cause health damage. Asbestos fibers are inert and when inhaled they move to the pleura where they remain present during a lifetime. There they cause chronic inflammation which eventually can result in neoplastic transformation of mesothelial cells. The role of the immune system in the development of this disease suggests that it may also play a role in the treatment of mesothelioma. The positive effect on survival of a large lymphocytic infiltrate in a tumor of patients with mesothelioma was noted already in 1982 [38] and spontaneous regression of mesothelioma does occur suggesting a role for the immune system. It was noted that mesothelioma patients treated with BCG vaccine immunotherapy had a better survival compared to those who only received best supportive care [39]. The positive effect of dendritic cell therapy [40, 41], has substantiated this hypothesis. The clinical results of our NivoMes trial with PD-1 inhibitor nivolumab in patients with mesothelioma, progressive after at least one line of systemic therapy, is described in chapter 6 (*PD-1 blockade with nivolumab in patients with recurrent Malignant Pleural Mesothelioma*). Translational research to find biomarkers that predict for response is ongoing and falls out of the scope of this thesis.

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CHAPTER 2

# 2

# Emerging Therapies for Malignant Pleural Mesothelioma

Josine M.M.F. Quispel-Janssen | Paul Baas

*Current Respiratory Care Reports (2012) 1:91*

**Abstract**

Malignant pleural mesothelioma continues to challenge clinicians and scientists, since its incidence is rising and prognosis is far from favourable. Currently, the standard treatment consists of a combination of cisplatin and pemetrexed. The role of surgery and multimodality treatment remains controversial, while new treatment approaches, such as immunotherapy and targeted therapies, ad promising and interesting options. This review provides a comprehensive evaluation of emerging therapies and predictive biomarkers that are being tested.

## Introduction



Malignant pleural mesothelioma (MPM) is an aggressive tumour posing major treatment challenges. Its widespread distribution on the pleural surface (see figure 1) does not easily permit an adequate resection: a radical resection inevitably compromises a large amount of normal lung tissue. Furthermore, MPM is resistant to the vast majority of systemic anticancer drugs.

The development of novel therapeutic strategies is hampered by several factors. Assessment of disease extent is complicated as is illustrated by the various staging systems for MPM (1). Due to this variability in staging, patient cohorts in trials are not entirely comparable, leading to heterogeneous study outcomes. To address this problem, the International Association for the Study of Lung Cancer (IASLC) and the International Mesothelioma Interest Group (IMIG) initiated the Prospective Staging Project in Malignant Pleural Mesothelioma. Recommendations are expected by January 2014.

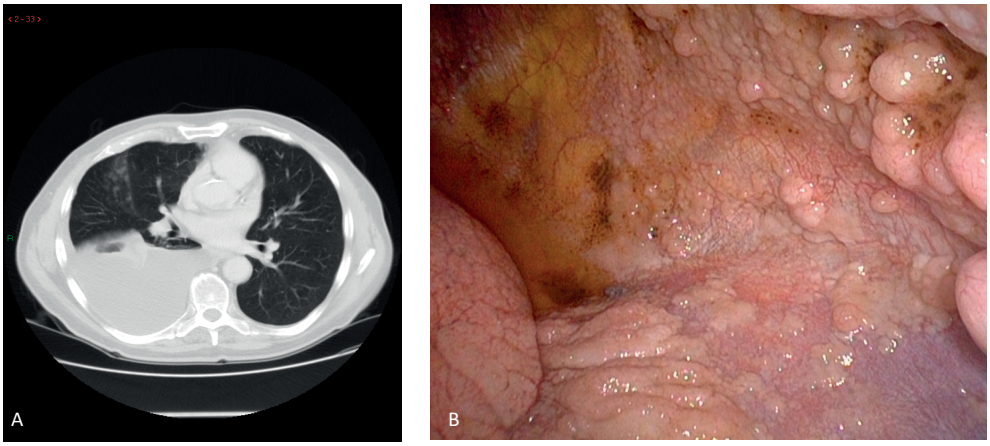


**Fig. 1.** (**A**) CT scan of a patient with mesothelioma showing right sided pleural fluid. (**B**) Thoracoscopic view of a patient with mesothelioma showing widespread distribution of tumor nodules on the pleural surface.

The modification of RECIST improved response evaluation, but still lacks sensitivity for adequate response assessment (2). Especially for thin tumor rinds, measurements are unreliable. Use of volumetric assessment is under investigation and seems promising for improving both staging and response evaluation (3-5).

Furthermore, MPM is a relatively rare and heterogeneous disease. The tumor comprises different histological subtypes: epitheloid, sarcomatoid and mixed (or biphasic), each of which are prognostically different. Recent pathologic studies have identified new prognostic factors like the pleiomorphic type, which is considered a subtype of epitheloid

mesothelioma, but has a prognosis similar to that of sarcomatoid MPM (6). Furthermore, stratification for nuclear grade, determined by nuclear atypia and mitotic count, enabled discrimination between 3 prognostic groups in a series of 323 MPM cases (7). Predictive biomarkers on the contrary, have not been identified. To date, no biomarker has proven to be sufficiently robust to apply in routine clinical practice. All of the above complicate validation of new therapeutic strategies in adequately powered randomized clinical trials.

In this review, we provide a comprehensive overview of current treatment options and the research that is ongoing in MPM with a focus on predictive biomarkers.

## Surgery

The role of surgery in MPM has been the subject of debate for many years. Cao et al. systematically reviewed all literature on extrapleural pneumonectomy (EPP) up to 2010 and concluded that EPP as part of multimodality treatment, may improve survival in a group of highly selected patients ( (8). However, only few trials have addressed this issue prospectively (9, 10) and retrospective trials typically suffer from selection bias. Two recent major publications assessed feasibility of multimodality treatment in early stage MPM. The MARS trial had patients undergo platinum-based induction chemotherapy and, if no signs of progression had occurred, randomized them to EPP followed by radiotherapy of the hemithorax, or to no EPP (11, 12). The primary endpoint, feasibility of randomizing 50 patients within one year, was not met. Patients were accrued in a three year time period. Only 45% of patients were eligible for randomisation and only 33% of the randomized patients were able to complete the full trimodality treatment. Median overall survival (OS) in the EPP group was 18 months (calculated from start of chemotherapy) versus 23.1 months in the no EPP group. Toxicity was higher in the EPP group and quality of life was lower. In the EORTC phase II multicentre trial on trimodality therapy, “success of treatment” was the primary endpoint. This was defined as undergoing the full protocol treatment within defined time-frames and still being alive 90 days after end of treatment, progression-free and without grade 3 or 4 toxicity (13). Only 42.1% of patients fulfilled these criteria. Median OS of the whole group was 18.4 months, but in those who completed trimodality therapy, it was as high as 33 months. Ninety-day mortality was 6.5%. Despite an encouraging 33 months’ median survival, neither study favours EPP in MPM patients.

Pleurectomy/decortication (P/D) on the contrary, may play a role in MPM treatment. Flores et al reported an improved survival in patients who underwent P/D, compared to those treated with EPP (14). However, this study was retrospective and selection bias is likely. In addition, the definition and surgical techniques of pleurectomy and decortication, vary

amongst different centers (15). Prospective trials with a uniform definition of lung sparing surgery for MPM are required to establish its role.



## Chemotherapy

Since 2003, chemotherapy consisting of cisplatin and the anti-folate pemetrexed is considered standard of care in MPM patients with an adequate performance status. Vogelzang et al. reported in their landmark study a response rate of 41% in patients treated with this combination (16). Compared to cisplatin monotherapy, the combination arm demonstrated a survival benefit of approximately 3 months, leading to a 12 months median survival time. To reduce the haematologic toxicity of pemetrexed, supplementation of vitamin B12 and folic acid has proven its value (17). Van Meerbeeck et al. reported similar progression-free survival (PFS) and OS results with raltitrexed, another anti-folate tested in a large randomized phase III EORTC trial combined with cisplatin (18). Response rate however, did not equal the cisplatin-pemetrexed combination (24% vs 41%). Registration of raltitrexed for this indication has therefore been limited to a few European countries.
Carboplatin may be a reasonable substitute for cisplatin in MPM treatment. Ceresoli et al. reported a time to progression (TTP) of 6.5 months and median OS of 12.7 months in chemotherapy naïve patients treated with carboplatin and pemetrexed (19).

Thymidylate synthase (TS), an enzyme involved in folate metabolism, was identified as a predictive biomarker for pemetrexed therapy. Righi et al. noted that low protein expression of TS predicted for better outcome in pemetrexed treated MPM patients (TTP 17.9 vs 7.9 months and OS 30 vs 16.7 months). In order to confirm these retrospective data, a prospective randomized trial should be conducted. However, this is not feasible since approximately 1700 patients would be required per study arm to power such a trial. High expression of the excision repair cross-complementation group 1 (ERCC1) protein in this group of patients, was a prognostic but not a predictive marker (20).

Anti-tumour activity of the gemcitabine-cisplatin combination was assessed in several phase II trials showing response rates between 12% and 48% (21-24) Although never tested in a randomized phase III trial, this regimen demonstrated survival outcomes similar to the pemetrexed-cisplatin combination in a retrospective study by Lee and coworkers (25).

## Second and further lines of treatment

Studies in second line treatment have yielded response rates between 10% and 20% with doxorubicin (26), pemetrexed alone (27, 28) pemetrexed in combination with carboplatin

(28), vinorelbine (29) or cisplatin in combination with irinotecan and mitomycin (30). A retrospective analysis of post-study treatment (PST) of patients included in the landmark study by Vogelzang, indicated that PST was associated with a better survival, regardless of the choice of chemotherapy (31). This may suggest a benefit of second or further lines of treatment in a subset of patients, although a clear survival benefit was not seen in any randomized trial (32). Retreatment with a pemetrexed-based regimen seems to be a valid option. A response rate of 19% has been noted in an observational study concerning patients that displayed an objective response or stable disease lasting for at least three months after first line pemetrexed-based chemotherapy (33). A similar response rate was observed in a second line phase II trial of patients receiving biweekly gemcitabine and docetaxel (34). With addition of granulocyte colony-stimulating factor (GCSF) to limit hematologic toxicity, this regimen proved to be well tolerated. Clinical activity of single agent taxanes however, is lacking (35). Surprisingly, gemcitabine combined with cisplatin did not elicit any objective responses in second line setting in another phase II study. Disease control rate was 67%, but toxicity was substantial with 35% of patients having grade 3 neutropenia and 47% having grade 3 or 4 trombocytopenia (36).

## Maintenance therapy

Only few studies have addressed the subject of maintenance therapy in MPM. A small single arm phase II study by Van den Bogaert et al. reported pemetrexed maintenance therapy to be feasible and capable of evoking an ongoing response after induction chemotherapy (37). The Cancer And Leukemia Group B (CALGB) currently runs a randomized phase II study, comparing maintenance pemetrexed to placebo in non-progressive patients after first-line chemotherapy, consisting of pemetrexed and cisplatin/carboplatin. Progression-free survival was defined as the primary endpoint (data collection to be completed by January 2012) (38). The histone deacetylase (HDAC) inhibitor vorinostat was investigated in maintenance setting and is discussed further on in this manuscript.

## Targeted therapies

In recent years, research has focused on exploring the molecular pathways involved in growth and progression of MPM. Several drugs that target these pathways, are being tested to define their role in MPM treatment (Table 1).

### Histone deacetylase inhibitors

Epigenetic modifications such as hypermethylation and histone regulation, play an important role in tumorigenesis. Histones are packaging proteins, clustering DNA to form chromatin. Gene transcription can only occur after decondensation of chromatin. Histone

**Table 1. Summary of drugs tested in MPM. n.a. = not assessed**

| Compound | Target | Phase | Line of treatment | Single agent/ combination therapy | Patients (no.) | RR (%) | PFS (months) | OS (months) | Biomarker tested | Predictive/ prognostic | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Belinostat | HDAC I&II | II | 2 | Single agent | 13 | 0 | 1 | 5 | None | | 38 |
| Vorinostat | HDAC | III | 2 | Single agent | 660 | 0.3 | 1.6 | 7.7 | Ongoing | | 39 |
| Valproate | HDAC | II | 2 | doxorubicin | 46 | 16 | 2.5 | 6.7 | None | | 40 |
| Bevacizumab | VEGF | II | 1 | cisplatin gemcitabine | 108 | 25 | 6.9 | 15.6 | VEGF | prognostic | 43 |
| Bevacizumab | VEGF | II | 2 | erlotinib | 24 | 0 | 2.2 | 5.8 | None | | 44 |
| Sorafenib | RAS/RAF/MEK VEGFR, C-kit | II | 1 or 2 | Single agent | 50 | 6 | 3.6 | 9.7 | p-ERK1/2 | prognostic | 46 |
| Sunitinib | VEGFR PDGFR | II | 2 | Single agent | 51 | 10 | 3.4 | 6.7 | None | | 47 |
| Cediranib | VEGFR PDGFR | II | 1 or 2 | Single agent | 50 | 10 | 1.9 | 4.4 | None | | 49 |
| Thalidomide | VEGF FGF | III | maintenance | Single agent | 220 | n.a. | 4 | 11 | None | | 51 |
| Bortezomib | proteasome | II | 1 or 2 | Single agent | 23 | 5 | 2 | 5.8 | NOXA | neither | 62 |
| Dasatinib | Src kinase PDGFR C-kit BCR-ABL | II | 2 | Single agent | 46 | 0 | 2.1 | 5.2 | ongoing | | 60 |
| CBP501 | G2 checkpoint | I | 1 | Single agent | 8 | 38 | n.a. | n.a. | ongoing | | 61 |
| ADI-PEG 20 | Arginine synthesis | II | 1 or 2 | Single agent | 66 | ongoing | ongoing | ongoing | ASS | predictive | 64 |
| Cetuximab | EGFR | II | 1 | Cis/carboplatin pemetrexed | 18 | ongoing | ongoing | ongoing | none | | 68 |
| MORAb-009 | mesothelin | I | 2 | Single agent | 13 | 0 | n.a. | n.a. | | | 78 |
| NGR-hTNF | h-TNF antivascular | II | 2 | Single agent | 57 | 1.8 | 2.8 | 12.1 | none | | 83 |

deacetylase (HDAC) inhibitors are a class of antitumor agents that modulate chromatin structure, thereby regulating gene transcription leading to apoptosis, inhibition of angiogenesis and cell cycle arrest. Preclinical data have suggested a promising role for these agents in MPM (39, 40). However, in a phase II study with HDAC inhibitor belinostat, no anti-tumour activity was noted (41). Recently, the results of a large randomized phase III trial comparing HDAC inhibitor vorinostat to placebo in pretreated patients, was presented at the ESMO conference in Stockholm. Despite encouraging response rates in an earlier phase I study (42), the randomized trial demonstrated only a minor improvement in PFS and no survival benefit at all (HR 0,98). (LBA L Krug oral presentation ESMO ECCO 2011) Valproic acid, another HDAC inhibitor, was tested in combination with doxorubicin in recurrent MPM (43). The response rate of 16% was higher than that of doxorubicin monotherapy (26). These data do not support the use of the currently tested HDAC inhibitors in routine clinical practice. The role of HDAC inhibitors in combination with chemotherapy needs further evaluation.

**Anti-angiogenic agents**

Angiogenesis, the process of new blood vessel formation, is essential for growth of solid tumours. Increase in angiogenesis, reflected by an increase in microvessel density (MVD) is a negative prognostic factor in MPM patients (44). Several regulators of angiogenesis, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor-2 (FGF-2) and transforming growth factor beta (TGF-β), may serve as targets for treatment. VEGF is the most potent regulator of growth, and expression in MPM tissue is high compared to that in benign mesothelial cells (45).

Bevacizumab, a monoclonal antibody that neutralizes VEGF, is being investigated in combination with several chemotherapeutic regimens. Previous phase II trials did not report clinical activity of bevacizumab when added to standard chemotherapy (46) or EGFR-TKI (47). Zalcman et al. described an increase in disease control rate in patients treated with bevacizumab and cisplatin and pemetrexed (73.5% vs 43.2% in placebo) in a phase II study in previously untreated patients (48). The final results of this and other trials have to be awaited to determine if bevacizumab has a role in the treatment of MPM.

Another method to block the VEGF pathway is to inhibit the tyrosine kinase activity of the VEGF receptor. Sorafenib targets the tyrosine kinase domain of both the VEGF- and PDGF-receptor and inhibits the RAS/RAF/MEK/ERK pathway. A phase II study of sorafenib as single agent in 50 chemotherapy naïve or pretreated MPM patients, showed a limited response rate of 6%. Median PFS and OS were 3.6 and 9.7 months, respectively. Low or negative phosphorylation status of ERK1/2 in tumor tissue was correlated with improved survival (49).

Sunitinib, another VEGF- and PDGF-receptor tyrosine kinase inhibitor (TKI), was tested in 53 previously treated MPM patients. Response rate was assessed by modified RECIST criteria

on CT-scan and by metabolic response on FDG-PET. The total response rate was 22%, with 10% of the responses confirmed by modified RECIST on CT (50). Metabolic response on FDG-PET may be a more accurate way than modified RECIST to assess response, but its clinical relevance remains to be proven. In this study however, the median TTP (3.4 months) and median OS (6.7 months) do not support the claim of modest activity. Furthermore, toxicity required dose reductions in 28% of patients. Another phase II study confirms the lack of clinical activity of sunitinib as single agent (51).



Campbell and coworkers presented their results of a phase II study involving Cediranib at the latest ASCO annual meeting. This tyrosine kinase inhibitor of VEGFR and PDGFR was poorly tolerated requiring dose reductions in 48% and discontinuation for toxicity in 26% of patients. The trial failed to meet its prespecified response endpoint with a response rate of 10% (52).

Thalidomide is an immunomodulating drug that also acts on promoter regions of growth factor genes such as VEGF and FGF-2 by intercalating into guanine (G) and cytosine (C) rich regions of DNA. Subsequently, VEGF and FGF expression levels decrease, thereby diminishing angiogenesis and tumor growth. After promising results from a phase I study in 40 MPM patients (53), a multicenter, randomized phase III study comparing thalidomide maintenance therapy to observation, was launched. In this large trial, 222 patients without disease progression after induction chemotherapy, were included. Despite only mild toxicity, there was no benefit of thalidomide in PFS or OS (54).

So far, clinical activity of anti-angiogenic drugs, is disappointing. Two major mechanisms of resistance to these drugs have been suggested by Bergers and Hanahan. Firstly, intrinsic resistance is determined by specific tumor microenvironment and secondly, evasive resistance is due to upregulation of alternative pro-angiogenic pathways (55). A strategy to combine anti-angiogenic drugs with targeted agents might be a way to move forward. For this we need predictive biomarkers for response or resistance. Furthermore, it is essential to get a better understanding of the processes that evolve during treatment. Therefore, we developed a study protocol with interim biopsy analysis for a randomized phase II trial combining cisplatin and pemetrexed with axitinib, a VEGFR and PDGFR TKI, or placebo (56). So far, patient accrual is satisfactory and performing a second thoracoscopy for interim biopsy analysis is feasible. Results of this study are awaited in 2012.

**PI3K/AKT/mTOR pathway**

The PI3K/AKT/mTOR pathway is involved in a number of cellular processes that regulate proliferation, survival and motility (57). In MPM this pathway is frequently dysregulated which makes it an interesting target for therapy (58). Several PI3K inhibitors are currently being developed and a randomized phase III study in recurrent MPM patients is in preparation.

The downstream effector of this pathway, mTOR can be inhibited by agents like sirolimus, temsirolimus and everolimus, currently used as immune suppressors in transplantation medicine. Everolimus is being tested in a phase II trial in MPM patients with disease recurrence. Loss of Merlin/NF2 will be evaluated as a biomarker to predict anti-tumour activity (59). The South West Oncology Group (SWOG) is also evaluating everolimus in recurrent MPM (60).

**Other targeted agents**

Bortezomib is a selective proteasome inhibitor that decreases nuclear factor-κB and upregulates proapoptotic BH3 proteins. A single agent phase II trial has evaluated efficacy of this drug in first and second line setting. As clinical activity is lacking, further investigation as monotherapy is not warranted (Fennell et al., submitted). The association of NOXA expression to response was assessed in this trial, showing that NOXA cannot be used as a predictive biomarker. Two trials regarding bortezomib are ongoing: one combining it to cisplatin (61) and the other to oxaliplatin (62).

Dasatinib a receptor TKI of Src family kinases, PDGFR, C-kit and BCR-ABL fusion protein, did not show activity in MPM and was poorly tolerated (63). Data on pre- and post-treatment plasma levels of several biomarkers will be available in due time.

Tumour cells that acquire DNA damage usually arrest cell cycles to repair damaged DNA. Most solid tumors have genetic alterations that disturb cell cycle checkpoint G1 which makes them dependent on checkpoint G2 for survival. CBP501 is a compound that abrogates the G2 checkpoint, resulting in tumor cell death. This compound has demonstrated promising activity in combination with cisplatin in patients with MPM and patients with ovarian cancer in a phase I trial. Three out of 8 MPM patients showed a response. In two of them, time to progression was more than 9 months. Dose limiting toxicity (DLT) consisted of a histamine-release syndrome (64). A phase II study with CBP501 in combination with cisplatin and pemetrexed is currently recruiting patients with MPM.

Arginine is an amino acid involved in tumor metabolism and essential for tumor growth. Arginine synthesis is regulated by the enzyme argininosuccinate synthetase (ASS) and is downregulated in a number of tumor types such as MPM, hepatocellular carcinoma, and melanoma. Loss of ASS results in dependence on extracellular arginine. In a study by Szlosarek et al, 63% of mesothelioma patients had reduced or absent levels of ASS (65). Pegylated arginine deiminase (ADI-PEG 20) is an arginine-depleting drug that has demonstrated interesting results in a phase I/II study in hepatocellular carcinoma and melanoma (66). A multicenter randomized phase II of single agent ADI-PEG 20TM was recently launched in MPM patients with ASS negative tumors (67). ASS expression may serve as a biomarker predictive for treatment response of ADI-PEG 20.

The epidermal growth factor receptor (EGFR) is overexpressed in more than 50% of MPM patients. Activating mutations in the EGF receptor however, are not prevalent in MPM (68). This is reflected by the lack of activity of EGFR-tyrosine kinase inhibitors gefitinib and erlotinib in patients with MPM (69, 70). Cetuximab is a monoclonal antibody binding to the EGF-receptor that has shown a survival benefit in non-small cell lung cancer (NSCLC) patients with high EGFR expression (71). A study exploring the role of cetuximab in combination with pemetrexed and cisplatin or carboplatin, is ongoing (72).



### Immunotherapy

Immunotherapy may be an attractive treatment approach for MPM for several reasons. The large lymphocyte infiltrate present in many cases of mesothelioma, and the spontaneous regression, occasionally occurring in MPM, suggest a role for the immune system in controlling tumor growth. Furthermore, several tumour-stroma generated cytokines (eg., TGF-β) suppress the local immune system, as do the abundant regulatory T cells in MPM (73). In the past, various passive immunotherapeutic approaches with cytokines such as IL-2, IL-12, INF-β and INF-Ɣ, were tested in murine models (74, 75) and some even in phase I-II clinical trials but with limited success (76-78). Hegmans et al. previously demonstrated efficacy of active immunotherapy in a murine MPM model using tumor lysate-pulsed dendritic cell vaccination (79). Recently, the results of a phase I trial testing this dendritic cell-based (DC) immunotherapy, were published. Ten patients received three vaccinations after completing standard chemotherapy. DC immunotherapy is feasible, well-tolerated and capable of inducing an immunological response to mesothelioma cells (80). It seems most effective in patients with modest tumour load. Applying DC immunotherapy after surgical debulking, is an interesting approach for future studies. A trial combining DC immunotherapy with cyclophosphamide, inhibiting T-regulatory lymphocytes and thereby enhancing immunological responses, is currently recruiting patients (81).

Mesothelin is a glycoprotein normally expressed on the surface of mesothelial cells lining the pleural and peritoneal cavity. Expression is upregulated in many solid tumors including MPM. Mesothelin can bind to CA-125, a cell surface mucin expressed on several types of tumor cells, thereby mediating tumor metastasis within pleural and peritoneal cavities (82). At least two different antibodies that target mesothelin, were developed and tested in phase I trials. MORAb-009 is a chimeric monoclonal antibody to mesothelin that was well tolerated and induced disease stabilization in patients with mesothelin-expressing tumors (83). An open-label clinical trial of MORAb-009 in combination with pemetrexed-cisplatin in patients with MPM, has completed accrual and results are awaited (84). SS1P (CAT-5001) is a recombinant immunotoxin linking an exotoxin of *Pseudomonas Aeruginosa* to mesothelin. Tolerability was demonstrated previously in a phase I study (85). Currently it is being tested

in combination with cisplatin and pemetrexed in MPM patients (86). Another phase I study is combining SS1P with an immune-depleting regimen consisting of pentostatin and cyclophosphamide (87).

Tumor necrosis factor α (TNF-α) is a potent anti-tumour agent. Systemic use however, is limited by severe toxicity (88). Asparagine-Glycine-Arginine–Human Tumor Necrosis Factor-α (NGR-hTNF) is a fusion protein of human TNF-α and Asparagine-Glycine-Arginine, a peptide that targets aminopeptidase N/CD13. This aminopeptidase N/CD13 is overexpressed by endothelial cells of the majority of solid tumors (89). NGR-hTNF was tested as single agent in triweekly and weekly dosing in MPM patients with disease recurrence. NGR-hTNF was well tolerated with short-lived chills being the most common side effects. Progression-free survival was 2.8 months and OS 12.1 months (90). A randomized double-blind phase II maintenance study of NGR-hTNF versus placebo, is currently recruiting patients with advanced MPM (91). A phase III study is also initiated comparing NGR-hTNF plus chemotherapy (best investigators choice (BIC)) to placebo in combination with chemotherapy BIC in patients previously treated with pemetrexed (92).

## Gene therapy

The purpose of gene therapy is to kill tumor cells by means of genetic modification. In general this implies that a therapeutic gene is inserted into tumor cells using a vector system. Several viruses such as adenovirus or vacciniavirus may serve as such. In MPM the vector can be administered locally via the pleural cavity. The inserted gene can either be a suicide or sensitivity gene (e.g. Herpes Simplex Virus thymidine kinase), an immune modulator (e.g. IL-6 or IFN-β) or a replacement for a tumor suppressor gene. Sterman et alI. recently published their results of intrapleural administration of an adenoviral vector expressing interferon β (93). Ten patients were treated with an intrapleural injection which was repeated after one week. Gene transfer was confirmed in the pleural fluid. One patient had a partial response and two patients disease stabilization. However, neutralizing antibodies were rapidly developed after the first dose, preventing effective gene transfer. An early second injection after three days is currently being tested.

## Conclusion and future perspectives

Despite ceaseless efforts to improve outcome in patients with MPM, the prognosis remains grim. The standard of care consisting of cisplatin-pemetrexed chemotherapy has not changed since 2003. Surgery should not be advocated outside clinical trials and targeted therapies have not entered clinical practice yet, due to lack of activity. In order to improve

prognosis, several measures are necessary. Firstly, we have to reconsider our current classification based on epitheloid vs non-epitheloid histology. Secondly, an improved system for staging and response assessment is required. In addition, we need better criteria to select patients that may benefit from surgery. The same applies to patient selection for targeted therapies as biomarkers predicting for treatment response are urgently needed. Furthermore, preclinical data suggest that in approximately half of MPM cases, more than one pathway is activated (94). Therefore, combining targeted agents is a treatment strategy worth exploring. Finally, to get a better understanding of the pathways involved in tumorigenesis, we advocate combining clinical trials with translational research.

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CHAPTER 3

# 3

# A Catalogue of Treatments and Technologies for Malignant Pleural Mesothelioma

Laurel M. Schunselaar | Josine M.M.F. Quispel-Janssen |
Jacques J. C. Neefjes | Paul Baas

*Expert Review of Anticancer Therapy 2016*

**Abstract**

Malignant pleural mesothelioma is an aggressive fatal malignancy with a prognosis that has not significantly improved in the last decades. This review summarizes the current state of treatment and the various attempts that are made to improve overall survival for patients with malignant pleural mesothelioma. It also discusses technologies and protocols to test new and hopefully more effective compounds in a more individualized manner. These developments are expected to improve the prognosis for this group of patients.

## Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumor that arises by neoplastic transformation of the mesothelial cells lining the pleural cavity [1–4]. In the United States, the incidence is approximately 1.05 cases per 100,000 persons [5]. In Europe, the incidence in males is higher, around 3 cases per 100,000 persons [6]. The occurrence of MPM is associated with asbestos exposure. There is a latency period of around 30-50 years between asbestos exposure and development of MPM. Even though all handling of asbestos is strictly regulated in Europe since 2005, the incidence is not expected to decrease before 2020[4–9]. In addition, outside Europe, some other developed countries have only controlled the import or still produce asbestos and less-developed countries still use or even expand the use of asbestos [5–7]. This results in an estimated 125 million asbestos-exposed people and 43,000 annual deaths due to asbestos-related diseases worldwide [4,9].



The prognosis for patients with MPM is poor. If untreated, most patients die in the first year after diagnosis [4,8]. First-line chemotherapy treatment consists of a platinum-based combination with pemetrexed [3,6,10]. This combination provides a 3-month survival benefit over cisplatin alone and a 6-month survival benefit over nontreated patients [11,12]. Around 40% of the patients with MPM respond to the combination [8,11,13,14]. For patients that do not respond to first-line chemotherapy or become progressive after treatment, there is no standard second-line regimen [6,14]. European Society for Medical Oncology Clinical Guidelines recommend enrolling eligible patients in clinical trials [6,7].

### First-line treatment in mesothelioma

Almost every chemotherapy regimen has been tested in mesothelioma [15–17]. The most effective anticancer drugs are cisplatin, antimetabolites (methotrexate and pemetrexed), and anthracyclines (doxorubicin and daunorubicin). Anticancer drugs with no or minor activity in MPM are the taxanes, topoisomerase inhibitors, alkylating agents, and the vinca-alkaloids with the exception of vinorelbine. The most studied anthracycline is doxorubicin. This drug showed some activity in a number of clinical trials with varying response rates [15–17].

Until 2000, nearly all studies tested single agents. In 2002, a meta-analysis suggested that combination therapy gave better response rates than single agent therapy [18]. The first clinical trial that compared single agent therapy to a combination was performed by Vogelzang et al. [11]. This resulted in the standard first-line treatment combination of cisplatin and pemetrexed. This combination therapy combines two drugs with different activities. Cisplatin is a platinum ion with two chloride atoms and two amine groups. One chloride is first removed for a hydroxyl group yielding $PtCl(H_2O)(NH_3)_2^+$. This form binds strongly to the G basis in deoxyribonucleic acid (DNA). Here, the second chloride atom can be removed yielding a cross-linking molecule between two G bases on different DNA strands. While the

majority of interactions are between two G–G bases, other interaction, such as G-A, can also be detected. DNA strand crosslinking obviously induces substantial problems with DNA strand separation during mitosis and is supposed to be the major mechanism of cell death [19]. Pemetrexed is an antifolate that inhibits the biosynthesis of purine and pyrimide nucleotides by inhibiting the enzymes dihydrofolate reductase, thymidylate synthase (TS), and glycinamide ribonucleotide formyltransferase (RNF). Pemetrexed enters the cell by the reduced folate carrier. Folylpolyglutamate synthetase polyglutamates pemetrexed to a form that has a 100-fold greater affinity for the enzymes TS and RNF. As a result, cell growth is attenuated due to a reduced amount of DNA bases available for DNA replication. Both drugs have serious side effects cisplatin can cause nephrotoxicity that is controlled by expanding the kidney fluid volume before treatment. Antifolates induce elevated levels of h omocysteine. Homocysteine accumulation causes severe toxicities such as neutropenia, thrombocytopenia, and diarrhea. With supplementation of vitamin B12 and folic acid, homocysteine can be recycled into methionine or converted into cysteine [11,20,21].

**The search for new treatment options for MPM**

A phase III trial by Vogelzang et al. showed patients receiving cisplatin with pemetrexed had an overall survival (OS) of 12.1 versus 9.3 months for patients receiving cisplatin. Also time to progression (TTP) was higher in the cisplatin with pemetrexed group (5.7 months) compared to the cisplatin group (3.9 months). Approximately 40% of the patients had a partial response (PR). A retrospective analysis of the follow-up data showed that patients receiving two or more lines of treatment had a significant longer survival. Sixty-two percent of the patients received single-agent therapy and 38% combination therapy. For patients with two or more lines of chemotherapy, the median survival time (MST) from start of first-line treatment was 15.3 months for those receiving first-line pemetrexed and cisplatin versus 12.2 months for patients that previously received first-line cisplatin. For patients that did not receive second-line chemotherapy, MST was 9.8 months in the cisplatin/pemetrexed group and 6.8 months in the cisplatin group. This analysis suggests that a selected group of eligible patients could benefit from a second-line treatment, but the most effective second-line treatment for this patient population has not yet been identified [22]. Since then, various other second-line phase II trials have been conducted as will be discussed below.

**Inhibitors of growth factors**

Growth factors and their receptors play an important role in the development of mesothelioma. The epidermal growth factor receptor (EGFR) plays a role in cell proliferation, differentiation, migration, adhesion, and survival. EGFR is highly overexpressed in mesothelioma. However, EGFR tyrosine kinase inhibitors erlotinib and gefitinib as well as the EGFR antibody cetuximab did not show any response. EGFR is not a tumor driver as suggested from the absence of sensitizing mutations in the EGFR tyrosine kinase domain, which may explain the lack of response to EGFR inhibitors [4,20,23].

Another transmembrane tyrosine kinase is activated by the platelet-derived growth factor (PDGF) and plays a role in cell proliferation. Imatinib and dasatinib are anticancer drugs that inhibit the kinase activity of the PDGF receptor, but phase II studies with these drugs in patients with MPM were disappointing [4,8,20,23].

**Inhibitors of angiogenesis**



A third growth factor activating kinase receptor is the vascular endothelial growth factor (VEGF) which plays a role in angiogenesis. VEGF expression levels are high in a large portion of MPM tumors and they may activate the VEGF receptor to induce angiogenesis in tumors. Therefore, different VEGF-receptor inhibitors were consequently tested in phase II studies. These include small kinase inhibitors sorafenib, sunitinib, vatalanib, and cediranib, which did not improve response rates or OS for patients with MPM [4,8,10,20,23]. Thalidomide was the most promising agent; however, no benefit in TTP or OS was observed in a large randomized phase III study [24]. Bevacizumab, an antibody binding VEGF, has recently been tested in a phase III trial in combination with cisplatin and pemetrexed. In patients who were able to receive bevacizumab, the OS was significantly extended in the pemetrexed/cisplatin/bevacizumab (PCB) group (18.8 months) versus the pemetrexed/cisplatin (PC) group (16.1 months). Second-line treatment with pemetrexed or with a platinum containing treatment was allowed in this study protocol and may have affected the OS. An improvement in progression-free survival (PFS) for the PCB group (9.2 months) versus the PC group (7.3 months) was also observed. Even though more patients stopped treatment in the PCB group due to toxicity, the quality of life in this group was considerably better than in the control group. However, absence of masking could have influenced the quality-of-life results, so these results should be interpreted with caution [25].

**Other targeted agents**

Other targeted agents investigated as second-line treatment are bortezomib, vorinostat, everolimus, defactinib, asparagine-glycine-arginine human tumor necrosis factor alpha (NGR-hTNFα), and amatuximab.

Bortezomib, an inhibitor of the 20S proteasome, was tested in two phase II studies. As a single agent in second-line treatment, it was not active. Also, in combination with cisplatin, bortezomib failed to meet the primary objectives [26,27].

Vorinostat is a histone deacetylase (HDAC) inhibitor. HDACs are regulatory enzymes that manipulate histone modifications resulting in changes in the cell epigenetics. Inhibiting HDACs results in expression of genes associated with cell cycle arrest, apoptosis, and tumor suppression [20,23]. Preclinical and phase I data showed promising results, which could not be confirmed in a randomized double-blind phase III study with single agent vorinostat [28].

A percentage of 35-40 of the patients with MPM have mutations in the neurofibromatosis type 2 (NF2) gene that encodes the protein merlin. Merlin downregulates the activity of the kinase mammalian target of rapamycin (mTOR) and blocks focal adhesion kinase (FAK) activation. Mutations in NF2 then results in activated mTOR and FAK [4,10]. Everolimus is an inhibitor of mTOR that was tested in patients with MPM, yet the phase II study did not meet its primary endpoint [29]. Another compound targeting the NF2-pathway is defactinib, a FAK-inhibitor. While preclinical data again were promising, the placebo-controlled phase II study was early terminated due to reasons of futility [30]. Possibly the inhibition of the NF2/mTOR/ FAK pathway was not sufficient to control MPM. Tumor necrosis factor alpha (TNF-α) is a secreted protein that induces apoptosis in endothelial-tumor cells via caspase activation. To target the protein to the tumor tissue and at the same time limit general side effects of TNF-α, TNF-α was fused to the tumor homing peptide sequence NGR [8,10,23]. A single agent phase II trial in 57 patients with MPM showed promising results [31]. In the following randomized phase III trial, patients who progressed on first-line treatment received weekly NGR hTNFα or placebo in combination with gemcitabine, vinorelbine, doxorubicin, or best supportive care. In the intention to treat analysis the OS was not significant different between the NGR-hTNFα group and placebo group [32]. Currently, a maintenance phase II trial with NGR-hTNFα is ongoing, the primary objective is TTP (NCT01358084) (Table 1).

Amatuximab (MORab-009) is a chimeric monoclonal antibody that binds with high affinity to mesothelin [8,10,20,33]. Mesothelin is a tumor-differentiating antigen, present at mesothelial cells lining the pleura, peritoneum, and pericardium. Its biological function is unknown [4,20,33]. Mesothelin is highly expressed in epithelial MPM, but not in sarcomatoid MPM. The limited expression in normal mesothelial cells and high expression in tumor cells makes i an attractive target [23,33–35]. Preclinical studies showed that amatuximab has activity against mesothelin expressing tumor cells [20,36]. In a single-arm phase II study, cisplatin and pemetrexed were combined with amatuximab for six cycles, which was followed by amatuximab-maintenance therapy in case of response or stable disease (SD). The primary endpoint, 3-month improvement in PFS compared to historical controls, was not met. However, with a PR in 39% of the patients and SD in 51% of the patients, the study concluded that amatuximab has activity in MPM [33]. Finding biomarkers to select patients for whom this drug would be effective is important. A randomized placebo-controlled study to investigate survival benefit is planned.

**Oncolytic viral therapy**

A different approach in cancer therapy employs oncolytic viruses that are emerged to selectively eliminate cells with particular driver mutations. Different viruses including adenovirus, measles virus, vesicular stomatitis virus, replication competent retrovirus, and the genetic engineered Newcastle disease virus have been tested in preclinical studies with good results [37–44]. To date, one phase I/IIa study is testing the safety, tolerability, and biological effect of the selectively replication-competent herpes simplex virus HSV1716 (NCT01721018) (Table 1).

**Immunotherapy in MPM**

There are reported cases of spontaneous regression of MPM, which were associated with lymphocyte infiltration in the tumor. Lymphocyte infiltration in MPM is also associated with improved survival [45–47]. These data suggest that MPM could be an immunogenic tumor, which makes immunotherapy an interesting therapeutic option [45,48,49].



There have been several different immunotherapy approaches tested. One of those is an antibody-drug conjugate. SS1P is a recombinant pseudomonas toxin coupled to the variable fragment of an anti-mesothelin antibody [35,50]. In phase I clinical trials, the vast majority of patients developed antibody responses to SS1P after one cycle of treatment, preventing further treatment unless this response is eliminated. Pentostatin and cyclophosphamide are drugs that deplete lymphocytes, preventing the formation of antitoxin antibodies. A phase II trial showed that pretreatment with these agents allowed patients to receive more cycles of treatment with SS1P, resulting in improved clinical responses [50].

While we discussed reagents directly targeting MPM, specific activation of immune responses in patients would be an alternative way of immunotherapy. A new wave of antibodies controlling checkpoints in immune cell control has shown strong responses in other tumors including non-small-cell lung cancer and melanoma [51–57]. These antibodies block the activities of programmed cell death protein 1 (PD-1), programmed death ligand 1 (PD-L1), and cytotoxic T-lymphocyte antigen 4 (CTLA-4).

PD-L1 is expressed in many tumor cells, including MPM [48,49,58–61]. Binding of PD-L1 to its receptor PD-1 on T cells inhibits proliferation and activation of T-cells and quenches immune responses against the tumor. As a result, tumors that express PD-L1 evade cytotoxic T-cell control. Consequently, blocking PD-1 with antibodies allows activation of cytotoxic T-cells. Mansfield et al. showed positive PD-L1 expression in 40% of MPM tissues by immunohistochemistry (IHC) staining. Cedres et al. reported that 20.8% of the cases are positive for PD-L1 expression. Both articles report a higher incidence of PD-L1 expression in sarcomatoid MPM than in epitheloid MPM and describe that PD-L1 expression is associated with a poor prognosis [48,49].

In a phase I study, pembrolizumab, a PD-1 receptor antibody, was not only safe and tolerable for patients, also a disease control rate (DCR) of 76% was observed. Twenty-five patients with MPM received pembrolizumab after first-line treatment. Seven patients had a PR and 12 experienced SD [62]. Recently, a phase II study with second-line pembrolizumab treatment in MPM has opened for patient accrual (NCT 02399371). The first primary objective is determining the overall response rate in an unselected patient population and in a patient population with PD-L1 positive MPM. The second primary objective is to determine the threshold for PD-L1 expression using 22C3 antibody-based IHC in correlation to tumor response (Table 1).

**Table 1: Ongoing phase II and III trials in mesothelioma.**

| | Drug | Clinical trial number | Primary outcome | Description |
|---|---|---|---|---|
| Growth factor inhibitor | IMC-A12 | NCT01160458 | CRR | Evaluate the safety and effectiveness of IMC-A12, an antibody blocking type I insulin like growth factor in patients that previously received chemotherapy |
| | cetuximab | NCT00996567 | PFS | Multicenter open phase II study testing cetuximab in combination with pemetrexed and cisplatin or carboplatin as first line treatment |
| Targeted agents | Alisertib | NCT02293005 | DCR | Evaluate the safety and effectiveness of alisertib an inhibitor of aurora kinase A protein |
| | Defactinib | NCT02004028 | Biomark respons | Assess biomarker response from tumor tissue of patients that received defactinib prior to surgery |
| | NGR-hTNFα | NCT01358084 | PFS | Randomized double blind phase II study to determine efficacy of NGR-hTNFα as maintenance treatment |
| | amatuximab | NCT02357147 | OS | Multicenter, double blind randomized phase II study evaluating the safety and efficacy of amatuximab in combination with pemetrexed and cisplatin as first line treatment. |
| Oncolytic viruses | HSV1716 | NCT01721018 | Safety, tolerability | Phase I/IIa of the safety, tolerability and biological effect of single and repeat administration of the herpes simplex virus |
| Immunotherapy | Pembrolizumab | NCT02399371 | Ability PD-L1 to predict response, OS | Phase II study to evaluate the effect of pembrolizumab on OS. |
| | Nivolumab | NCT02497508 | DCR | Single arm phase II study to determine if nivolumab will improve DCR from 20% to 40% at 12 weeks. |
| | Tremelimumab | NCT01843374 | OS | Phase IIb, randomized double blind study to determine the effect of tremelimumab on OS. |
| | Tremelimumab + MEDI4736 | NCT02588131 | ORR | NIBIT-MESO1 is a phase II, open label, single arm study evaluating the efficacy of tremelimumab in combination with the αPD-L1 MEDI4736 |
| Vaccine | DC vaccination | NCT02649829 | Number patients * | MESODEC is a phase I/II trial to show the feasibility and safety of WT-1 targeted DC vaccination in combination with chemotherapy prior to surgery. |
| | WT-1 vaccination | NCT01890980 | One year PFS | Phase II study determining if PFS is extended for patients receiving WT1 vaccine and montanide + GM-CSF after multimodality treatment compared to patients receiving montanide + GM-CSF after multimodality treatment |
| | WT-1 vaccination | NCT01265433 | One year PFS | Phase II study determining if PFS is extended for patients receiving WT1 vaccine and montanide + GM-CSF after multimodality treatment compared to patients receiving montanide + GM-CSF after multimodality treatment |

NGR-hTNFα: peptide asparagine-glycine-arginine – human tumor necrosis factor alpha, DC: dendritic cell, CRR: clinical response rate, PFS: progression free survival, DCR: disease control rate, OS: overall survival, ORR: objective response rate.* number of resectable patients with feasible and safe DC vaccine product and the number of patients receiving DC vaccination in combination with chemotherapy within the proposed time frame of surgery.

Nivolumab, another PD-1 receptor antibody, is currently evaluated in a single-arm phase II study in patients with recurrent MPM (NCT02497508). The primary objective of this study is the DCR at 12 weeks, which is expected to increase from 20% to 40% (Table 1).

Tremelimumab is a monoclonal antibody against CTLA-4. Blocking CTLA-4 will activate cytotoxic T-cells directly. Two single-arm phase II studies have been conducted, both showing encouraging clinical activity [63,64]. Therefore, a randomized double-blind placebo-controlled phase II study is now evaluating the efficacy of tremelimumab. The primary objective is demonstrating a 50% improvement in OS from 7 to 10.5 months (NCT01843374). Tremelimumab is also tested in combination with the anti-PD-L1 checkpoint inhibitor durvalumab. The primary outcome of this phase II study is immune-related objective response rate (NCT02588131) (Table 1).



While these checkpoint inhibitors allow an OS improvement of 20% in melanoma patients, the current studies should show whether these could be reproduced for mesothelioma patients or whether it predominantly induces PRs with only limited survival benefit.

**Vaccines**

Vaccines against mesothelioma cells may increase immune responses against the tumor. In 2005, Hegmans et al. reported that vaccination with antigen-pulsed dendritic cells (DCs) prevented tumor outgrowth in mice [65]. In the following phase I study, 10 patients received mature DCs, pulsed with the patient's own tumor lysate after chemotherapy. The treatment was feasible and safe and in some patients antitumor immune responses were detected. Whether this has any effects on survival of patients with mesothelioma should be further tested [66]. The DCs in this study were pulsed with tumor extracts in which only a minor portion of the antigens are tumor specific and relevant for the immune system. Pulsing DCs with only one tumor-associated antigen should provide more specific responses. The MESODEC study is a phase I/II trial in which patients are treated with DCs that are loaded with Wilms tumor 1 (WT-1) antigens. WT-1 is a transcription factor, which is highly overexpressed in mesothelioma cells. The general objective of the MESODEC study is to show the feasibility and safety of WT-1-targeted DC vaccination in combination with chemotherapy. Whether this treatment enables the induction of a systemic or immune response is also evaluated (NCT02649829) (Table 1). Another strategy focusing on WT-1 is vaccination of patients with ynthetic peptides derived from the WT-1 protein sequence. WT-1 could be targeted with a T-cell-based immunotherapeutic approach because it is processed and presented at the cell surface in the context of major histocompatibility complex class I molecules. A pilot study showed that the vaccine gave minimal toxicity and induced immune responses against WT-1 in a high proportion of patients [67]. Currently, two phase II studies with WT-1 vaccination are ongoing. In both studies, WT-1 vaccination in combination with granulocyte-macrophage-colony-stimulating-factor with or without

the vaccine adjuvant (montanide), is given after combined modality therapy. Primary outcome is 1-year PFS (NCT01890980 and NCT01265433) (Table 1).

Immunotherapy against cancer is a fast-developing treatment strategy with antibody-drug conjugates, new reagents to overcome immune checkpoints in order to boost immune responses, and vaccination strategies that are all tested in phase II studies on patients with mesothelioma. The prospects are bright for a subgroup of patients but these have to be selected.

**Preclinical models in translational research for MPM**

If clinical trials reveal one thing, it is that many drugs fail in phase II studies. Most of the drugs described in this review were active in preclinical studies, but lacked antitumor activity in the clinical setting. It is apparently difficult to predict clinical outcome with preclinical models. Selection of compounds for further clinical development is challenging. This is even more urgent in MPM since the disease is heterogeneous, the patient population is small and many new drugs are generated. Preclinical models are essential for a better selection process. Several factors are important in a good preclinical model. First of all, the preclinical model should resemble the patients' tumor, ideally with a representation of the stroma surrounding the tumor cells, the surrounding immune cells and vasculature. With many new drugs generated, it is important to be able to test multiple drugs at the same time; therefore, the preclinical model should be easy to handle and reproducible in its readout. Another factor is time; it is important to get results within a short period of time, so a preclinical model should not be time-consuming. There are many preclinical models available, each with their own advantages and disadvantages.

**Cell lines**

Most preclinical models are based on cell-line experiments. Cell lines are typically passaged for many years, making them highly selected clonal subpopulations of the original tumor, with many additional genetic aberrations. They then become a relatively poor representation of the original tumor [68–71]. Cell lines can be cultured in monolayer or in spheroids. Spheroids are tumor cells organized in a three-dimensional (3D) arrangement [70]. Monolayer cultures are easy to handle and suitable for large scale drug testing. Spheroids are more laborious but may better reflect the natural conditions of the tumor. They are not suitable for large-scale drug testing since read out of cell survival and quantification is challenging. MPM is a tumor extremely resistant to chemotherapy, mostly due to resistance to apoptosis [70,72]. Spheroids acquire multicellular resistance to a variety of treatments, which mimics the chemoresistance in patients [73,74]. Some drugs exhibit sensitivity in monolayer culture but resistance in spheroids. The proteasome inhibitor bortezomib, for example was found to be very effective in monolayer MPM cell-line cultures [75–77]. However, the phase II studies with this drug were disappointing.

Lack of activity was also observed in spheroid cultures [26,27]. Barbone et al. showed that spheroids treated with bortezomib were resistant due to upregulation of Noxa, a BH3-protein that displaces Bim and thereby mediates apoptosis [73].

Perfused microfluidic systems in combination with spheroids, may better reflect the in vivo situation, because regulation of drug exposure and mass transport is possible. Ruppen et al. compared static 3D-cultures with perfused 3D-cultures. For perfused 3D-cultures, a microfluidic chip was used. This chip contained two identical channels, each with eight trap ping sections and in each section a spheroid. Spontaneous formed spheroids were trapped in the sections, after which nutrients, oxygen, and drugs were delivered by diffusion from the main channel. Interestingly, perfused spheroids were twice as resistant to cisplatin compared to static spheroids [74].



**Primary tumor cultures**

Primary tumor cultures are cultures of single cells isolated from patients, which are propagated for a short period of time in order to prevent formation of clonal subpopulations. Multiple groups generated primary tumor cultures from cells isolated from pleural effusions of patients with MPM. These cultures resemble the original tumor closely regarding histological and molecular features [14,71,78,79]. Szulkin et al. used primary tumor cultures for chemosensitivity assays and observed a large patient-to-patient variability in sensitivity to drugs. Many cultures were resistant to drugs as was also observed in the clinical setting [14].

Xiang et al. generated spheroids from primary tumor cells. The spheroid of one primary cell line resembled cell line spheroids, while the spheroid of another primary cell line formed mostly loose aggregates [79]. It was not reported how long these primary cells were cultured and how often they were passaged, which makes it difficult to conclude that single cell spheroid formation from primary tumor cultures is a reproducible system. Tumor fragment spheroids are small biopsies of the tumor cultured on a collagen layer in order to grow out as spheroids. These tumor fragment spheroids exhibit the same complexity of cell types and extracellular matrix as the tumor. They retain many characteristics of the original tumor. Chemosensitivity assays on these tumor fragment spheroids are possible, but only for a very limited number of conditions [72,73,80,81].Techniques allowing a simple, individual tumor-based drug screen remain challenging.

**Mouse models**

Animal models are also very important in preclinical drug development. One advantage of animal models is that they can mimic the 3D-structure of a tumor and the vasculature around it. Furthermore, it also considers the pharmacokinetics, pharmacodynamics, and toxicity of a compound and in some models even the contribution of the immune system. There are different types of models reported, most of them mouse- based. In older

models, mesothelioma tumors were induced by intrapleural or intrabronchial exposure to carcinogens-like asbestos fibers, other natural and synthetic fibers and metals. Mouse models with mesothelial specific expression of oncogenes like SV40, NF2, or p53 were used to accelerate the induction of MPM in asbestos-exposed mice [82–84]. While these models resembled human mesothelioma in terms of latency, superficial growth, shedding of tumor cells, and growth as spheroids, these models had no loss of function of genes known to be inactivated in human MPM. This made it difficult to understand the molecular mechanism underlying the tumor [82]. Jongsma et al. developed the first genetic mouse model of MPM. Knockout-mice, deficient in the NF2 gene, were crossed with INK4A/ARF or p53-deficient mice. The offspring mice rapidly developed mesothelioma, with a high incidence and without further exposure to carcinogens [82,84]. The tumors that arise in these mice are not representative of the human tumor, but can be constructed with genetic mutations common to most of the patients with MPM. With increasing knowledge about genetic mutations in human mesothelioma, it is important to introduce the most prevalent mutations in these genetic mouse models. This will better resemble the human tumor. In other animal models, cell lines were injected in the pleural cavity of the mice. Most available cell lines however, do not form tumors in mice [71]. Those that do, may be selected for survival under mouse conditions and may not reflect human MPM. Patient-derived xenografts (PDX) are tumor biopsies or tumor cells from pleural effusions transplanted in nude mice. Kalra et al. showed that a PDX-mouse-model for MPM resembles the primary tumor culture and primary tumor regarding both histological and molecular features [71]. A disadvantage of this type of model is that it can only be generated in immune-deficient mice. The immune system may have a role in tumor clearance and sometimes chemotherapy responses, which complicates evaluation of the PDX-mouse-models. Although there are drawbacks, PDX-mouse-models could be very useful in evaluating efficacy of therapeutic agents.

We summarized various cell-based models and mousemodels that are available to improve translational research (Table 2). Each model has its own advantages and disadvantages and no model is perfect. Which model should be used depends on the aim of the research. Most important, none of the models have been validated by a strong corresponding chemotherapy response between the model and the corresponding patient.

**Expert commentary and five year view**

The prognosis for patients with MPM has not improved over the last decade. The current standard of care, cisplatin in combination with pemetrexed, has not been replaced by another treatment regimen in 12-year time. Although many therapies have been tested on patients with MPM, none were effective in phase II trials. There are various reasons for the limited progress in the treatment of mesothelioma. The first reason is the relatively small size of the patient population. This limits the interest of the pharmaceutical industry but

also complicates the execution of large randomized studies. This may be further complicated when mesothelioma is a more diverse tumor than anticipated. It is very difficult to define personalized treatment options unless obvious biomarkers related to treatment success are defined. These are currently lacking.

**Table 2. Overview of the available preclinical models and the features based on resembling the tumor, drug testing, and time**



| Preclinical model | | Resemble patient cells of tumor | Resemble natural conditions of tumor | Drug testing | Time |
|---|---|---|---|---|---|
| Cell line models | Monolayer | No | No | Multiple | Fast |
| | 3D spheroids | No | Only to chemo resistance | View | Slow |
| Primary tumor models | Monolayer | Yes | No | Multiple | Fast |
| | 3D spheroids | Yes | Only to chemoresistance | View | Slow |
| | Tumor fragments | Yes | Stroma composition | View | Slow |
| | | | chemoresistance | | |
| Mouse models | Asbestos induced | No | Yes | One | Slow |
| | Genetic | No | Yes | One | Fast |
| | Xenograft cell lines | No | Yes, however, no immune system | One | Slow |
| | Patient-derived xenograft | Yes | Yes, however, no immune system | One | Slow |

Yet there are a number of developments that can be expected to improve the prospects for, at least a subgroup of, patients with MPM. First, the genome of many mesothelioma tumors is being sequenced and defines genes that are often mutated, including the gene encoding the breast cancer-associated protein 1 (BAP1) [85–87]. BAP1 loss may affect the activity of the histone-methyltransferase EZH2 resulting in unusually high H3K27me3 modifications [88]. This epigenetic marker is also observed in other tumors and suggests that drugs affecting this epigenetic marker may be more selective and effective against MPM. This is indeed suggested in preclinical models. Second, drug screens can be performed on primary tumor cultures of MPM cells or, possibly, spheroids of these cells [14]. The detected drugs responses could be coupled to the patient that donated these tumor cells. This will allow personalized treatment for patients with MPM and ex vivo testing of larger series of anticancer drugs to select the best combination for the individual patient. Prediction should be accurate to prevent false-negative predictions and inadequate treatment of patients with MPM. This is critical before personalized screening on basis of patients tumor cells will be introduced in the clinic. Third, the latest addition to the cancer-drug repertoire, is immunotherapy with check-point inhibitors. Proteins like

PD-1, PD-L1, and CTLA-4 can dampen the adaptive immune response against tumors. Antibodies blocking these proteins establish the local immune responses against cancer, in fact starting a controlled auto-immune response. This new therapy can be effective for tumors with a high mutational load, which does not include MPM. Yet, the unique and high expression of proteins in tissues or tumors may also unleash an immune response and this will be tested for MPM in the near future.

Although the prospects for MPM treatment have not improved over the last decade, there are various developments that may finally lead to a step forward in the treatment of this tumor. The next decade will show serious progress in the fundamental understanding of MPM which in turn will improve the prospects of these patients.

**Key issues**

- MPM is an aggressive tumor with a poor prognosis. For patients that do not respond to first-line treatment or become progressive after treatment there is no standard second-line treatment available.
- Many inhibitors of growth factors are tested in MPM, most with negative results. Bevacizumab is the most promising agent.
- For other targeted agents, large phase II and phase III trials have been conducted.
- Immunotherapy is a new development in MPM, studies testing antibodies against PD-1 and CTLA-4 are ongoing.
- Other ongoing trials are focusing on primed DC-vaccination and WT-1 vaccination.
- Many drugs that were active in preclinical models, fail in phase II studies, indicating it is difficult to predict clinical outcome with preclinical models.
- A good preclinical model resembles the patients' tumor, is able to test multiple drugs at the same time and generate results within a short period of time.
- Each model, cell-based or mouse, has its own advantages and disadvantages; no model is perfect. Which model should be used depends on the aim of the research.
- Genomesequencing, drug screens performed on primary MPM cells, and immunotherapy with checkpoint inhibitors, are developments that can be expected to improve MPM.

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CHAPTER 4

# Chemical Profiling of Primary Mesothelioma Cultures Defines Subtypes with Different Expression Profiles and Clinical Responses

Josine M.M.F. Quispel-Janssen[2‡] | Laurel M. Schunselaar[1‡] | Yongsoo Kim[1] | Constantine Alifrangis[3] | Wilbert Zwart[1] | Paul Baas[2] | Jacques Neefjes[4]

‡ Both authors contributed equally

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## Abstract

**Purpose:** Finding new treatment options for patients with malignant pleural mesothelioma is challenging due to the rarity and heterogeneity of this cancer type. The absence of druggable targets further complicates the development of new therapies. Current treatment options are therefore limited and prognosis remains poor.

**Experimental Design:** We performed drug screening on primary mesothelioma cultures to guide treatment decisions of corresponding patients that were progressive after first or second line treatment.

**Results:** We observed a high concordance between *in vitro* results and clinical outcomes. We defined three subgroups responding differently to the anti-cancer drugs tested. In addition, gene expression profiling yielded distinct signatures that segregated the differently responding subgroups. These gene signatures involved various pathways, most prominently the fibroblast growth factor pathway.

**Conclusions:** Our primary mesothelioma culture system has proved to be suitable to test novel drugs. Chemical profiling of primary mesothelioma cultures allows personalizing treatment for a group of patients with a rare tumor type, where clinical trials are notoriously difficult. This personalized treatment strategy is expected to improve the poor prospects of mesothelioma patients.

**Statement of translational relevance**

Mesothelioma or asbestos cancer is a tumor with a poor prognosis. Three mesothelioma subtypes have been defined based on morphology and no effective treatment is available. Here we describe a system allowing the culture of primary mesothelioma cells for drug testing and genetic analyses. On the basis of drug sensitivitie s, we define three new mesothelioma subtypes with a concomitant different gene expression profile, including the FGF-pathway. Translating the results of the primary cultures to treatment of a small set of patients correctly predicted clinical responses. Chemical profiling of patients with mesothelioma allows identification of subgroups separated by the feature most relevant to patients: drug responses. The corresponding genetic analysis identifies the FGF-pathway for targeting in a defined mesothelioma subgroup.

## Introduction

Malignant pleural mesothelioma (MPM) is a rare but aggressive tumor arising from mesothelial cells in the pleural cavity. It usually presents with pain or dyspnea, caused by pleural fluid or shrinkage of the hemithorax (1). Palliative chemotherapy consisting of a platin and anti-folate combination is considered standard of care and gives a modest survival advantage of around three months (2). Further systemic treatment can be offered to fit patients, but thus far, studies in second line failed to detect a survival benefit. Response rates in different second line therapies range between 0 and 20% (3), which urges the need for more effective treatments.



Using genetic profiling to define drivers in cancer amendable to targeting by small molecular drugs, has been successful in other types of tumors. MPM however, has only a few mutations and none of these present as a likely target for therapy. Most genetic mutations found in MPM are loss of tumor suppressor genes, like CDKN2A, NF2 and BAP1, rather than activation of oncogenes (4). The absence of druggable molecular targets in MPM hinders the development of more dedicated and effective therapies (5-9).

Based on histology, three types of mesothelioma are recognized: an epithelioid, a sarcomatoid, and a biphasic or mixed type (10). Epithelioid mesothelioma comprises the largest group and has a better outcome than the sarcomatoid and mixed type. Regarding response to treatment, epithelioid mesothelioma is a heterogeneous disease. To increase the effectivity of current therapies, it is vital to find ways to more accurately profile this group of patients for personalized treatment and new therapeutic options.

Long-established cell lines are commonly used for *in vitro* drug screens to select compounds for further clinical development (11). However, their resemblance to primary tumors is questionable since cells change pheno- and genotypically during their adaptation to tissue culture conditions (12-15). This can have a profound influence on their responses to anti-cancer drugs (16,17). The use of cell lines in drug development programs did not yield any active drugs for mesothelioma patients. One example is the VANTAGE-014 trial which was based on positive results from established cell lines (18). This study exemplifies the difficulty of conducting clinical trials in a rare disease like mesothelioma (19). In this placebo-controlled trial that evaluated the HDAC-inhibitor vorinostat in second or third line, the time to accrue 661 patients with mesothelioma from 90 international centers, was 6 years. Unfortunately, there was no clinical benefit from treatment with vorinostat in this very large study (20). This trial stresses the need for *in vitro* drug testing conditions that reflect genuine mesothelioma tumors more accurately. Primary mesothelioma cultures may provide a valuable model for personalized drug selection for patients with mesothelioma

since they recapitulate the original tumor far more accurately than long-established MPM cell lines (21,22).

We established a method of profiling primary mesothelioma cultures with commonly-used anticancer drugs and validated the results in corresponding patients. We distinguished three groups, not by means of genetic parameters, but based on the drug response patterns which are ultimately more relevant to the patient. We found that the three 'chemical' profiles were associated with three distinct gene expression profiles relating to the FGFR pathway. Indeed, FGFR inhibition blocked proliferation of primary mesothelioma cultures, providing proof-of-concept of chemical profiling as a method to reveal novel sensitivities to targeted agents.

## Materials and Methods

### Patients

All patients provided written informed consent for the use and storage of pleural fluid, tumor biopsies and germ line DNA. Separate informed consent was obtained to use the information from the drug screens for making treatment decisions. The study was conducted in accordance with the Declaration of Helsinki and approved by Netherlands Cancer Institute review board. Diagnosis was determined on available tumor biopsies and confirmed by the Dutch Mesothelioma Panel, a national expertise panel of certified pathologists who evaluate all patient samples suspected of mesothelioma.

### Culture method

Short-term primary mesothelioma cultures were generated by isolating tumor cells from pleural fluid. Within half an hour after drainage, the pleural fluid was centrifuged at 1500rpm for five minutes at room temperature (RT). When the cell pellet was highly contaminated with erythrocytes, it was incubated with erythrocyte lysis buffer (containing 150mM $NH_4Cl$, 10mM potassium bicarbonate and 0.2mM EDTA) for 10 minutes at RT. Cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with penicillin/streptomycin and 8% fetal calf serum. The cells were seeded in T75 flasks at a quantity of $10x10^6$, $15x10^6$ or $20x10^6$ cells and incubated at 37°C at 5% $CO_2$. Medium was refreshed depending on metabolic activity of the cells, usually twice a week. Cells were cultured for a maximum period of four weeks.

**Comparative genome hybridization**

To ensure that our cultures consisted mainly of tumor cells, we performed comparative genome hybridization (CGH) on a number of cultures. CGH was performed as described by Schouten et al (23). Tumour DNA was labelled with Cy3 and female pooled reference DNA (G1521, Promega) was labelled with Cy5 using the ENZO labelling kit for BAC arrays (ENZ-42670, ENZO Life Sciences). Unincorporated nucleotides were removed with the Qiagen MinElute PCR Purification Kit (28004, Qiagen). Subsequently, tumour and reference DNA were pooled and pelleted using an Eppendorf Concentrator (5301, Eppendorf). The pellets were resuspended in hybridisation mix (NimbleGen Hybridization Kit, Roche Nimblegen) and the sample loaded on the array. Hybridisation was at 42°C for 40–72h. (Maui Hybridization System, BioMicro Systems). Slides were washed three times (Roche NimbleGen Wash Buffer Kit) and scanned at 2µm double pass using an Agilent High Resolution Microarray Scanner (Scanner model: G2505C, Agilent). The resulting image files were further analyzed using NimbleScan software (Roche Nimblegen). Grids were aligned on the picture manually and per channel pair files generated. The NimbleScan DNA Copy algorithm was applied at default settings and the unaveraged DNA copy text files were used for further analyses.



**Drug screens**

Drug screens were performed in biological duplicate after one and two weeks of culture. Seven single agents (cisplatin, carboplatin, oxaliplatin, vinorelbine, gemcitabine, pemetrexed and doxorubicin) and five combinations (cisplatin+pemetrexed, cisplatin+gemcitabine, carboplatin+pemetrexed, oxaliplatin+gemcitabine and oxaliplatin+vinorelbine) were used. Cells were seeded in a flat bottom 96 wells plate at a density of 5000 cells/well. After overnight incubation, chemotherapeutics in a concentration range of 50µM-5nM were added in technical triplicates. After 72 hours of incubation with the drugs, the cytotoxicity was measured with a metabolic activity assay (Cell Titer blue G8081, Promenga). Fluorescent readout was performed with the Envision Multilabel Reader (Perkin Elmer).

**Interpretation dose-response curves**

***Classification of cultures in three groups***

The classification of cultures in three groups was based on results from all drugs and drug-combinations screened. For three concentrations (10 nM, 1 mM and 50 mM) cell survival cut-off was determined in. Cell survival cut-off for a drug concentration of 10 nM was set at ≥90% cell survival, for 1 mM at ≥70% and for 50 mM at ≥50%. For each concentration the number of drugs above the cut-off value was counted. A culture was defined as non-responsive when for <u>all</u> three concentrations, 5 or more drugs were above the cell survival cut-off value. A culture was defined as an intermediate responder when for <u>one or two</u> concentrations, 5 or more drugs were above the cell survival cut-off value. When for all concentrations, less than 5 drugs were above the cell survival cut-off value, the culture was classified as a responder.

***In vitro response prediction***

An *in vitro* response prediction was made for each drug or drug-combination individually. The *in vitro* response was correlated to the clinical response defined by RECIST modified for mesothelioma, thereby identifying patients with progressive disease, stable disease and partial response. A test set of dose-response curves was used to determine cut-off points for AUC values to predict clinical responses. Very low or very high drug concentrations were not expected to be clinically relevant. Therefore the AUC was determined in a concentration range of 50-5000nM (GraphPad Prism). An AUC level of less than 1485 predicted a partial response. An AUC level higher than 2970 predicted progressive disease. All AUC levels between these numbers predicted stable disease.

**RNA isolation**

Total RNA was extracted using TRIzol reagent (15596-018, Ambion life technologies) according to the manufacturer's protocol. Typically 1 mL of TRIzol reagent was used per $1 \times 10^6$ cells. The total RNA pellet was air-dried for 8 minutes, dissolved in an appropriate volume of nuclease-free water (AM9937, Ambion life technologies) and quantified using Nanodrop UV-VIS Spectrophotometer. Total RNA was further purified using the RNeasy MinElute Cleanup Kit (74204, Qiagen) according to the manufacturer's instructions. Quality and quantity of the total RNA was assessed by the 2100 Bioanalyzer using a Nano chip (Agilent, Santa Clara, CA). Total RNA samples having RIN>8 were subjected to library generation.

**RNA sequencing**

Strand-specific libraries were generated using the TruSeq Stranded mRNA sample preparation kit (Illumina Inc., San Diego, RS-122-2101/2) according to the manufacturer's instructions (Illumina, Part # 15031047 Rev. E). The libraries were analyzed on a 2100 Bioanalyzer using a 7500 chip (Agilent, Santa Clara, CA), diluted and pooled equimolar into a 10nM multiplexed sequencing pool and stored at -20 °C. The libraries were sequenced with 65bp paired-end reads on a HiSeq2500 using V4 chemistry (Illumina Inc., San Diego).

**Gene expression analysis**

The raw sequencing data was aligned to a human reference genome (build hg38) using *tophat 2.0*, followed by measuring gene expression using our own protocol based on htseq count (Icount). Normalized count-per million (CPM) was measured using library sizes corrected wurg Trimmed mean of M-values (TMM) normalization with edgeR package (24). For differential expressed gene (DEG) identification, we used voom transformation (25) followed by empirical Bayes method with *limma* r package. Then, DEGs were identified as the genes with P-values less than 0.005 and log2 fold changes larger than 2. The voom transformed log-CPM of DEGs were used in Principal Component Analysis (PCA). For heatmap generation

voom transformed log- CPM of DEGs were standardized by mean centering and scaling with standard deviation. Genes were ordered based on hierarchical clustering with Pearson correlation as a similarity measure and ward linkage. ID number and corresponding fold changes of DEGs were uploaded in ingenuity pathway analysis (IPA) (Qiagen Bioinformatics, Redwood City). Analysis was performed with 224 mapped IDs.

**Stability assessment of differential gene expression analysis**

To assess the reliability of DEGs, we performed differential expression analysis with leaving out each of the responders and non-responders. The P-values and rankings of DEGs that were obtained with this analysis were used in the down-stream analysis. Further, for each of the held-out experiments, we obtained DEGs using same P-values and fold-change cut-offs. For each of the DEG lists, hierarchical clustering analysis was performed, after which consensus of the clustering is obtained.



## Results

**Profiling and characterization of primary mesothelioma cultures**

Between February 2012 and July 2016, 155 pleural fluids from 102 patients with a confirmed histological diagnosis of mesothelioma, were collected for early passage primary cultures. Eighty-nine patients (87%) were male, the mean age was 67 years and most patients had an epithelial subtype, similar to the conventional distribution of mesothelioma subtypes. Forty-one patients were chemotherapy naïve at the time of cell isolation and 61 patients had received one or more lines of treatment (Supplementary Table S1A). Fig. 1A shows a flow chart of the pleural fluid pipeline depicting *in vitro* drug testing and subsequent clinical testing in patients. Eighty-one of the 155 isolations were suitable for further culture and drug screening, resulting in a take rate of 52%. These 81 isolations were derived from 57 patients. We failed to perform a drug screen for 45 patients. Patients' characteristics for both groups are given in Supplementary Table S1B and C. There was no significant difference between the two groups for age (p=0.05), prior lines of treatment (p=0.54) or histology (p=0.42). There was a significant difference in gender (p=0.03), however the number of female patients was too low to make conclusions about any effect of gender on success rate. Failure was mainly due to too low tumor cell count isolated from the pleural fluid. The time between isolation of pleural fluid and the start of the first drug screen was generally one week. A biological duplicate screen was performed in the following week (Fig. 1B).

Because cultures may change over time, we assessed the stability of our cultures using comparative genome hybridization (CGH). While mesothelioma is generally characterized by very few mutations, they frequently show loss of the gene CDKN2A, located at the p16

locus on chromosome 9 (26-28). This can be detected by CGH. There was no deletion of the p16 locus detected in samples of two patients. In the pleural fluid of three other patients, deletion of the p16 locus was detected in the first culture passages. At later passages, this deletion could not be detected anymore in two of the three patients. Since deletions cannot be repaired spontaneously, this suggests overgrowth of reactive mesothelial cells co-isolated with the mesothelioma cells (Supplementary Fig. S2). These experiments validated the isolation and culture of primary mesothelioma cells and showed that drug screens should be performed during the first 3 weeks after isolation from patients, before overgrowth of other cells could be expected.

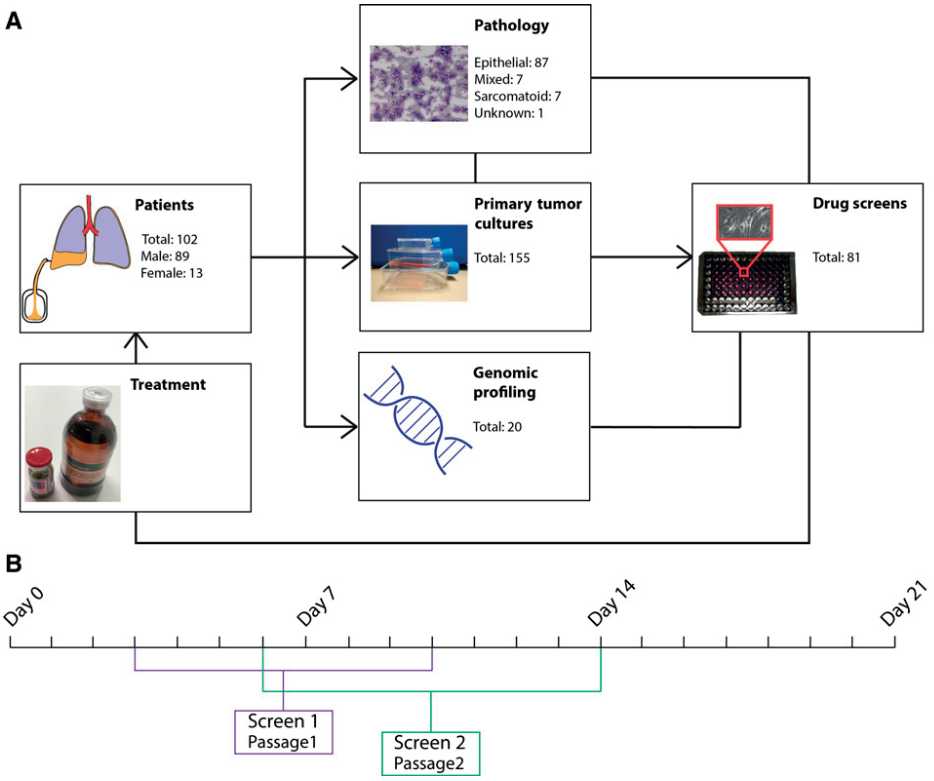


**Fig. 1. Flow chart and timeline of the chemical and genetic profiling of primary mesothelioma cultures.**

(**A**) Flow chart of the pleural fluid pipeline. Pleural fluid was extracted from 102 patients diagnosed with mesothelioma. The cultures were diagnosed with pathology and primary cultures were made. Twenty primary tumor cultures were genetically profiled. Eighty-one cultures were suitable for drug screening. The results from 11 drug screens were used in patient treatment. (**B**) Timeline of drug screens using primary mesothelioma cultures. The first screen was started within 10 days after isolation (day 0), the biological duplicate screen was performed within one week after the first screen. The drug screening assays took five days and primary cultures were analyzed within three weeks after cell isolation from the pleural fluid.

**Chemical profiling identifies 3 mesothelioma subgroups**

Drug screening was performed on 81 different primary cultures with compounds selected on the basis of their current or historical use as treatment of patients with mesothelioma (2,29-33). Cisplatin, carboplatin, oxaliplatin, gemcitabine, vinorelbine, pemetrexed and doxorubicin have been tested as single agent and/or in combination. The different cultures showed marked differences in the dose-response profiles. This allowed clustering of the primary cultures in three different groups: so called 'responders', 'non-responders' and 'intermediate responders' (see Materials and Methods). The clustering is based on all drugs and drug-combinations screened. We defined a 'responder' as a culture responding to most of the chemotherapeutics screened (Fig. 2A and supplementary Fig. S3A). We defined a 'non-responder' as a culture failing to respond to more than 5 of the drugs screened (Fig. 2B and supplementary Fig. S3B). An 'intermediate responder' responded to some of the drugs, but not to all of them and visually did not fit in one of the other two categories (Fig. 2C and supplementary Fig. S3C). From the 81 cultures, six cultures classified as 'responder', 27 as 'non-responder' and 48 as 'intermediate responders'. Thirty-one drug screens were performed on chemo-naïve cells. Fifty drug screens were performed on cells from patients that received one or more lines of treatment. The clustering in the 3 groups was not significantly different for cells isolated from patients that had or had not received prior treatment (p=0.72) (supplementary Table S4A). These data suggested that primary mesothelioma cultures allow subdivision of tumors based on drug sensitivity without significant effects of earlier treatments of the corresponding patients.

**Transcriptomic analyses reveals distinct genomic subclasses through chemical profiles**

Between primary mesothelioma cultures, divergent responses to chemotherapeutic intervention were observed. To test whether there was a genomic basis for these three groups identified by chemical profiling, we performed RNA-seq on 20 primary mesothelioma samples, taken immediately after isolation and representing four 'responder' samples, nine 'non-responder' samples and seven samples from the 'intermediate' group. We first identified a set of differentially expressed genes (DEGs) between responders and non-responders with P-values less than 0.005 and log2 fold changes larger than 2 (see Material and Methods). A total of 133 genes were downregulated and 152 genes were upregulated in the 'responder' group compared to the 'non-responder' group (supplementary Table S5). In differential gene expression analysis with leave-one-out cross validation, we confirmed that the 285 DEGs were consistently highly ranked and the cut-offs (P-value<0.005 and log2 fold changes >2) provided genes that stably separated patients by response (supplementary Fig. S6). The 'intermediate' group shows a signature that differs from both 'responders' and 'non-responders', also genetically defining it as a separate group (Fig. 3A). We observed the same trend in Principal Component Analysis on expression levels of DEGs (Fig. 3B; Materials and Methods). Ingenuity pathway analysis on DEGs revealed 10 networks containing at least 7 DEGs. The top network with 23 DEGs contained the fibroblast growth factor (FGF) pathway

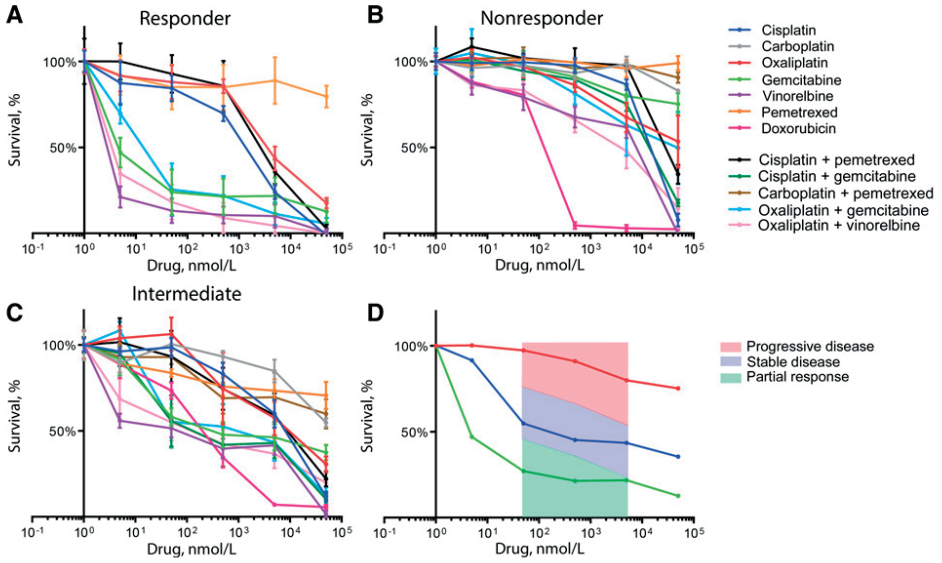


**Fig. 2. Dose response curves for various drugs depicted for the differently responding subgroups.**

(**A-C**) Dose- response curves of a responder, a non-responder and an intermediate responder are shown, as indicated. Drug screens were performed on chemo-naïve cells. Survival (mean ±SD) is shown in relation to increasing concentrations of single agents and combinations, as indicated. (**D**) Dose response curves for the drug gemcitabine screened in 3 different patients, a responder (green), an intermediate responder (blue) and a non-responder (red). Boxes indicate the AUC from which progressive disease (red), stable disease (blue) and partial response (green) is predicted. The AUC surface is pictured in the trend of the gemcitabine curves.

(Fig. 3C). FGF9 was significantly upregulated in the non-responder group (Fig. 3D). Since this pathway has been described previously in MPM (34), we analyzed gene expression of the preferred receptors for FGF9: FGFR3 and FGFR1. Gene expression of these receptors was also upregulated in the non-responder group (Fig. 3D). The paired-end RNA-sequencing analysis did not reveal mutated expressed genes.

To test the relevance of the various components of the FGF-pathway, primary mesothelioma cultures were exposed to compound PD-173074, a FGFR inhibitor with a high affinity for FGFR3 and FGFR1. Two 'non-responder' primary mesothelioma cultures were sensitive to the FGFR-inhibitor (Fig. 3E). In mesothelioma cell lines we also found a statistically significant correlation between elevated FGF9 mRNA expression and IC50 to PD-173074 (p=0.0117) (Quispel et al. submitted to Clinical Cancer Research CCR-17-1172). These experiments show that chemical profiling of primary mesothelioma cultures allows identification of subgroups that are characterized by different expression profiles. In addition, new targets for treatment of mesothelioma subgroups can be identified, as is illustrated here for the FGF-pathway.

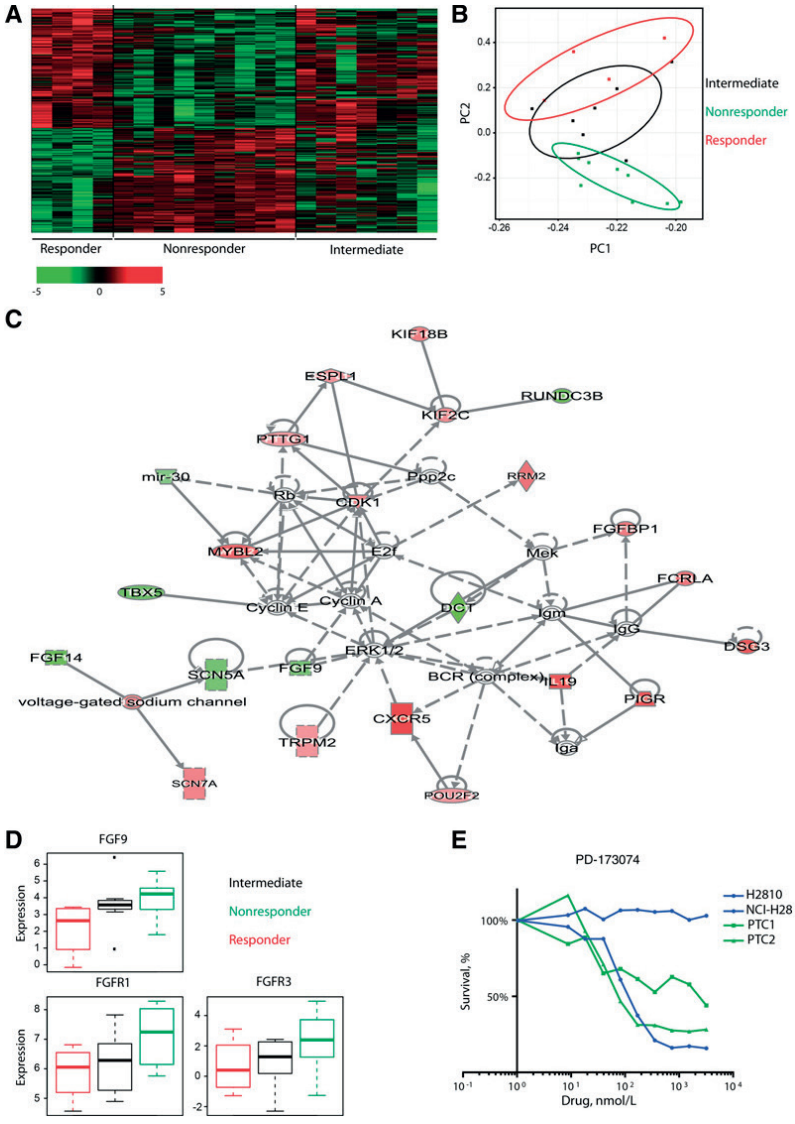


**Fig. 3. Gene expression profiling of the differently responding mesothelioma subgroups.**

(A) Heatmap showing 285 genes that are differentially expressed between 'responders' and 'non-responders'. Green bars depict genes that are downregulated, while red bars depict upregulated genes in 'non-responders'. The gene expression profile of the intermediate group is different from the expression profile of 'responders' and 'non-responders'. The list of genes is shown in Supplemental table. 2. (B) Principal Component Analysis separates responders (red) from 'non- responders' (green). The intermediate group (black) locates between these groups. (C) Ingenuity pathway analysis illustrating the most significant network containing 23 DEGs between 'responders' and 'non-responders'. Green: upregulated, red: downregulated DEGs in non-responders. (D) Boxplot depicting gene expression of FGF9 and interaction partners FGFR1 and FGFR3 in 'responders' (red), 'non-responders' (green) and 'intermediate responders' (black). The level of gene expression is indicated on the y-axis. Boxplot shows mean expression level with 75$^{th}$ (top) and 25$^{th}$ (bottom) percentile value. Whiskers indicate range of values. (E) Dose-response curves of two non-responder cultures and reference cell lines NCI-H28 and H2810, treated with increasing concentrations of FGFR inhibitor PD-173074. Cell viability is measured.

**Clinical implication of in vitro drug screens**

To study the correlation between *in vitro* drug screens and clinical outcome, we quantified drug sensitivity by calculating the area under the curve (AUC) values of dose-response curves. The AUC was determined in a concentration range between 50-5000nM. Lower or higher concentrations were not expected to be clinically relevant. *In vitro* response was determined for each drug or drug-combination and was classified as the clinical responses: partial response, stable disease or progressive disease. Fig. 2D illustrates dose-response curves for the drug gemcitabine in 3 different patients. The boxes indicate the AUC in which progressive disease, stable disease and partial response were predicted. We treated ten patients that were progressive after first or second line treatment, with the drug that was most effective based on the *in vitro* drug screen, that was performed on the patient's primary mesothelioma cells (Table 1). Patient 1 was a 61-year-old woman with an epithelial type mesothelioma. Her frontline treatment consisted of the standard first-line combination of cisplatin and pemetrexed, which was followed by a surgical procedure consisting of a pleurectomy/decortication. Upon progression, the *in vitro* drug screen demonstrated oxaliplatin and vinorelbine as the most effective compounds and we predicted a partial response (Fig. 4A, patient 1). She was treated accordingly resulting in a partial response, as is shown in Fig. 4B. The second patient, a 52-year-old male with epithelial mesothelioma, was treated with cisplatin and pemetrexed, followed by a pleurectomy/decortication. Progression occurred 7 months after completion of his first-line therapy.

**Table 1: Overview of patients treated based on their in vitro drug screen.**

Ten patients were treated based on their in vitro drug screen. Gender, histology, chemotherapeutic given, in vitro response prediction and actual patient response are given. Patient 5 was treated twice based on his in vitro drug screen. F: Female, M: Male, green: PR partial response, yellow: SD- stable disease, red: PD - progressive disease

| Patient | Gender | Histology | Drug | In vitro predicted response | Patient response |
|---|---|---|---|---|---|
| 1 | F | Epithelial | Oxaliplatin + vinorelbine | PR | PR |
| 2 | M | Epithelial | Oxaliplatin + gemcitabine | SD | SD |
| 3 | F | Mixed | Oxaliplatin + vinorelbine | PD | PD |
| 4 | M | Epithelial | Oxaliplatin + gemcitabine | SD | PR |
| 5-1 | M | Epithelial | Gemcitabine | SD | SD |
| 5-2 | | | Vinorelbine | PR | SD |
| 6 | M | Epithelial | Oxaliplatin + vinorelbine | PD | SD |
| 7 | M | Epithelial | Oxaliplatin + gemcitabine | PD | PR |
| 8 | M | Epithelial | Doxorubicine | SD | SD |
| 9 | M | Epithelial | Oxaliplatin + gemcitabine | PD | PD |
| 10 | M | Epithelial | Oxaliplatin + gemcitabine | SD | SD |

The combination of oxaliplatin and gemcitabine was the most effective one and stable disease was predicted (Fig 4A, patient 2), which was indeed observed after clinical treatment with these drugs (Fig. 4B). Patient 3, a 36-year-old female patient with a mixed type of mesothelioma, had disease progression four months after her initial treatment with cisplatin, pemetrexed and a pleurectomy/decortication. The *in vitro* drug screen showed a 'non-responder' profile and progressive disease was to be expected from treatment (Fig. 4A, patient 3). She was treated with consecutive courses of the best combination observed (carboplatin/gemcitabine and oxaliplatin/vinorelbine) but experienced disease progression after 2 courses of each combination (Fig. 4B) and died shortly thereafter. *In vitro* drug screen results and CT scans before and after treatment of patients 4-10 are depicted in Supplementary Fig. S7. For patient 8-10 *in vitro* response prediction correlated with the actual patient response. For patient 4, 6 and 7 the patient response was better than predicted. Patient 5, a 71-year-old man with epithelial mesothelioma, was treated twice based on his chemosensitivity screen. After front-line treatment with carboplatin and pemetrexed, he was first treated with gemcitabine and later with vinorelbine. The clinical response for both treatments was stable disease. For gemcitabine this was predicted based on the *in vitro* screen. For vinorelbine however, the observed response was not as pronounced as was expected based on the *in vitro* results (Supplementary Fig. S7). For patient 6 vinorelbine was selected as the best option to which oxaliplatin was added. Patient 7, 9 and 10 did not receive the most potent drug based on *in vitro* drug screen because of contra-indications for treatment with doxorubicin. Due to the patients history vinorelbine or a combination with vinorelbine could not be given. From eleven drug screens, seven *in vitro* response predictions were correct. For the four that were not correctly predicted, the actual clinical response was better in three patients. These results suggest that the *in vitro* drug screens had added value in predicting actual individual patient responses to selected drugs.

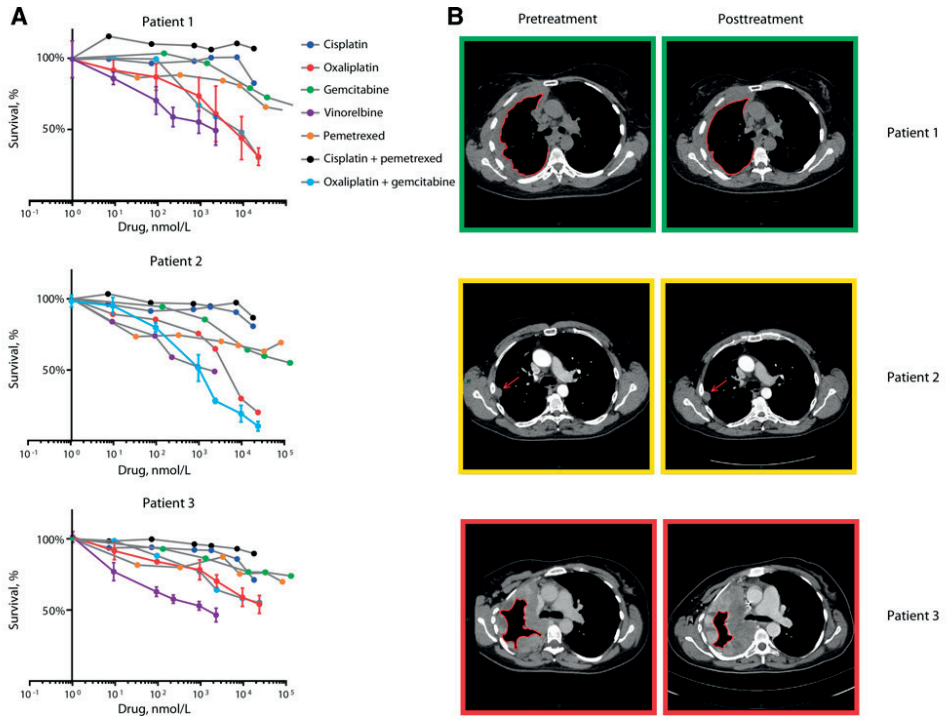


**Fig. 4. Dose-response curves and clinical responses of three patients.**

(**A**) Dose-response curves of primary mesothelioma cells isolated from patients 1-3 and treated with several single agents and combinations of cytotoxic drugs, as indicated. Cell viability measured after 72 hours of drug exposure as a function of increasing concentrations of several drugs and combinations is depicted. (**B**) CT-scans of patient 1-3 before and after treatment with the drugs selected based on the *in vitro* drug screens. Response evaluation was done using modified RECIST for mesothelioma. Colored boxes around CT-scans indicate in vitro response prediction before treatment and the actual response after treatment. Green: partial response, yellow: stable disease, red: progressive disease. Patient 1 was treated with a combination of oxaliplatin and vinorelbine. The tumor rind indicated by the red line is irregular on her pre-treatment scan and is smaller and smoother on her post-treatment scan, indicating a partial response. Patient 2 received a combination of oxaliplatin and gemcitabine. The tumor nodule indicated by the red arrow, remains similar between the scans indicating stable disease. Patient 3 received successively carboplatin/gemcitabin and oxaliplatin/vinorelbine. The grey tumor rind on the pre-treatment scan -encircled by the red line- is larger on the post-treatment scan, which illustrates progressive disease.

## Discussion

Cancer treatment strategies are changing from general therapy regimens to more personalized treatment, often based on the genetic make-up of the tumor. Unfortunately, no druggable driver mutations have been identified in mesothelioma (5,6,8,9,35). Therefore, we 'chemically' profiled primary mesothelioma cultures with common chemotherapeutic drugs and subsequently treated ten patients with the most effective drug or drug combination. This strategy has previously been successfully applied in lung cancer (36-38), ovarian cancer (39,40) and breast cancer (41) and showed that in vitro drug responsiveness bears clinically relevant information for patient treatment efficacy.

For the patients treated in this study, we observed considerable overlap between the predicted drug responses *in vitro* and the corresponding clinical responses. Although the number of patients is too small to make definite conclusions, we present a system that can personalize the treatment of patients with mesothelioma, a heterogeneous disease, with a limited number of patients available for clinical trials and only one registered systemic therapy option.

In addition to predicting the best chemotherapeutic option for an individual patient, we identified 'chemical profiles' corresponding to gene signatures that distinguished tumors resistant to most tested therapeutics, from tumors that were largely responsive. A third group with intermediate responses to drugs had an expression profile that was different from the responding and non-responding group. We expected that drug screens performed on chemo-naïve cells would give a different chemosensitivity profile compared to drug screens performed on pre-treated cells. However, no significant differences were detected in the three 'chemical profiles' between these groups. This corresponds to results of Mujoomdar et al. who described similar results for chemo- naïve and pre-treated biopsies treated in vitro with three single agents (42).



The different 'chemical profiles' that we identified could not have been identified based on pathology without prior knowledge. In cancer types like prostate and breast cancer, gene expression profiles were successfully used to define subclasses. These were usually retrospectively correlated with prognostic features (43,44), although one such a profile -the 70-gene signature in breast cancer- has recently been validated on the basis of a prospective study (45). Our prospectively determined chemical profiles have predictive value, which -from the patients' perspective- is the most important factor and clinically more relevant than prognostic values.

Of note, there are some limitations to our pipeline. The drug screening system was unable to test pemetrexed. Pemetrexed is an antifolate that inhibits multiple enzymes involved in the formation of nucleotides (46-49). Pemetrexed activity is competed away by folate (46,47,50,51). The culture medium used in this system contained folate, probably at supra-physiological levels. Serum also contains a variety of folate, nucleosides and nucleotides, which is expected to circumvent growth inhibition by pemetrexed (46,52). The presence of folate, nucleosides and nucleotides in the culture system could explain why primary cultures were not sensitive to pemetrexed. Another limitation of the system is that the culture does not include pharmacokinetics and dynamics of the different drugs. As every cell-based model it lacks features of the original tumor like vasculature and tumor micro-environment which makes it impossible to simulate pharmacokinetics and –dynamics. On logical grounds, our system can also not be used for the testing of the recently introduced classes of Immuno-Oncology drugs. Our in vitro response prediction method is arbitrary

and expanding with more patients would provide data to further define cut-offs for better drug response prediction.
Thus far, we have tested only chemotherapeutics that are commonly used in clinical practice because these allowed validation of the results in patients with mesothelioma. By further expanding the number and classes of compounds in the drug screen, we may not only be able to further characterize the more heterogeneous intermediate group, but also identify more suitable therapeutic options for the non-responder patient population.

Our model will enable us to select drugs or drug-combinations that are more likely to give a response in subgroups of patients. Since mesothelioma is a rare tumor type, such subgroups would probably not have been detected in clinical trials. Preselection of drugs and patients will help to optimize the design and success of clinical trials in this patient group.

We already have one example of a new drug selected on the basis of our method. Based on gene expression profiling, the FGF pathway appeared upregulated in the non-responder patient population, for whom at this stage no active therapeutic options are available. Deregulated FGF signaling has been linked to cancer pathogenesis (53) and several groups have reported involvement of the FGF signaling cascade in mesothelioma (34,54). Since this pathway appeared selectively upregulated in the non-responder patient population, preselected patients may derive specific benefit from therapeutic intervention using FGFR inhibitors, as we successfully illustrate in our primary cultures (Fig. 3E). Chemical profiling of primary mesothelioma cultures revealed three response groups corresponding to distinct gene signatures involving the FGF signaling cascade. We demonstrated considerable overlap between *in vitro* and *in vivo* responses suggesting that our pipeline represents a feasible method to personalize treatment that could ultimately improve the prospects of mesothelioma patients.

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**Author Contributions**: J.Q. and L.S. designed, conducted, and interpreted the majority of the experiments and prepared the manuscript. C.A. performed the experiments in Fig. 3C. Y.K. performed statistical and bioinformatics analyses. W.Z. discussed the results throughout the project. P.B. and J.N. supervised the project. All authors edited the manuscript.

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## Supplemental Data

**Table S1A Patient characteristics**

| Characteristics of all patients where cells could be isolated from pleural fluid | |
|---|---|
| Patients no. | 102 |
| Male/female no. (%) | 89/13 (87%/13%) |
| Mean age in years | 67 |
| Prior treatment lines: 0/1/2/unknown (%) | 41/40/19/2 (40%/39%/19%/2%) |
| Histology: epithelioid/sarcomatoid/mixed/unknown (%) | 87/7/7/1 (85%/7%/7%/1%) |

For patients who had multiple cultures at different time points, the number of prior treatment lines was determined at the first successful culture. When we failed to perform a drug screen, the number of prior treatment lines was set at the first culture.

**Table S1B**

| Characteristics of patients with a successful drug screen | |
|---|---|
| Patients no. | 57 |
| Male/female no. (%) | 46/11 (81%/19%) |
| Mean age in years | 65 |
| Prior treatment lines: 0/1/2/unknown (%) | 26/19/11/1 (46%/33%/19%/2%) |
| Histology: epithelioid/sarcomatoid/mixed/unknown (%) | 50/4/2/1 (88%/7%/4%/2%) |

**Table S1C**

| Characteristics of patients where the drug screen failed | |
|---|---|
| Patients no. | 45 |
| Male/female no. (%) | 43/2 (96%/4%) |
| Mean age in years | 68 |
| Prior treatment lines: 0/1/2/unknown (%) | 15/21/8/1 (33%/47%/18%/2%) |
| Histology: epithelioid/sarcomatoid/mixed/unknown (%) | 37/3/5/0 (82%/7%/11%/0%) |

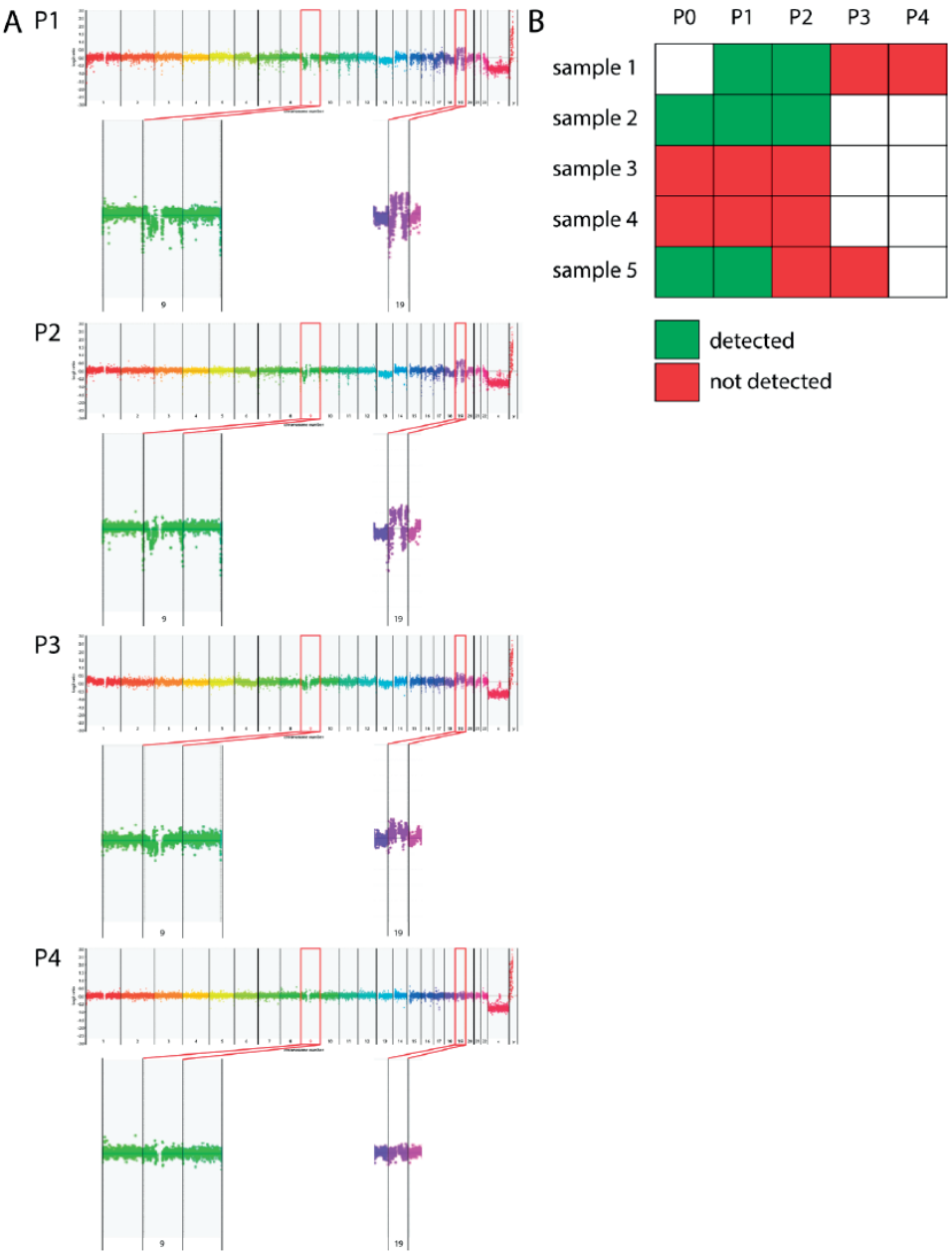


**Fig. S2. CGH profiles at different passages of a primary mesothelioma culture.**

(**A**) The log2 ratio of copy number variations (CNV) is depicted for different chromosomes visualized on the X-axis, each chromosome in a different color. The overall profiles in the first two passages indicate the presence of malignant cells as is illustrated by deletion of the P16 locus on chromosome 9 (shown as a zoom-in in the inset). After more passages the CNV is normalized indicating overgrowth by normal mesothelial cells. (**B**) Overview of CDKN2A deletion for 5 patients. P1: passage 1, P2: passage 2, P3: passage 3, P4: passage 4. Green: detected, red: not detected, white: not assessed. For patient 3 and 4 no deletion could be detected. For patient 1,2 and 5 the CDKN2A deletion was detected in early passages.

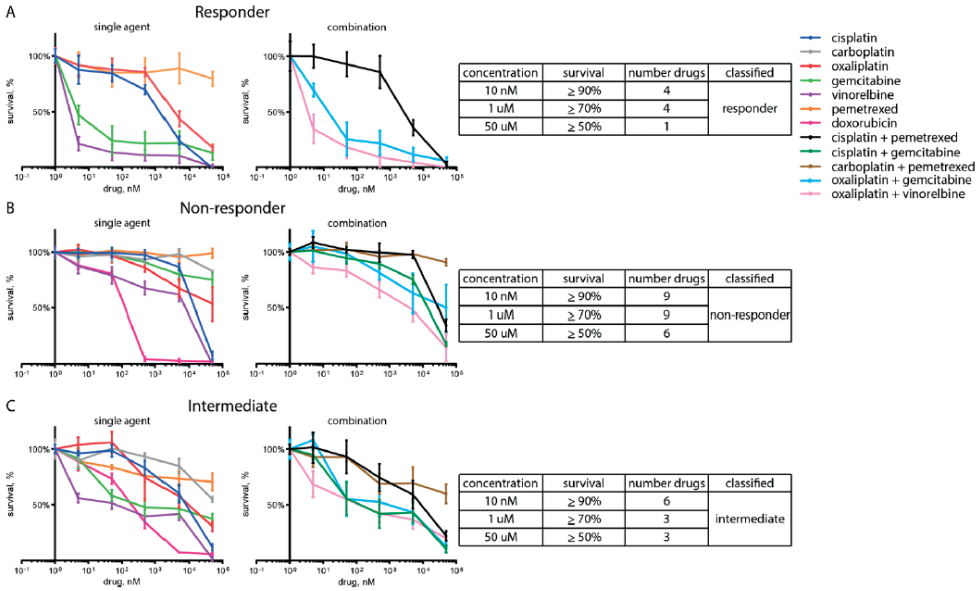


**Fig. S3. Dose-response curves of single agents and combinations depicted for the differently responding subgroups.**

Dose-response curves of figure 2 separated to single agents and combinations are depicted for (**A**) a responder, (**B**) a non-responder and (**C**) an intermediate responder. Explanation of the subgroup definition is depicted next to the dose-response curves.

**Table S4: Drug screen classification characteristics**

| | Non-treated | | Treated | |
|---|---|---|---|---|
| | Number | Percentage | Number | Percentage |
| Drug screens | 31 | 38% | 50 | 62% |
| Responder | 3 | 10% | 3 | 6% |
| Intermediate | 19 | 61% | 29 | 58% |
| Non-responder | 9 | 29% | 18 | 36% |

There was no significant difference between the treated and the non-treated group (p=0.72)

**Table S5: List of differentially expressed genes.**

| Gene ID | Gene Symbol | Gene Name |
|---|---|---|
| ENSG00000163995 | ABLIM2 | actin binding LIM protein family member 2 |
| ENSG00000100312 | ACR | acrosin |
| ENSG00000174837 | ADGRE1 | adhesion G protein-coupled receptor E1 |
| ENSG00000116771 | AGMAT | agmatinase |
| ENSG00000165695 | AK8 | adenylate kinase 8 |
| ENSG00000215267 | AKR1C7P | aldo-keto reductase family 1 member C7, pseudogene |
| ENSG00000244301 | AOX3P | |
| ENSG00000006453 | BAIAP2L1 | BAI1 associated protein 2 like 1 |
| ENSG00000197299 | BLM | Bloom syndrome RecQ like helicase |
| ENSG00000229106 | BTBD6P1 | BTB domain containing 6 pseudogene 1 |
| ENSG00000221953 | C1orf229 | chromosome 1 open reading frame 229 |
| ENSG00000128346 | C22orf23 | chromosome 22 open reading frame 23 |
| ENSG00000225940 | C5orf67 | chromosome 5 open reading frame 67 |
| ENSG00000118307 | CASC1 | cancer susceptibility candidate 1 |
| ENSG00000246228 | CASC8 | cancer susceptibility candidate 8 (non-protein coding) |
| ENSG00000168491 | CCDC110 | coiled-coil domain containing 110 |
| ENSG00000274736 | CCL23 | C-C motif chemokine ligand 23 |
| ENSG00000272398 | CD24 | CD24 molecule |
| ENSG00000170312 | CDK1 | cyclin dependent kinase 1 |
| ENSG00000100162 | CENPM | centromere protein M |
| ENSG00000259430 | CERS3-AS1 | CERS3 antisense RNA 1 |
| ENSG00000197748 | CFAP43 | cilia and flagella associated protein 43 |
| ENSG00000172361 | CFAP53 | cilia and flagella associated protein 53 |
| ENSG00000122966 | CIT | citron rho-interacting serine/threonine kinase |
| ENSG00000144619 | CNTN4 | contactin 4 |
| ENSG00000273509 | CNTNAP3P1 | contactin associated protein-like 3 pseudogene 1 |
| ENSG00000124749 | COL21A1 | collagen type XXI alpha 1 chain |
| ENSG00000050767 | COL23A1 | collagen type XXIII alpha 1 chain |
| ENSG00000158525 | CPA5 | carboxypeptidase A5 |
| ENSG00000109472 | CPE | carboxypeptidase E |
| ENSG00000150938 | CRIM1 | cysteine rich transmembrane BMP regulator 1 |
| ENSG00000169429 | CXCL8 | C-X-C motif chemokine ligand 8 |
| ENSG00000160683 | CXCR5 | C-X-C motif chemokine receptor 5 |
| ENSG00000080166 | DCT | dopachrome tautomerase |
| ENSG00000165325 | DEUP1 | deuterosome assembly protein 1 |
| ENSG00000267432 | DNAH17-AS1 | DNAH17 antisense RNA 1 |
| ENSG00000118997 | DNAH7 | dynein axonemal heavy chain 7 |
| ENSG00000007174 | DNAH9 | dynein axonemal heavy chain 9 |
| ENSG00000134757 | DSG3 | desmoglein 3 |
| ENSG00000198842 | DUSP27 | dual specificity phosphatase 27 (putative) |
| ENSG00000165891 | E2F7 | E2F transcription factor 7 |
| ENSG00000186976 | EFCAB6 | EF-hand calcium binding domain 6 |
| ENSG00000135373 | EHF | ETS homologous factor |
| ENSG00000188316 | ENO4 | enolase family member 4 |
| ENSG00000204334 | ERICH2 | glutamate rich 2 |
| ENSG00000171320 | ESCO2 | establishment of sister chromatid cohesion N-acetyltransferase 2 |
| ENSG00000264527 | ESP33 | uncharacterized locus ESP33 |
| ENSG00000135476 | ESPL1 | extra spindle pole bodies like 1, separase |
| ENSG00000229007 | EXOSC3P1 | exosome component 3 pseudogene 1 |
| ENSG00000198780 | FAM169A | family with sequence similarity 169 member A |
| ENSG00000125804 | FAM182A | family with sequence similarity 182 member A |
| ENSG00000175170 | FAM182B | family with sequence similarity 182 member B |
| ENSG00000104059 | FAM189A1 | family with sequence similarity 189 member A1 |
| ENSG00000269881 | FAM234A | family with sequence similarity 234 member A |
| ENSG00000164616 | FBXL21 | F-box and leucine rich repeat protein 21 (gene/pseudogene) |
| ENSG00000132185 | FCRLA | Fc receptor like A |
| ENSG00000181617 | FDCSP | follicular dendritic cell secreted protein |
| ENSG00000230316 | FEZF1-AS1 | FEZF1 antisense RNA 1 |
| ENSG00000275340 | FGD5P1 | FYVE, RhoGEF and PH domain containing 5 pseudogene 1 |
| ENSG00000102466 | FGF14 | fibroblast growth factor 14 |
| ENSG00000102678 | FGF9 | fibroblast growth factor 9 |

| | | |
|---|---|---|
| ENSG00000137440 | FGFBP1 | fibroblast growth factor binding protein 1 |
| ENSG00000232774 | FLJ22447 | uncharacterized LOC400221 |
| ENSG00000105255 | FSD1 | fibronectin type III and SPRY domain containing 1 |
| ENSG00000123689 | G0S2 | G0/G1 switch 2 |
| ENSG00000197093 | GAL3ST4 | galactose-3-O-sulfotransferase 4 |
| ENSG00000227135 | GCSAML-AS1 | GCSAML antisense RNA 1 |
| ENSG00000139278 | GLIPR1 | GLI pathogenesis related 1 |
| ENSG00000140478 | GOLGA6A (includes others) | golgin A6 family member C |
| ENSG00000170775 | GPR37 | G protein-coupled receptor 37 |
| ENSG00000138271 | GPR87 | G protein-coupled receptor 87 |
| ENSG00000167914 | GSDMA | gasdermin A |
| ENSG00000111305 | GSG1 | germ cell associated 1 |
| ENSG00000075218 | GTSE1 | G2 and S-phase expressed 1 |
| ENSG00000164588 | HCN1 | hyperpolarization activated cyclic nucleotide gated potassium channel 1 |
| ENSG00000162639 | HENMT1 | HEN1 methyltransferase homolog 1 |
| ENSG00000235527 | HIPK1-AS1 | HIPK1 antisense RNA 1 |
| ENSG00000183598 | HIST2H3D | histone cluster 2, H3d |
| ENSG00000212769 | HMGN2P8 | high mobility group nucleosomal binding domain 2 pseudogene 8 |
| ENSG00000276975 | HYDIN2 | HYDIN2, axonemal central pair apparatus protein (pseudogene) |
| ENSG00000146678 | IGFBP1 | insulin like growth factor binding protein 1 |
| ENSG00000142224 | IL19 | interleukin 19 |
| ENSG00000254294 | IMPDH1P6 | inosine monophosphate dehydrogenase 1 pseudogene 6 |
| ENSG00000123999 | INHA | inhibin alpha subunit |
| ENSG00000183856 | IQGAP3 | IQ motif containing GTPase activating protein 3 |
| ENSG00000170549 | IRX1 | iroquois homeobox 1 |
| ENSG00000176049 | JAKMIP2 | janus kinase and microtubule interacting protein 2 |
| ENSG00000184408 | KCND2 | potassium voltage-gated channel subfamily D member 2 |
| ENSG00000235262 | KDM5C-IT1 | KDM5C intronic transcript 1 |
| ENSG00000186185 | KIF18B | kinesin family member 18B |
| ENSG00000116852 | KIF21B | kinesin family member 21B |
| ENSG00000142945 | KIF2C | kinesin family member 2C |
| ENSG00000237649 | KIFC1 | kinesin family member C1 |
| ENSG00000124743 | KLHL31 | kelch like family member 31 |
| ENSG00000137812 | KNL1 | kinetochore scaffold 1 |
| ENSG00000205426 | KRT81 | keratin 81 |
| ENSG00000233930 | KRTAP5-AS1 | KRTAP5-1/KRTAP5-2 antisense RNA 1 |
| ENSG00000133317 | LGALS12 | galectin 12 |
| ENSG00000186152 | LILRP1 | leukocyte immunoglobulin-like receptor pseudogene 1 |
| ENSG00000170858 | LILRP2 | leukocyte immunoglobulin-like receptor pseudogene 2 |
| ENSG00000180422 | LINC00304 | long intergenic non-protein coding RNA 304 |
| ENSG00000214851 | LINC00612 | long intergenic non-protein coding RNA 612 |
| ENSG00000237945 | LINC00649 | long intergenic non-protein coding RNA 649 |
| ENSG00000242258 | LINC00996 | long intergenic non-protein coding RNA 996 |
| ENSG00000271856 | LINC01215 | long intergenic non-protein coding RNA 1215 |
| ENSG00000249667 | LINC01259 | long intergenic non-protein coding RNA 1259 |
| ENSG00000249911 | LINC01265 | long intergenic non-protein coding RNA 1265 |
| ENSG00000251396 | LINC01301 | long intergenic non-protein coding RNA 1301 |
| ENSG00000227467 | LINC01537 | long intergenic non-protein coding RNA 1537 |
| ENSG00000079435 | LIPE | lipase E, hormone sensitive type |
| ENSG00000260868 | LOC100128905 | uncharacterized LOC100128905 |
| ENSG00000234432 | LOC100129484 | uncharacterized LOC100129484 |
| ENSG00000278909 | LOC100130057 | uncharacterized LOC100130057 |
| ENSG00000237499 | LOC100130476 | uncharacterized LOC100130476 |
| ENSG00000257545 | LOC100287944 | uncharacterized LOC100287944 |
| ENSG00000250365 | LOC101927124 | uncharacterized LOC101927124 |
| ENSG00000226747 | LOC101927196 | uncharacterized LOC101927196 |
| ENSG00000250548 | LOC101927780 | uncharacterized LOC101927780 |
| ENSG00000235834 | LOC101928389 | uncharacterized LOC101928389 |
| ENSG00000255337 | LOC101928424 | uncharacterized LOC101928424 |
| ENSG00000261465 | LOC102723385 | uncharacterized LOC102723385 |

**Table S5: Continued**

| | | |
|---|---|---|
| ENSG00000270171 | LOC105376689 | uncharacterized LOC105376689 |
| ENSG00000233593 | LOC105378853 | |
| ENSG00000256050 | LOC107984678 | uncharacterized LOC107984678 |
| ENSG00000234665 | LOC403323 | uncharacterized LOC403323 |
| ENSG00000236780 | LOC644838 | uncharacterized LOC644838 |
| ENSG00000230445 | LRRC37A6P | leucine rich repeat containing 37 member A6, pseudogene |
| ENSG00000240720 | LRRD1 | leucine rich repeats and death domain containing 1 |
| ENSG00000235448 | LURAP1L-AS1 | LURAP1L antisense RNA 1 |
| ENSG00000187391 | MAGI2 | membrane associated guanylate kinase, WW and PDZ domain containing 2 |
| ENSG00000234456 | MAGI2-AS3 | MAGI2 antisense RNA 3 |
| ENSG00000078018 | MAP2 | microtubule associated protein 2 |
| ENSG00000008735 | MAPK8IP2 | mitogen-activated protein kinase 8 interacting protein 2 |
| ENSG00000199094 | mir-30 | microRNA 30a |
| ENSG00000208018 | mir-645 | microRNA 645 |
| ENSG00000263463 | MIR378I | microRNA 378i |
| ENSG00000162006 | MSLNL | mesothelin-like |
| ENSG00000101057 | MYBL2 | MYB proto-oncogene like 2 |
| ENSG00000250174 | MYLK-AS2 | MYLK antisense RNA 2 |
| ENSG00000272916 | NDST2 | N-deacetylase and N-sulfotransferase 2 |
| ENSG00000247809 | NR2F2-AS1 | NR2F2 antisense RNA 1 |
| ENSG00000167693 | NXN | nucleoredoxin |
| ENSG00000119547 | ONECUT2 | one cut homeobox 2 |
| ENSG00000099985 | OSM | oncostatin M |
| ENSG00000083454 | P2RX5 | purinergic receptor P2X 5 |
| ENSG00000257950 | P2RX5-TAX1BP3 | P2RX5-TAX1BP3 readthrough (NMD candidate) |
| ENSG00000174740 | PABPC5 | poly(A) binding protein cytoplasmic 5 |
| ENSG00000107719 | PALD1 | phosphatase domain containing, paladin 1 |
| ENSG00000231806 | PCAT7 | prostate cancer associated transcript 7 (non-protein coding) |
| ENSG00000248383 | PCDHAC1 | protocadherin alpha subfamily C, 1 |
| ENSG00000262576 | PCDHGA4 | protocadherin gamma subfamily A, 4 |
| ENSG00000056487 | PHF21B | PHD finger protein 21B |
| ENSG00000164530 | PI16 | peptidase inhibitor 16 |
| ENSG00000153823 | PID1 | phosphotyrosine interaction domain containing 1 |
| ENSG00000162896 | PIGR | polymeric immunoglobulin receptor |
| ENSG00000127564 | PKMYT1 | protein kinase, membrane associated tyrosine/threonine 1 |
| ENSG00000122861 | PLAU | plasminogen activator, urokinase |
| ENSG00000137841 | PLCB2 | phospholipase C beta 2 |
| ENSG00000136040 | PLXNC1 | plexin C1 |
| ENSG00000240694 | PNMA2 | paraneoplastic Ma antigen 2 |
| ENSG00000028277 | POU2F2 | POU class 2 homeobox 2 |
| ENSG00000184486 | POU3F2 | POU class 3 homeobox 2 |
| ENSG00000185250 | PPIL6 | peptidylprolyl isomerase like 6 |
| ENSG00000119938 | PPP1R3C | protein phosphatase 1 regulatory subunit 3C |
| ENSG00000158528 | PPP1R9A | protein phosphatase 1 regulatory subunit 9A |
| ENSG00000068489 | PRR11 | proline rich 11 |
| ENSG00000112812 | PRSS16 | protease, serine 16 |
| ENSG00000206549 | PRSS50 | protease, serine 50 |
| ENSG00000225706 | PTPRD-AS1 | PTPRD antisense RNA 1 |
| ENSG00000164611 | PTTG1 | pituitary tumor-transforming 1 |
| ENSG00000076344 | RGS11 | regulator of G-protein signaling 11 |
| ENSG00000253006 | RN7SKP283 | |
| ENSG00000263974 | RN7SL121P | RNA, 7SL, cytoplasmic 121, pseudogene |
| ENSG00000242853 | RN7SL749P | |
| ENSG00000164197 | RNF180 | ring finger protein 180 |
| ENSG00000251819 | RNU6-322P | RNA, U6 small nuclear 322, pseudogene |
| ENSG00000221340 | RNU6ATAC18P | |
| ENSG00000201558 | RNVU1-6 | RNA, variant U1 small nuclear 6 |
| ENSG00000213228 | RPL12P38 | ribosomal protein L12 pseudogene 38 |
| ENSG00000243422 | RPL23AP49 | ribosomal protein L23a pseudogene 49 |
| ENSG00000171848 | RRM2 | ribonucleotide reductase regulatory subunit M2 |
| ENSG00000160188 | RSPH1 | radial spoke head 1 homolog |
| ENSG00000105784 | RUNDC3B | RUN domain containing 3B |
| ENSG00000160307 | S100B | S100 calcium binding protein B |

| | | |
|---|---|---|
| ENSG00000183873 | SCN5A | sodium voltage-gated channel alpha subunit 5 |
| ENSG00000136546 | SCN7A | sodium voltage-gated channel alpha subunit 7 |
| ENSG00000135094 | SDS | serine dehydratase |
| ENSG00000012171 | SEMA3B | semaphorin 3B |
| ENSG00000232352 | SEMA3B-AS1 | SEMA3B antisense RNA 1 (head to head) |
| ENSG00000167680 | SEMA6B | semaphorin 6B |
| ENSG00000057149 | SERPINB3 | serpin family B member 3 |
| ENSG00000206073 | SERPINB4 | serpin family B member 4 |
| ENSG00000101049 | SGK2 | SGK2, serine/threonine kinase 2 |
| ENSG00000129946 | SHC2 | SHC adaptor protein 2 |
| ENSG00000171241 | SHCBP1 | SHC binding and spindle associated 1 |
| ENSG00000188991 | SLC15A5 | solute carrier family 15 member 5 |
| ENSG00000103257 | SLC7A5 | solute carrier family 7 member 5 |
| ENSG00000227258 | SMIM2-AS1 | SMIM2 antisense RNA 1 |
| ENSG00000206754 | SNORD101 | small nucleolar RNA, C/D box 101 |
| ENSG00000163071 | SPATA18 | spermatogenesis associated 18 |
| ENSG00000150628 | SPATA4 | spermatogenesis associated 4 |
| ENSG00000184005 | ST6GALNAC3 | ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 3 |
| ENSG00000127954 | STEAP4 | STEAP4 metalloreductase |
| ENSG00000169302 | STK32A | serine/threonine kinase 32A |
| ENSG00000144834 | TAGLN3 | transgelin 3 |
| ENSG00000182521 | TBPL2 | TATA-box binding protein like 2 |
| ENSG00000089225 | TBX5 | T-box 5 |
| ENSG00000240280 | TCAM1P | testicular cell adhesion molecule 1, pseudogene |
| ENSG00000253304 | TMEM200B | transmembrane protein 200B |
| ENSG00000165685 | TMEM52B | transmembrane protein 52B |
| ENSG00000118503 | TNFAIP3 | TNF alpha induced protein 3 |
| ENSG00000050730 | TNIP3 | TNFAIP3 interacting protein 3 |
| ENSG00000188001 | TPRG1 | tumor protein p63 regulated 1 |
| ENSG00000170893 | TRH | thyrotropin releasing hormone |
| ENSG00000142185 | TRPM2 | transient receptor potential cation channel subfamily M member 2 |
| ENSG00000157570 | TSPAN18 | tetraspanin 18 |
| ENSG00000214391 | TUBAP2 | tubulin alpha pseudogene 2 |
| ENSG00000276043 | UHRF1 | ubiquitin like with PHD and ring finger domains 1 |
| ENSG00000093134 | VNN3 | vanin 3 |
| ENSG00000075702 | WDR62 | WD repeat domain 62 |
| ENSG00000154764 | WNT7A | Wnt family member 7A |
| ENSG00000177752 | YIPF7 | Yip1 domain family member 7 |
| ENSG00000169064 | ZBBX | zinc finger B-box domain containing |
| ENSG00000221886 | ZBED8 | zinc finger BED-type containing 8 |
| ENSG00000091656 | ZFHX4 | zinc finger homeobox 4 |
| ENSG00000229956 | ZRANB2-AS2 | ZRANB2 antisense RNA 2 (head to head) |

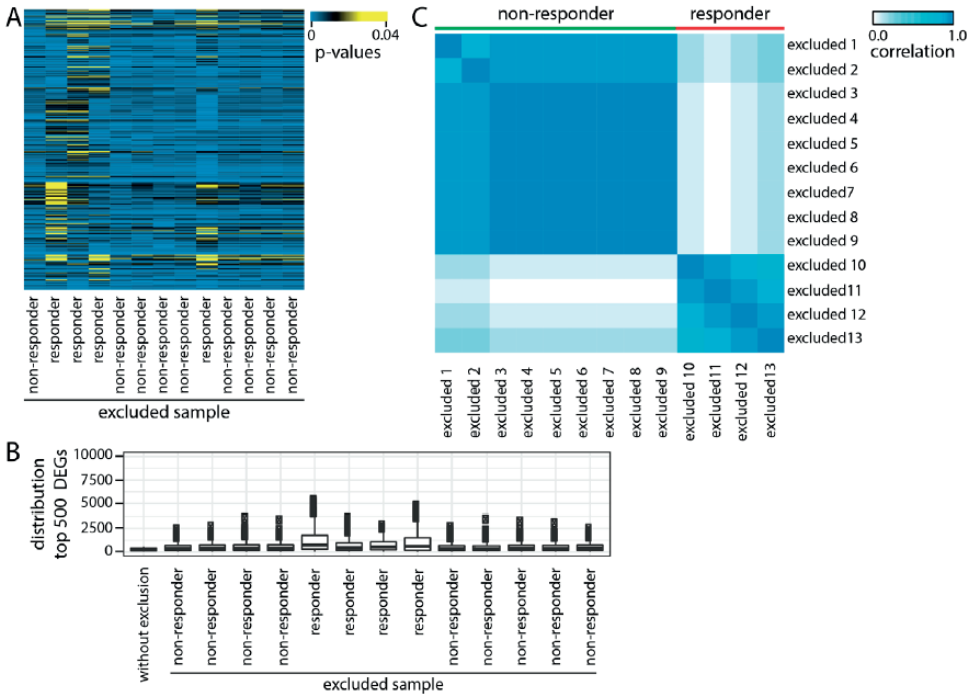


**Fig. S6. Stability assessment of differential gene expression analysis.**

(**A**) Heat map indicating P-values with leave-one-out cross validation experiment. Columns are held-out samples and rows are held-out genes. (**B**) Ranks of Differentially Expressed Genes (DEGs) in terms of P-values in the held-out experiment. (**C**) Consensus clustering of samples with DEGs obtained from each of the held-out experiment. Color bars indicate patient groups.

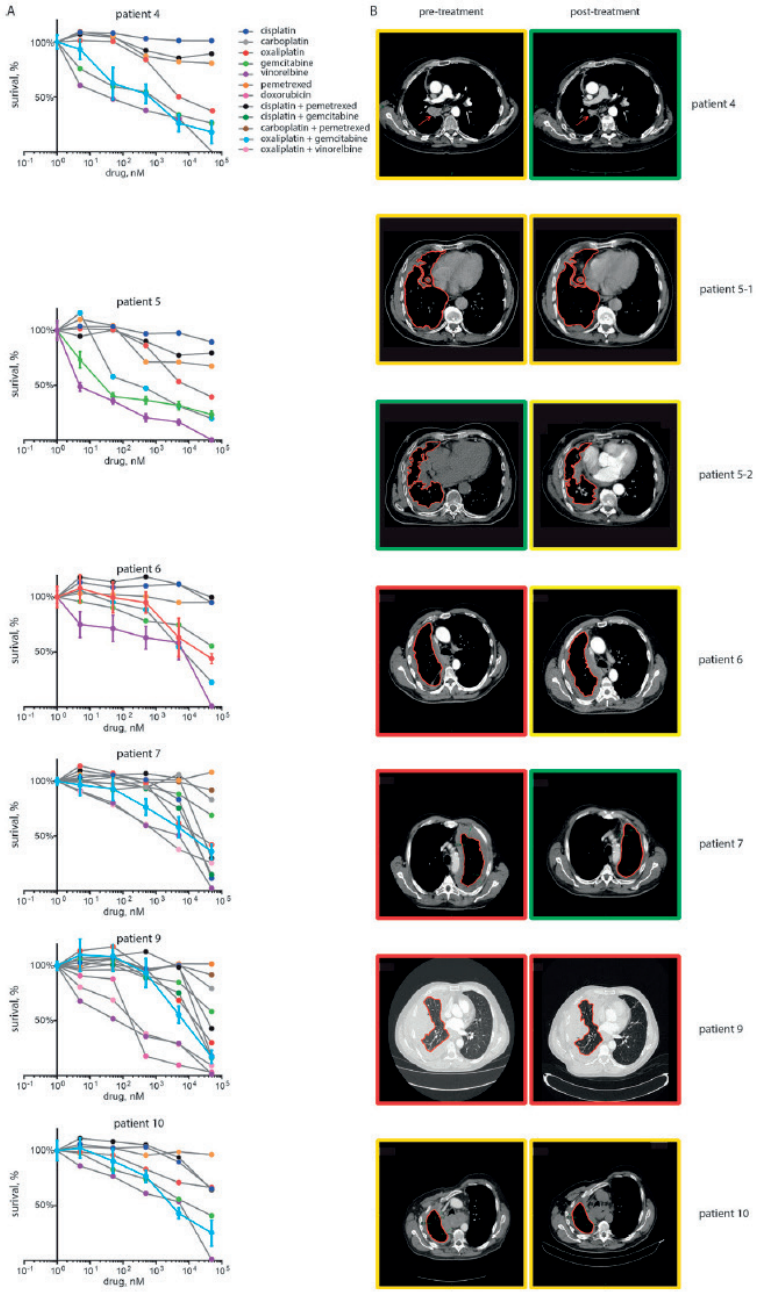


**Fig. S7. Dose-response curves and clinical responses.**

(**A**) Dose-response curves of primary tumor cultures performed for patients 4-7, 9 and 10. The chemotherapeutic agents that were administered to the patient are depicted in color, the other chemotherapeutics used in the screen are depicted with gray lines and colored dots. (**B**) CT-scans of patients 4-7, 9 and 10 before and after treatment with the chemotherapeutic agents selected by the drug screens. Response evaluation was done using RECIST modified for mesothelioma. Colored boxes around CT-scans indicate responses are as predicted by the drug screens. Green: partial response, yellow: stable disease, red: progressive disease. Tumor rinds are circumscribed by red lines.

CHAPTER 5

# 5

# Comprehensive Pharmacogenomic Profiling of Malignant Pleural Mesothelioma Identifies a Subgroup Sensitive to FGFR Inhibition

Josine M. Quispel-Janssen[1] | Jitendra Badhai[1] | Laurel Schunselaar[1] | Stacey Price[2] | Jonathan Brammeld[2] | Francesco Iorio[3] | Krishna Kolluri[4] | Matthew Garnett[2] | Anton Berns[1], Paul Baas[1] | Ultan McDermott[2] | Jacques Neefjes[1] | Constantine Alifrangis[2]

*Clinical Cancer Research; 24(1); 84–94 2017*

## Abstract

**Purpose:** Despite intense research, treatment options for patients with mesothelioma are limited and offer only modest survival advantage. We screened a large panel of compounds in multiple mesothelioma models and correlated sensitivity with a range of molecular features to detect biomarkers of drug response.

**Experimental design:** We utilized a high-throughput chemical inhibitor screen in a panel of 889 cancer cell lines, including both immortalized and primary early-passage mesothelioma lines, alongside comprehensive molecular characterization using Illumina whole-exome sequencing, copy-number analysis and Affymetrix array whole transcriptome profiling. Subsequent validation was done using functional assays such as siRNA silencing and mesothelioma mouse xenograft models.

**Results:** A subgroup of immortalized and primary MPM lines appeared highly sensitive to FGFR inhibition. None of these lines harbored genomic alterations of FGFR family members, but rather BAP1 protein loss was associated with enhanced sensitivity to FGFR inhibition. This was confirmed in an MPM mouse xenograft model and by BAP1 knockdown and overexpression in cell line models. Gene expression analyses revealed an association between BAP1 loss and increased expression of the receptors FGFR1/3 and ligands FGF9/18. BAP1 loss was associated with activation of MAPK signaling. These associations were confirmed in a cohort of MPM patient samples.

Conclusions: A subgroup of mesotheliomas cell lines harbor sensitivity to FGFR inhibition. BAP1 protein loss enriches for this subgroup and could serve as a potential biomarker to select patients for FGFR inhibitor treatment. These data identify a clinically relevant MPM subgroup for consideration of FGFR therapeutics in future clinical studies.

### Translational Relevance

Malignant pleural mesothelioma (MPM) has limited treatment options and a dismal prognosis. To date, targeted therapies have proved ineffective, and no druggable genetic alterations have been identified. Selecting compounds for further clinical evaluation in this small and heterogeneous patient group is challenging. By combining high-throughput drug screens, comprehensive molecular characterization and functional assays in multiple mesothelioma models, we were able to identify an FGFR inhibitor-sensitive subgroup with BAP1 loss as a potential predictive biomarker. Loss of BAP1 is found in up to 64% of MPM tumors. These data suggest that a significant group of patients with mesothelioma may benefit from FGFR inhibition.

## Introduction

Malignant pleural mesothelioma (MPM) is a tumor arising from the pleural cavity and is strongly associated with occupational exposure to asbestos. Although strict regulation is in place in more than 50 countries, in parts of the world where there is still widespread usage of asbestos, most notably in South America, Russia, and states of the former Soviet Republic, China, and South-East Asia, the incidence of this disease is rising (1, 2). MPM is highly refractory to conventional anticancer therapies, and the prognosis is poor; most patients die within a year of diagnosis. Surgery with curative intent is only possible in a highly selected group of patients and needs to be combined with chemotherapy. The only approved treatment, a combination of the cytotoxic agents cisplatin and pemetrexed, yields at best modest improvements in survival (3, 4). Despite many clinical studies utilizing novel biological therapies, there are as yet no effective targeted therapies for this cancer (5, 6).



A recent comprehensive genomic analysis of 216 MPM samples found BAP1, NF2, TP53, SETD2, and CDKN2A to be recurrently mutated or structurally rearranged (7). The landscape is thus one of mutated tumor suppressor genes and alterations in pathways as diverse as Hippo, mTOR, and TP53, as well as histone methylation. Such loss-of-function oncogenic events are typically considered "undruggable," but downstream programs of genes, activated as a consequence of such mutations, may themselves be tractable therapeutic targets. This is illustrated by NF2-deficient tumors with activated focal adhesion kinase (FAK). Defactinib, a FAK inhibitor, demonstrated efficacy in NF2-deficient tumors in vitro (8) but a subsequent clinical trial in mesothelioma was halted due to lack of efficacy. Other drugs tested to date that have failed to improve the outcome in MPM include EGFR inhibitors (9), Bcr–Abl inhibitors (10), thalidomide (11), bortezomib (12), and vorinostat (13). In many of these studies, a subgroup of patients appeared to derive some benefit. However, in MPM, it has been difficult to elucidate reproducible biomarkers that identify these sensitive subgroups. Some research groups have demonstrated coactivation of multiple RTK pathways in MPM tumors, which may provide a rationale for combination therapies with kinase inhibitors (14).

We aimed to utilize high-throughput chemical screening platforms alongside molecular characterization of immortalized and early-passage cell line models of MPM to uncover critical signaling pathways that may be amenable to therapeutic interrogation.

## Materials and Methods

### Cell lines and tissue culture

Cells are grown and maintained in either RPMI or DMEM F/12 supplemented with 10% FBS and 1% penicillin/streptomycin. Cell lines were maintained at 37$^{o}$ C at 5% $CO_2$. All cell lines have been verified by genotyping using short tandem repeat (STRs) profiling and Sequenom profiling of a panel of 92 single-nucleotide polymorphisms.

### Cell viability assays

Cells are trypsinized and counted before seeding at the optimal density for the well size (either 96-or 384-well plates were used) and duration of the assay. Seeding density was optimized by titration of the cells such that upon visual inspection of the control wells at the end of the assay, a confluency of 70% to 90% was observed allowing cells to grow in a linear phase. Adherent cell lines were seeded 24 hours before drug addition. The high-throughput chemical inhibitor screen was carried out using 384-well plates, and viability was measured 72 hours after drug addition with a 5-point serial fourfold concentration range of 265 compounds. All other viability assays were carried out using 96-well plates and a 9-point twofold dilution of the drugs. Drugs were all dissolved in DMSO, and DMSO was used only as a control condition. At the end of the experiment, cells were fixed with 4% paraformaldehyde. Following two washes with $dH_2O$, 100 mL of Syto60 nucleic acid stain (Invitrogen) was added to a final concentration of 1 mmol/L (a 1/5,000 stock dilution), and plates were fixed for 1 hour at room temperature. Quantification of fluorescent signal was achieved using a Paradigm (BD) plate reader using excitation/emission wavelengths of 630/695 nm. Data were analyzed by adjusting for background signals and normalizing each well to the DMSO-treated control.

### High-throughput screening compounds

Compounds were acquired from academic collaborators or commercial vendors. Each compound, its therapeutically relevant target substrate and pathway, and the minimum and maximum screening concentrations are listed in Supplementary Table S1. Compounds were stored as 10 mmol/L aliquots at -80$^{o}$ C and were subjected to a maximum of 5 freeze–thaw cycles. Each of the agents was screened at a 5-point serial fourfold dilution to provide a 256-fold range from the lowest to highest concentration. The concentrations selected for each compound were based on in vitro data to cover the range of concentrations known to inhibit relevant kinase activity and cell viability.

### Apoptosis assay

Cells were seeded in a flat-bottom 384 wells plate at optimal cell density. After 24 hours, PD173074 and AZD 4547 in a concentration range between 0.007813 and 1 mmol/L were added using a Tecan HP D300 Digital Dispenser. Five replicate wells were assayed for each

condition. Phenylarsine oxide (20 mmol/L) was used as positive control condition. To assess apoptosis, 5 mmol/L of IncuCyte caspase-3/7 green apoptosis assay reagent was added to the cells. Confluence and apoptosis levels were quantified by IncuCyte Zoom live-cell imaging systems from Essen Bioscience. Relative apoptosis was calculated by dividing the confluence of fluorescent apoptotic cells by total confluence and normalized to the positive control condition.

**Western blots**

Cell monolayers were lysed on ice in NP40 Cell Lysis Buffer (Invitrogen) containing fresh protease and phosphatase inhibitors (Roche). Lysates were centrifuged at 13,000 rpm for 10 minutes and the supernatant used for analyses. Protein concentration was calculated from a standard curve of BSA using the BCA assay (calbiotech) according to the manufacturer's instructions. Equal protein concentrations were loaded on pre-cast 4% to 12% Bis-Tris SDS-PAGE Gels (Invitrogen), run at 200 V for 1 hour. Proteins were transferred onto a methanol activated PVDF membrane at 100 V for 1 hour or overnight at 30 V. Membranes were blocked in 5% milk for 1 hour before the addition of primary antibody at a concentration recommended. After overnight incubation with the primary antibody at 4$^{O}$ C, the membrane was washed three times in 0.1% TBS-T followed by incubation with the secondary antibody according to the supplier's description at 1/2,500 dilution). Immunoblots were imaged using Pierce Supersignal Plus chemiluminescent kit on a gel imager (Syngene). Antibodies against BAP1, pERK, ERK, pFGFR (total), and pFGFR1 (all from Cell Signaling Technologies) and the polyclonal p-FGFR3 antibody sc-33041 (Santa Cruz Biotechnology) were used. Beta Tubulin was used as a loading control for Western blots. Phospho-RTK arrays (RD Systems) and caspase-Glo 3/7 assay were used according to the manufacturer's instructions.

**Establishment of early-passage primary mesothelioma tumor cell cultures**

All patients whose materials were used provided written informed consent for the use and storage of pleural fluid, tumor biopsies, and germline DNA. Diagnosis was made on tumor biopsies according to local IHC protocols and confirmed by the Dutch mesothelioma panel, a national expert panel of certified pathologists that evaluate all suspected mesothelioma patient samples. Early-passage primary mesothelioma cultures were generated from tumor cells isolated from pleural fluid of patients at the Netherlands Cancer Institute. The pleural fluid was centrifuged at 1,500 rpm for 5 minutes at room temperature. Erythrocyte lysis buffer was used to remove erythrocytes if many were present. Cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with peniciline/streptomycin and 8% fetal calf serum. The cells were seeded in T75 flasks at a density of 1 x $10^6$ cells/mL and incubated at 37$^{O}$ C at a humidified 5% $CO_2$ atmosphere. Medium was refreshed depending on cell growth, usually twice a week. At seeding and during the first two passages, cytospins were made and stained with HE and reviewed by our pathologist to determine the percentage of tumor cells. If the tumor percentage was over 70%, usually

reached after one passage, living cell cultures were transported to the Wellcome Trust Sanger Institute within 6 hours for drug screening and genetic analysis. Cells were cultured for a maximum period of 4 weeks.

**RNA interference and transfection**

Lipofectamine RNAiMAX (Thermofisher) was used according to product guidelines for transfection with siRNA against FGFR3 (Thermo Fisher Silencer Select s5167 and s5169) or BAP1 (s15822) utilizing the protocol "forward transfection of mammalian cell lines." KIF11 siRNA (s7902) was used as a transfection (positive) control. Viability or protein expression was assayed as described above, at specified time points. H226 cell expressing a BAP1 stable construct, and BAP1 C91A mutant lines were a kind gift from K Kolluri (UCL, London).

**Gene expression analyses**

Microarray data were generated on the Human Genome U219 96-Array Plate using the Gene Titan MC instrument (Affymetrix). The robust multi-array analysis (RMA) algorithm (15) was used to establish intensity values for each of 18562 loci (BrainArray v.10). We discarded transcripts with low sample variance and consolidated duplicated genes by averaging their expression values across duplicates. The resulting data were subsequently normalized ($\mu$=0; $\sigma$= 1) sample-wise and gene-median centered. Raw data were deposited in ArrayExpress (accession E-MTAB-3610). The RMA processed dataset is available at www.cancerrxgene.org/gdsc1000/GDSC1000_WebResources/Home.html. The expression-level signal of each gene was normalized using a nonparametric kernel estimation of its cumulative density function as described in ref. 16. Additionally, the normalized expression values were further tissue-centered using as grouping factors the cell line tissue labels of ref. 17.

**MPM mouse xenograft models**

All animal experiments were conducted according to institutional guidelines under protocol approved by the animal ethics committee of the Netherlands Cancer Institute. To establish xenografts, 3 million human mesothelioma cells (H2731 and MSTO211H) were implanted subcutaneously into the right dorsal flank of 6- to 7-week-old female nude SCID mice. Mice were randomized into vehicle and drugs treatment groups, and treatment was initiated once the tumor volumes reached approximately 200 $mm^3$. Tumor size was measured with calipers twice a week, and tumor volume was determined as $a \times b^2 \times 0.5$, where $a$ and $b$ were the large and small diameters, respectively.

## Results

### High-throughput chemical inhibitor screens in immortalized cell lines

A panel of 889 cancer cell lines was screened with 265 compounds that included targeted and cytotoxic compounds (for detail see http://www.cancerrxgene.org/). It was observed that three of 19 MPM lines (H2795, H2591, and MSTO-211H) had $IC_{50}$ values among the top 5% of cell lines showing highest sensitivity to the compound PD-173074, an FGFR1 and FGFR3 kinase inhibitor (Fig. 1A; ref. 15). These three cell lines, together with two additional MPM lines (NCI-H28, resistant; MPP-89, partially sensitive) and an FGFR-dependent lung cancer cell line harboring amplification of FGFR1 (NCI-H1581), were rescreened with PD-173074 and were as sensitive to PD-173074 as the FGFR1-dependent lung cancer line NCI-1581 (Fig. 1B). Furthermore, this sensitivity was also seen with two more selective FGFR inhibitors, NVP-BGJ398 and AZD4547 (Supplementary Fig. S1). Sensitivity to PD-173074 in the MPM cell lines was confirmed by clonogenic survival assays (Fig. 1C). Although some sensitive lines died by apoptosis, as is shown by activated caspase activity with both PD-173074 and the multi-FGFR-targeted inhibitor AZD4547 (Fig. 1D and E), not all sensitive lines showed a dose incremental increase in this marker. These data confirm previous findings (18) that a subset of MPM cell lines require FGF pathway activation for growth and survival, and that targeting this pathway could be a critical step in the control of these tumors.



### Drug sensitivity in early-passage MPM cultures

To test whether these observations could be reproduced in an independent cohort of primary mesothelioma cell lines, a panel of 11 pleural fluid-derived early-passage cultures from patients with MPM tumors were obtained and screened for viability using a panel of 48 small molecule inhibitors including PD-173074. Most of the early-passage cultures were resistant to virtually all agents (Supplementary Fig. S2). However, one MPM early-passage culture (NKI04) did demonstrate marked sensitivity to PD-173074. The sensitivity of NKI04 to FGFR inhibition was confirmed in a longer duration clonogenic survival assay, and the effect on cell viability was comparable with that seen in the FGFR1-amplified NCI-H1581 lung cancer cell line (Fig. 2A-C).

### Molecular characterization of FGF pathway signaling in cell lines and patient samples

In order to understand the basis for the observed sensitivity to FGFR inhibition, we analyzed whole-exome sequence and copy number array data for 21 MPM lines (http://cancer.sanger.ac.uk/cell_lines). There was no evidence of activating mutations or whole gene amplifications in any FGFR family member. RNA sequencing has been undertaken and shows no evidence of a fusion transcript involving any member of the FGFR family in any of the MPM cell lines (personal communication, M. Garnett). We then analyzed the corresponding gene expression data and focused on differential expression of FGFR and FGF family members in PD-173074-sensitive and -resistant MPM cell lines. Normalized expression of each of the FGF and FGFR

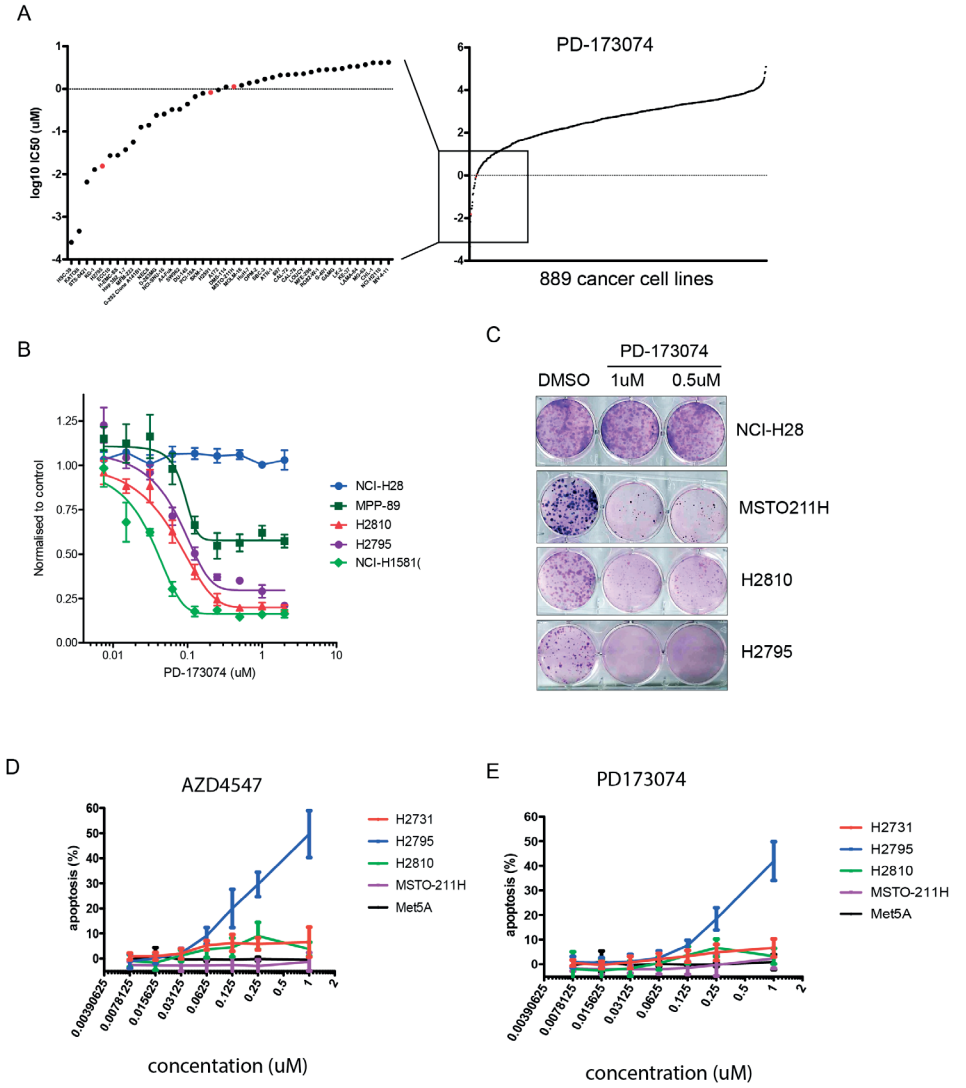


**Figure 1. Sensitivity to FGFR inhibition in established mesothelioma cell lines.**

(**A**) Sensitivity to FGFR inhibitor PD173074 expressed as logIC$_{50}$ value (inhibiting concentration that kills 50% of the cells) of each different cell line. The enlargement shows the 5% most sensitive cell lines with amongst them mesothelioma cell lines depicted in red. (**B**) Dose–response curves depicting the cell viability (mean ±SD) of different cell lines (y-axis) as a function of the dose of FGFR inhibitor PD-173074. NCI-H28, MPP-89, H2810, and H2795 are mesothelioma cell lines, while NCI-H1581 is an FGFR-dependent lung cancer cell line. (**C**) Fourteen-day clonogenic survival assay of selected mesothelioma cell lines (NCI-H28, MSTO-211H, H2810, and H2795), treated with FGFR inhibitor PD-173074 at concentrations of 500 nmol/L and 1 mmol/L. (**D**) FGFR inhibitor AZD4547 kills mesothelioma cell lines via induction of apoptosis as is demonstrated by an increase in caspase 3/7 activity after 48 hours of treatment with different doses of AZD4547 in a panel of MPM cell lines. (**E**) FGFR inhibitor PD173074 kills mesothelioma cell lines via induction of apoptosis as is demonstrated by an increase in caspase 3/7 activity after 48 hours of treatment with different doses of PD-173074 a panel of MPM cell lines.

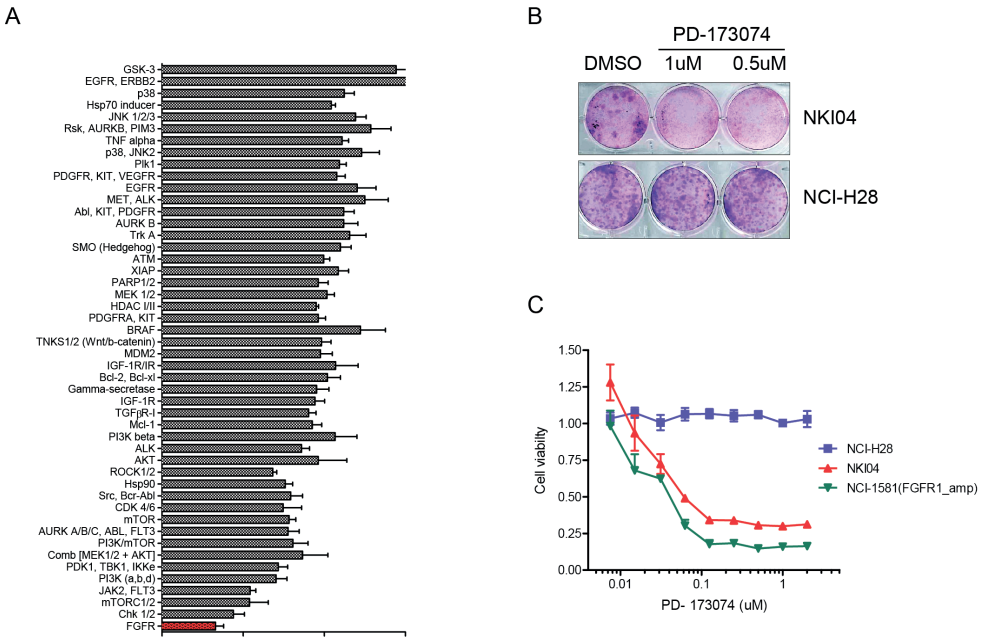


**Figure 2. Sensitivity to FGFR inhibitors in primary mesothelioma lines.**

(**A**) Cell viability (mean ±SD) of primary mesothelioma line NKI04 after treatment with a fixed does of 48 different small molecule inhibitors. This cell line is most sensitive to FGFR inhibition. (**B**) Fourteen-day clonogenic survival assay of primary mesothelioma line NKI04 compared with immortalized mesothelioma line NCI-H28 treated with FGFR inhibitor PD-173074 at concentrations of 500 nmol/L and 1 mmol/L. (**C**) Cell viability (mean ±SD) of primary mesothelioma line NKI04 compared with immortalized mesothelioma line NCI-H28 and FGFR-dependent lung cancer cell line NCI-H1581 (y-axis), as a function of the concentration of FGFR inhibitor PD-173074.

family genes was correlated with sensitivity to PD-173074 to explore whether the variation in any single family member, either ligand or receptor, was associated with response to FGFR inhibition. We found a statistically significant correlation between elevated FGF9 mRNA expression and response to PD-173074 (P=0.0148) and AZD4547 treatment (P=0.0098; Fig. 3A). FGF9 is a secreted, high-affinity ligand for the FGFR3 receptor, with low affinity for the FGFR1 and FGFR2 receptors (19). To determine whether a subset of MPM exhibits elevated expression of the FGF9 ligand in patients, we analyzed gene expression from a panel of 53 assorted MPM and matched normal lung clinical samples (Fig. 3B; ref. 20). Overall, we observed significantly higher FGF9 transcript levels in MPM tumors compared with pleura and lung normal tissue (P< 0.0001). Therefore, similar to our observation in the MPM cell lines, a subset of patient samples also demonstrates high levels of FGF9 expression.

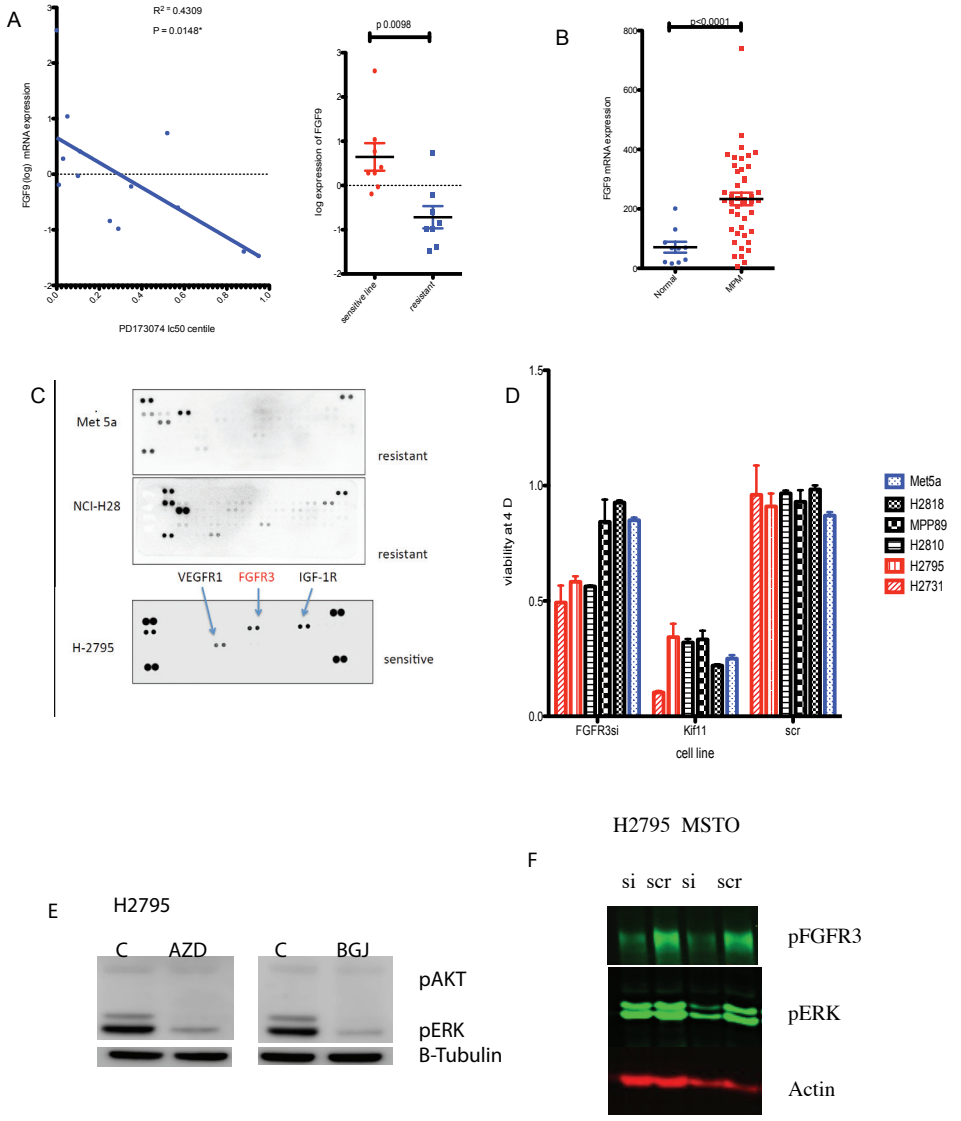


**Figure 3. FGFR inhibitor sensitivity is mediated by FGF axis signaling through FGF9 and FGFR3.**

(**A**) Scatterplot depicting sensitivity to FGFR inhibitor PD-173074 as a function of expression of FGF9. mRNA. Y-axis depicting log mRNA expression of FGF9 and x-axis showing centile of $IC_{50}$ to PD173074 of individual MPM cell line in cell line screen. High FGF9 gene expression is significantly correlated to high sensitivity to FGFR inhibition. Right hand scatterplot showing FGF9 expression correlates with sensitivity to AZD4547. (**B**) Expression of FGF9 in a set of MPM tumors, compared with normal lung and pleura, derived from GE0 dataset GSE2549. The mean expression in MPM tumors is significantly higher than that of normal lung and pleura. (**C**) Phospho-RTK array reveals phosphorylated-FGFR3 in FGFR inhibitor–sensitive cell line H2795 that is absent in two resistant lines (NCI-H28 and Met5a). (**D**) Cell viability of MPM cell lines after silencing of the FGFR3 transcript demonstrates reduced viability of FGFR inhibitor–sensitive cell lines H2795, H2810, and H2731 compared with FGFR inhibitor–resistant lines Met5A, NCI-H2052, H2818, and MPP89. Viability at 4 days post transfection is compared with Kif11–positive control siRNA and scrambled negative control. (**E**) Modulation of pERK signaling in H2795 cell line following 6 hours of exposure to DMSO (C) or 500 nmol/L AZD4547 or DMSO and 100 nmol/L BGJ398. (**F**) siRNA-mediated knockdown of pFGFR3 in H2795 and MSTO211H, showing effect on pFGFR3 and pERK versus scrambled control.

**Modulation of FGF/FGFR function in MPM lines**

A possible premise for the observed sensitivity of MPM lines that express high levels of FGF9 would be activation of the FGFR3 receptor kinase in an autocrine loop and subsequent engagement of prosurvival downstream signaling pathways. Indeed, a comparison of phosphorylation status of 42 receptor tyrosine kinases between a small sample of MPM cell lines demonstrated increased phosphorylation of FGFR3 in the sensitive line H2795 but not in resistant lines Met-5A and NCI-H28 (Fig. 3C).

To further confirm a critical role for FGFR3, this transcript was silenced by siRNA in a panel of MPM cell lines and the direct effect on cell viability was measured. Transient siRNA-mediated silencing of the FGFR3 transcript reduced cell viability in all 3 FGFR inhibitor-sensitive cell lines, but not in the FGFR inhibitor-resistant lines. This indicates a dependency on FGFR3 mediated signaling of the FGFR inhibitor-sensitive lines (Fig. 3D). As would be expected, inhibition of FGFR3 by the specific inhibitors AZD4547 and BJG398 decreased pERK levels (Fig. 3E), and this was also seen following siRNA-mediated silencing of FGFR3 in H2795 and MSTO-211H (Fig. 3F). The addition of the FGF9 ligand to MPM cells lacking baseline FGFR3 activation was able to induce phosphorylation of FGFR3 and a change in the growth kinetics of this cell line in a dose-dependent fashion (Supplementary Fig. S5).



**Role of BAP1 in modulating FGF pathway signaling**

Although we failed to identify genomic alterations in any member of the FGFR family that might explain the sensitivity to FGFR inhibition, we reasoned that this dependency might also be the consequence of other gene aberrations up- or downstream of FGFR3 signaling. We evaluated the gene expression and mutation database for other statistical associations explaining sensitivity to the FGFR inhibitor AZD4547 in the panel of MPM cell lines. We focused on driver mutations or copy-number alterations in three of the most frequently mutated genes in MPM, namely BAP1, NF2, and CDKN2A (7). We detected a weak but non-significant association between AZD4547 sensitivity and BAP1 mutations in the sensitive cell lines (Fig. 4A). Given that loss of BAP1 protein expression might also occur through nonmutational mechanisms as previously described (21), we additionally characterized BAP1 protein status in these lines by Western blot analysis (Supplementary Figs. S3 and S4). When sensitivity to the AZD4547 was correlated with BAP1 protein expression (low/absent vs. expressed), there was a significant correlation between loss of BAP1 expression and sensitivity (P=0.0208; Fig. 4B).

**Functional consequences of BAP1 modulation on FGFR signaling.**

Because silencing FGFR3 reduced cell viability in a subset of MPM lines, we next investigated whether this dependency on FGFR signaling was regulated by BAP1. BAP1 is a nuclear deubiquitinating enzyme with many unelucidated functions that might include modulation of the FGFR pathway. Silencing of BAP1 expression resulted in increased phosphorylation

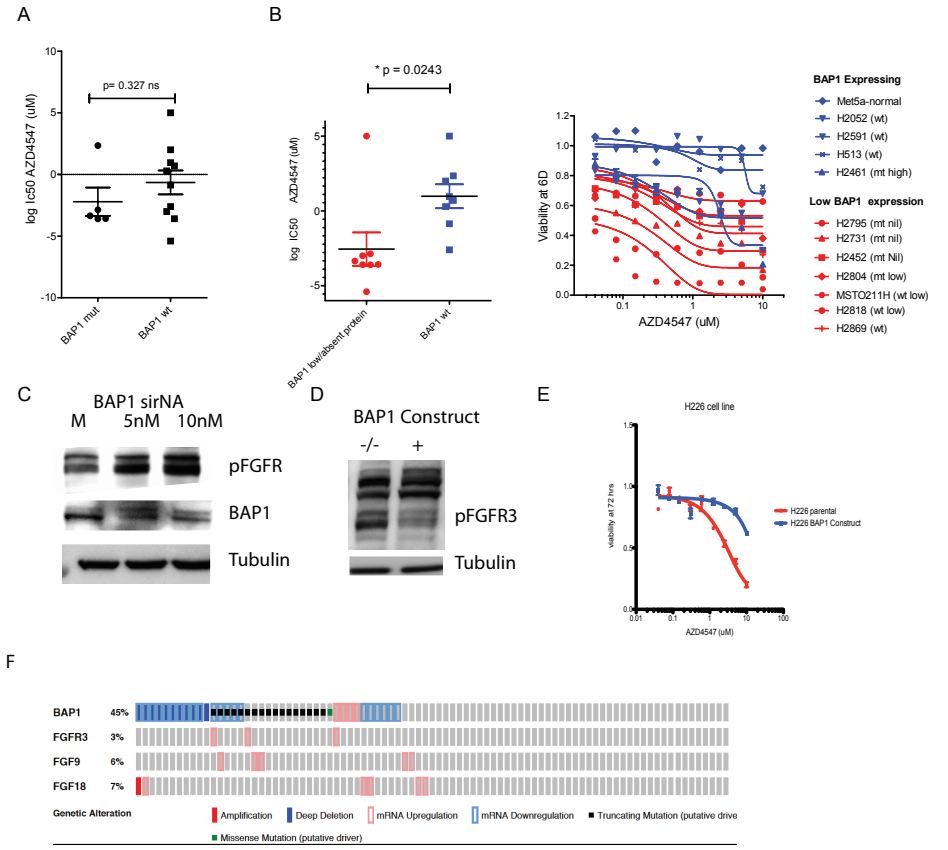


**Figure 4 Loss of BAP1 protein expression is correlated to FGFR inhibitor sensitivity.**

(**A**) Sensitivity to FGFR inhibitor AZD4547—expressed as logIC50 value—of cell lines, grouped according to BAP1 mutation status. The mean logIC50 value is not significantly different between the two groups. (**B**) Sensitivity to FGFR inhibitor AZD4547 according to BAP1 protein expression. Red are cell lines with low or absent BAP1 protein. Blue lines have normal BAP1 protein expression. Sensitivity (left) is expressed as logIC50 value (y-axis). The difference between the two groups is statistically significant. Cell viability (right) of different mesothelioma lines (y-axis) after treatment with FGFR inhibitor AZD4547 (x-axis). wt, wild-type; mt, mutant; high, high protein expression; low, low protein expression; nil, no protein expression. Right-hand panel showing dose–response curves of MPM cell lines treated with FGFR inhibitor AZD4547. Cell lines in red are lines with low or absent BAP1 protein expression. Blue lines have normal BAP1 protein expression. (**C**) SiRNA-mediated depletion of BAP1 in H2052 at increasing siRNA doses of 5 and 10 nmol/L versus mock transfected (M) control. Western blot comparing pFGFR3 and BAP1 expression at these conditions. Tubulin as loading control. (**D**) BAP1 overexpression in BAP1-null cell line H226. Western blot of BAP1 construct versus parental cell line baseline pFGFR levels with tubulin as loading control. (**E**) Cell viability after treatment with increasing doses of FGFR inhibitor AZD4547 in parental cell line H226 BAP1-null (red) and in the same cell line with BAP1 construct (red). BAP1 overexpression increases cell viability after FGFR inhibition. (**F**) Co-occurence of somatic mutations in BAP1 and FGFR family members in MPM tumors in the TCGA cohort.

of FGFR3 (Fig. 4C). Conversely, restoring BAP1 expression in the BAP1-null MPM line (Fig. 4D) H226 resulted in a decrease in pFGFR and a modest increase in resistance to the FGFR inhibitor AZD4547 (Fig. 4E).

We observed increased expression at the protein level in the BAP1 mutant cell lines of other RTK receptor genes and their appropriate ligands also known to be important in cell survival signaling in MPM such as PDGFRB, IGF1-R, and MET (22) using phospho-RTK arrays (Supplementary Fig. S4A and S4B). The H226-null MPM cell line was transfected with a wild-type BAP1 construct and a functionally inactive C91A-mutant BAP1 construct. Gene expression analysis on these two lines was performed and Signaling Pathway Impact Analysis (SPIA) of the data (Supplementary Table S) demonstrated that among the most significantly activated pathways in BAP1-inactive cells is the "Bladder Cancer" pathway including FGFR3 (arrow, Supplementary Fig. S6A) illustrated in Supplementary Figure S6B (23). In summary, the gene expression analysis demonstrates that BAP1 loss of function is associated with a transcriptional response upregulating not only FGFR signaling but also other RTKs such as PDGFRB, CMET, and IGF1R, that may be important mediators of cell growth and survival. However, only FGFR inhibitors showed a significant viability effect as single agents. We analyzed gene expression data from a study of 51 mesothelioma tumor samples to see if a similar effect on the FGFR pathway was seen *in vivo* (40 BAP1 wild-type and 11 mutant; GEO GSE29354; ref. 24). Amongst members of the FGFR signaling family, BAP1-mutant tumors did indeed demonstrate increased expression of FGF18, FGFR2, and FGFR3 relative to BAP1 wild-type tumors (Supplementary Table). To explore this association further in human tumors, we analyzed the available TCGA data and looked for the incidence of genetic and mRNA alterations of these genes in MPM tumors by BAP1 status (Fig. 4F). This showed the majority of dysregulation (10 of 14) events in FGF9, FGF18, and FGFR3 occurred in the context of BAP1 gene or mRNA dysregulation.



***FGFR inhibition in MPM xenograft model***

To assess the in vivo efficacy of targeting FGFR in MPM, we established a xenograft model using the FGFR inhibitor-sensitive MPM lines H2795 and MSTO-211H. Mice were treated with AZD4547, a selective inhibitor of FGFR1/2/3, which is currently being evaluated in clinical trials. We observed that treatment with AZD4547 resulted in significant growth inhibition in the H2795- and MSTO-211H-derived tumors (Fig. 5A). Furthermore, AZD45457 treated tumors showed a reduction in pERK signaling by immunohistochemistry compared with vehicle control-treated tumors (Fig. 5B), indicating target engagement by the drug in this model. Caspase activation was also seen in drug-treated tumors suggesting apoptosis (Supplementary Fig. S7).

***Combination therapeutic screen***

As the single-agent efficacy of FGFR inhibition was seen only in a subset of MPM cell lines, and because persistent pAKT pathway activation was seen in cell lines not responsive to FGFR inhibition, we hypothesized that a combination screen utilizing a PI3 Kinase inhibitor may reveal useful synergies. We undertook an anchor-based combination screen in 15 MPM cell lines using 95 small-molecule inhibitors (see Supplementary Table for details) selected

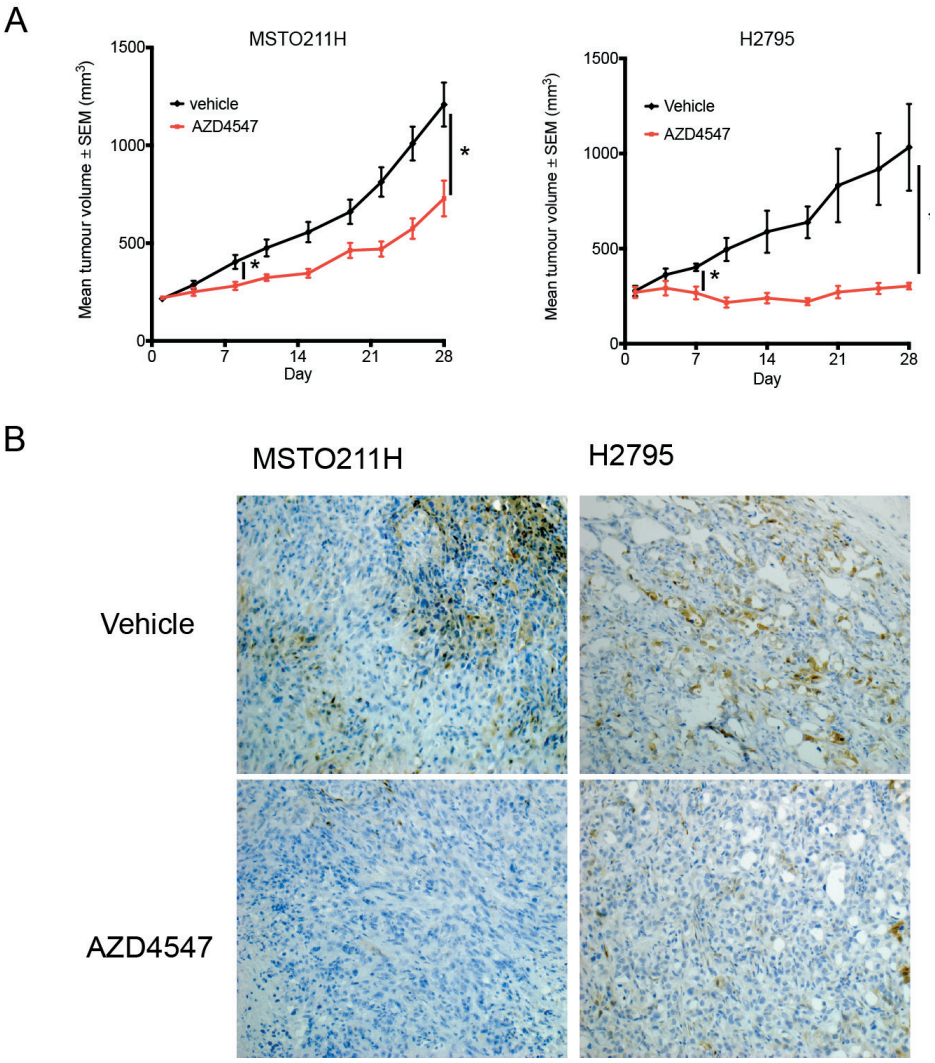


**Figure 5. Xenograft mouse model shows FGFR inhibition efficacy in vivo.**

(**A**) Xenograft mouse model using mesothelioma cell lines H2795 and MSTO211H. Mean tumor volume is depicted on the y-axis as a function of time (x-axis). Red lines indicate tumor growth in mice treated with FGFR inhibitor AZD4547, while the black lines indicate growth in vehicle-treated mice. (**B**) Immunohistochemistry of AZD4547- versus vehicle control-treated xenograft tumors. ppERK expression in representative tumors in drug-treated versus vehicle control groups.

to target many critical pathways in cancer, both as single agents and in combination with a fixed dose of the PI3 Kinase inhibitor AZD6482. The resulting difference in area under the curve (AUC) between single agent small-molecule inhibitor and the combination with AZD6482 was used to calculate synergy. The most recurrent synergistic interactions were seen with IGF1R inhibitor BMS-536924 and FGFR inhibitor PD-173074 (Supplementary Fig.

S8A) with synergy observed in seven and six of 15 lines, respectively. Supplementary Fig. S8B shows a validation dose-response curve of the FGFRi-resistant NCI H28 cell lines showing minimal effect of BMS 536824 or AZD6482 alone, but reduced viability and pAKT reduction with the combination. This cytotoxicity is not seen in the mesothelial control cell line Met5a, suggesting that the synergy is not generic but cell line specific.

## Discussion



Because MPM is a rare and heterogeneous tumor, it is notoriously difficult to identify and characterize responding subgroups in clinical trials. Our work illustrates the application and possibilities of comprehensive pharmacogenomic profiling approaches in intractable cancers such as MPM. The finding of FGFR inhibitor sensitivity in a subgroup of immortalized MPM cell lines represents a potentially novel therapeutic approach for this tumor type. As immortalized cell lines may undergo genetic drift, we also confirmed our findings in primary mesothelioma early-passage lines.

Dysregulation of the FGFR pathway has been described in many cancer types (25, 26). FGF9 signaling through FGFR3 has been shown to have a role in the development and progression of tumor cells in mouse models for NSCLC and prostate cancer (27). In MPM cell line models, we observed that high levels of the ligand FGF9 were strongly correlated with sensitivity to the FGFR inhibitor PD-173074 and AZD4547. We hypothesize that the effects of FGF9 are mediated through FGFR3 signaling, as illustrated by modulation of downstream ERK phosphorylation upon chemical inhibition with small-molecule inhibitors of FGFR3 and knockdown of FGFR3. FGFR3 is conversely not phosphorylated in cell lines insensitive to FGFRi, and this phosphorylation can be induced by the addition of synthetic FGF9 ligand. Interestingly, there was variability in FGF9 mRNA expression levels among the MPM cell lines, similar to what is observed in tumors in previously published studies. Recently, other groups demonstrated efficacy of FGFR inhibition in preclinical models of MPM mediated by other FGF-pathway members such as FGFR1 (18, 28, 29). We confirm the efficacy of a clinically utilized FGFR inhibitor including AZD4547 *in vivo* in MPM xenograft models. Furthermore, since undertaking these studies, early-phase clinical work with pharmacokinetic data has been published (30, 31) on AZD4547 and BGJ398. These have confirmed that the doses used in the *in vitro* work (100 nmol/L to 1 μmol/L) here are achievable in plasma *in vivo* and are able to modulate the target, with pharmacodynamic end points of target engagement with FRS2 downregulation and changes in serum phosphate levels seen.

FGF-receptors and -ligands are being targeted in clinical trials by both selective and nonselective FGFR TKI's and monoclonal antibodies (32) and AZD4547 has shown modest clinical activity in tumors with FGFR-pathway aberrant activation (33). In MPM dovitinib, a

multitargeting kinase inhibitor with activity against FGFR has been trialed and has failed in small cohort of patients with MPM (34). Because the data across tumor types demonstrate only a small group of patients responds to FGFR inhibition, it is crucial to find biomarkers that predict response to FGFR inhibition. Guagnano et al. integrated genomic and transcriptomic data of about 500 tumor cell lines with drug-sensitivity data to find predictive biomarkers for response to FGFR inhibitor NVP-BGJ398. A genetic alteration in one of the four FGF-receptors was found in 7% of cell lines, but only about half of the cell lines with such an alteration was found to be sensitive (35).

We did not find any mutation, amplification, or fusion transcripts of the FGFR-family in the inhibitor-sensitive MPM cell lines. The genes that were most recurrently altered in our MPM cell lines include CDKN2A, BAP1, and NF2. The frequency at which these genes were mutated is broadly similar to those previously described in clinical MPM samples (6, 7).

We show that loss of BAP1 expression was associated with sensitivity to FGFR inhibition. This finding was further validated with modulation of pFGFR-signaling and dose-response kinetics to FGFR inhibition following siRNA-mediated knockdown and BAP1 overexpression in MPM cell lines. Caveats with this association were also observed: NCI-H28 was one of the most resistant cell lines to FGFR inhibition but carried a BAP1 homozygous deletion, suggesting that BAP1 loss may enrich for FGFR inhibitor-sensitive cell lines but that some heterogeneity of drug response may still be observed. BAP1 (BRCA-associated protein 1) is a nuclear deubiquinating enzyme that controls gene expression by interaction with numerous transcription factors and other complexes, including those of the double strand DNA-break repair machinery (36). BAP1 thus influences cell-cycle progression (37) and double-strand DNA break repair (38). We show here that its loss may also affect gene expression of FGF pathway members, thereby enhancing signaling through this pathway.

The BAP1 gene is inactivated by somatic mutation in 23% to 64% of patients with MPM and between 1% and 47% in other tumor types (24, 39-43). Furthermore, BAP1 protein levels are undetectable in about 25% of MPM with normal BAP1 gene status, likely by epigenetic modification (24). BAP1 loss was observed to enrich for FGFR inhibitor-sensitive MPM lines, and expression of C91 hydrolase inactive mutant versus wild-type BAP1 protein in the H226 cell line induced activation of FGFR3 signaling. We hypothesize that inactivation of BAP1 in MPM, possibly through its function as a ubiquitin hydrolase, induces changes in gene expression of both FGF-family ligands and receptors to stimulate cell growth and survival.

We performed a combination drug screen to assess the impact of novel combinations of targeted therapies on MPM cell lines. On the 15 MPM cell lines screened, we found that FGFR and IGF1R inhibitors were the most recurrently synergistic with the PI3-Kinase inhibitor AZD6482. This is the first time, to our knowledge, that both a single agent and

combination therapeutic screen have been performed, which point to the primacy of the FGFR signaling pathway in MPM. Interestingly, one of the most resistant cell lines to FGFR inhibition was amenable to treatment with AZD6482 plus IGF1R inhibition with evidence of ablation of pAKT with the combination of drugs but not with either alone, implying true synergy. Previous studies have identified that multiple RTK's are active in MPM (14), and this has provided some rationale to consider combination therapies to overcome innate resistance to targeted therapies. It is also interesting to speculate as to whether IGF1R plus Pi3K inhibition would be of use in acquired resistance to FGFR inhibitors.

## Conclusion



High-throughput drug screening revealed a subset of both immortalized and primary mesothelioma cell lines to be highly sensitive to FGFR inhibition. This sensitivity was mediated through FGFR3 and was associated with loss of BAP1 protein expression. The high incidence of BAP1 protein loss in MPM tumors implies potential benefit from FGFR inhibition for a substantial subset of this patient group. In addition, our anchor-based screens revealed synergistic combinations that helped to overcome innate resistance to FGFR inhibition.

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## Supplemental Data

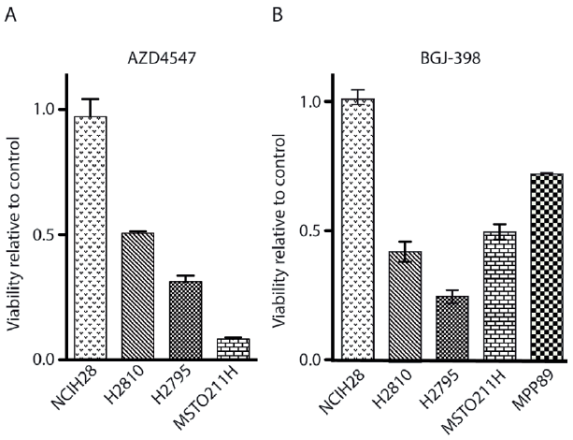



**Figure. S1. A subset of MPM cell lines respond to FGFR inhibition.**

Cell viability of selected mesothelioma cell lines (NCI-H28, H2810, H2795, MSTO-211H and MPP-89) after 72 hours of treatment with (A) AZD4547 at a fixed dose of 500 nmol/L and (B) BGJ398 at a fixed dose of 300 nmol/L

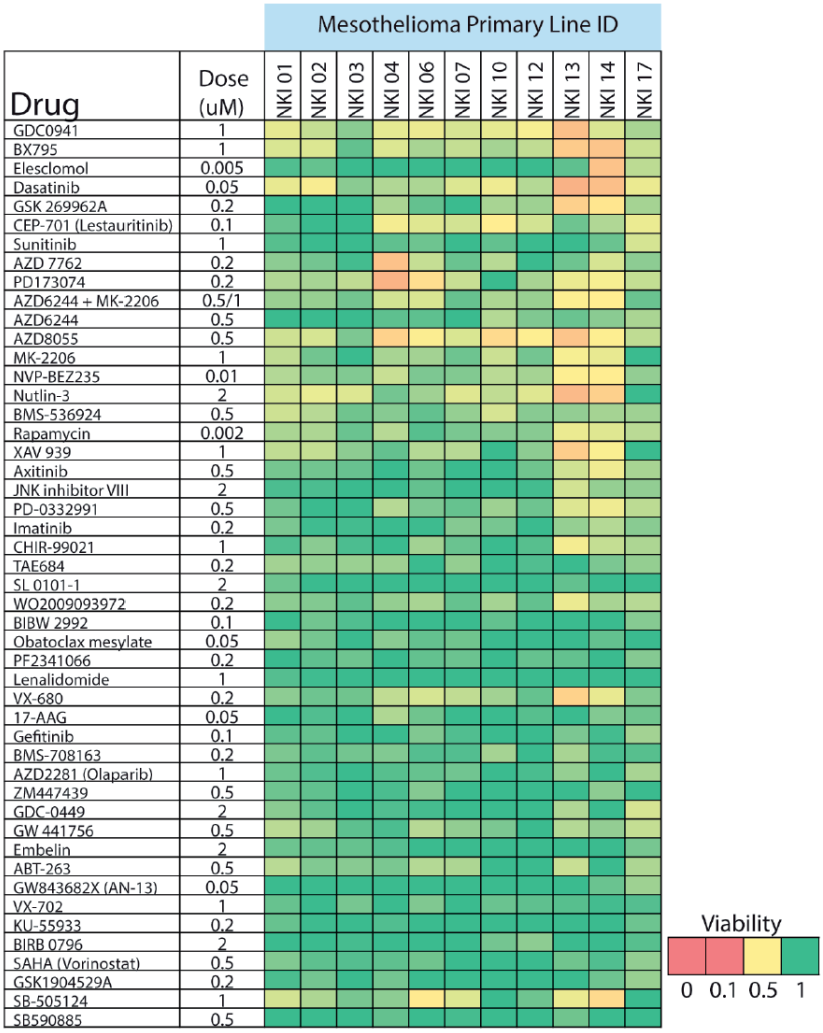
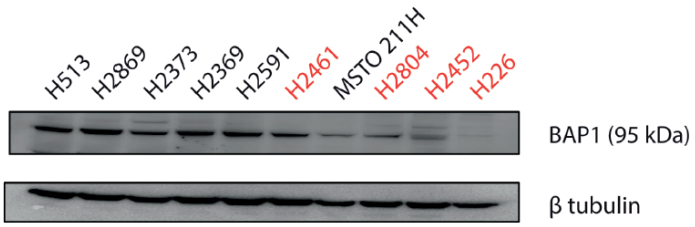



**Figure. S2. A subset of pleural fluid derived early passage primary cultures (EPL) respond to FGFR inhibition.**

Cell viability of 11 early passage primary cultures (columns) after treatment with a fixed dose of 48 small molecule inhibitors (rows), depicted in a color scale (green: 100% cell viability; red: 0% cell viability).

A




B

| Cell line | CHR | GENE_NAME | CDS_SYNTAX | AA_MUT_SYN | Class | DESCRIPTION |
|---|---|---|---|---|---|---|
| H2461 | 3 | BAP1 | c.65delT | p.F22fs*50 | indel | Deletion - Frameshift |
| H2722 | 3 | BAP1 | c.? | p.0 | indel | HomDel |
| H2731 | 3 | BAP1 | c.123-15_131( | p.? | indel | Essential Splice |
| H2795 | 3 | BAP1 | c.38-7_49del1 | p.? | indel | Essential Splice |
| H2804 | 3 | BAP1 | c.37+4_37+5in | p.? | indel | Essential Splice |
| IST-MES1 | 3 | BAP1 | c.1983+1_198 | p.? | indel | Essential Splice |
| NCI-H2452 | 3 | BAP1 | c.284C>A | p.A95D | Missense | Substitution - Missense |
| NCI-H28 | 3 | BAP1 | c.438-19_441( | p.? | indel | Essential Splice |

**Figure. S3. BAP1 mutation status does not correlate fully with protein expression.**

(A) Western Blot showing BAP1 protein expression in several MPM cell lines, both BAP1 wild type (black) and mutant lines (red). Beta-tubulin represents the protein loading control. (B) List of somatic mutations in BAP1 seen in MPM cell lines.

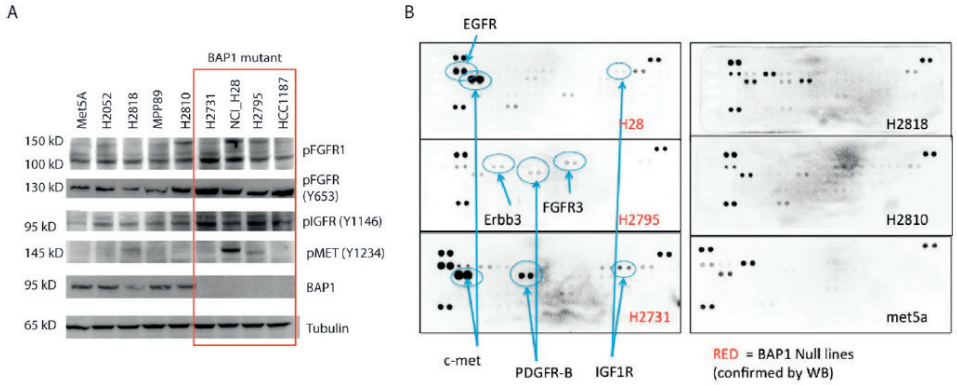



**Figure. S4. BAP1 null cell lines show increased activity of multiple tyrosine kinases.**

(A) Western Blot showing BAP1 protein expression in several MPM cell lines as well as activation in IGFR, MET and FGFR. (B) Phospho-RTK array panel showing baseline RTK-activation of BAP1 mutant (highlighted in red) versus wild type mesothelioma cell lines.

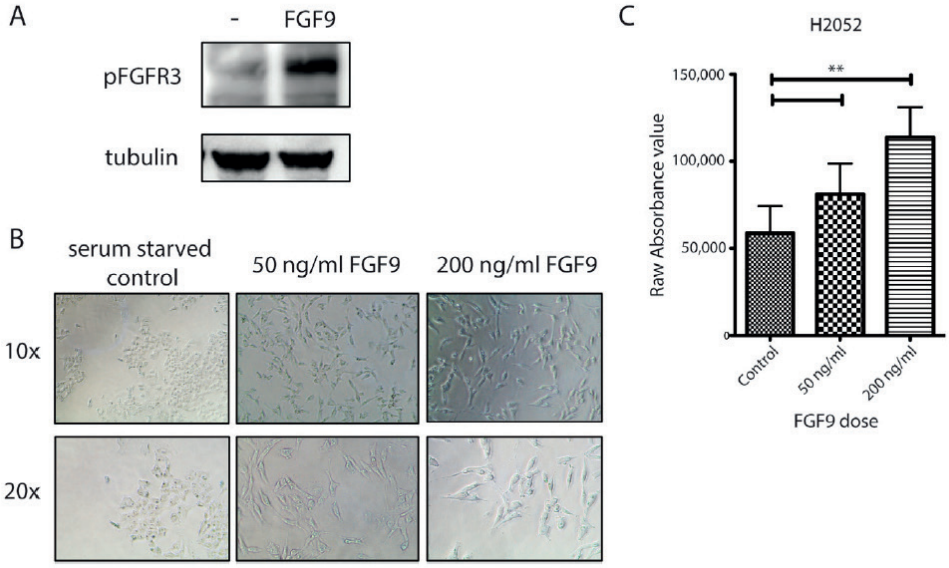



**Figure. S5. FGF9 activated FGFR3 modulates growth and phenotype.**

(A) Western Blot of pFGFR in serum-starved H2052 MPM cell line at baseline and following the addition of recombinant FGFR9 ligand (50 ng/mL ) after 1 hour. (B) Light microscopy at 10x and 20x magnification of H2052 cell line under serum-starved conditions and with the addition of FGF9 ligand at 2 concentrations. (C) Comparative viability of H2052 by SYTO60 assay at baseline and following the addition of FGF9 ligand at 50ng/mL and 200ng/mL.

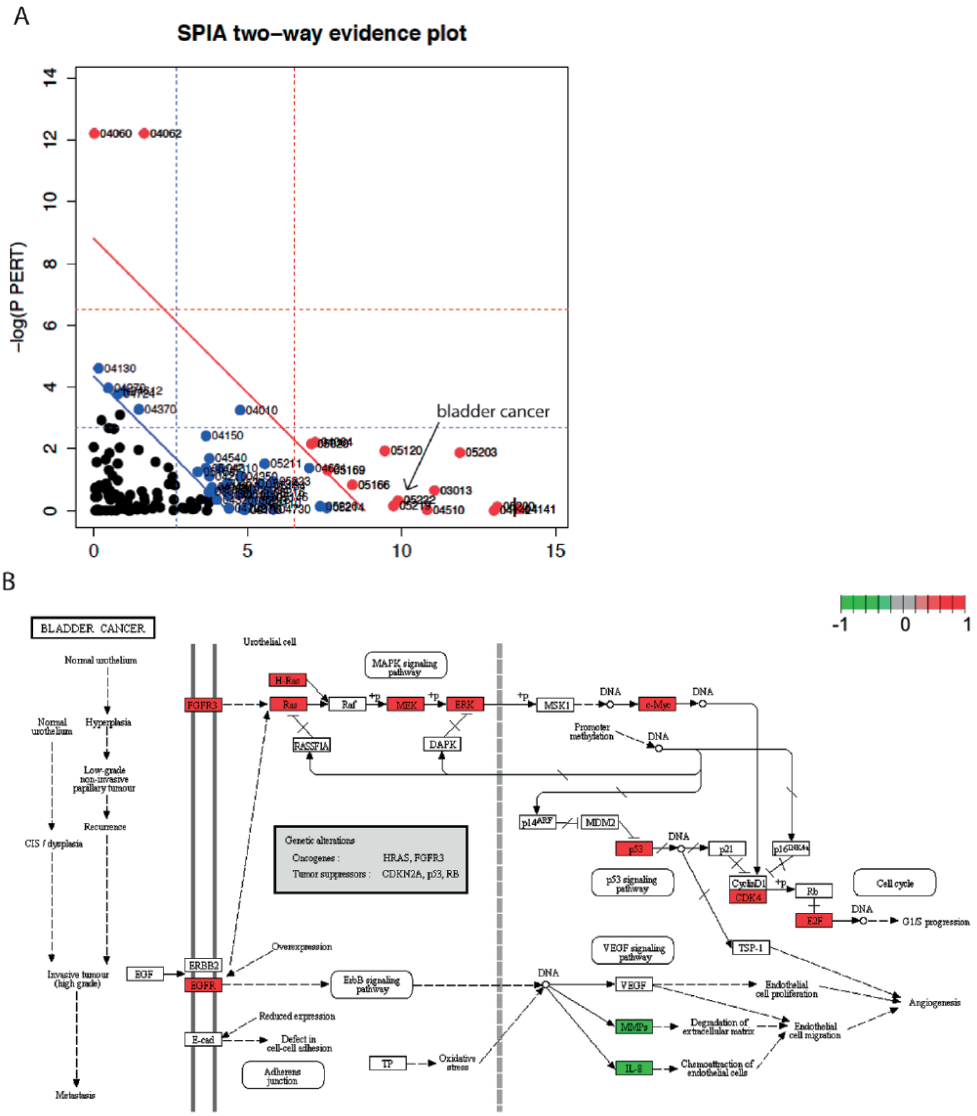



**Figure. S6. BAP1 modulation and FGFR pathway activation by gene expression.**

(A) Gene expression analysis of H226 cell line (BAP1 null) transfected with wild type BAP1 construct versus BAP1 inactive (C91A) construct. SPIA pathway analysis of C91A versus wild type cell line revealed the KEGG 'bladder cancer' pathway to be significantly activated in C91A cell line. (B) 'Bladder cancer' pathway showing genes that are overexpressed in the C91A line in red.

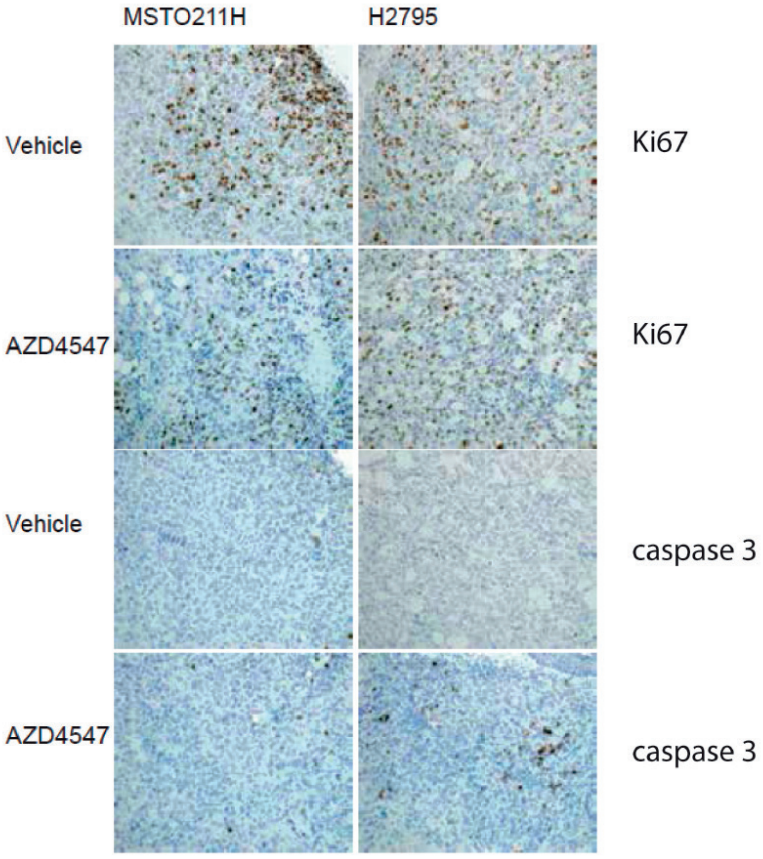



**Figure. S7. Xenograft tumor immunohistochemistry.**

Immunohistochemistry for Caspase3 and Ki67 in MPM xenograft tumors AZD4547-treated conditions compared to vehicle control.

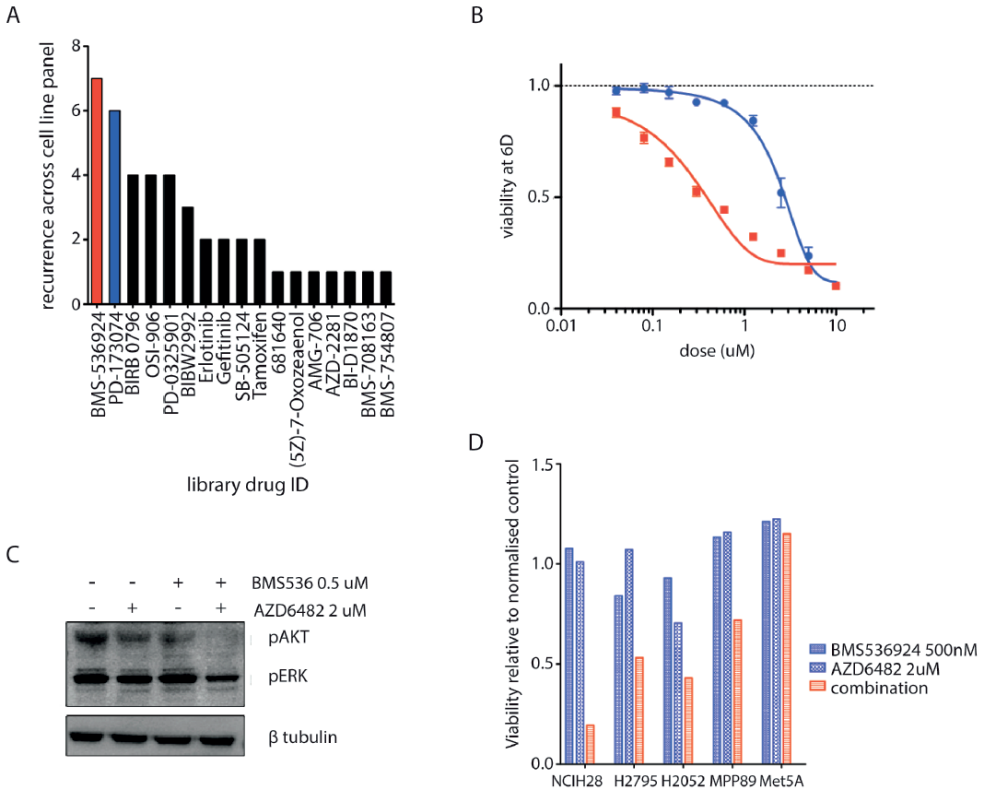



**Figure. S8. Combination drug screen of PI3Kinase inhibitor plus drug library in MPM cell lines.**

(A) Bar chart showing recurrent synergistic events in a combination screen with PI3K inhibitor AZD6482 plus 95 small molecule inhibitors across 15 MPM cell lines. (B) Validation of synergy between IGF1-R inhibitor BMS-536924 and PI3K inhibitor AZD6482 in NCO-H28 (FGFRi resistant cell line). Dose-response kinetics of BMS-536924 alone (blue) or with fixed dose (2μM) of AZD6482 (red). (C) Immunoblot of NCI-H28 FGFRi resistant cell line treated with a combination of IGF-1R inhibitor BMS-536924 and PI3K inhibitor AZD6482 showing loss of pAKT with combination treatment. (D) Cell Titer Blue quantification of 2 week clonogenic survival assay of 5 MPM cell lines with of IGF-1R inhibitor BMS-536924 alone and in combination with PI3K inhibitor AZD6482.

**Table S1. List of compounds used in the chemical inhibitor screen**

| Identifier | Name | Synonyms | Brand name | Action | Clinical Stage | Putative Target | Targeted process/ pathway |
|---|---|---|---|---|---|---|---|
| 1 | Erlotinib | AY-22989,Sirolimus,WY-090217 | Tarceva | targeted | clinically approved | EGFR | EGFR signaling |
| 3 | Rapamycin | | Rapamune | targeted | clinically approved | MTOR | TOR signaling |
| 5 | Sunitinib | | Sutent | targeted | clinically approved | PDGFRA, PDGFRB, KDR, KIT, FLT3 | RTK signaling |
| 6 | PHA-665752 | zLLL | | targeted | experimental | MET | RTK signaling |
| 9 | MG-132 | BMS-181339-01 | | targeted | experimental | Proteasome | other |
| 11 | Paclitaxel | 11-deoxojervine | Taxol | cytotoxic | clinically approved | Microtubules | cytoskeleton |
| 17 | Cyclopamine | | | targeted | experimental | SMO | other |
| 29 | AZ628 | BAY-43-9006 | | targeted | experimental | BRAF | ERK MAPK signaling |
| 30 | Sorafenib | MK-045,MK-0457,VX-68 | Nexavar | targeted | clinically approved | PDGFRA, PDGFRB, KDR, KIT, FLT3 | RTK signaling |
| 32 | VX-680 | STI-571 | MK-0457 | targeted | in clinical development | AURKA, AURKB, AURKC, FLT3, ABL1, JAK2 | mitosis |
| 34 | Imatinib | KIN001-017 | Gleevec | targeted | clinically approved | ABL, KIT, PDGFR | ABL signaling |
| 35 | NVP-TAE684 | PF-02341066 | TAE684 | targeted | experimental | ALK | RTK signaling |
| 37 | Crizotinib | Saracatinib,KIN001-045 | Xalkori | targeted | in clinical development | MET, ALK | RTK signaling |
| 38 | AZD-0530 | NSC 83265 | | targeted | in clinical development | SRC, ABL1 | ABL signaling |
| 41 | S-Trityl-L-cysteine | Z-L-Norleucine-CHO | | targeted | experimental | KIF11 | mitosis |
| 45 | Z-LLNle-CHO | KIN001-005 | na | targeted | experimental | g-secretase | other |
| 51 | Dasatinib | KIN001-013 (GNF-2 / 3-(6-(4-(trifluoromethoxy) phenylamino)pyrimidin-4-yl) benzamide) | Sprycel | targeted | clinically approved | ABL, SRC, KIT, PDGFR | ABL signaling |

**Table S1. Continued**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 52 | GNF-2 | KIN001-019 | | targeted | experimental | ABL [T315I] | ABL signaling |
| 53 | CGP-60474 | CINK4,KIN001-021 | | targeted | experimental | CDK1,CDK2,CDK5, CDK7,CDK9 | cell cycle |
| 54 | CGP-082996 | A770041,KIN001-111 | | targeted | experimental | CDK4 | cell cycle |
| 55 | A-770041 | KIN001-112 | A770041 | targeted | experimental | SRC family | other |
| 56 | WH-4-023 | KIN001-123 | | targeted | experimental | SRC family, ABL | ABL signaling |
| 59 | WZ-1-84 | KIN001-124 | | targeted | experimental | BMX | other |
| 60 | BI-2536 | KIN001-126 | NPK33-1-98-1 | targeted | in clinical development | PLK1, PLK2, PLK3 | mitosis |
| 62 | BMS-536924 | KIN001-127 | BMS-536924 | targeted | experimental | IGF1R | IGFR signaling |
| 63 | BMS-509744 | KIN001-128 | BMS-509744 | targeted | experimental | ITK | other |
| 64 | CMK | | Chloromethylketone Rsk inhibitor | targeted | experimental | RSK | ERK MAPK signaling |
| 71 | Pyrimethamine | | Daraprim | cytotoxic | clinically approved | Dihydrofolate reductase (DHFR) | DNA replication |
| 83 | JW-7-52-1 | KIN001-139 | | targeted | experimental | MTOR | TOR signaling |
| 86 | A-443654 | KIN001-134 | | targeted | experimental | AKT1, AKT2, AKT3 | PI3K signaling |
| 87 | GW843682X | MS275 | GW843682X (AN-13) | targeted | experimental | PLK1 | mitosis |
| 88 | MS-275 | | | targeted | in clinical development | HDAC | chromain histone acetylation |
| 89 | Parthenolide | KIN001-135 | | targeted | in clinical development | NFKB1 | other |
| 91 | KIN001-135 | | | targeted | experimental | IKKE | other |
| 94 | TGX221 | LDP-341, PS-341 | | targeted | experimental | PI3Kbeta | PI3K signaling |
| 104 | Bortezomib | XMD8-85 | Velcade | targeted | clinically approved | Proteasome | other |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 106 | XMD8-85 | Seliciclib | | targeted | experimental | MAP2K5 (ERK5) | other |
| 110 | Roscovitine | 3-Phenyl-N-[2,2,2-trichloro-1-[[(8-quinolinylamino) thioxomethyl]amino]ethyl]-2-propenamide | | targeted | in clinical development | CDKs | cell cycle |
| 111 | Salubrinal | Tykerb, Tyverb | | targeted | experimental | GADD34-PP1C | other |
| 119 | Lapatinib | KIN001-155 | Tykerb, Tyverb | targeted | clinically approved | ERBB2, EGFR | EGFR signaling |
| 127 | GSK269962A | Doxil,Rubex | | targeted | experimental | ROCK1, ROCK2 | cytoskeleton |
| 133 | Doxorubicin | VP-16 | Adriamycin | cytotoxic | clinically approved | DNA intercalating | DNA replication |
| 134 | Etoposide | LY-188011 | Etophophos | cytotoxic | clinically approved | TOP2 | DNA replication |
| 135 | Gemcitabine | | Gemzar | cytotoxic | clinically approved | DNA replication | DNA replication |
| 136 | Mitomycin C | | | cytotoxic | clinically approved | DNA crosslinker | DNA replication |
| 140 | Vinorelbine | | Navelbine | cytotoxic | clinically approved | Microtubules | cytoskeleton |
| 147 | NSC-87877 | ICI-176334 | | targeted | experimental | PTPN6 (SHP-1), PTPN11 (SHP-2) | other |
| 150 | Bicalutamide | QS11 | Casodex | targeted | clinically approved | ANDR (androgen receptor) | other |
| 151 | QS11 | [2-(6,7-dimethoxyquinazolin-4-yl)-5-(pyridin-2-yl)-2H-1,2,4-triazol-3-amine] | | targeted | experimental | ARFGAP | other |
| 152 | CP466722 | PKC 412 | | targeted | experimental | ATM | Genome integrity |
| 153 | Midostaurin | CT 99021 | | targeted | in clinical development | KIT | RTK signaling |
| 154 | CHIR-99021 | KIN001-192 | | targeted | experimental | GSK3B | WNT signaling |
| 155 | AP-24534 | KIN001-193 | Ponatinib | targeted | in clinical development | ABL | ABL signaling |
| 156 | AZD6482 | KIN001-204 | | targeted | in clinical development | PI3Kbeta | PI3K signaling |
| 157 | JNK-9L | (KIN001-205) | | targeted | experimental | JNK | JNK and p38 signaling |
| 158 | PF-562271 | KIN001-206 | | targeted | experimental | FAK | cytoskeleton |

**Table S1. Continued**

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|---|---|---|---|---|---|---|---|
| 159 | HG-6-64-1 | JQ1 | | targeted | experimental | BRAFV600E, TAK, MAP4K5 | ERK MAPK signaling |
| 163 | JQ1 | JQ12 | | targeted | experimental | BRD4 | chromatin other |
| 164 | JQ12 | Dimethyloxalylglcine | | targeted | experimental | HDAC | chromain histone acetylation |
| 165 | DMOG | | | targeted | experimental | Prolyl-4-Hydroxylase | other |
| 166 | FTI-277 | AR-12 | | targeted | experimental | Farnesyl transferase (FNTA) | other |
| 167 | OSU-03012 | Shikonin | | targeted | experimental | PDPK1 (PDK1) | PI3K signaling |
| 170 | Shikonin | | | not defined | in clinical development | unknown | other |
| 171 | AKT inhibitor VIII | | | targeted | in clinical development | AKT1, AKT2, AKT3 | PI3K signaling |
| 172 | Embelin | FH535 | | targeted | in clinical development | XIAP | apoptosis regulation |
| 173 | FH535 | PAC-1 | | not defined | experimental | unknown | other |
| 175 | PAC-1 | IPA-3 | | targeted | in clinical development | CASP3 agonist | apoptosis regulation |
| 176 | IPA-3 | | | targeted | experimental | PAK1, PAK2, PAK3 | cytoskeleton |
| 177 | GSK-650394 | | | targeted | experimental | SGK3 | other |
| 178 | BAY 61-3606 | 5-Fluorouracil | | targeted | experimental | SYK | other |
| 179 | 5-Fluorouracil | | | cytotoxic | clinically approved | DNA antimetabolite | DNA replication |
| 180 | Thapsigargin | | | targeted | experimental | sarco-endoplasmic reticulum Ca2+-ATPases | other |
| 182 | Obatoclax Mesylate | | GX15-070 | targeted | in clinical development | BCL2, BCL2L1, MCL1 | apoptosis regulation |
| 184 | BMS-754807 | OSI-906 | | targeted | in clinical development | IGF1R | IGFR signaling |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 185 | OSI-906 | LG-100069,LGD-1069 | | targeted | in clinical development | IGF1R | IGFR signaling |
| 186 | Bexarotene | | Targretin | targeted | clinically approved | Retinioic acid X family agonist | other |
| 190 | Bleomycin | DDE-28 | | cytotoxic | clinically approved | DNA damage | DNA replication |
| 192 | LFM-A13 | GW-2580 | | targeted | experimental | BTK | other |
| 193 | GW-2580 | VER-52296,NVP-AUY922 | | targeted | experimental | CSF1R (cFMS) | RTK signaling |
| 194 | AUY922 | Phenformin | | targeted | in clinical development | HSP90 | other |
| 196 | Phenformin | Bryostatin 1 | imidodica_ rbonimidi_c diamide_, N-(2-ph_enylethyl_)- | targeted | experimental | AAPK1 (AMPK) agonist | metabolism |
| 197 | Bryostatin 1 | GW786034 | NSC 339555 | targeted | in clinical development | PRKC | other |
| 199 | Pazopanib | Dacinostat, NVP-LAQ824 | Votrient | targeted | in clinical development | VEGFR, PDGFRA, PDGFRB, KIT | RTK signaling |
| 200 | LAQ824 | GNF-PF-193 | | targeted | in clinical development | HDAC | chromain histone acetylation |
| 201 | Epothilone B | GSK1904529A | EPO906 (ixabepilone, Patupilone) | cytotoxic | in clinical development | Microtubules | cytoskeleton |
| 202 | GSK-1904529A | | | targeted | experimental | IGF1R | IGFR signaling |
| 203 | BMS-345541 | | | targeted | experimental | IKBKB | other |
| 204 | Tipifarnib | Avagacestat | Zarnestra,IND58359, R115777 | targeted | in clinical development | Farnesyl-transferase (FNTA) | other |
| 205 | BMS-708163 | INCB-18424 | | targeted | in clinical development | g-secretase | other |
| 206 | Ruxolitinib | AS601245 | Jakafi | targeted | clinically approved | JAK1, JAK2, TYK2 | other |
| 207 | AS601245 | Ispinesib Mesylate | | targeted | experimental | JNK | JNK and p38 signaling |

**Table S1. Continued**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 208 | SB-715992 | | | targeted | in clinical development | KIF11 | mitosis |
| 211 | TL-2-105 | | | targeted | experimental | CRAF | ERK MAPK signaling |
| 219 | AT-7519 | KIN001-201 | | targeted | in clinical development | CDK9 | cell cycle |
| 221 | TAK-715 | KIN001-175 | | targeted | in clinical development | p38a | JNK and p38 signaling |
| 222 | BX-912 | KIN001-167 | | targeted | experimental | PDPK1 (PDK1) | PI3K signaling |
| 223 | ZSTK474 | KIN001-173 | | targeted | in clinical development | PI3K | PI3K signaling |
| 224 | AS605240 | | | targeted | experimental | PI3Kgamma | PI3K signaling |
| 225 | Genentech Cpd 10 | | | targeted | experimental | AURKA, AURKB | mitosis |
| 226 | GSK1070916 | | | targeted | in clinical development | AURKB | mitosis |
| 228 | KIN001-102 | Enzastaurin | | targeted | experimental | AKT1 | PI3K signaling |
| 229 | LY317615 | | | targeted | in clinical development | PRKCB (PKCbeta) | other |
| 230 | GSK429286A | KIN001-242 | | targeted | experimental | ROCK2 | cytoskeleton |
| 231 | FMK | | | targeted | experimental | RSK | ERK MAPK signaling |
| 235 | QL-XII-47 | | | targeted | experimental | BTK, BMX | other |
| 238 | CAL-101 | | | targeted | clinically approved | PI3Kdelta | PI3K signaling |
| 245 | UNC0638 | Cabozantinib | | targeted | experimental | G9a(EHMT2), GLP(EHMT1) | chromatin histone methylation |
| 249 | XL-184 | | Cometriq | targeted | clinically approved | VEGFR, MET, RET, KIT, FLT1, FLT3, FLT4, Tie2,AXL | RTK signaling |
| 252 | WZ3105 | | | targeted | experimental | CLK2, CNSK1E, FLT3, ULK1 | other |
| 253 | XMD14-99 | Quizartinib, AC-220 | | targeted | experimental | EPHB3, CAMK1 | RTK signaling |

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|---|---|---|---|---|---|---|
| 254 | AC220 | | targeted | in clinical development | FLT3 | RTK signaling |
| 255 | CP724714 | | targeted | in clinical development | ERBB2 | EGFR signaling |
| 256 | JW-7-24-1 | | targeted | experimental | LCK | other |
| 257 | NPK76-II-72-1 | | targeted | experimental | PLK3 | mitosis |
| 258 | STF-62247 | | not defined | experimental | stimulates autophagy | other |
| 260 | NG-25 | | targeted | experimental | MAP3K7 (TAK1) | other |
| 261 | TL-1-85 | | targeted | experimental | MAP3K7 (TAK1) | other |
| 262 | VX-11e | | targeted | experimental | ERK | ERK MAPK signaling |
| 263 | FR-180204 | | targeted | experimental | ERK | ERK MAPK signaling |
| 265 | Tubastatin A | | targeted | experimental | HDAC6 | chromain histone acetylation |
| 266 | Zibotentan, ZD4054 | Sepantronium bromide | targeted | in clinical development | Endothelin A Receptor | other |
| 268 | YM155 | XI-006 | targeted | in clinical development | BIRC5 (Survivin) | apoptosis regulation |
| 269 | NSC-207895 | 4-(Butanoyloxymethyl)phenyl-(2E,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoate | targeted | experimental | MDM4 | p53 pathway |
| 271 | VNLG/124 | HDAC-42 | targeted | experimental | HDAC, RAR | chromain histone acetylation |
| 272 | AR-42 | | targeted | in clinical development | HDAC | chromain histone acetylation |
| 273 | CUDC-101 | | targeted | in clinical development | HDAC, EGFR | chromain histone acetylation |

**Table S1. Continued**

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|---|---|---|---|---|---|---|
| 274 | PXD101, Belinostat | GSK525762A, | targeted | clinically approved | HDAC | chromain histone acetylation |
| 275 | I-BET 151 | | targeted | experimental | BRD2, BRD3, BRD4 | chromatin other |
| 276 | CAY10603 | | targeted | experimental | HDAC6 | chromain histone acetylation |
| 277 | ABT-869 | Linifanib | targeted | in clinical development | VEGFR and PDGFR family | RTK signaling |
| 279 | BIX02189 | | targeted | experimental | MAP2K5 (MEK5) | other |
| 281 | CH5424802 | | targeted | in clinical development | ALK | RTK signaling |
| 282 | EKB-569 | Pelitinib | targeted | in clinical development | EGFR | EGFR signaling |
| 283 | GSK2126458 | EX-8678 | targeted | in clinical development | PI3K, MTOR | PI3K signaling |
| 286 | KIN001-236 | | targeted | experimental | TIE2 | other |
| 287 | KIN001-244 | | targeted | experimental | PDPK1 (PDK1) | PI3K signaling |
| 288 | KIN001-055 | WHI-P97, AC1L1GQE | targeted | experimental | JAK3, MNK1 | other |
| 290 | KIN001-260 | Bayer IKKb inhibitor | targeted | experimental | IKK | other |
| 291 | KIN001-266 | | targeted | experimental | MAP3K8 (COT) | other |
| 292 | Masitinib | AB1010 | targeted | clinically approved | KIT | RTK signaling |
| 293 | MP470 | | targeted | in clinical development | PDGFR | RTK signaling |
| 294 | MPS-1-IN-1 | | targeted | experimental | MPS1 | mitosis |
| 295 | NVP-BHG712 | | targeted | experimental | EPHB4 | RTK signaling |
| 298 | OSI-930 | | targeted | in clinical development | KIT, VEGFR, PDGFR | RTK signaling |

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|---|---|---|---|---|---|---|
| 299 | OSI-027 | activebiochem A-1065 | targeted | in clinical development | MTORC1/2 | TOR signaling |
| 300 | CX-5461 | | targeted | experimental | RNA Pol I | other |
| 301 | PHA-793887 | | targeted | experimental | CDK-pan | cell cycle |
| 302 | PI-103 | | targeted | experimental | PI3Ka, PRKDC (DNAPK) | PI3K signaling |
| 303 | PIK-93 | | targeted | experimental | PI4K, PI3K | PI3K signaling |
| 304 | SB52334 | | targeted | experimental | ALK5 | RTK signaling |
| 305 | TPCA-1 | | targeted | experimental | IKK | other |
| 306 | TG101348 | | targeted | in clinical development | JAK2 | other |
| 308 | XL-880 | GSK1363089, foretinib | targeted | in clinical development | MET | RTK signaling |
| 309 | Y-39983 | | targeted | experimental | ROCK | cytoskeleton |
| 310 | YM201636 | | targeted | experimental | FYV1 | other |
| 312 | AV-951 | Tivozanib | targeted | in clinical development | VEGFR | RTK signaling |
| 326 | GSK690693 | | targeted | experimental | AKT | PI3K signaling |
| 328 | SNX-2112 | | targeted | experimental | HSP90 | other |
| 329 | QL-XI-92 | | targeted | experimental | DDR1 | RTK signaling |
| 330 | XMD13-2 | | targeted | experimental | RIPK | other |
| 331 | QL-X-138 | | targeted | experimental | MNK2, PRKDC (DNAPK), MTOR, BTK, JAK3 | other |
| 332 | XMD15-27 | | targeted | experimental | CAMK2B, CLK2, DYRK1A, MAST1, STK39 | other |
| 333 | T0901317 | | targeted | experimental | LXR | other |
| 341 | EX-527 | | targeted | experimental | SIRT1 | other |
| 344 | THZ-2-49 | | targeted | experimental | CDK9 | cell cycle |

**Table S1. Continued**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 345 | KIN001-270 | | | targeted | experimental | CDK9 | cell cycle |
| 346 | THZ-2-102-1 | | | targeted | experimental | CDK7 | cell cycle |
| 1001 | AICAR | N1-(b-D-Ribofuranosyl)-5-aminoimidazole-4-carboxamide | AICAR | targeted | in clinical development | AAPK1 (AMPK) agonist | metabolism |
| 1003 | Camptothecin | 7-Ethyl-10-Hydroxy-Camptothecin,SN-38 | SN-38 | cytotoxic | clinically approved | TOP1 | DNA replication |
| 1004 | Vinblastine | Vinblastine sulphate | Vinblastine | cytotoxic | clinically approved | Microtubules | cytoskeleton |
| 1005 | Cisplatin | cis-Diammineplatinum(II) dichloride | Cisplatin | cytotoxic | clinically approved | DNA crosslinker | DNA replication |
| 1006 | Cytarabine | Ara-Cytidine,Arabinosyl Cytosine,U-19920 | Cytarabine (AraC) | cytotoxic | clinically approved | DNA synthesis | DNA replication |
| 1007 | Docetaxel | RP-56976 | Taxotere | cytotoxic | clinically approved | Microtubules | cytoskeleton |
| 1008 | Methotrexate | | Methotrexate | cytotoxic | clinically approved | Dihydrofolate reductase (DHFR) | DNA replication |
| 1009 | ATRA | Tretinoin | Vesanoid | targeted | clinically approved | Retinoic acid and retinoid X receptor agonist | other |
| 1010 | Gefitinib | ZD-1839 | Iressa | targeted | clinically approved | EGFR | EGFR signaling |
| 1011 | ABT-263 | | | targeted | in clinical development | BCL2, BCL2L1, BCL2L2 | apoptosis regulation |
| 1012 | Vorinostat | SAHA | Zolinza | targeted | clinically approved | HDAC inhibitor Class I, IIa, IIb, IV | chromain histone acetylation |
| 1013 | Nilotinib | | Tasigna | targeted | clinically approved | ABL | ABL signaling |
| 1014 | RDEA119 | RDEA119 | | targeted | in clinical development | MAP2K1 (MEK1), MAP2K2 (MEK2) | ERK MAPK signaling |
| 1015 | CI-1040 | PD-18435,PD-184352 | | targeted | in clinical development | MAP2K1 (MEK1), MAP2K2 (MEK2) | ERK MAPK signaling |
| 1016 | Temsirolimus | CCI-779 | Torisel | targeted | clinically approved | MTOR | TOR signaling |
| 1017 | Olaparib | KU-0059436, AZD-2281 | Lynparza | targeted | in clinical development | PARP1, PARP2 | Genome integrity |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 1018 | ABT-888 | ABT-888 | | targeted | in clinical development | PARP1, PARP2 | Genome integrity |
| 1019 | Bosutinib | SKI-606 | Bosulif | targeted | clinically approved | SRC, ABL, TEC | ABL signaling |
| 1020 | Lenalidomide | | Revlimid | targeted | clinically approved | TNFA | other |
| 1021 | Axitinib | AG-013736 | Axitinib | targeted | in clinical development | PDGFR, KIT, VEGFR | RTK signaling |
| 1022 | AZD7762 | AZD 7762 | | targeted | in clinical development | CHEK1, CHEK2 | Genome integrity |
| 1023 | GW 441756 | | | targeted | experimental | NTRK1 | RTK signaling |
| 1024 | CEP-701 | CEP-701 | Lestaurtinib | targeted | in clinical development | FLT3, JAK2, NTRK1, RET | RTK signaling |
| 1025 | SB 216763 | SB 216763 | | targeted | experimental | GSK3A, GSK3B | WNT signaling |
| 1026 | 17-AAG | 17-AAG | Telatinib | targeted | in clinical development | HSP90 | other |
| 1028 | VX-702 | | | targeted | in clinical development | p38 | JNK and p38 signaling |
| 1029 | AMG-706 | AMG-706 | Motesanib Diphosphate | targeted | in clinical development | VEGFR, RET, c-KIT, PDGFR | RTK signaling |
| 1030 | KU-55933 | | | targeted | experimental | ATM | Genome integrity |
| 1031 | Elesclomol | | | targeted | in clinical development | HSP70 | other |
| 1032 | Afatinib | Tovok, BIBW2992 | Gilotrif | targeted | clinically approved | ERBB2, EGFR | EGFR signaling |
| 1033 | Vismodegib | GDC-0449 | Erivedge | targeted | in clinical development | SMO | other |
| 1036 | PLX4720 | Vemurafenib (derivative) | Zelboraf (derivative) | targeted | clinically approved | BRAF | ERK MAPK signaling |
| 1037 | BX-795 | BX 795 | | targeted | in clinical development | TBK1, PDPK1, IKK, AURKB, AURKC | other |
| 1038 | NU-7441 | NU-7432,KU-57788 | | targeted | experimental | PRKDC (DNAPK) | Genome integrity |

**Table S1. Continued**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 1039 | SL 0101-1 | | | targeted | experimental | RSK, AURKB, PIM3 | ERK MAPK signaling |
| 1042 | BIRB 0796 | | Doramapimod | targeted | experimental | p38, JNK2 | JNK and p38 signaling |
| 1043 | JNK Inhibitor VIII | JNK Inhibitor VIII | | targeted | experimental | JNK | JNK and p38 signaling |
| 1046 | 681640,00 | 681640,00 | | targeted | experimental | WEE1, CHEK1 | cell cycle |
| 1047 | Nutlin-3a | Nutlin-3a (-) enantiomer | | targeted | in clinical development | MDM2 | p53 pathway |
| 1049 | PD-173074 | PD-173074 | | targeted | experimental | FGFR1, FGFR3 | RTK signaling |
| 1050 | ZM-447439 | ZM447439 | | targeted | experimental | AURKB | mitosis |
| 1052 | RO-3306 | | | targeted | experimental | CDK1 | cell cycle |
| 1053 | MK-2206 | | | targeted | in clinical development | AKT1, AKT2 | PI3K signaling |
| 1054 | PD-0332991 | PD-0332991 | | targeted | in clinical development | CDK4, CDK6 | cell cycle |
| 1057 | NVP-BEZ235 | BEZ235 | | targeted | in clinical development | PI3K (Class 1) and MTORC1/2 | PI3K signaling |
| 1058 | GDC0941 | | | targeted | in clinical development | PI3K (class 1) | PI3K signaling |
| 1059 | AZD8055 | AZD8055 | pp242 | targeted | in clinical development | MTORC1/2 | TOR signaling |
| 1060 | PD-0325901 | PD-0325901 | | targeted | in clinical development | MAP2K1 (MEK1), MAP2K2 (MEK2) | ERK MAPK signaling |
| 1061 | SB590885 | | | targeted | experimental | BRAF | ERK MAPK signaling |
| 1062 | AZD6244 | | | targeted | in clinical development | MAP2K1 (MEK1), MAP2K2 (MEK2) | ERK MAPK signaling |
| 1066 | AZD6482 | WO2009093972 | | targeted | in clinical development | PI3Kbeta | PI3K signaling |
| 1067 | CCT007093 | | | targeted | experimental | PPM1D | other |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 1069 | EHT 1864 | | | targeted | experimental | Rac GTPases | cytoskeleton |
| 1072 | BMS-708163 | Avagacestat | | targeted | in clinical development | g-secretase | other |
| 1091 | BMS-536924 | BMS-536924 | | targeted | experimental | IGF1R | IGFR signaling |
| 1114 | Cetuximab | Cetuximab | Erbitux | targeted | clinically approved | EGFR | EGFR signaling |
| 1129 | PF-4708671 | | | targeted | experimental | RPS6KB1 (p70S6KA) | TOR signaling |
| 1133 | JNJ-26854165 | | Serdemetan | targeted | in clinical development | MDM2 | p53 pathway |
| 1142 | HG-5-113-01 | | | targeted | in clinical development | LOK, LTK, TRCB, ABL(T315I) | ABL signaling |
| 1143 | HG-5-88-01 | | | targeted | experimental | EGFR, ADCK4 | EGFR signaling |
| 1149 | TW 37 | | | targeted | experimental | BCL2, BCL2L1 | apoptosis regulation |
| 1158 | XMD11-85h | | | targeted | experimental | BRSK2, FLT4, MARK4, PRKCD, RET, SPRK1 | other |
| 1161 | ZG-10 | | | targeted | experimental | IRAK1 | other |
| 1164 | XMD8-92 | | | targeted | experimental | MAP2K5 (ERK5) | other |
| 1166 | QL-VIII-58 | | | targeted | experimental | MTOR, ATR | TOR signaling |
| 1170 | CCT018159 | | | targeted | experimental | HSP90 | other |
| 1175 | AG-014699 | PF-01367338 | | targeted | experimental | PARP1, PARP2 | Genome integrity |
| 1192 | GSK269962A | KIN001-155 | | targeted | experimental | ROCK1, ROCK2 | cytoskeleton |
| 1194 | SB-505124 | 2-(5-Benzo[1,3]dioxol-5-yl-2-tert-butyl-3H-imidazol-4-yl)-6-methylpyridine hydrochloride hydrate | | targeted | experimental | TGFR1 (ALK5) | other |
| 1199 | Tamoxifen | | | targeted | clinically approved | ER | other |
| 1203 | QL-XII-61 | | | targeted | experimental | BTK | other |
| 1218 | JQ1 | | | targeted | experimental | BRD2, BRD3, BRD4 | chromatin other |

**Table S1. Continued**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 1219 | PFI-1 | | | targeted | experimental | BRD2, BRD3, BRD4 | chromatin other |
| 1230 | IOX2 | | | targeted | experimental | EGLN1 | other |
| 1236 | UNC0638 | | | targeted | experimental | G9a(EHMT2), GLP(EHMT1) | chromatin histone methylation |
| 1239 | YK 4-279 | | | targeted | experimental | RNA helicase A | other |
| 1241 | CHIR-99021 | CT 99021 | | targeted | experimental | GSK3B | WNT signaling |
| 1242 | (5Z)-7-Oxozeaenol | | | targeted | experimental | MAP3K7 (TAK1) | other |
| 1243 | piperlongumine | | | not defined | experimental | Increases ROS levels | other |
| 1248 | FK866 | APO866 | | targeted | experimental | NAMPT | metabolism |
| 1259 | BMN-673 | | | targeted | experimental | PARP1 | Genome integrity |
| 1261 | rTRAIL | | | targeted | experimental | TR10A (DR4), TR10B (DR5) | apoptosis regulation |
| 1262 | UNC1215 | | | targeted | experimental | LMBL3 | other |
| 1264 | SGC0946 | | | targeted | experimental | Q8TEK3 (DOT1L) | chromatin histone methylation |
| 1268 | XAV 939 | NVP-XAV 939 | | targeted | experimental | TNKS1 (tankyrase-1) | WNT signaling |
| 1371 | PLX4720 (rescreen) | Vemurafenib (derivative) | Zelboraf | targeted | experimental | BRAF | ERK MAPK signaling |
| 1372 | Trametinib | GSK1120212 | Mekinist | targeted | clinically approved | MAP2K1 (MEK1), MAP2K2 (MEK2) | ERK MAPK signaling |
| 1373 | Dabrafenib | GSK2118436 | Tafinlar | targeted | clinically approved | BRAF | ERK MAPK signaling |
| 1375 | Temozolomide | Temodar | | cytotoxic | clinically approved | DNA akylating agent | DNA replication |
| 1377 | Afatinib (rescreen) | Tovok, BIBW2992 | Gilotrif | targeted | clinically approved | ERBB2, EGFR | EGFR signaling |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 1378 | Bleomycin (50 uM) | | | cytotoxic | clinically approved | DNA damage | DNA replication |
| 1494 | SN-38 | 7-ETHYL-10-HYDROXY-CAMPTOTHECIN | | cytotoxic | experimental | TOP1 | DNA replication |
| 1495 | Olaparib | Olaparib | | targeted | clinically approved | PARP1, PARP2 | Genome integrity |
| 1498 | AZD6244 | | | targeted | in clinical development | MAP2K1 (MEK1), MAP2K2 (MEK2) | ERK MAPK signaling |
| 1502 | Bicalutamide | ICI-176334 | Casodex | targeted | clinically approved | ANDR (androgen receptor) | other |
| 1526 | RDEA119 (rescreen) | | | targeted | experimental | MAP2K1 (MEK1), MAP2K2 (MEK2) | ERK MAPK signaling |
| 1527 | GDC0941 (rescreen) | | | targeted | in clinical development | PI3K | PI3K signaling |
| 1529 | MLN4924 | | | targeted | in clinical development | NEDD8-activating enzyme | other |

**Table S2. SPIA pathway analysis performed highlighting significantly upregulated/downregulated pathways between BAP1 mutant and BAP1 wild type lines.**

| Name | ID | pSize | NDE | pNDE | tA | pPERT | pG |
|---|---|---|---|---|---|---|---|
| Complement and coagulation cascades | 4610 | 67 | 1 | 0,239757502 | 16,87317213 | 0,004 | 0,007623894 |
| Gap junction | 4540 | 85 | 2 | 0,047099152 | 6,9486621 | 0,03 | 0,010684996 |
| MAPK signaling pathway | 4010 | 260 | 5 | 0,004188945 | 1,872639843 | 0,393 | 0,01219752 |
| Glioma | 5214 | 62 | 2 | 0,026481172 | 5,769688959 | 0,125 | 0,022213608 |
| Prostate cancer | 5215 | 88 | 2 | 0,05011322 | 5,902911432 | 0,068 | 0,022769284 |
| Melanoma | 5218 | 71 | 2 | 0,033994969 | 7,652553716 | 0,169 | 0,035386672 |
| Protein processing in endoplasmic reticulum | 4141 | 161 | 1 | 0,483342609 | 2,760670737 | 0,025 | 0,06544349 |
| Focal adhesion | 4510 | 198 | 2 | 0,193196516 | 5,353545369 | 0,141 | 0,125390148 |
| HTLV-I infection | 5166 | 259 | 2 | 0,284858199 | 2,25223501 | 0,151 | 0,178344625 |
| Cytokine-cytokine receptor interaction | 4060 | 248 | 2 | 0,268225286 | 1,4263907 | 0,163 | 0,180563656 |
| Bladder cancer | 5219 | 40 | 1 | 0,150861921 | 0,28656377 | 0,294 | 0,182539356 |
| Regulation of actin cytoskeleton | 4810 | 212 | 3 | 0,055678487 | -1,134243636 | 0,798 | 0,182782389 |
| Neurotrophin signaling pathway | 4722 | 117 | 2 | 0,082459008 | -2,062560979 | 0,586 | 0,194728203 |
| Pancreatic cancer | 5212 | 69 | 1 | 0,24596437 | 1,146255078 | 0,256 | 0,237079519 |
| Endometrial cancer | 5213 | 52 | 1 | 0,191570826 | 0,764170052 | 0,339 | 0,242511753 |
| Pathways in cancer | 5200 | 321 | 2 | 0,377272508 | 3,238496438 | 0,188 | 0,258607838 |
| Non-small cell lung cancer | 5223 | 54 | 1 | 0,198165792 | 0,818753627 | 0,381 | 0,270566523 |
| ErbB signaling pathway | 4012 | 86 | 1 | 0,29674309 | 1,152623162 | 0,262 | 0,276335005 |
| Oocyte meiosis | 4114 | 106 | 1 | 0,352175452 | -2,13630758 | 0,242 | 0,295091787 |
| Cell cycle | 4110 | 122 | 1 | 0,393397279 | 1,022863241 | 0,41 | 0,45557714 |
| Apoptosis | 4210 | 87 | 1 | 0,299622651 | -1,11934781 | 0,547 | 0,46030136 |
| Vasopressin-regulated water reabsorption | 4962 | 44 | 1 | 0,164651424 | 0 | 1 | 0,461670182 |
| Hepatitis C | 5160 | 129 | 1 | 0,410607473 | 0,28656377 | 0,452 | 0,498171235 |
| Mineral absorption | 4978 | 51 | 1 | 0,188253298 | 0 | 1 | 0,502630073 |
| Tuberculosis | 5152 | 171 | 1 | 0,504199822 | 0,780592299 | 0,377 | 0,505677304 |
| Amyotrophic lateral sclerosis (ALS) | 5014 | 52 | 1 | 0,191570826 | 0 | 1 | 0,508141173 |
| Insulin signaling pathway | 4910 | 134 | 1 | 0,422604562 | 1,11934781 | 0,46 | 0,512792474 |
| Axon guidance | 4360 | 127 | 1 | 0,405740054 | 2,22E-16 | 0,535 | 0,548653527 |
| Adipocytokine signaling pathway | 4920 | 68 | 1 | 0,242867213 | 0 | 1 | 0,586582712 |
| PPAR signaling pathway | 3320 | 70 | 1 | 0,249049025 | 0 | 1 | 0,595253447 |
| Phosphatidylinositol signaling system | 4070 | 77 | 1 | 0,270295671 | 0 | 1 | 0,623906966 |
| Lysosome | 4142 | 119 | 1 | 0,385870539 | 0 | 1 | 0,753317055 |
| Measles | 5162 | 127 | 1 | 0,405740054 | 0 | 1 | 0,771734861 |
| Alcoholism | 5034 | 129 | 1 | 0,410607473 | 0 | 1 | 0,776096401 |
| RNA transport | 3013 | 146 | 1 | 0,450423166 | 0 | 1 | 0,809666166 |
| Transcriptional misregulation in cancer | 5202 | 156 | 1 | 0,47259166 | 0 | 1 | 0,826810244 |
| Herpes simplex infection | 5168 | 173 | 1 | 0,508270435 | 0 | 1 | 0,852238193 |
| Calcium signaling pathway | 4020 | 178 | 1 | 0,51830324 | 0 | 1 | 0,858929435 |

| pGFdr | pGFWER | Status | KEGGLINK |
|---|---|---|---|
| 0,154501919 | 0,289707958 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa04610+7035 |
| 0,154501919 | 0,406029842 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa04540+1950+5154 |
| 0,154501919 | 0,463505756 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa04010+4915+8912+5154+1950+51347 |
| 0,173046556 | 0,844117116 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa05214+1950+5154 |
| 0,173046556 | 0,865232778 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa05215+1950+5154 |
| 0,224115586 | 1 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa05218+1950+5154 |
| 0,355264658 | 1 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa04141+258010 |
| 0,569205517 | 1 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa04510+1950+5154 |
| 0,569205517 | 1 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa05166+9184+5154 |
| 0,569205517 | 1 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa04060+1950+5154 |
| 0,569205517 | 1 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa05219+1950 |
| 0,569205517 | 1 | Inhibited | http://www.genome.jp/dbget-bin/show_pathway?hsa04810+10152+1950+5154 |
| 0,569205517 | 1 | Inhibited | http://www.genome.jp/dbget-bin/show_pathway?hsa04722+4915+397 |
| 0,5833739 | 1 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa05212+1950 |
| 0,5833739 | 1 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa05213+1950 |
| 0,5833739 | 1 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa05200+1950+5154 |
| 0,5833739 | 1 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa05223+1950 |
| 0,5833739 | 1 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa04012+1950 |
| 0,590183573 | 1 | Inhibited | http://www.genome.jp/dbget-bin/show_pathway?hsa04114+9748 |
| 0,721707926 | 1 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa04110+9184 |
| 0,721707926 | 1 | Inhibited | http://www.genome.jp/dbget-bin/show_pathway?hsa04210+5575 |
| 0,721707926 | 1 | Inhibited | http://www.genome.jp/dbget-bin/show_pathway?hsa04962+397 |
| 0,721707926 | 1 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa05160+1950 |
| 0,721707926 | 1 | Inhibited | http://www.genome.jp/dbget-bin/show_pathway?hsa04978+26872 |
| 0,721707926 | 1 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa05152+9902 |
| 0,721707926 | 1 | Inhibited | http://www.genome.jp/dbget-bin/show_pathway?hsa05014+4747 |
| 0,721707926 | 1 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa04910+5575 |
| 0,744601216 | 1 | Activated | http://www.genome.jp/dbget-bin/show_pathway?hsa04360+64221 |
| 0,7539877 | 1 | Inhibited | http://www.genome.jp/dbget-bin/show_pathway?hsa04920+2182 |
| 0,7539877 | 1 | Inhibited | http://www.genome.jp/dbget-bin/show_pathway?hsa03320+2182 |
| 0,764789184 | 1 | Inhibited | http://www.genome.jp/dbget-bin/show_pathway?hsa04070+3628 |
| 0,858929435 | 1 | Inhibited | http://www.genome.jp/dbget-bin/show_pathway?hsa04142+2581 |
| 0,858929435 | 1 | Inhibited | http://www.genome.jp/dbget-bin/show_pathway?hsa05162+9367 |
| 0,858929435 | 1 | Inhibited | http://www.genome.jp/dbget-bin/show_pathway?hsa05034+4915 |
| 0,858929435 | 1 | Inhibited | http://www.genome.jp/dbget-bin/show_pathway?hsa03013+9939 |
| 0,858929435 | 1 | Inhibited | http://www.genome.jp/dbget-bin/show_pathway?hsa05202+5154 |
| 0,858929435 | 1 | Inhibited | http://www.genome.jp/dbget-bin/show_pathway?hsa05168+6431 |
| 0,858929435 | 1 | Inhibited | http://www.genome.jp/dbget-bin/show_pathway?hsa04020+8912 |

**Table S3. GEO data analysis of 40 BAP1 wild type versus 11 BAP1 mutant mesothelioma tumors showing fold change in mRNA expression.**

| ID | adj.P.Val | P.Value | t | B | logFC | Gene.symbol | Gene.title |
|---|---|---|---|---|---|---|---|
| 206987_x_at | **0,0467** | 8,40E-06 | -4,9297914 | 2,074 | -1,55210916 | **FGF18** | fibroblast growth factor 18 |
| 211029_x_at | **0,0331** | 3,23E-06 | -5,200056 | 2,71569 | -1,46628317 | **FGF18** | fibroblast growth factor 18 |
| 211485_s_at | **0,0173** | 7,78E-07 | -5,5953934 | 3,66039 | -1,32935104 | **FGF18** | fibroblast growth factor 18 |
| 203638_s_at | 0,8387 | 9,17E-02 | -1,7172855 | -4,20483 | -0,58459186 | **FGFR2** | fibroblast growth factor receptor 2 |
| 208228_s_at | 0,8261 | 7,39E-02 | -1,822761 | -4,07017 | -0,57807812 | **FGFR2** | fibroblast growth factor receptor 2 |
| 205110_s_at | 0,7406 | 2,47E-02 | -2,3120189 | -3,35944 | -0,57359639 | **FGF13** | fibroblast growth factor 13 |
| 203639_s_at | 0,8385 | 8,69E-02 | -1,7439568 | -4,17143 | -0,42430659 | **FGFR2** | fibroblast growth factor receptor 2 |
| 204379_s_at | 0,7207 | 1,73E-02 | -2,4574403 | -3,12282 | -0,30363692 | **FGFR3** | fibroblast growth factor receptor 3 |
| 214284_s_at | 0,7144 | 8,97E-03 | -2,7123752 | -2,68264 | -0,2416635 | **FGF18** | fibroblast growth factor 18 |
| 215404_x_at | 0,7195 | 1,54E-02 | 2,5044305 | -3,04406 | 0,32050106 | **FGFR1** | fibroblast growth factor receptor 1 |

CHAPTER 6

# 6

# PD-1 blockade with Nivolumab in Patients with Recurrent Malignant Pleural Mesothelioma

Josine Quispel-Janssen MD[a*] | Vincent van der Noort PhD[b] | Jeltje F. de Vries PhD [b] | Marion Zimmerman[a] | Ferry Lalezari MD[c] | Erik Thunnissen MD PhD[d] | Kim Monkhorst MD PhD[e] | Robert Schouten MD[a] | Laurel Schunselaar MS[g] | Maria Disselhorst MD[a] | Houke Klomp MD PhD[f] | Sjaak Burgers MD PhD[a] | Wieneke Buikhuisen MD[a] | Paul Baas MD PhD[a]

[a] Department of Thoracic Oncology, Netherlands Cancer Institute, Amsterdam, The Netherlands
[b] Biometrics Department, Netherlands Cancer Institute, Amsterdam, The Netherlands
[c] Department of Radiology, Netherlands Cancer Institute, Amsterdam, The Netherlands
[d] Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands
[e] Department of Pathology, Netherlands Cancer Institute, Amsterdam, The Netherlands
[f] Department of Surgery, Netherlands Cancer Institute, Amsterdam, The Netherlands
[g] Division of Oncogenomics, Oncode Institute within Netherlands Cancer Institute, Amsterdam, The Netherlands

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## Abstract

**Background:** Malignant Pleural Mesothelioma (MPM) has limited treatment options and a poor outcome. PD-1/PD-L1 checkpoint inhibitors have proven efficacious in several cancer types. Nivolumab is a fully humanized monoclonal antibody against PD-1 with a favorable toxicity profile. In MPM, the immune system is considered to play an important role. We therefore tested nivolumab in recurrent MPM.

**Methods:** In this single center trial, patients with MPM received nivolumab 3mg/kg i.v. every two weeks. Primary endpoint was the disease control rate (DCR) at 12 weeks. Pre- and on-treatment biopsies were taken to analyze biomarkers for response.

**Results:** Of the 34 patients included, eight patients (24%) had a partial response at 12 weeks and another eight had stable disease (SD) resulting in a DCR at 12 weeks of 47%. One reached a PR at 18 weeks. In four patients with SD, the tumor remained stable for more than 6 months. Treatment-related adverse events (TR-AE) of any grade occurred in 26 patients (76%), most commonly fatigue (29%) and pruritus (15%). Grade 3 and 4 TR-AE were reported in 9 patients (26%), with pneumonitis, gastro-intestinal disorders and laboratory disorders mostly seen. One treatment-related death was due to pneumonitis and probably initiated by concurrent amiodarone therapy. PD-L1 was expressed on tumor cells in 9 samples (27%), but did not correlate with outcome.

**Interpretation:** Single agent nivolumab has meaningful clinical efficacy and a manageable safety profile in pretreated patients with mesothelioma. PD-L1 expression does not predict for response in this population.

**Keywords:** Mesothelioma; Immunotherapy; PD-L1; Nivolumab; Checkpoint Inhibitor

## Introduction

Malignant Pleural Mesothelioma (MPM) is an aggressive tumor arising from mesothelial cells of the pleural cavity and is strongly related to (occupational) asbestos exposure. Although the use of asbestos is banned in most western countries, this disease will continue to score victims over the next decade, due to the long latency time [1].

MPM is refractory to the vast majority of drugs and has a dismal prognosis: most patients die within two years after diagnosis. The standard treatment for patients with advanced disease is chemotherapy consisting of a platinum- anti-folate combination [2]. There is no registered second-line therapy, since no study demonstrated a survival benefit in this setting [3]. Improving outcome is urgently needed, but remains a huge challenge due to the difficulty of response evaluation and the heterogeneity of the disease. The success of new treatment approaches such as immunotherapy in other cancer types, gives hope to these patients.



Immunotherapy enhances the ability of the patients own immune system to recognize and destroy tumor cells. Tumors can evade this immunosurveillance by upregulating inhibitory signals such as the PD-1/PD-L1 pathway [4]. Blockade of this pathway by PD-1 inhibitors resulted in long-lasting responses, as was first demonstrated in melanoma [5]. It has shown efficacy in many other cancer types, including lung cancer [6,7] and renal cell carcinoma [8].

Nivolumab (BMS-936558) is a fully human monoclonal antibody that binds PD-1 on activated immune cells and disrupts binding of PD-1 to its ligand PD-L1. This process will prevent downregulation of cytotoxic T-cells and augment the host-antitumor response. Nivolumab is registered in several countries for the treatment of advanced melanoma and is approved for the second-line treatment of NSCLC after previous platinum-containing chemotherapy. To date, nivolumab shows a mild toxicity profile as hematologic toxicities are rare and the majority of non-hematological toxicities are low grade and manageable. The safety profile of nivolumab monotherapy is similar across tumor types.

In spite of all the positive reports about checkpoint inhibitors, not all tumors respond well to this treatment. Therefore, it is crucial to find predictive biomarkers that enable us to withhold treatment from patients that are unlikely to respond and thus prevent time loss and unwanted side effects. The most frequently studied biomarker is PD-L1 expression. In MPM, expression of PD-L1 was demonstrated by several groups, especially on sarcomatoid MPM [9-12]. PD-L1 expression is also present on immune cells as is assessed in several tumor types [13]. Emerging data reveal that other factors like mutational load, general immune status and the tumor micro-environment may play an important role in evoking a response. Therefore, we designed this single arm phase II trial with an emphasis on biomarker research.

## Methods

### Study design and participants

In this prospective, single arm, single center, phase II trial, a Simons' minimax design was used. Patients aged 18 years or older with MPM were eligible for study participation if they had disease recurrence after at least one chemotherapy regimen, WHO performance status 0 or 1, measurable disease and adequate liver, renal and bone marrow functions including lactate dehydrogenase (LDH). In addition, C-reactive protein (CRP), amylase, lipase, thyroid stimulating hormone (TSH) and free Thyroxine 4 (fT4) were measured. Tumors had to be accessible for repeated biopsies by thoracoscopy or a CT- or ultrasound guided transthoracic approach. Key exclusion criteria were symptomatic central nervous system (CNS) metastasis, autoimmune disease or systemic immunosuppressive therapy.

The study protocol was approved by the institutional ethics committee and conducted in accordance with the Good Clinical Practice guidelines and the Declaration of Helsinki. Written informed consent was obtained from all participants. The trial was registered at ClinicalTrials.gov, number NCT02497508.

### Procedures

Treatment consisted of bi-weekly intravenous administration of Nivolumab 3mg/kg, a fully humanized IgG4 antibody targeting PD-1 (Opdivo, Bristol-Meyers Squibb). Dose and treatment schedule were based on data from a phase I trial [14]. No dose escalations or reductions were allowed. Dose delays were permitted for protocol-defined reasons. Treatment continued for a maximum of 1 year or until disease progression or unacceptable toxicity.

Tumor response was assessed with CT-scans every six weeks (every 8 weeks after 24 weeks of treatment) using a combination of Response Evaluation Criteria In Solid Tumors (RECIST) modified for mesothelioma [15] and RECIST modified for immunotherapeutic agents [16]. A partial response (PR) was defined as a decrease of ≥30% of the sum of target lesions, measured according to RECIST modified for mesothelioma (unidimensional measurements of tumor thickness perpendicular to the chest wall or the mediastinum). Progressive disease (PD) was defined as an increase of ≥20% of target lesions, confirmed by another CT-scan at least 4 weeks apart. Patients were allowed to continue treatment beyond initial radiologic progression in the absence of clinical deterioration. If the subsequent CT scan did not confirm progression, the initial progression was considered to be pseudoprogression, and the patient was allowed to continue treatment with nivolumab. New lesions did not define progression, but were added to the total sum of tumor burden, according to RECIST modified for immunotherapeutic agents. Non-target lesions could contribute to the designation of overall progression, but PD was never concluded solely on the basis of increased lymph nodes. Stable disease (SD) was defined as having neither complete response (CR), PR nor PD.

Laboratory testing was performed before each nivolumab administration. Pulmonary function was assessed at baseline and after 6 weeks. Tumor tissue specimens were obtained prior to and after 3 courses of nivolumab by means of thoracoscopy or ultrasound- or CT-guided transthoracic biopsies.
PD-L1 expression on formalin-fixed, paraffin-embedded tissue samples was assessed with immunohistochemistry using monoclonal antibody 28-8 according to the manufacturer (Dako Autolink PD-L1 28-8, Rb Monoclonal, detection with Rabbit Linker and Envision). At least 100 neoplastic cells were scored for membranous staining and a tissue sample was considered positive if more than 1% of tumor cells stained positive. Expression was quantified in five categories: 1-5% positive cells, 5-10%, 10-25%, 25-50% and ≥50% positive cells.



**Outcomes**
The disease control rate (DCR) at 12 weeks was the primary endpoint of this study. DCR was defined by the number of patients with CR, PR and SD, as a percentage of the total number of patients in the study. Secondary endpoints included DCR at 6 months, clinical benefit rate, objective response rate (ORR), progression-free survival (PFS), overall survival (OS) and safety. Patients with CR, PR and patients with long-term SD (≥6 months) were considered to have clinical benefit. PFS was defined as the time interval from the date of start of treatment to the date of the first documented tumor progression or death due to any cause, whichever occurred first. OS was defined as the time interval from the date of start of treatment to the date of death due to any cause. Safety was assessed by incidence of adverse events, reported according to the NCI Common Terminology Criteria for Adverse Events, version 4.03.

**Statistical analysis**
Based on our hypothesis that treatment with nivolumab will increase the DCR at 12 weeks from 20 to 40%, a Simon mini-max design with a sample size of 33 patients was chosen with an interim analysis for futility after 18 patients, allowing the study to continue only if at least 5 of the first 18 patients had disease control. This design with an early stop for futility was chosen because of the limited number of patients with this rare tumor type. Treatment with Nivolumab was deemed successful if the study was not stopped at the interim analysis and at least 11 patients out of the 33 showed disease control. When the true DCR in the population is 40%, the chosen numbers guarantee that the power of declaring success will be 80% while the probability of making a type I error (defined as declaring success when the true DCR was 20% or less) is controlled at 0.05. PFS and OS were calculated using the Kaplan-Meier method. All patients that received at least one dose of nivolumab and had at least one radiologic evaluation were considered evaluable. All patients that received at least one dose of nivolumab and had at least one follow up visit were included in the safety analysis.

Cut-off for survival analysis was January 2018. Fisher's exact test was used to analyze the correlation between PD-L1 expression and response.

**Role of the funding source**
The study was designed by the authors and financially supported by Bristol-Meyers Squibb which included medication supply.

## Results

Between July 2015 and June 2016, 38 patients gave informed consent. Of these, 34 patients fulfilled the entry criteria and received study treatment. Thirty-three patients were evaluated; one patient died due to cardiac disease prior to the response evaluation (Fig. 1). At the interim analysis, five out of 18 patients had a partial response and four had stable disease. Disease control was thus reached in more than 5 patients allowing the trial to continue. Baseline characteristics are shown in table 1. With a median age of 67 years, a male predominance (82%) and a majority of epithelial subtype, our study population was representative for the general mesothelioma population.

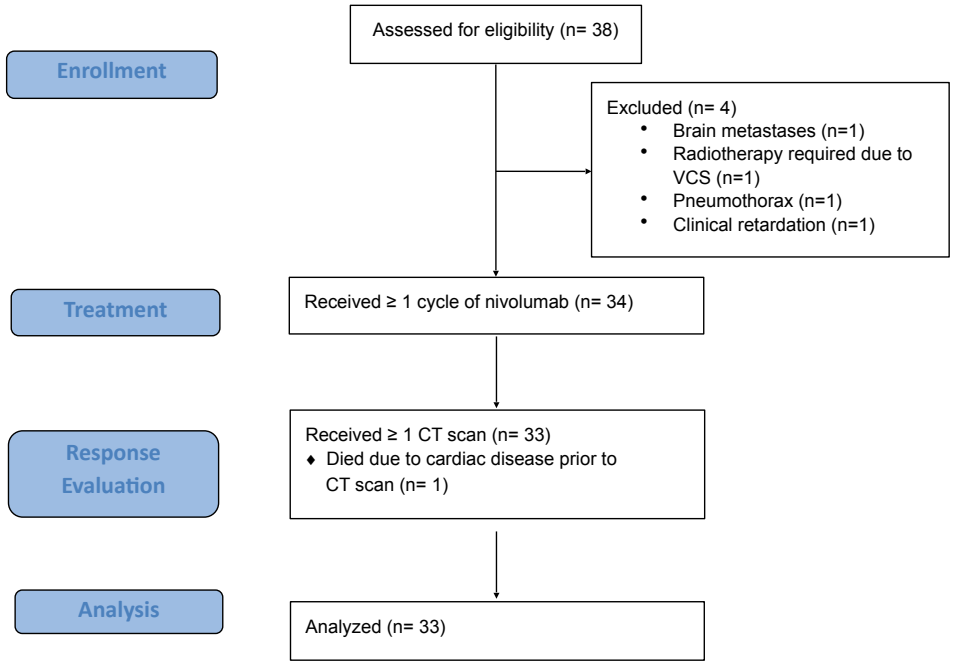


**Figure 1. CONSORT Flow Diagram.**

**Table 1. Patient characteristics.**

| Demographic Variable | Patients (n=34) |
|---|---|
| Age, median in years (range) | 67 (50-81) |
| Sex | |
| Male | 28 (82%) |
| Female | 6 (18%) |
| WHO performance score | |
| 0 | 18 (53%) |
| 1 | 16 (47%) |
| Histologic subtype | |
| Epithelioid | 28 (82%) |
| Sarcomatoid | 2 (6%) |
| Mixed | 4 (12%) |
| Previous local therapy | |
| Surgery | 3 (9%) |
| Radiotherapy | 5 (15%) |
| Disease stage | |
| I-III | 24 (71%) |
| IV | 10 (29%) |



Most patients received one prior line of systemic treatment; one patient received two lines. Pleurectomy/decortication was performed in four patients. Five patients received radiotherapy prior to start of study treatment. Median time from the initial diagnosis of mesothelioma to the start of study enrolment was 12.3 months. One quarter of patients started nivolumab treatment within 3 months after completing their previous chemotherapy. The median number of doses nivolumab administered was 7 (IQR 3 – 17.25) and the median duration of treatment was 2.8 months (95% CI 1.8 – 6). Dose delays occurred 11 times in 9 patients. In 7 cases in 6 patients this was due to toxicity. Administrative or personal requests caused the other dose delays. Post-study treatment was given in 9 patients (27%), mostly gemcitabine or vinorelbine.

At 12 weeks, a PR was observed in eight patients of the 34 in the intention to treat group (24%, 95% CI: 11% - 42%). Eight patients had SD, resulting in a DCR of 47% (95% CI: 30%-65%). Seventeen patients had PD after 12 weeks. One patient with SD at 12 weeks eventually reached a PR after 18 weeks resulting in a total of 9 patients (26%) with a PR. In four patients with SD at 12 weeks, the tumor remained stable for more than 6 months. In total, 13 patients (9 with PR and 4 with long-term SD; 39%) were considered to have clinical benefit from their treatment with nivolumab.
Three patients had an initial increase in tumor burden of more than 20% followed by a PR which was considered to be pseudoprogression.

The median follow up was 27.5 months (95% CI: 19.3-upper boundary of CI not attained); the minimum follow up was 1.9 months. Median time to response in the nine responders was 2.6 months (95% CI: 2.3-upper boundary of CI not attained). The median duration of response was 7.0 months (95% CI: >3.0). Two patients with a PR had to discontinue treatment due to adverse events (pneumonitis and pneumonitis in combination with nausea). Their responses lasted 3 and 8 months. One of the responding patients received only one dose of nivolumab. Five patients with clinical benefit discontinued study treatment after one year according to protocol rules, with two of them having ongoing clinical benefit. Responses and duration of treatment of all patients are visualized in the swimmer plot in figure 2.

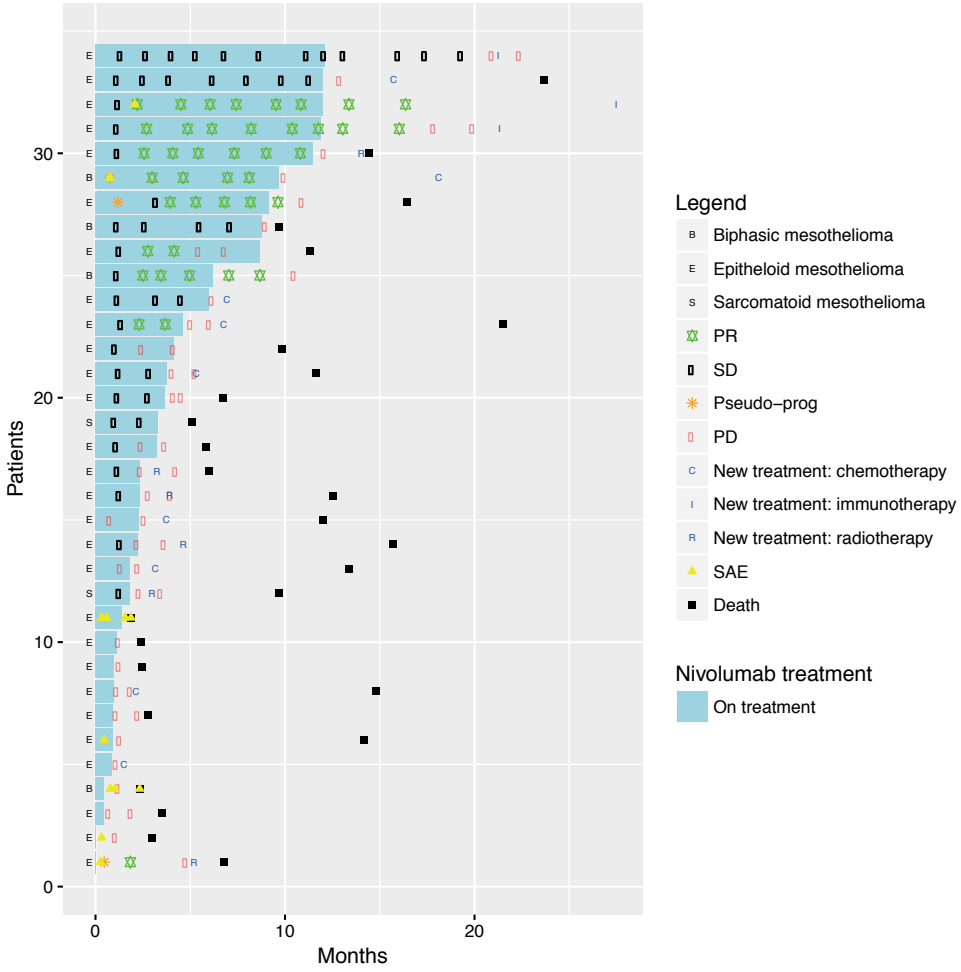


**Figure 2. Efficacy of Nivolumab in swimmerplot organized by treatment duration.**

Median PFS was 2.6 months (95% CI: 2.23 – 5.49) and at six months, 29% of patients (95% CI 18% - 50%) were free of progression (figure 3A). Median OS was 11.8 months (95% CI:

9.7-15.7) (figure 3B). At 6 months the OS was 74% (95% CI 60% - 90%) and after one year 50% (95% CI: 36% - 70%).

**Biomarkers**

Pre-treatment biopsies were taken from all patients according to study protocol and 33 out of the 34 patients that received at least one course of nivolumab were evaluable for PD-L1 expression. PD-L1 expression on > 1% of tumor cells was seen in 9 samples (27%) of which 7 (78%) were epithelioid, 1 (11%) sarcomatoid and 1 (11%) mixed type. PD-L1 expression was positive in 4 of the 9 patients (44%) with a PR. Of all 13 patients that experienced clinical benefit 5 (38%) had PD-L1 expression while PD-L1 expression was demonstrated in 4 (20%) out of 20 patients without clinical benefit (Table 2A). On-treatment biopsies were obtained from 31 patients with 27 samples being evaluable. In four cases there was no accessible tumor left to biopsy, or no viable tumor was found in the specimen. Of the 13 patients with clinical benefit, 11 samples were evaluable and 3 (27%) were PD-L1 positive. Of the patients without clinical benefit, 3 out of 16 evaluable samples (19%) were PD-L1 positive (Table 2B). There was no correlation between PD-L1 expression in pre-treatment biopsies compared to on-treatment biopsies. PD-L1 expression in neither pre-treatment nor on-treatment biopsies correlated with outcome (p-values 0.43 and 0.66 respectively).



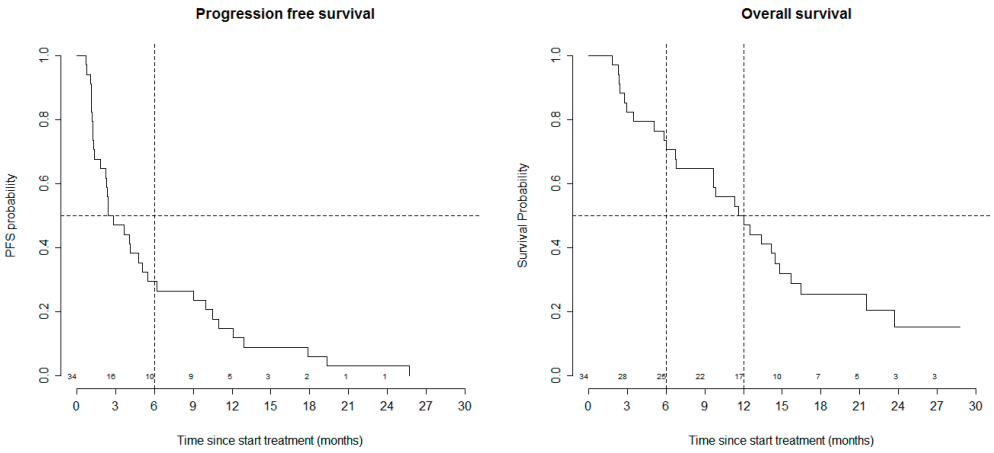


**Figure 3. A Progression Free Survival**
**Figure 3. B Overall Survival**

**Table 2 PD-L1 Expression**

| Pre-treatment biopsy | PD-L1 + 1-5% | PD-L1 + 5-10% | PD-L1 + 10-25% | PD-L1 + 25-50% | PD-L1 + >50% | PD-L1 - | Biopsy not evaluable | Total |
|---|---|---|---|---|---|---|---|---|
| **Clinical benefit +** | 1 | 0 | 0 | 2 | 2 | 8 | 0 | 13 |
| **Clinical benefit -** | 1 | 0 | 1 | 1 | 1 | 15 | 1 | 20 |
| **Pt not evaluable** | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 |
| **Total** | 2 | 0 | 1 | 3 | 3 | 24 | 1 | **34** |

**A** PD-L1 expression in pre-treatment biopsies of 34 patients that were included. Patients with a PR and patients with long-term SD (≥6 months) were considered to have clinical benefit. Expression was quantified in five categories: 1-5% positive cells, 5-10%, 10-25%, 25-50% and ≥50% positive cells. PD-L1 expression did not correlate with outcome (p = 0.43).

| On-treatment biopsy | PD-L1 + 1-5% | PD-L1 + 5-10% | PD-L1 + 10-25% | PD-L1 + 25-50% | PD-L1 + >50% | PD-L1 - | Biopsy not evaluable | Total |
|---|---|---|---|---|---|---|---|---|
| **Clinical benefit +** | 2 | 0 | 0 | 0 | 1 | 8 | 2 | 13 |
| **Clinical benefit -** | 1 | 0 | 1 | 1 | 0 | 13 | 2 | 18 |
| **Pt not evaluable** | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 3 |
| **Total** | 3 | 0 | 1 | 1 | 1 | 21 | 7 | **34** |

**B** PD-L1 expression in on-treatment biopsies. PD-L1 expression did not correlate with outcome (p = 0.66).

Blood biomarkers such as LDH, CRP, lymphocytes and neutrophil-to-lymphocyte ratio (NLR) were analyzed with respect to outcome. LDH, CRP, and absolute leucocyte count at baseline and at six weeks did not predict response or progressive disease. Neither was a change from baseline to week six in these parameters related to outcome. However, an increase in NLR of > 25% from baseline to week six correlated with non-response.

**Toxicity**

All 34 patients that started study treatment were included in the safety analysis. Treatment-related adverse events of any grade occurred in 26 patients (76%), most commonly fatigue (29%) and pruritus (15%) (Table 3). Grade 3 and 4 treatment related adverse events were reported in 9 (26%) patients. There was one treatment related death. This patient received amiodarone for atrial fibrillation and developed respiratory symptoms and radiologic changes, consistent with pneumonitis within 4 weeks after start of treatment. In retrospection, subtle signs of interstitial lung disease were already discernable prior to nivolumab treatment, which suggests that amiodarone initiated the pneumonitis. Both amiodarone and nivolumab were stopped immediately and the patient was treated with corticosteroids. Over the course of several weeks, he deteriorated and died, while at that time, disease progression was also suspected.

**Table 3. Treatment-related Adverse Events.**

| Adverse Events | Any grade | Grade 3-4 | Grade 5 |
|---|---|---|---|
| Any | 26 (76%) | 9 (26%) | 1 (3%) |
| General disorders | | | |
| Fatigue | 10 (29%) | 0 | |
| Fever | 3 (9%) | 0 | |
| Infusion related reaction | 2 (6%) | 0 | |
| Pruritus | 5 (15%) | 0 | |
| Allergic reaction | 2 (6%) | 1 | |
| Respiratory disorders | | | |
| Pneumonitis | 4 (12%) | 2 | 1 |
| Gastrointestinal disorders | | | |
| Nausea | 3 (9%) | 1 | |
| Vomiting | 1 (3%) | 1 | |
| Colitis | 0 (0%) | | |
| Laboratory abnormalities | | | |
| Liver biochemistry | 2 (6%) | 2 | |
| Other | | | |
| Acute kidney injury | 1 (3%) | 1 | |
| Pericardial effusion | 1 (3%) | 1 | |

Pneumonitis was reported in three other cases. One of these patients, who had a PR, developed grade 2 pneumonitis that resolved with corticosteroid treatment, but recurred after restart of nivolumab. Study treatment was therefore discontinued permanently. Two patients were admitted to the hospital with respiratory symptoms and radiologic changes suggestive of pneumonitis in combination with disease progression. After start of treatment with corticosteroids, both turned out to have pseudoprogression. One of the patients successfully restarted nivolumab after resolution of symptoms and had a PR that lasted 9.5 months. The other experienced worsening of his pre-existing nausea, simultaneously with his respiratory symptoms and therefore, study treatment was discontinued. In spite of discontinuation after only one course, he developed a PR. One patient died prior to response evaluation due to cardiac disease, unrelated to study treatment.

## Discussion

Until now, results in second-line MPM therapy have been disappointing with response rates varying between 7 and 20% [3,17]. Our study shows that single agent nivolumab has promising anti-cancer activity in this PD-L1–unselected population of patients with progressive

MPM after previous systemic treatment. With a DCR of 47% at 12 weeks, our trial met its primary endpoint. In addition to the 9 patients with a PR, there were 4 patients that had SD for a period longer than six months, suggesting a clear clinical benefit. This makes the 26% ORR in this trial encouraging for a disease that is notoriously difficult to treat. At first glance, a median PFS of 2.6 months does not seem spectacular, but the median OS of 11.8 months is very promising in this cohort of pretreated patients. These results are in line with outcomes of other immuno-oncology trials where OS is mainly driven by a small group of patients with long lasting responses. Furthermore, our results are consistent with those of the recently published phase I study with pembrolizumab that reported a response rate of 20% [18]. Patients in that trial were selected to have more than 1% PD-L1 expression. The subsequent phase II study was performed in an unselected group of mesothelioma patients and showed a comparable response rate of 21% [19]. The reported DCR of 76% at 12 weeks in this pembrolizumab trial may look superior to our results, but the limited number of patients in these trials is likely to render the difference not significant. We consider the efficacy of pembrolizumab and nivolumab to be comparable as is the case in second-line studies in NSCLC [7,20]. The Javelin trial reported 9.4% responders with avelumab, a PD-L1 inhibitor. Thus far, there is no good explanation for this difference other than a variation in patient selection [21].

Despite a higher rate of pneumonitis, the safety profile in our study was similar to those noted in previous nivolumab trials and to the phase II study with pembrolizumab. The fatal case with pneumonitis was most likely initiated by use of amiodarone and enhanced by nivolumab. A detailed retrospective analysis of the CT scans identified a barely noticeable interstitial lung disease already present before start of nivolumab. Amiodarone is well known for its risk of drug interactions and pneumonitis. To our knowledge, this is the first observation of a fatal outcome of this combination. Of the three other patients with pneumonitis, only one had a typical presentation; two others had pneumonitis simultaneously with pseudoprogression, which is likely to have aggravated respiratory symptoms. All three cases recovered completely. Pseudoprogression was seen in 3 patients (9%), which is within the expected range [22]. We did not see any cases of hyperprogression as was recently defined as time-to-treatment failure (TTF) <2 months, >50% increase in tumor burden compared to pre-immunotherapy imaging, and >2-fold increase in progression pace [23,24]. Most adverse events were manageable with established guidelines.

PD-L1 expression as a biomarker of response has been analyzed in various studies using different antibodies and staining procedures. Studies comparing different PD-L1 assays, suggest that three assays do not differ a lot from each other (SP263, 28-8, 22C3), but none give 100% interchangeable results [25,26]. In our trial, the 28-8 assay was used showing PD-L1 expression in 27% of tumors, which is consistent with previous reports of MPM [9-12]. Responses were seen irrespective of PD-L1 expression and pre-treatment PD-L1 expression

did not correlate with on-treatment expression levels. Several clinical trials demonstrated that PD-L1 expressing tumors enrich for response [7,20]. However, PD-L1 is frequently expressed non-homogenously throughout a tumor, which may lead to sampling errors. In addition, PD-L1 expression on tumor cells can be a result of innate [27] or adaptive [28,29] immune resistance. In case of innate resistance, tumors express PD-L1 without the presence of active immune cells in the tumor micro-environment and as a consequence, PD-1 blockade will not be able to elicit a response. Both factors compromise the predictive value of PD-L1 expression as a biomarker.

Due to these concerns about PD-L1, several other biomarkers are currently evaluated for their predictive value in cancer immunotherapy. Blank and Haanen designed the Cancer Immunogram that takes into account parameters such as mutational load, lymphocyte count, CRP and LDH to describe a comprehensive immune status [30]. We investigated the possibility to predict response by using blood biomarkers, including a selection of biomarkers from the Cancer Immunogram. LDH, CRP and absolute lymphocyte count did not correlate with response in our patient set. However, a rise in NLR from baseline to week six did predict for non-response. None of the patients with an increase had a response except for one. In this patient, the rise in NLR was caused by use of corticosteroids which is known to induce an increase in neutrophil levels [31]. After discontinuation of corticosteroids, the NLR decreased sharply in this patient. NLR has prognostic value in several tumor types including MPM [32] but its merit as a predictive parameter has to be validated in a larger patient cohort. Since time to response is fairly long in immunotherapy, it may be convenient to have a marker that predicts non-response at an early time point in order to withhold a potentially toxic treatment. It should be noted however, that in our cohort no meaningful difference in NLR increase was observed between patients with progression and those with SD.

In conclusion, nivolumab has meaningful clinical activity and an acceptable safety profile in second line in an unselected population of patients with mesothelioma. Further studies with a combination of checkpoint inhibitors (ipilimumab and nivolumab) are ongoing.

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CHAPTER 7

# Discussion and Future Perspectives

## Mesothelioma research

Research in mesothelioma is notoriously difficult for several reasons. The patient population is small -around 500 new patients a year in the Netherlands- and heterogeneous in presentation. The three main histological types of mesothelioma each have their own disease course in time and response to treatment. Within the epithelial type, there can be large differences in prognosis and responses to therapy. It is likely that genetic variation in the tumor contributes to this heterogeneity. Commonly, the physical condition of patients with mesothelioma is negatively affected by disease symptoms and this reduces the – already small- number of patients eligible for clinical trials and research. The majority of mesothelioma patients has been exposed to asbestos. This material is evidently carcinogenic but it takes a long time to induce cancer; the latency period between asbestos exposure and a diagnosis of mesothelioma is somewhere between 30 and 50 years.



In sophisticated mouse models, the time needed to develop mesothelioma has been reduced significantly [1], but tumor induction still takes several months. Moreover, most mice develop sarcomatoid mesothelioma while in humans, the vast majority has epithelial type, making a mouse model not representative for the bulk of human mesothelioma patients. Cell lines grow faster and are easier to handle than tumors in mice. Long established cell lines however, acquire changes that adapt the cells to life in an artificial medium on plastic. In addition, selection for the fastest growing cell occurs. The longer cells are cultured, the less they resemble the original tumor due to selection pressure. This phenomenon is called genetic drift. We aimed for an *in vitro* model more representative of the original tumor and better reflecting the genetic diversity seen in mesothelioma tumors. Therefore, we developed a short-term primary tumor culture model from tumor cells derived from pleural fluid of patients with mesothelioma.

## Mesothelioma short-term primary tumor cultures

The diagnosis of malignant pleural mesothelioma is often complicated. Many different conditions present with pleural fluid and mesothelial cells are shed in this fluid regardless of the underlying condition. On cytological examination, the distinction between reactive and malignant cells cannot be made by hematoxylin eosin (HE) staining. For a definitive diagnosis of mesothelioma, invasive growth on a histologic specimen is required. A pleural fluid sample of a patient diagnosed with mesothelioma contains a mixture of both reactive and malignant mesothelial cells. A known feature of a tumor cell is continuous growth potential. Therefore, one would expect that tumor cells outgrow reactive mesothelial cells, when cultured *in vitro*. However, this does not seem to be the case. We propagated cells derived from pleural fluid and analyzed them by comparative genome hybridization

(CGH). We found that after many passages the CGH patterns normalized and deletions in the genome disappeared, indicating overgrowth of normal mesothelial cells in favor of tumor cells. For this reason, we use our primary tumor cultures only for a short period of time to assure that we have tumor cells in our experiments. Another disadvantage of our model is that we only culture tumor cells from patients that actually have pleural fluid. The sarcomatoid type usually does not produce pleural fluid and in the scarce sarcomatoid cases that do present with pleural fluid, only few tumor cells are shed into this fluid. Therefore, this type is underrepresented in our model. However, the sarcomatoid type represents less than 10% of all mesothelioma [2], so we miss out on only a fraction of patients.

## Chemical and pharmacogenomic profiling

Each model is a simplified version of its original and simplification can lead to certain drawbacks. Tumor cells in pleural fluid are easier to extract from the patient than tumor cells that grow in solid tissue. However, cells that have shed into pleural fluid may have different properties than cells that are strongly attached to a solid tumor. The group of Broaddus demonstrated that tumor cells grown in 2 dimensional layers respond differently to certain drugs than 3 dimensional growing tumors; a phenomenon called multicellular resistance [3]. The dual intention of our culture model was 1) to predict the best chemotherapy for an individual patient by testing sensitivity of its tumor cells to a small number of clinically used chemotherapy regimens (chemical profiling to personalize treatment as described in chapter 4) and 2) to expand the number of existing mesothelioma cell lines with several short-term tumor cultures for screening a large number of different drugs and correlating the results with genomic data (pharmacogenomic profiling as described in chapter 5).

Culturing in 3D models is more challenging and time consuming than in 2D models and large-scale drug screening is not possible. Therefore, we accepted the limitations of our 2D model and demonstrated that multicellular resistance was not an issue with the drugs that were found to be effective (FGFR inhibitors) by also testing them in an *in vivo* model. As for the chemical profiling and prediction of the best chemotherapeutic drug(s) for a patient, multicellular resistance is not a problem either since all drugs tested have already proven their value in clinical trials and practice. Several other factors can influence the outcome of our drug sensitivity screens, for example the time of drug exposure and the cut-off levels that were set. Ideally, one would use a test cohort and a validation cohort for determining the cut-off levels but patient numbers were too small for this. That our cut-off levels are indeed well chosen is demonstrated by the RNA sequencing data that demonstrate the 3 groups to be distinctly different.

## FGFR inhibition in mesothelioma

Exome sequencing has demonstrated a low mutational load in MPM when compared to other tumor types (Figure 1) [4-6]. The chance of a targetable mutation is highest in tumor types with a high mutational load like non-small cell lung cancer (NSCLC) and melanoma. Several sequencing studies demonstrated loss of tumor suppressor genes as the most common type of mutation in mesothelioma [5-7]. Our pharmacogenomic profiling study confirmed this. Furthermore, we saw increased sensitivity to inhibition of the FGF pathway, both in immortalized cell lines as in short-term cultures. This is previously described in mesothelioma cell lines [8, 9]. FGFR inhibitors so far are mostly 'dirty' drugs targeting not only FGFR but also PDGF and VEGF. Several clinical trials studied the efficacy of FGFR inhibitors in mesothelioma. A study using dovitinib, inhibiting both VEGF and FGFR, was halted prematurely due to lack of activity and poor tolerability [10]. The LUME-meso trial, a large double-blind, randomised, placebo-controlled phase lll study using the multi-RTK inhibitor nintedanib showed no difference in PFS between the study group and the placebo group [11]. A phase Ib trial combining cisplatin and pemetrexed with a FGF ligand trap was recently published and showed a response rate of 44% and PFS of 7,4 months in the group using 15mg weekly. Four out of 36 patients had durable responses lasting over a year [12]. In comparison, the trial by Vogelzang in 2003 setting the standard in mesothelioma treatment, showed an ORR of 41,3% and a time to progression (TTP) of 5,7months [13]. These results show that FGFR inhibition in an unselected population has only minimal activity. However, the durable responses in the trial with the FGF ligand trap suggest that a selected group of patients may benefit from FGF pathway inhibition. The FGF pathway is complex and finding the right biomarker for selecting patients sensitive to FGFR inhibition is challenging. We found a correlation between loss of BAP1 expression and sensitivity to FGFR inhibition but this is not a straight forward biomarker as is, for example, an activating EGFR mutation in NSCLC predicting for sensitivity to EGFR-TKI's. Other, possibly still unknown, factors may play a role in the FGF pathway. Schelch recently described that loss of micro RNA (miR) 15/16 in MPM leads to loss of post-transcriptional control of the FGF-axis [14]. This suggests that combination of miRNA mimics and FGF pathway inhibitors may have synergistic effects but above all, it illustrates the importance of fundamental research to elucidate all aspects of growth and development of cancer cells.

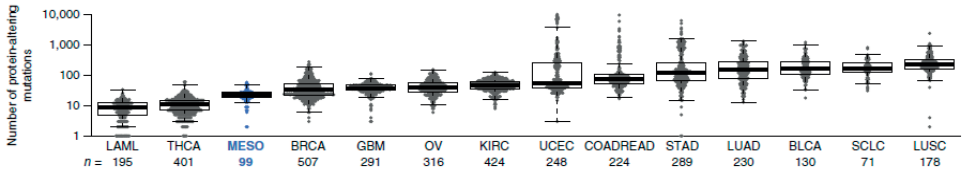


**Figure 1. Mutational load for different tumor types.**

Bueno *et al*. Nature Genetics 2016

## BAP1 in mesothelioma

The most common genomic alterations in mesothelioma are found in the genes *CDKN2A (56%),* NF2 (74%), BAP1 (57%) [5, 6, 15]. The BAP1 protein is a deubiquinating enzyme located in the nucleus. Ubiquitination and deubiquitination are post-translational protein modifications with a number of effects: they can affect protein activity, alter their cellular localization or mark them for degradation. BAP1 protein interacts with several proteins or protein complexes involved in transcription regulation, DNA damage repair, cell differentiation and cell cycle control [16]. Although its function is not fully elucidated, there is clear evidence that loss of BAP1 protein can contribute to cancer development. In mesothelioma, absence of BAP1 protein occurs most commonly through chromosomal deletions of the 3p21.1 region or somatic inactivating mutations of the BAP1 gene [7]. Germline BAP1 mutations give rise to a tumor predisposition syndrome with increased risk of developing melanomas, mesotheliomas and renal cell carcinomas [17-21]. In most cell types BAP1 deficiency causes apoptosis by suppressing expression of prosurvival genes such as bcl2 and mcl1, but not in melanocytes and mesothelial cells explaining the tumor predisposition sites [22]. In our *in vitro* experiments we found a correlation between low BAP1 expression and sensitivity to FGFR inhibition. Although BAP1 loss was not predictive for FGFR inhibitor sensitivity in 100% of cases and the exact mechanism cannot be explained with our current knowledge, the correlation was demonstrated to be plausible by functional assays using BAP1 knock outs and BAP1 constructs by *in vitro* and by *in vivo* experiments. Loss of BAP1 protein expression is easy to assess by immunohistochemistry [23] and thus BAP1 meets one of the requirements of a predictive biomarker. Ideally, a predictive biomarker explains how it predicts for sensitivity. Unfortunately, reality is that biomarkers like activating EGFR mutations in NSCLC where the exact mechanism is known, are extremely rare. Further research to unravel the complexity of the FGF pathway and the multiple functions of BAP1 and validation of BAP1 as a biomarker in a large patient cohort -challenging given the small patient population in mesothelioma- is needed.

## Immunotherapy In mesothelioma

Immunotherapy has brought a remarkable improvement in quality of life to those patients that respond to it. In our NivoMes trial, we reported a response rate of 26% which is in line with the response rates in NSCLC and other tumor types [24-27]. Apart from the patients with a significant decrease in tumor volume, there was a group of patients that demonstrated long-term stable disease (>6 months), adding to a total of 39% of patients considered to have clinical benefit from treatment with nivolumab. Compared to the tolerability and response rates of second line cytotoxic therapy in mesothelioma (ranging between 7% and 20% [28, 29]), immunotherapy is a tremendous asset for this disease. But since clinical benefit is still limited to a small group of patients, there is a pressing need for a biomarker that predicts for response, especially given the long median time to response (2,6 months in our trial with one patient reaching response only after 18 weeks) and the phenomenon of pseudoprogression. Several different biomarkers are under investigation. Expression of PD-L1 is amongst the most studied ones. In our NivoMes trial, we detected responses irrespective of PD-L1 expression. High tumor mutational load was reported to predict for response to immune checkpoint inhibition across several tumor types [30]. In mesothelioma however, mutational load is extremely low [5]. Microsatellite instability (MSI) is known to cause a multitude of somatic mutations in tumor cells resulting in a high tumor mutational load, a large lymphocytic infiltrate and increased neoantigen expression, all correlated to response to checkpoint inhibition [31]. Based on these results, the FDA has granted accelerated approval to pembrolizumab in tumor types with MSI. Evidence is emerging that loss of BAP1 expression is correlated to an inflamed tumor microenvironment [32]. In uveal melanoma, CD3 and CD8 positive T cells were more abundantly present in the tumor microenvironment of BAP1 deficient tumors [33]. In peritoneal mesothelioma, BAP1 loss was associated with increased expression of several immune checkpoint molecules [34]. Analysis of 74 pleural mesothelioma samples from The Cancer Genome Atlas (TCGA) revealed upregulation of IRF pathways in BAP1 deficient samples. IRF8 is involved in CD103-positive dendritic cells that have a role as antigen-presenting cells in stimulating cytotoxic T cells in the tumor microenvironment [6]. A gene called VISTA (V-type immunoglobulin domain-containing suppressor of T-cell activation) was recently found to repress activation of T-cells and to be highly expressed in epithelioid mesothelioma. High expression of this gene may thus serve as a negative predictor for immunotherapy [6]. Loss of the gene PBMRI, involved in epigenetic regulation, was recently described to correlate to increased T-cell infiltration and efficacy of checkpoint inhibition [35, 36]. Given the complexity of the immune system and the genomic variation that exists among different cancers, it is likely that we will need sets of biomarkers to predict response to immunotherapy, rather than one biomarker that is applicable in all tumor types. Combinations of several types of immunotherapy and immuno- and chemotherapy hold a strong promise for the future.

A combination of immunotherapy and cytotoxic chemotherapy is currently investigated in mesothelioma in the PreCOG trial (NCT0289919). Results have to be awaited.

## Future perspectives

The Netherlands houses a lot of knowledge on mesothelioma. First of all, tumor samples of each patient suspected of having mesothelioma, are validated by a panel of expert pathologists (Nederlands Mesotheliomen Panel NMP) making the diagnoses highly reliable. Secondly, there is an institute for asbestos victims (Instituut Asbest Slachtoffers IAS) that documents the extent of asbestos exposure and performs epidemiological research. Furthermore, there is a national cancer registry (Integraal Kankercentrum Nederland IKNL) including data of all cancer patients in the Netherlands that is very accurate. International acknowledged scientists perform high quality research with international collaborations with several outstanding institutes. In addition, a motivated working group of the Dutch association of pulmonologists (Nederlandse Vereniging voor Artsen voor Longziekten en Tuberculose NVALT) with members across the whole country, form a network to improve quality of care and research by composing guidelines and performing clinical trials. Patients are keen on participating in trials which can be illustrated by the quick accrual of the NivoMes trial for which patients had to have 1 or 2 extra surgical interventions. It would be fantastic to build a large biobank for research by gathering biopsies from all new patients –since the samples are all sent to the NMP for diagnosis, the infrastructure is already in place- together with a sample of blood and basic clinical data. Financial support is usually the limiting factor in propositions like these. Perhaps the Dutch government can provide this to compensate for their past and current omissions, namely 1. installing a ban on the use of asbestos only as late as 1993 while the health threats have been known much earlier and 2. keeping the unethical statute of limitations of 30 years in legal liability cases for a disease that presents commonly only after 30-50 years .

A financial and logistic challenge that our society faces is to get rid of all the asbestos that is used in the Netherlands during the last centuries. The system that is built to asses the extent of asbestos pollution and remove it, has grown to be a complicated industry that keeps prices high by sticking to excessive and incomprehensible rules. As much as eighty percent of all mesothelioma patients have had verifiable asbestos exposure. The risk of getting mesothelioma after extensive exposure is, on the other hand, as low as 5%. This number is calculated from a large cohort (6489 men and 419 women) of heavily exposed asbestos workers who were employed in the asbestos mine or mill in Wittenoom, Australia and were followed for over a period of 50 years [37]. This suggests that additional factors including genetic predisposition may be critical to develop mesothelioma since 95% of asbestos exposed workers did not develop mesothelioma. The previously mentioned BAP1 predisposition syndrome is in line with this hypothesis. It is not reasonable that we fear each

individual asbestos fiber. Instead we would better reform the asbestos renovation industry and screen their workers for this BAP1 tumor predisposition syndrome, and if present, persuade them to abandon this industry and re-educate. A minimum age of 55 years for asbestos renovation workers may also reduce the risk of developing mesothelioma given the long latency period. These propositions can only be introduced after a serious discussion in society.

It is astonishing that while all this effort and money is put into research to treat mesothelioma, asbestos - the main causative agent- is still used and produced in the majority of countries worldwide. Prevention of this disease should be key and this can only be achieved by a complete ban on mining and use of asbestos globally.

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# APPENDICES

A

Nederlandse Samenvatting
List of Publications
Dankwoord
Curriculum Vitae

## Nederlandse Samenvatting

In dit proefschrift worden de uitkomsten beschreven van onderzoeken die verricht zijn om de behandeling van patiënten met de ziekte mesothelioom -in de volksmond ook wel longvlieskanker of asbestkanker genoemd- te verbeteren door te kijken naar individuele tumorkenmerken en de behandeling zo mogelijk daarop aan te passen.

**Hoofdstuk 1**

Mesothelioom is een vorm van kanker die ontstaat uit mesotheelcellen, de dunne laag cellen waaruit het borstvlies -de binnenbekleding van de borstholte-, het longvlies -de buitenbekleding van de long- en het buikvlies -de binnenbekleding van de buikholte- bestaan. De ziekte verspreid zich meestal lokaal en veroorzaakt een verdikking van het vlies of ophoping van vocht, of beiden wat in de borstholte kan leiden tot klachten van pijn en kortademigheid, en bij het buikvlies tot verstopping en pijn. Onbehandeld gaan de meeste patiënten met deze ziekte dood binnen 2 jaar na het begin van de klachten. In dit proefschrift zal ik mij beperken tot het mesothelioom van het borst- en longvlies, het zogenaamde pleurale mesothelioom.



Ingeademde asbestvezels zijn de belangrijkste veroorzakers van de ziekte pleuraal mesothelioom. Asbest is een verzamelnaam voor een groep van 6 verschillende minerale vezels die over de hele wereld in de natuur voorkomen. De vuurbestendige eigenschappen van asbest waren al in de oudheid bekend, wat blijkt uit archeologische vondsten van kleipotten waarin asbest is verwerkt om ze vuurbestendig te maken. Een bekende toepassing van asbest bij de Romeinen is het gebruik in de lonten van de Vestaalse maagden die moesten zorgen voor het brandend houden van het eeuwige vuur in de tempel van de godin Vesta. Asbest werd ten tijde van de industriële revolutie steeds populairder omdat het materiaal bestendig was tegen hitte, elektriciteit en chemicaliën en daarom ideaal was om stoommachines mee te isoleren die in die tijd ontwikkeld werden. Om aan de groeiende vraag naar asbest te voldoen werden er commerciële asbestmijnen geopend in landen als Canada, Rusland, Schotland, Engeland, Duitsland en Italië. Rond 1900 werd er een procedé ontwikkeld waarbij asbest werd vermengd met cement waarmee er een keur aan nieuwe toepassingsmogelijkheden in de bouw ontstond.

Laat in de 19$^{de}$ eeuw werd al vermoed dat asbest schadelijke effecten op de gezondheid kon hebben. In 1924 werd voor het eerst gepubliceerd over asbestose, een ziekte waarbij het longweefsel verlittekent als gevolg van asbest. Het vermoeden dat asbest tot kanker kon leiden werd gepubliceerd in 1938 en in 1955 werd door middel van dierexperimenten het wetenschappelijke bewijs hiervoor geleverd. Vanaf 1960 werd duidelijk dat asbest niet alleen longkanker, maar ook de tot dan toe zeer zeldzame ziekte mesothelioom kon veroorzaken. De Nederlandse arts Stumphius deed onderzoek naar deze ziekte en vond een

uitzonderlijk hoog aantal patiënten onder arbeiders van een scheepswerf waar veel asbest werd gebruikt. In zijn proefschrift in 1969 riep hij op tot beschermende maatregelen bij het gebruik van asbest. Pas in 1993 werd in Nederland het gebruik van asbest verboden.

Het aantal mensen met mesothelioom is sinds 1969 verzesvoudigd tot 550 nieuwe patiënten per jaar. De latentietijd van mesothelioom -de periode tussen blootstelling aan asbestvezels en de eerste symptomen van de ziekte- bedraagt 30 tot 50 jaar. Door deze lange latentietijd wordt verwacht dat na het verbod op het gebruik van asbest in 1993, het aantal nieuwe patiënten per jaar in Nederland pas vanaf 2021 zal gaan dalen. In de rest van de wereld varieert het aantal nieuwe patiënten met mesothelioom sterk. Dat heeft een aantal oorzaken, waaronder de mate waarin asbest werd en wordt gebruikt. De meeste geïndustrialiseerde landen hadden in het verleden een hoge asbestconsumptie maar nu -net als Nederland- het gebruik van asbest verboden. Er zijn echter nog circa 140 landen wereldwijd waar er nauwelijks of geen regels omtrent asbest zijn en dit dus nog veelvuldig verwerkt wordt. De ziekte is met 550 nieuwe patiënten per jaar zeldzaam, zeker in vergelijking met de meer dan 13.000 mensen waarbij in Nederland jaarlijks longkanker wordt gediagnosticeerd. Het vaststellen van mesothelioom is lastig en omdat de ziekte zeldzaam is, is er in Nederland een panel van experts (het Nederlands Mesothelioom Panel NMP) samengesteld om zoveel mogelijk zekerheid te hebben over de diagnose. Er zijn echter veel landen waar, door het ontbreken van een dergelijke infrastructuur, de diagnose en daarmee de cijfers over het aantal patiënten met de ziekte, veel minder betrouwbaar zijn. In totaal zijn er in Nederland circa 70 werkbranches waarbij asbestexpositie kan hebben plaatsgevonden. De meeste patiënten hebben blootstelling gehad tijdens hun werk in de bouw, op een scheepswerf of bij de auto-industrie waar asbest gebruikt werd in de remblokjes. Dit verklaart dat de ziekte veel vaker bij mannen voorkomt dan bij vrouwen.

**Hoofdstuk 2**

Onderzoek naar nieuwe behandelingen voor de ziekte mesothelioom is ingewikkeld om meerdere redenen. Het is lastig om de hoeveelheid tumor bij een mesothelioom te meten doordat de tumor niet als een bol in een orgaan groeit maar zich verspreidt over een groot dun oppervlak. Daardoor is het meten van het effect van een behandeling, de zogenaamde responsevaluatie (dit doe je door de hoeveelheid tumor voor en na een behandeling met elkaar te vergelijken) gecompliceerd. Om groepen patiënten in klinische onderzoeken goed vergelijkbaar te maken wordt hun ziekte ingedeeld in stadia waarmee de uitgebreidheid van de tumor vastgelegd wordt. Het lastig kunnen meten van de hoeveelheid tumor maakt dus ook het onderling vergelijken van patiënten moeilijk. Andere maten voor de effectiviteit van een behandeling zijn de zogenaamde progressievrije overleving (Progression Free Survival PFS, de tijd sinds start van de behandeling waarin de kankergroei tot stilstand is gebracht) en de algemene overleving (Overall Survival OS, de tijd sinds start van de behandeling tot aan overlijden). Er zijn binnen het reeds zeldzame pleurale mesothelioom meerdere subtypes

die verschillende groeisnelheden en dus verschillende prognoses kennen wat vergelijking van de progressievrije en algemene overleving in studieverband bemoeilijkt.

Bij de behandeling van kanker in het algemeen geeft het chirurgisch verwijderen van een tumor de beste kansen op genezing als de tumor radicaal -dat wil zeggen in zijn geheel- kan worden weggehaald. Bij mesothelioom is radicale verwijdering extreem lastig door de verspreiding over een groot oppervlakte in de borstholte. Verscheidene artikelen beschrijven series van patiënten waarbij het borstvlies inclusief de hele long (extrapleurale pneumonectomie) werd verwijderd waarbij een lange overleving werd gezien. De patiënten die voor zo'n operatie werden geselecteerd waren meestal jonge patiënten met een beperkte hoeveelheid tumor en een uitstekende conditie; factoren die op zichzelf al tot een langere overleving kunnen leiden. Deze 'selectie bias' zoals deze vertekening door selectie wordt genoemd, kan worden vermeden door patiënten door middel van loting te verdelen in 2 groepen waarbij de ene groep een behandeling wel en de andere groep een behandeling niet krijgt; het zogenaamde gerandomiseerde onderzoek. In de 'MARS' trial kregen patiënten met pleuraal mesothelioom een behandeling met alleen chemotherapie, of chemotherapie in combinatie met een extrapleurale pneumonectomie en bestraling van het gehele operatiegebied. De onderzoekers concludeerden dat een dergelijke ingrijpende operatie geen voordeel bood en misschien zelfs nadeel ten opzichte van alleen chemotherapie. In Nederland worden patiënten met mesothelioom alleen geopereerd in het kader van een klinisch onderzoek. Nieuwe studies met longsparende operaties geven hopelijk een antwoord op de vraag of een operatie zorgt voor een verbeterde overleving. Wat in ieder geval duidelijk is geworden uit deze onderzoeken is dat voor de meeste patiënten een operatie niet haalbaar is omdat hun conditie te slecht is of de ziekte te uitgebreid.



De standaardbehandeling van pleuraal mesothelioom bestaat uit een combinatie van 2 soorten chemotherapie, te weten cisplatin en pemetrexed. In een studie uit 2003 werd met deze combinatie bij 41% van de patiënten een respons gezien (respons wordt gedefinieerd als een afname van de hoeveelheid tumor van 30% of meer). De combinatie van cisplatin en gemcitabine laat vergelijkbare resultaten zien maar is nooit in een gerandomiseerde studie getest. Met een chemotherapeutische behandeling is genezing niet mogelijk; doel is om de ziekte zolang mogelijk te remmen en klachten die erdoor veroorzaakt worden te verminderen. Iedere behandeling kan echter ook bijwerkingen hebben. Een chemotherapieschema bestaat uit vier toedieningen (kuren) met telkens een interval van drie weken. Bij een uitzonderlijk goede respons en goede tolerantie worden wel eens zes kuren gegeven. Daarna wordt elke 3 maanden met CT scans gemonitord of de tumor nog stabiel is. Het zou prettig zijn om voor de individuele patiënt te kunnen voorspellen welke chemotherapeutische behandeling de grootste kans van slagen heeft. Er wordt dan ook veel onderzoek gedaan naar specifieke kenmerken van de tumor of de patiënt -zogenaamde biomarkers- die dit kunnen voorspellen. Hierbij wordt onderscheid gemaakt tussen predictieve biomarkers -biomarkers

die de respons op een bepaalde behandeling voorspellen- en prognostische biomarkers die iets zeggen over het beloop van de ziekte los van een behandeling. Voor de behandeling met pemetrexed is in een retrospectieve ('terugkijkende') studie een biomarker gevonden die voorspellend lijkt. Voordat een dergelijke biomarker echter gebruikt kan worden moet de waarde ervan bevestigd worden in een prospectieve gerandomiseerde studie waarvoor duizenden patiënten nodig zijn om een statistisch significant oordeel te kunnen geven. Vanwege de zeldzaamheid van het pleurale mesothelioom blijken dit soort grote studies in de praktijk niet haalbaar.

Aangezien een chemotherapeutische behandeling niet genezend is zal bij iedere patiënt met mesothelioom, de tumor korte of langere tijd na de eerste behandeling weer gaan groeien. Als de conditie van de patiënt dit toelaat kan dan een zogenaamde tweedelijns behandeling worden gegeven. Als de progressievrije overleving na de eerstelijns chemotherapie lang is (>6m) kan hetzelfde schema met cisplatin en pemetrexed overwogen worden. Verschillende andere soorten chemotherapie zijn onderzocht als tweedelijns behandeling waarbij de kansen op respons liggen tussen de 10 en 20%. Geen van deze studies liet echter een overlevingsvoordeel zien. Er is dan ook geen officiële standaard tweedelijns behandeling geregistreerd voor mesothelioom. Geadviseerd wordt patiënten zoveel mogelijk in studieverband te behandelen.

Er wordt ook onderzoek gedaan naar de resultaten van onderhoudsbehandelingen met chemotherapie waarbij de behandeling wordt voortgezet zolang deze goed verdragen wordt en de tumor onder controle houdt. Dit blijkt alleen het geval met 'monotherapie', een behandeling met één enkele soort chemotherapie; onderhoudsbehandeling met een combinatie van 2 soorten chemotherapie geeft te veel bijwerkingen en is daarom niet lang vol te houden. Tot nu toe lijken ook bij monotherapie de voordelen niet op te wegen tegen de nadelen zoals bijwerkingen en de last van een driewekelijks bezoek aan het ziekenhuis.
Chemotherapie doodt snel-delende cellen -wat tumorcellen bij uitstek zijn- maar kan geen onderscheid maken tussen tumorcellen en andere snel-delende cellen in het lichaam zoals de cellen van het beenmerg (rode en witte bloedcellen en bloedplaatjes) en cellen van de binnenbekleding van ons maagdarmstelsel. Doordat deze cellen beschadigd raken krijg je bijwerkingen zoals bloedarmoede, verhoogde vatbaarheid voor infecties, bloedingen, misselijkheid, braken en diarree. Door onderzoek wordt er steeds meer bekend over de verschillende mechanismen in een cel die leiden tot de ontwikkeling van kanker. Er worden medicijnen ontwikkeld waarmee specifiek deze mechanismen kunnen worden aangegrepen om tumorgroei te remmen, de zogenoemde doelgerichte therapie (targeted therapy). Deze doelgerichte behandelingen zijn veelal in tabletvorm beschikbaar en worden meestal beter verdragen waardoor ze langere tijd gegeven kunnen worden. Diverse doelgerichte therapieën worden in klinische studies getest bij patiënten met mesothelioom maar tot op heden zonder succes.

Immunotherapie is een behandeling waarbij het eigen immuunsysteem van de patiënt gestimuleerd wordt door een medicijn om tumorcellen op te ruimen. Dit lijkt een veelbelovende behandeling voor het mesothelioom omdat er rond de tumorcellen veel immuuncellen te vinden zijn. Dit is niet bij alle tumortypen het geval. Daarnaast zijn er meerdere patiënten beschreven met mesothelioom waarbij de tumor vanzelf kleiner werd wat ook suggereert dat het immuunsysteem van de patiënt een rol speelt. Momenteel zijn er diverse onderzoeken gaande waarbij op verschillende plekken in het immuunsysteem wordt aangegrepen.

**Hoofdstuk 3**

In de loop der jaren zijn er veel medicijnen getest in klinische studies voor patiënten met mesothelioom maar helaas bleek het overgrote deel niet werkzaam. In preklinisch onderzoek (studies met bijvoorbeeld cellijnen of muizen) waren deze medicijnen veelal wel effectief. Hieruit blijkt dat het lastig is om op basis van preklinisch onderzoek goed te voorspellen welke middelen in de mens daadwerkelijk werkzaam zullen zijn. Er zijn diverse preklinische modellen die gebruikt worden voor onderzoek; ieder met eigen voor- en nadelen. Een goed model moet lijken op de oorspronkelijke tumor zoals die in de mens groeit en idealiter niet alleen tumorcellen bevatten maar ook de overige cellen die normaal gesproken in een tumor voorkomen zoals bijvoorbeeld immuuncellen. Een model moet makkelijk zijn in het onderhoud en reproduceerbare resultaten geven. Verder is het prettig als er meerdere medicijnen tegelijkertijd getest kunnen worden aangezien er continu nieuwe middelen ontwikkeld worden. Helaas bestaat er niet één model waarin al deze eigenschappen verenigd zijn. Cellijnen bijvoorbeeld zijn cellen van één bepaalde tumorsoort die oneindig door kunnen groeien in het laboratorium. Deze cellen zijn makkelijk te onderhouden en kunnen gebruikt worden om heel veel nieuwe medicijnen tegelijkertijd te testen. Een belangrijk nadeel van cellijnen is dat de cellen zich toch geleidelijk aanpassen aan hun nieuwe leefomgeving in plastic kweekflessen en na jarenlang gebruik genetisch niet meer zo goed lijken op de oorspronkelijke tumorcel waar ze van afstammen. Verder groeien cellijnen als één cellaag in een kweekfles terwijl een tumor 3-dimensionaal groeit. Sommige medicijnen zijn wel effectief in een 2-dimensionaal systeem zoals een cellijnmodel maar blijken vervolgens niet te werken in een 3-dimensionaal systeem. Een 3-dimensionaal model lijkt dus meer op een tumor zoals die in een mens voorkomt. Deze modellen -ook wel spheroïden of organoïden genoemd- zijn echter zeer bewerkelijk en niet geschikt om grote hoeveelheden medicijnen tegelijk op te testen.

Primaire tumorkweken zijn tumorcellen uit pleuravocht van een patiënt die gedurende enkele weken in kweekflessen in het laboratorium groeien en zich vermenigvuldigen. Door ze maar korte tijd te kweken voorkom je dat er veranderingen aan de tumorcellen ontstaan door het kweken zelf zoals wel gebeurt bij cellijnen. Deze primaire tumorkweken zijn goed

te gebruiken voor het testen van relatief grote hoeveelheden medicijnen en weerspiegelen de heterogeniteit binnen een tumortype beter dan cellijnen.

Muizenmodellen zijn erg belangrijk bij het ontwikkelen van medicijnen omdat hiermee ook factoren zoals farmacokinetiek (de processen die de absorptie, distributie en eliminatie van een geneesmiddel in het lichaam bewerkstelligen), farmacodynamiek (de werkingsmechanismen van een geneesmiddel in het lichaam) en bijwerkingen beoordeeld kunnen worden. Verder groeit een tumor in een muis 3-dimensionaal en lijkt daarmee meer op een tumor in de mens. Aanvankelijk werden mesotheliomen bij muizen geïnduceerd door de dieren bloot te stellen aan asbest. Hierbij duurde het echter vrij lang voordat de mesotheliomen zich ontwikkeld hadden. Recent zijn er genetische muizenmodellen ontwikkeld waarbij de muizen DNA mutaties hebben die ervoor zorgen dat zich in korte tijd mesotheliomen in de muis ontwikkelen. Daarnaast bestaan er modellen waarbij een klein stukje van een tumor uit een patiënt in een muis wordt geplaatst en gaat groeien (Patient-derived xenograft). Zo'n stukje tumor gaat echter alleen groeien als de muis genetisch is bewerkt zodat hij geen eigen afweersysteem meer heeft. In een dergelijk muizenmodel is het echter niet mogelijk de invloed van het afweersysteem op een tumor te bestuderen. Verder is dit model erg tijdrovend. Zo heeft ieder model zijn voor- en nadelen en blijkt het ideale model helaas niet te bestaan.

**Hoofdstuk 4**

In dit hoofdstuk beschrijf ik de manier waarop we in het laboratorium primaire tumorkweken hebben ontwikkeld van tumorcellen uit het pleuravocht van patiënten met mesothelioom. Deze primaire tumorkweken hebben we gebruikt om meerdere chemotherapeutica, zowel afzonderlijk als in combinaties te testen. Hiervoor kozen we middelen en combinaties die in de klinische praktijk reeds gebruikt worden voor de behandeling van patiënten met mesothelioom. Vervolgens hebben we 10 patiënten behandeld met het middel of de combinatie waarvoor hun eigen tumorcellen in het laboratorium het meest gevoelig waren. De resultaten van de klinische behandeling bleken in hoge mate overeen te komen met de uitkomsten in het laboratorium. We zagen dat een klein deel van de patiënten gevoelig was voor veel van de in het laboratorium geteste chemotherapeutica, de zogenaamde 'responders'. Het grootste deel van de patiënten bleek helaas ongevoelig voor het merendeel van de geteste middelen, de 'non-responders'. Daarnaast was er nog een groep zogenaamde 'intermediate responders'. Bij het genetisch vergelijken van deze 3 groepen (ingedeeld op basis van hun 'chemische' profiel- het zogenaamde 'chemical profiling') bleken hun genexpressieprofielen duidelijk verschillend. Een opvallend verschil was te zien bij de 'fibroblast-groeifactor 9 ' (FGF9) en de 'fibroblast-groeifactor-receptoren 1 en 3' (FGFR1 en FGFR3) die bij de non-responders in hogere mate tot expressie kwamen. Het blokkeren van deze FGF-route in een non-responder zou er toe kunnen leiden dat de tumorcellen dood gaan. Dat bleek ook het geval toen we enkele primaire tumorcelkweken van non-responders

behandelden met een FGFR-remmer. Hiermee hebben we nieuw bewijs in handen dat de FGF-route een mogelijk doelwit is bij mesothelioom om een doelgerichte therapie tegen te ontwikkelen.

**Hoofdstuk 5**

De primaire tumorkweken uit hoofdstuk 4 zijn toegevoegd aan een panel van 889 geïmmortaliseerde cellijnen van allerlei tumortypes die in het Sanger Instituut in Engeland zijn gebruikt voor 'drug screens' met 265 verschillende medicijnen. De uitkomsten van deze drug screens werden gekoppeld aan genetische informatie die van alle cellijnen werd verkregen. Hieruit bleek wederom dat een deel van de primaire tumorkweken maar ook van de geïmmortaliseerde mesothelioomcellijnen opvallend gevoelig was voor remming van de FGF-route door middel van FGFR-remmers. Deze resultaten werden in een muizenmodel bevestigd. Vergelijking van genexpressie van de FGFR remmer gevoelige cellijnen met die van de ongevoelige cellijnen toonde een verhoogde expressie van FGF9 en FGFR3 in de gevoelige cellijnen. Bij verder genetisch onderzoek vonden we dat het eiwit BAP1 (Breast-cancer Associated Protein 1) de mate van FGFR3 expressie kan reguleren. Dit BAP1 eiwit is in mesothelioomtumoren vaak afwezig door genmutaties: veranderingen in het DNA. (Afwezigheid van) BAP1 zou kunnen dienen als biomarker om patiënten met mesothelioom te selecteren die baat kunnen hebben bij behandeling met een FGFR-remmer.



**Hoofdstuk 6**

Zoals al eerder geschreven lijkt het immuunsysteem een rol te spelen in de controle van tumorgroei bij het mesothelioom. Bij tumortypes zoals longkanker en melanoom -een agressieve vorm van huidkanker- blijkt immunotherapie met het medicijn nivolumab succesvol bij een deel van de patiënten. Tumorcellen kunnen door bepaalde eiwitten op hun celoppervlak te ontwikkelen signalen afgeven aan immuuncellen. Met een eiwit genaamd PD-L1 (Programmed Death-Ligand 1) kan de tumorcel binden aan het eiwit PD-1 op het oppervlak van een immuuncel en daarmee het signaal uitzenden dat hij niet schadelijk is en niet opgeruimd hoeft te worden. Nivolumab is een medicijn dat bindt aan het PD-1 eiwit op het oppervlak van de immuuncel en daarmee voorkomt dat de tumorcel kan binden. Hierdoor wordt deze laatste WEL als schadelijk herkend en opgeruimd. In de NivoMes studie (Nivolumab in Mesothelioma) die in het Antoni van Leeuwenhoek is opgezet en uitgevoerd hebben we 34 patiënten met mesothelioom behandeld met het medicijn nivolumab. Dit medicijn werd elke 2 weken op de dagbehandeling via een infuus gegeven. De behandeling kon worden doorgezet zolang deze effectief was en goed verdragen werd tot een maximum van 1 jaar. Bij 24% van de patiënten zagen we dat de tumor kleiner werd (>30% afname) en bij nog eens 8 patiënten was de tumor 3 maanden na start van de behandeling nog steeds stabiel. Bij 3 patiënten zagen we initieel een toename van de tumor van meer dan 20%, gevolgd door afname van meer dan 30%; dit wordt pseudoprogressie genoemd. De meest voorkomende bijwerkingen waren moeheid (29% vd patiënten) en jeuk (15%). Ernstige

bijwerkingen kwamen voor bij 26% van de patiënten. Een voorbeeld van zo'n ernstige bijwerking is een longontsteking die kan ontstaan doordat het immuunsysteem te actief wordt en zicht richt tegen eigen organen zoals de longen. Bij de meeste patiënten is een dergelijke bijwerking te behandelen met medicijnen die het immuunsysteem weer remmen. Eén patiënt is helaas overleden aan zo'n longontsteking die mede veroorzaakt werd door gebruik van een ander medicijn. De hoeveelheid van het eiwit PD-L1 op de tumorcellen werd onderzocht als biomarker maar bleek niet goed te kunnen voorspellen bij wie de behandeling zou aanslaan. Concluderend is het medicijn nivolumab effectief bij een deel van de patiënten met mesothelioom en wordt het door de meeste patiënten goed verdragen.

Al deze onderzoeken brengen de personalisering van de behandeling voor patiënten met de ziekte mesothelioom een klein stapje dichterbij.

## List of Publications

### Publications that are part of this thesis

**Quispel-Janssen** J, van der Noort V, de Vries JF, Zimmerman M, Lalezari F, Thunnissen E, Monkhorst K, Schouten R, Schunselaar L, Disselhorst M, Klomp H, Hartemink K, Burgers S, Buikhuisen W, Baas P
*Programmed Death 1 Blockade With Nivolumab in Patients With Recurrent Malignant Pleural Mesothelioma*
Journal of Thoracic Oncology 2018 Oct 13(10):1569-1576

**Quispel-Janssen JM*,** Schunselaar L*, Kim Y, Alifrangis C, Zwart W, Baas P, Neefjes J.
* equal contribution
*Chemical profiling of primary mesothelioma cultures defines subtypes with different expression profiles and clinical responses*
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* equal contribution
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*Emerging Therapies for Malignant Pleural Mesothelioma*
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Disselhorst MJ, **Quispel-Janssen J**, Lalezari F, Monkhorst K, de Vries JF, van der Noort V, Harms E, Burgers S, Baas P
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*Differential expression of estrogen receptors alpha and beta mRNA during differentiation of human osteoblast SV-HFO cells*
Endocrinology 1997 Nov 138(11):5067-70

A

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-Lieve Rutger, samen met jou kan ik de wereld aan! Dank voor ontelbaar veel dingen maar tijdens dit promotietraject vooral ook voor je 'vingers die me wijzen op mezelf'.

## Curriculum Vitae

Josina Martine Maria Franscisca Janssen werd op 31 januari 1975 geboren in Sevenum, Noord-Limburg. Zij doorliep het gymnasium aan het Boschveldcollege in Venray en slaagde hiervoor in 1993 cum laude. Aansluitend begon zij met haar studie geneeskunde aan de Erasmus Universiteit in Rotterdam. Een jaar later startte zij op het Rotterdams Conservatorium een studie klassiek saxofoon met als bijvak piano. Naast beide studies deed zij wetenschappelijk onderzoek op de afdeling microbiologie en het LASER centrum van het AMC naar de effecten van photodynamische therapie op de groei van Pseudomonas Aeruginosa en in het laboratorium van de afdeling endocrinologie van de Erasmus Universiteit naar expressie van oestrogeenreceptoren in een humaan osteoblastenmodel. Tussen 1998 en 2000 doorliep zij haar coschappen in diverse ziekenhuizen in de regio Zuid-Holland en Zeeland. Zij volgde een keuze-coschap in de Local Authority Clinic in Khayelitsha, een township nabij Kaapstad, Zuid-Afrika. Na een periode als ANIOS gewerkt te hebben op de afdeling interne geneeskunde van het Onze Lieve Vrouwe Gasthuis in Amsterdam startte zij in 2002 met haar opleiding tot internist aan het UMC Utrecht onder leiding van Prof. dr. D.W. Erkelens en later van mw Prof. dr. E. van der Wall. In 2005 maakte zij de overstap naar de opleiding longziekten in het Onze Lieve Vrouwe Gasthuis in Amsterdam onder leiding van dr. H.B. Kwa. Zij volgde een academische stage in het Leids Universitair Medisch Centrum onder leiding van Prof. Dr. K.F. Rabe. Eind 2009 voltooide zij haar opleiding tot longarts en na een reis van enkele maanden begon zij met haar promotieonderzoek in het Nederlands Kanker Instituut-Antoni van Leeuwenhoek onder leiding van Prof. dr. P. Baas en Prof. dr. J.J.C. Neefjes. Het onderzoek werd gedurende het hele promotietraject gecombineerd met patientenzorg. Vanaf 2016 werkte zij als longarts in het Maasstad Ziekenhuis en het Erasmus MC in Rotterdam. Momenteel is zij werkzaam als longarts-oncoloog in het Haaglanden Medisch Centrum in Den Haag. Josine is getrouwd met Rutger Quispel en samen met hun 3 kinderen -Michiel, Teun en Lola- wonen zij in Oegstgeest.