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Immune checkpoint inhibitors in mesothelioma

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Table of contents

1

Chapter 1

General introduction and outline of thesis

Malignant pleural mesothelioma (MPM) is an aggressive tumor originating from the mesothelial cells of the pleural cavity. It has a causal relation with (occupational) asbestos exposure (1).

Asbestos is a group of 6 different mineral fibers naturally occurring throughout the world; all are composed of long and thin fibrous crystals. Two large subgroups are known as the serpentine and amphibole subgroup. Chrysotile (white asbestos) is a serpentine mineral, of which the fibers are relatively large and curly and it is the most commonly used type of asbestos. Amphibole minerals are needle-like and members of this class are amosite (brown asbestos), crocidolite (blue asbestos), tremolite, actinolite and anthophyllite. Asbestos is being used since prehistoric times due to its fire-resistant properties (2). In the last century it has been used extensively in buildings and ship-building, because of its strength, fire-resistance and isolating properties. Furthermore it is cheap. All types of asbestos fibers can cause mesothelioma.

Asbestos is banned from most countries in the world, but it is estimated that approximately 43,000 people will die from this disease worldwide (3). The survival is poor, with a 5 year survival rate in Europe of 7% (4). In the Netherlands, spray asbestos was banned in 1978 and complete use of asbestos in 1993. Unfortunately, exposure is still possible since it is incorporated in many buildings and sheds. With a latency time between asbestos exposure and diagnosis of mesothelioma of 20 to 50 years (1, 5) we are still confronted with 600 patients per year in the Netherlands.

The carcinogenic mechanism of how asbestos can cause MPM is not completely understood. Chronic inflammation may predispose individuals to develop this malignancy as is concluded from microscopic examinations. In the tumor microenvironment (TME) inflammation promotes proliferation and survival of malignant cells (6). Asbestos can cause an influx of mononuclear phagocytic cells into the tumor that internalize asbestos fibers. These phagocytic cells will release proinflammatory cytokines. In combination with chronic inflammation, oxygen radical release and DNA damage, these processes promote malignant transformation. In combination with the immunosuppressive environment, this promotes cancer growth. It has been shown that CXCR3 (the chemokine receptor on the surface of T helper cells) and the production of interferon gamma (IFN-γ) were reduced in peripheral CD4+ cells of asbestos-exposed patients, thereby showing the decreased antitumor immunity of asbestos (7).

Only a minority of asbestos exposed people develop mesothelioma. This might for some cases be explained by genetic susceptibility. Germline mutations in (BRCA1) associated protein-1 (BAP1) tumor suppressor gene cause the BAP1 tumor predisposition syndrome. Carriers have an increased risk of developing

mesothelioma, (uveal) melanoma, renal cell, basal cell and hepatocellular carcinoma. It is thought that loss of BAP1 may predispose to mesothelioma after asbestos exposure. Homozygous deletion of CDKN2A, loss of NF2 or germline PALB2 deletions may also favor the development of MPM (8, 9). Genetic susceptibility can predispose to MPM via chronic exposition.

MPM is classified in 3 histological subtypes, epithelioid, biphasic and sarcomatoid. The sarcomatoid subtype is composed of malignant spindle cells and occurs in 10-15% of MPM, is chemotherapy-resistant and has the worst survival. The epithelioid subtype is the most common variant. It accounts for 50-70% of all mesotheliomas, and is composed of epithelioid polygonal cells. The biphasic subtype has features of both epithelioid and sarcomatoid subtype, larger biopsies are needed to demonstrate both components. Examination of both tumor and surrounding stroma has revealed that features such as inflammation, cellular diversity and vacuolization within the stroma all have a prognostic effect, besides the histopathological findings (10).

Diagnosis

First step in diagnostic process is usually a contrast-enhanced computed tomography (CT) of chest or a positive-emission tomography (PET) with CT, showing pleural enlargement, pleural fluid and sometimes thoracic wall invasion.

A cytological diagnosis of mesothelioma is often difficult when thoracocentesis is used to obtain the pleural fluid. This material provides a diagnosis in 20-50% of patients and only in epithelioid subtype, but it can often exclude other diagnoses. Histological biopsies by thoracoscopy or ultrasound or CT-guided have a high diagnostic accuracy. Immunohistochemistry markers usually include calretinine, cytokeratin 5/6, Wilms Tumor 1 antigen (WT1), those should be positive. Markers for adenocarcinoma should be negative (TTF-1, CEA, Ber-EP4). The sensitivity for sarcomatoid subtype is poor. Absence of BAP1 expression could be an important extra tool, it is lost in up to 60% of cases, most often in epithelioid subtype (11, 12).

In the Netherlands, nearly all mesothelioma diagnoses (and possible diagnoses) are centrally reviewed by an expert pathology board, the "Nederlands Mesotheliomen Panel" because of the rareness of the disease and the difficulty of the diagnosis.

Comprehensive genomic and transcriptomic sequencing of MPM revealed large heterogeneity between patients. Most mutations found inactivation of tumor suppressor genes (f.e. BAP1, CDKN2A, NF2, TP53, SETD2) (13-15). Heterogeneity has been reported

within the tumor location in the chest cavity. Kiyotani examined biopsies of patients at 3 different sites and showed intratumoral heterogeneity in somatic mutations and unique TCRβ clonotypes of TILs (16).

Clinical

Patients with MPM are typically men and older than 65. Symptoms are gradually worsening and include dyspnea, chest pain, cough, night sweats, fatigue and weight loss. Tumor spreads throughout the pleural cavity, and can result in pleural effusions. Metastases are rare, but can involve the lungs, bone, liver and CNS. Most patients present with advanced disease, which is incurable.

Treatment

Surgical treatment for MPM remains controversial in many parts of the world, since it is always incomplete. Whether cytoreductive surgery prolongs overall survival is unclear, studies did not provide a clear positive outcome that outweighs the risk, with high morbidity for surgery. This is beyond the scope of this thesis, which is focused on systemic treatment.

For almost 20 years, platinum containing chemotherapy combined with an antifolate has been the standard of care for patients. Leading to a median overall survival of about 12 to 16 months. Unfortunately, the mean progression free survival (PFS) is only 6 months (17, 18).

The MAPS trial (Mesothelioma Avastin plus Pemetrexed-Cisplatin) showed that standard of care chemotherapy combined with bevacizumab (a monoclonal antibody targeting vascular endothelial growth factor), improved survival over chemotherapy alone (18.8 vs 16.1 months). Although there is a survival improvement, there is also an increased adverse event profile for bevacizumab. So it failed to be approved as standard treatment (19). Other anti-angiogenetic drugs also failed to show benefit (20).

In the past it has been observed that installation of BCG (Bacillus Calmette-Guérin) vaccine immunotherapy could have an improved survival rate for MPM (21).

This led to the idea that the immune system could play an important role in the biology and treatment of MPM. Cancer immunotherapy makes use of the host system to induce

or enhance an effective immune response against cancer cells. Different types of immunotherapy use different parts of the immune system to evoke effect on tumor cells.

Immune checkpoint proteins are crucial for maintenance of self-tolerance. Expression of these proteins is dysregulated in tumor cells, thereby making the tumor cell immune resistant. Immune checkpoint inhibitors (ICI) can block inhibitory checkpoints, thereby restoring immune system function and evoking an anti-cancer immune response.

Anti-Cytotoxic T-Lymphocyte-associated protein 4 (CTLA-4) antibodies impact the lymphoid compartment; increasing the number and broadening the tumor antigen reactive T cells; stimulating priming of naive T cells and enhancing antigen presentation. PD-L1 checkpoints are mainly expressed in activated lymphocytes and exhausted T cells. Anti-PD-(L)1 antibodies can promote T cell activation during the effector phase and can restore exhausted T cell functionality, mainly in the tumor microenvironment.

These immune checkpoint inhibitors are the most widely used agents of cancer immunotherapy and completely changed treatment of many cancer types over the last decade. In 2011, ipilimumab was the first checkpoint inhibitor approved by the FDA for treatment of melanoma (22). Ipilimumab blocks immune checkpoint molecule CTLA-4. After that PD-1 (nivolumab, pembrolizumab, cemiplimab), and PD-L1 (atezolizumab, durvalumab, avelumab) checkpoint inhibitors are approved for many cancer types.

For mesothelioma some promising data on ICI treatment have been reported in the second or later lines, mostly in single arm trials. Single agent PD-1 ICI have consistent objective response rates of about 20%, and disease control rates (DCR) between 48 and 72% in mainly phase II trials (23-28). The single agent CTLA-4 checkpoint inhibitor tremelimumab however, did not show any benefit compared to placebo (29).

The first randomized trial of pembrolizumab (PD-1 antibody) failed to improve PFS or OS over single agent chemotherapy (vinorelbine or gemcitabine) in later lines. Although pembrolizumab did have a higher overall response rate (ORR), 22% versus 6% (P=0.004) (30).

The second phase III trial of monotherapy of anti-PD-1 (nivolumab)showed a survival benefit of nivolumab over best supportive care in relapsed MPM, mOS was 10.2 months (95% CI 8.5-12.1) in the nivolumab group versus 6.9 months (5.0-8.0) in the placebo group (adjusted HR 0.69 [95% CI 0.52-0.91]; p=0.0090). Placebo was used for the comparator arm since no approved second line therapy exists (31).

Combining aPD-(L)1 and aCTLA-4 therapy has been shown to induce synergistic effects in preclinical and clinical trials (32, 33). Combining them can induce a more potent antitumor immune response (34).

This led to setting up a clinical trial in MPM with combination therapy, the INITIATE trial, which is described in chapter in this thesis (35).

For combination treatment with anti-PD-1 plus anti-CTLA-4, the ORR is around 27% and mPFS 6 months in single arm phase II trials, in recurrent disease (27, 35, 36).

In 2021, the Checkmate 743 trial was published. This international randomized phase III trial compared standard of care chemotherapy with combined nivolumab plus ipilimumab. ICI treatment significantly increased overall survival compared to chemotherapy by 4 months (mOS 18.1 months [95% CI 16.8 – 21.4] versus 14.1 months [95% CI 12.4-16.2], HR 0.74 [p=0.0020]). This lead to approval of nivolumab plus ipilimumab as first line therapy for MPM by the FDA and EMA. The benefit is most prominent in the non-epithelioid subgroup, as revealed by a post-hoc subgroup analysis, epithelioid subgroup HR 0.86 (95% CI 0.69–1.08) and non-epithelioid subgroup HR 0.46 (95% CI 0.31–0.68) (37).

Tumor microenvironment

The mesothelioma tumor microenvironment (TME) is composed of heterogeneous stromal, endothelial and immune cells.

The TME in MPM is known to be highly immunosuppressive, with large numbers of tumor associated macrophages (TAMs), myeloid-derived suppressor cells (MDSC) and regulatory T cells (Tregs) (38-41).

Macrophages are plentiful present in MPM, with large heterogeneity, in both the epithelial and non-epithelial subtype. Mesothelial cells produce cytokines, which give chemotactic and stimulatory signals to immune cells of the myeloid lineage and recruit monocytes. In the tumor mass the monocytes differentiate into macrophages. Interleukins such as IL-1, IL-4, and IL-10 produced by tumor infiltrating lymphocytes (TILs) promote differentiation of macrophages towards a certain phenotype. This phenotype is pro-tumorgenic and promotes tumor growth by production of multiple cytokines. Higher percentages of macrophages are negatively correlated with overall survival and are positively correlated to the number of Tregs in tumor microenvironment (42-45).

MDSCs are immature myeloid cells and have immunosuppressive properties. They induce Tregs and produce nitric oxide and arginase, which leads to loss of function of CD4+ and CD8+ T cells (46).

T-Lymphocytes play an important role in the immune defense in cancer. These immune cells may influence tumor growth, but also mediate response to therapy. Twenty to 42% of the cells in the immune infiltrate consist of CD3+ T-lymphocytes. Besides the CD8+ T-lymphocytes, regulatory CD4+ FoxP3+ T-cells are frequently observed (39, 47, 48). Some studies suggest that higher levels of CD8+ T-cells have a favorable prognostic impact, while others found that high CD4+ and CD20+ and low FoxP3+ cells are linked to a better outcome (47-49).

The composition of the TME is different between subtypes, between individuals and within individuals (16, 40).

Biomarkers

Although a number of patients with cancer benefit from ICI treatment, many patients do not. Different mechanisms are proposed to explain these (non)responses to ICI treatment in cancers in general and in mesothelioma specifically.

The number of non-synonymous single nucleotide variants, referred to as the tumor mutation burden (TMB) may affect the odds of generating immunogenic peptides and thereby influence ICI response. In different tumor types the response to ICI treatment is positively correlated to TMB; a higher TMB resulting in a higher overall response rate (50). However, some tumor types respond better (51) and some worse (52) than would be expected based on TMB alone. And even within a specific tumor type some patients respond better than others. So although the association between TMB and ICI response is pretty robust, other factors are involved. MPM shows a rather low mutation rate, so TMB alone does not explain the response rates (13, 14, 16, 53).

It is hypothesized by Mansfield et al. that the number of alterations actually targeted by T cells, may have a stronger association with ICI response than does TMB (54). This includes immunogenic translocations or insertions/deletions, called chromoplexy and chromothrypsis.

A strong expression of the immune-checkpoint gene VISTA was found on tumor cells in epithelioid subtype. VISTA is a negative checkpoint regulator, possibly it avoids

an antitumor immune response (15, 55). The immunoregulatory impact needs to be elucidated.

In MPM, PD-L1 expression on tumor cells is observed in about 40% and is frequently associated with non-epithelioid subtype. PD-L1 expression is a prognostic marker and associated with worse outcome, when used in patients that are not treated with IO agents (56-61).

In NSCLC, PD-L1 expression is predictive of response to PD-1 checkpoint inhibitors. Tumors with higher PD-L1 expression usually respond better to IO treatment. But responses occur even in PD-L1 negative tumors and not all patient with high PD-L1 expression respond to treatment. In different other tumor types PD-L1 expression on tumor cells is not associated with response, whereas PD-L1 expression on tumor-infiltrating immune cells is (62-64).

In some phase II trials with ICI treatment for MPM, a (poor) correlation of PD-L1 expression with objective response rate and/or survival is shown. But in most other trials no correlation was found. Data are inconsistent (24-27, 31).

The predictive role of PD-L1 expression for dual agent ICI treatment has not been established either.

In the Checkmate 743 trial a relatively large amount of patients had PD-L1 positive tumors (77%), PD-L1 expression did not correlate with outcome. However survival with chemotherapy was better in patients with PD-L1 expression of less than 1% than in those with expression higher than 1%, this is probably more prognostic than predictive (35, 37).

In several tumor-types it is shown that density of tumor-infiltrating lymphocytes (TIL) is a positive prognostic indicator (regardless of ICI treatment) (65). In melanoma it is shown that pre-treatment TIL-density at the invasive margin is associated with response to anti-PD-1 treatment (62). Standardization is difficult, especially in MPM, which does not even have a distinct invasive margin.

Not only density of TILs impacts ICI outcomes, but also the type of immune cells. In melanoma, response to ICI treatment relies on pre-treatment infiltration of activated CD8 T-effector cells (62). In many more different cancers the association of infiltrating CD8+ cytotoxic T cells with longer disease free survival and/or overall survival has been demonstrated (66). In NSCLC, CD8 cell infiltration was positively correlated with ORR and PFS in patients treated with PD-1 blockade (67). A positive correlation of CD8+ T cells with overall survival has been reported, but not in all studies. One study even described opposite negative correlation (47-49, 68). This might be caused by sampling bias due to a

heterogeneous distribution in tumors, more advanced stage or from functional variability. The CD8+ cells could be exhausted cytotoxic T cells, with relatively high expression of multiple inhibitory receptors.

One study reported more CD8+ cells in PD-L1+ tumors versus PD-L1- tumors (69). Another study showed a higher ratio of cytotoxic T cells to malignant cells in the sarcomatoid subtype (70). Furthermore, CD8+ cells increased after administration of platinum plus pemetrexed, examined in paired biopsies (71).

For further analyses of mechanism of effect of ICI treatment, longitudinal tumor biopsies are needed. However these are not always possible to obtain, since in patients having a complete or partial response it is no longer possible to biopsy.

Peripheral blood T cells can provide insight of understanding immunological responses induced by ICI treatment. It also can provide biomarkers to monitor or predict response to ICI treatment. In lung cancer, it is shown that an increase in Ki-67+ PD-1+ CD8+ T cells is seen after ICI treatment in most patients. This may indicate activation of tumor-specific CD8+ T cells. These cells co-expressed CTLA-4 after PD-1 antibody treatment (72). In melanoma, presence of neoantigen specific T cells in peripheral blood is shown. Mainly in CD8+ PD-1+ T cells, which account for < 5% of all peripheral blood lymphocytes, patient specific neoantigens that target mutant and/or shared tumor neoantigens in all the melanoma patients (73).

Other blood biomarkers have been a focus in biomarker research, since they are easily accessible, are independent from intra- and inter-tumor heterogeneity, and reflect multiple factors (e.g. tumor cells, tumor-microenvironment and patient's immune system). Inflammation is a mechanism of immune-resistance in patients with cancer, promoting cancer growth and dissemination, based on activating oncogenic signaling pathways. Proposed inflammatory biomarkers that might be prognostic or predictive include LDH, CRP, white blood cells, absolute neutrophil count, neutrophil to lymphocyte ratio (NLR) derived neutrophil to lymphocyte ratio (dNLR; absolute neutrophil count/(white blood cell concentration – absolute neutrophil)). In melanoma a pro-inflammatory status is correlated with poor outcomes in patients treated with ICIs (74, 75). In NSCLC pretreatment Lung Immune Prognostic Index (LIPI), combining dNLR greater than 3 and LDH greater than ULN was correlated with worse outcome for ICI, but not for chemotherapy (76).

Exhaled breath analysis has shown potential as a non-invasive and easy-to-use technology for diagnosis and phenotyping of a wide range of diseases including mesothelioma and lung cancer. Electronic nose (eNose) technology can be used for this breath analysis (77-81). This eNose could be used for immunotherapy response in lung cancer. In lung cancer

it has been shown that exhaled breath analysis before start of treatment could identify patients that show progressive disease to anti-PD-1 therapy, thereby ICI treatment could possibly be with-held (82). In addition, it can identify patients with an objective response to anti-PD-1 therapy early during treatment (83).

Outline of thesis

This thesis aims to contribute to a better treatment of malignant pleural mesothelioma, and is specifically evaluating dual checkpoint inhibitor treatment. Besides the clinical effect of ICI treatment also the search for an explanation for the effect of this treatment, thereby aiming to predict response to treatment.

Part I summarizes what is known about treatment of mesothelioma

Chapter 2 is a review that discusses optimal systemic therapy for patients with advanced MPM. Including first-line, maintenance and second-line therapy, as well as antibody drug conjugates and targeted agents.

Chapter 3 is a review that focusses more in detail on novel treatment options in MPM, including immune checkpoint inhibitors.

Chapter 4 is a chapter from the ESMO handbook Immuno-Oncology on mesothelioma, describing what is known about immune checkpoint inhibition in MPM.

Part II is the clinical part of this thesis.

Chapter 5 describes the single center, single arm, phase 2 clinical INITIATE trial of nivolumab plus ipilimumab. This combination of checkpoint inhibitors shows marked efficacy in MPM, with no new safety concerns.

Part III is the translational research part of the thesis.

In chapter 6 immune cell profiling was performed on screening and on treatment peripheral blood samples of MPM patients treated with nivolumab (anti PD-1 antibody) monotherapy or a combination of nivolumab and ipilimumab (anti-CTLA-4 antibody). High proportions of effector memory CD8 T cells that re-expressed RA (TEMRA) and cytokine production by TEMRAs before treatment was associated with a better clinical outcome.

In chapter 7 exhaled breath analysis of volatile organic compounds by electronic technology (eNose) is performed in patients treated with nivolumab plus ipilimumab. An eNose is able to discriminate between responders and non-responders to treatment at baseline.

In chapter 8 immunohistochemistry analysis was performed on baseline and on-treatment biopsies from INITIATE trial and from a clinical trial using nivolumab in MPM. Cell density of CD4+, CD8+, and FoxP3+ cells is higher in patients having a response to nivolumab plus ipilimumab compared to patients having progressive disease at 24 weeks.

In chapter 9 RNA and whole genome sequencing was performed on the same biopsies as described above. A particular gene set demonstrated an interaction with tumor junction burden and was predictive of overall survival. Thus, analysis of structural variants and gene expression may facilitate patient selection for immune checkpoint inhibitors.

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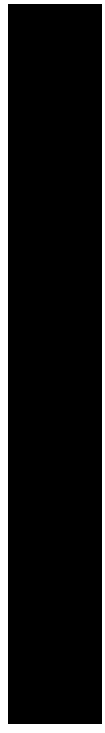

PART I

Mesothelioma treatment

2

Chapter 2

Optimal therapy of advanced stage mesothelioma

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Introduction

Malignant pleural mesothelioma (MPM) is a cancer of the pleural cavity. It has a well-documented causal relation with (occupational) asbestos exposure (1). The latency time between exposure and presentation of the malignancy varies from less than 20 to more than 50 years. It is estimated that approximately 43,000 people will die from this disease worldwide (2). The survival of patients with MPM is poor, with a 5-year survival rate in Europe of 7.2% according to the age-standardized relative survival data from the Eurocare-5 study (3).

The Eurocare-5 data also point out that the prognosis of MPM has only shown a slight improvement over the last decades. The poor survival data of the general popu- lation contrast with the median overall survival estimates from study populations, which range from 7 to 8 months to 16 months for untreated cases (4, 5). This emphasizes the selection of patients within the studies, and has implications for patient selection for chemotherapeutic treatment in general practice. One should beware for a certain therapeutic nihilism based on these data.

The low incidence of mesothelioma has impaired the realization of larger randomized studies for many years. Mainly, based on patient series and retrospective analysis of single center data, surgery is a possible therapy in MPM, being embedded in a multimodality protocol with chemotherapy and radiotherapy. Surgery by itself does not seem to be of benefit for the patient (6). There is an ongoing discussion whether extrapleural pneumonectomy or extended pleurectomy/decortication is the procedure of choice.

Radiation therapy has been implemented in multimodality studies. The routine use of hemithorax irradiation as part of a trimodality regime with neoadjuvant chemotherapy and extrapleural pneumonectomy was recently debated by a randomized trial, showing that hemithorax irradiation (median dose 55.9 Gy) did not significantly improve the median locoregional relapse-free survival from surgery (7). The use of radiotherapy is primarily confined to palliation of local pain or tumor invasion. Pain relief was achieved in more than 50% of the patients in a 189-patients study (8).

Optimization of the therapy in the patient with advanced MPM

Chemotherapy in first line

It took more than 10 years before further progress was made in the first line setting after the initial studies showed that cisplatin combined with antifolate improved survival of the non-surgical patients with MPM (9, 10). The most promising data to date is the additional

effect of bevacizumab to the standard of care in patients who were amenable for a treatment with chemotherapy and an anti-vascular agent (5).

The French intergroup study reported a 2.7-month gain in OS from 16.1 months in the control group to 18.8 months in the bevacizumab group, which was a statistically significant difference (HR 0.77 (0.62–0.95); p = 0.0167). In this study, the median overall survival in the control group was considerably better than the OS reported in the earlier registration studies. This is most likely an effect of the inclusion criteria: better PS, better selection of patients without cardiovascular diseases or a country specific effect. So far, the results of this study have not led to a change of the standard approach in most countries except France.

In this perspective it should be noted that the phase II study randomizing MPM-patients who had a first line therapy with gemcitabine-cisplatin failed to show any improvement in PFS or OS when bevacizumab or placebo was added to this regimen (11). Whether this is a consequence of differences in specific drug-drug interactions or not still remains to be resolved.

Nintedanib, oral, triple angiokinase inhibitor of VEGFR, PDGFR, and FGFR, has been investigated in a phase II study randomizing patients who received first line pemetrexed-cisplatin between nintedanib or placebo. Recently, preliminary data were reported on the additional effect of nintedanib added to cisplatin/pemetrexed. The PFS significantly increased from 5.7 to 9.4 months (HR 0.56 (0.34–0.91) p = 0.017), which was promising enough to proceed to a phase III study (12, 13).

Maintenance therapy

Both the bevacizumab and the nintedanib trial mentioned above are examples of continuation maintenance therapy in mesothelioma. Continuation treatment with pemetrexed is feasible, as described in an observational study by Van den Bogaert et al. (14). The study design does not allow to conclude whether the better PFS and OS in patients who had maintenance treatment, compared to those who had not, was due to patient selection or the actual therapy.

The alliance for clinical trials in oncology is performing a randomized phase II trial in the USA to determine whether pemetrexed maintenance after 4 cycles of chemotherapy for malignant mesothelioma has a better progression-free survival than observation only. The study is ongoing but not recruiting any patients and results are expected. (NCT01085630).

Switch maintenance with thalidomide, a drug with anti-angiogenic properties, in patients who did not progress on the standard first line chemotherapy, did not result in improved PFS or OS in a 222 patient randomized phase III study (15). Currently, a randomized phase II switch maintenance study with gemcitabine, which has antitumor activity as shown in several phase II studies, is accruing patients (NVALT19, Netherlands Trial Register NTR4132). The command study compared the impact of the focal adhesion kinase inhibitor defactinib in patients with MPM. Unfortunately, the study has been terminated prematurely due to ineffectiveness (16). The full data are now being awaited shortly.

Chemotherapy in second line

Twenty-five to 30% of patients are refractory to first line chemotherapy, and most patients will have a recurrence of disease within 6 months. All these patients are possible candidates for second line treatment. Over the last three decades, different drugs have been tested in second or third line. Unfortunately, until now there is no therapeutic modality with a proven clinical benefit. Patients in a good performance status should therefore be advised to participate in clinical trials (6).

In an ideal situation, the outcome of chemotherapy could be predicted for each individual patient, but so far, all techniques have failed to do so. The NCI developed a platform with a series of cell lines where responses as well as the corresponding genome sequence are provided (the NCI60 platform). This was further explored in wide analyses to correlate drug responses to the genetic profile (17). Correlations were observed between particular drugs and the cell's genetic makeup, yet it is hard to translate this into clinical practice. How that translates to primary tumor tissue or real patient treatment is now being investigated in patients with MPM (18). In Fig. 1, it is shown how such an approach can be done in the lab using fresh pleural fluid, extracted from patients with MPM.

A difference in the metabolic state of the cancer cell is another approach of selecting patients for a specific treatment. The sarcomatoid subtype of MPM seems to lack an enzyme arginine succinate synthetase 1 (ASS1) (19). These cells are unable to generate arginine for the metabolic processes and fully depend on its availability in the bloodstream. When pegylated arginine deiminase (ADI-PEG) is administered in these cases, arginine will catalytically degrade and apoptosis will be the result when ASS1 levels are low. The initial study proved this concept to be important (20). The sarcomatoid type of MPM accounts for only 20% of all cases, which will make this approach possible in the minority of patients.

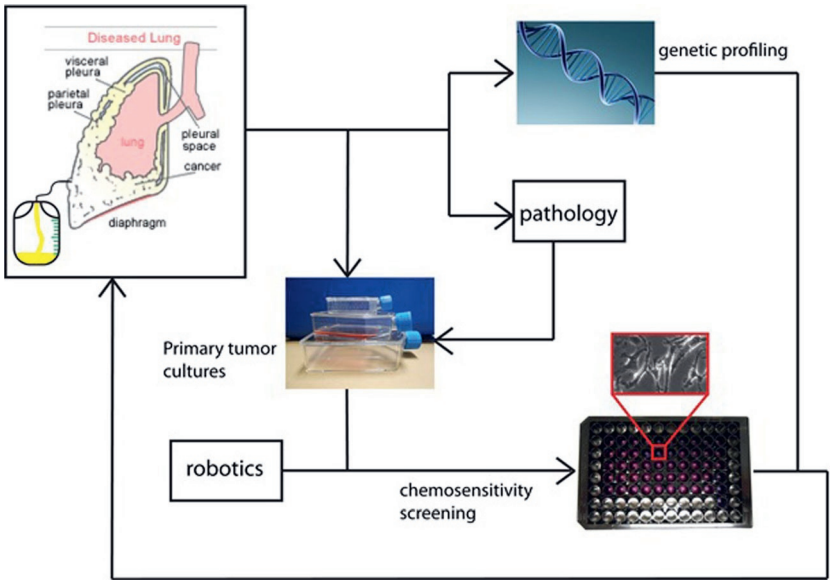

Figure 1 Pleural fluid is collected from patients with a MPM. In the cases where tumor cells are shed, these can be cultured and tested to different doses and types of chemotherapy. The best results of the exposure can be used to select the most promising treatment for the patient, or when resistance is seen with all known drugs other avenues can be chosen.

In a multicenter randomized phase 2 clinical trial, 68 out of 201 patients with newly diagnosed or recurrent MPM were identified with a ASS1 deficiency. The administration of weekly ADI-PEG20 i.m. plus best supportive care (BSC) was compared with BSC alone. The primary endpoint PFS was determined in this patient population with a follow-up of up to 38 months. With a randomization of 2:1, 44 patients completed 2 × 4-week cycles and were compared to the 22 patients receiving only best supportive care. No partial or complete responses were observed, and the PFS in the active treatment arm was 3.2 months compared to 2.0 months for the control arm. These figures met the predefined statistical endpoint with a HR of 0.56 to 0.60 for patients treated without or with prior cisplatin containing therapy. A clear relationship was observed for the patients who had a higher (975%) depletion of ASS1 and the PFS (19). Currently this treatment is now tested in a phase III multicenter study.

Immunotherapy

Cancer immunotherapy has emerged in the last decade as the most promising new cancer treatment approach. Immune checkpoints are crucial for the maintenance of self-tolerance, but the expression of immune checkpoint proteins can be dysregulated in

tumor cells as a major mechanism of immune resistance. Thus, in recent years, checkpoint inhibitors have emerged as primary agents in clinical testing to manipulate antitumor immunity (21).

Recent data suggest a moderate expression of PD-L1 in mesotheliomas, in particular, the sarcomatoid subtype. Cedrés et al. analyzed tumor samples from 119 chemotherapy naïve patients with MPM. The data were collected between January 2000 and April 2014. In 77 samples, with adequate tumor tissue, IHC analysis of PD-L1 was performed, giving 16 (20.8%) positive and 61 (79.2%) negative results. All patients presented TILs (tumor infiltrating lymphocytes) in the tumor specimen, without any predominant cell line. The univariate analysis demonstrated a correlation between PD-L1 expression and histology: in the non-epithelioid histology group a significantly higher rate of patients was PD-L1 positive, compared to the epithelioid MPM group (respectively: 9/24 pts., 37.5% vs 7/53pts, 13.2%; $p = 0.033$). Moreover, PD-L1 expression was associated with outcome, with PD-L1 positive patients having a shorter survival (22).

Similar results were achieved in another case series including patients diagnosed with MPM between 1987 and 2003 at the Mayo Clinic of Rochester, Minnesota (23). Forty-two (40%) out of the 106 patients who were considered eligible expressed PD-L1 (i.e., PD-L1 expression ≥5% cells). PD-L1 expression was cytoplasmic in 18 patients (43%), membranous in 10 patients (24%) and both cytoplasmic and membranous in 14 patients (33%). All the sarcomatoid MPMs were found to be PD-L1-positive, except for one single case. Moreover, patients in the PD-L1 positive cohort were characterized by a significantly shorter survival compared to PD-L1 negative MPMs (median OS: 5 months, range 2–9.5; vs 14.5 m, range 9.25–19; $p < 0.0001$) and the results were confirmed in the multivariate analysis (risk ratio for PD-L1 expression: 1.71, 95% CI 1.03–2.78; $p = 0.04$) (23). Among patients with epithelioid MPM, PD-L1 positive patients showed a trend toward a worse prognosis compared to PD-L1 negative ones, although not statistically significant (23).

Single agent treatment with PD-1 or CTLA-4 IO blocking drugs have been tested in MPM. For both pembrolizumab and nivolumab promising data have been reported. Unfortunately a large phase III study with tremelimumab (anti CTLA-4) was negative after initial promising data (24).

At the 16th World Conference Lung Cancer in Vienna results of one phase 1 and two phase 2 studies with single agent PD-1 blockers were reported (25–28). In line with other tumor types, a response percentage of around 25% was observed, occasionally with long-term survivors.

CTLA-4 and PD-1/PD-L1 pathway blockade enhance T cell activity through complementary mechanisms. CTLA-4 inhibition enhances the activity of early stage T cells, leading to enhanced T cell activation and proliferation. PD-1 inhibitors can enhance T cells activity in peripheral tissue, by preventing PD-1 interaction with its ligands. However, many tumors can escape immune-destruction, by PD-L1 and/ or PD-L2 overexpression that can inhibit T cell activity in peripheral tissues.

Preclinical data suggest synergistic effect of CTLA-4 and PD-1 blockade versus these agents alone. Curran et al. described an enhanced rejection of B16 melanoma in mice with the combination therapy rather than with the single agent therapy (rejection of 50% of melanomas in animals with the combination blockade of CTLA-4 and PD-1). Moreover, they showed that the inhibition of a single pathway led to enhanced infiltration of effector T cells in the tumors, but that these T cells accumulated high levels of negative co-receptors that eventually could limit their activity. Blockade of multiple pathways allowed CD4+ and CD8+ to proliferate and carry out their activity within the tumor. This study also demonstrated that the double blockade increases the ratio of effector T cells to regulatory T cells, thus reducing inhibitory signals and promoting inflammation in the tumor microenvironment (29). The efficacy of the combination has recently been confirmed for the treatment of advanced melanoma. These results have led to the start of different phase II studies in MPM. The French intergroup has already completed the randomized study in second line of nivolumab vs. nivolumab plus ipilimumab, and its results are eagerly awaited (NCT02716272) (Table 1).

Table 1. Drugs in development

Drug groups	Positive study	Negative study	Ongoing study
Immunotherapy	Phase II: pembrolizumab (interim analysis) Phase IB: pembrolizumab (Keynote 28) Phase II: nivolumab (NivoMes)	Phase III: tremelimumab	Combination PD-1 and CTLA-4 checkpoint inhibitor
Antibody drug conjugate	Anti-mesothelin. Phase II: anetumab avtansine Phase II: amatuximab		Phase III: amatuximab Phase III: anetumab ravtansine

Antibody drug conjugates

Mesothelin is a tumor antigen that is highly expressed in MPM and other tumors. It can be targeted, and therefore act as a new therapeutic target in MPM. Besides its expression on malignant cells, normal mesothelial cells also show the expression, but these cells are dispensable (30). Several antibody-based therapeutic agents directed at mesothelin are currently under clinical evaluation. Other approaches are vaccine and T cell therapies.

The anti-mesothelin immunotoxins have extensively been studied in the NCI lab. The tested com- pounds were SS1P and RG7787/LMB-100, chimeric anti-mesothelin antibody (amatuximab), mesothelin-directed antibody drug conjugates (anetumab ravtansine, DMOT4039A, BMS-986148), live attenuated Listeria monocytogenes-expressing mesothelin (CRS-207, JNJ-64041757), and chimeric antigen receptor T cell therapies. Two anti-mesothelin drugs are currently in phase III clinical registration trials for malignant mesothelioma; amatuximab and anetumab ravtansine have both shown promising results in the phase II setting (31) (Table 1). The development of the CRS-207 in MPM has not yet matured enough to start randomized studies in MPM (32). It is foreseen that these agents will also be tested in combination with checkpoint inhibitors.

BRCA1-associated protein-1 (BAP1) inactivation

BRCA1-associated protein-1 (BAP1) has a role in DNA repair, control of gene expression through histone modification. It also enhances the progression through the G1-S checkpoint (33). In MPM, BAP1 is inactivated in around 25% of the tumors and can be considered to be a potential target. A number of different mutations have been identified that inhibit the function of BAP1 (34). The role of BAP1 in histone modification is of interest because it could allow histone deacetylase inhibitors (HDAC) to influence the disease. Unfortunately, a large randomized phase 3 trial of the HDAC inhibitor vorinostat in second and third line MPM did not show any activity (16). However, it must be noted that no correlation with BAP1 was made in this study.

Ongoing is a phase II, two-stage trial of tazemetostat. In part 1, patients will be treated regardless of BAP1 status, and in part 2, only patients who are BAP1 deficient (NCT02860286) are included.

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Chapter 3

Chemotherapy options versus "novel" therapies: how should we treat patients with malignant pleural mesothelioma

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Abstract

Today there are several options for the treatment of patients with malignant pleural mesothelioma (MPM). The therapeutic arsenal has expanded from only chemotherapy with or without surgery in selected cases to a variety of new compounds that target the malignant cell or its micro-environment. Immunotherapy has been the latest achievement and now single arm and randomized studies are being presented. A renewed interest has occurred in the combination of surgery, chemotherapy and radiation therapy.

In this review we present the available data on previous and running studies and try to give a recommendation how to select the best patient for the most optimal therapy.

Introduction

Until the change of the century patients with malignant pleural mesothelioma (MPM) were treated with best supportive care (BSC) and offered mostly single agent chemotherapy as part of a study. The median overall survival of this group was 7-8 months and only a hand full of chemotherapeutic agents gave responses of 15-20%. One of the most informative studies before 2000 was the MS-01 study from the UK. In this three-arm study, BSC was compared to vinorelbine and to mitomycin plus vinblastine plus cisplatin. No statistical benefit was observed but a slight survival benefit for the vinorelbine arm was noted.(1) It was until 2003 that the study by Vogelzang showed a clear benefit from the combination of cisplatin plus pemetrexed versus cisplatin monotherapy. This raised the median overall survival (OS) from 10 to 13 months.(2) The combination has been the standard now for over 15 years and is considered the backbone to which other combinations can be tested. The basic idea of the success of this combination is based on the low expression of Thymidilate Synthase (TS) in patients with MPM allowing the multitarget antifolate (pemetrexed) to inhibit the generation of nucleotides in the malignant cells. This, combined with the DNA disrupting effect of cisplatin during the cell division lead to an improved mOS and a response rate of 35%.(3)

In this review we describe the literature and latest data and try to recommend the best possible treatment.

Angiogenesis inhibitors

For growth of MPM cells, the vasculature plays an important role. Examination of histologic specimen have indicated that a variety of vascular growth factors play a role. A high micro-vessel count is often seen in patients with active growth of the tumor and are correlated with a worse prognosis. Many receptors have been identified that are activated in patients with MPM, like VEGF-R1 to 4, PDGF and PGF.(4-6) Vascular endothelial growth factor (VEGF) is expressed in MPM, can promote tumor angiogenesis, but also directly stimulate tumor growth.(7,8) These observations have led to a variety of studies using anti-angiogenic drugs as shown in Table 1. Not only new compounds like small molecules have been tested but also older angiogenic inhibiting drugs like thalidomide. The latter has been tested in a phase III maintenance setting, where patients who did not progress after 4-6 courses of platinum plus pemetrexed, were randomized to receive observation or thalidomide until progression. Although the drug was well tolerated, there was no sign of activity at all compared to observation alone.(9) Many phase 2, non-randomized studies have been performed with small molecules directed against the VEGF receptors. Most of these compounds had shown activity in other tumors like kidney cancer. Unfortunately,

most of the studies did not show consistent activity in patients with MPM and response rates of 6-12% were noted. The toxicities were often reason for dose reduction or even discontinuation of the therapy. To date no small molecule has been identified to be used on larger scale.(10-19)

A special note must be made for the addition of bevacizumab to the standard of care. In the MAPS study in France, patients were randomized to receive the standard of care with or without bevacizumab in a dose of 15 mg/kg i.v. every 3 weeks. The drug could be given as a maintenance after a maximum of 6 courses of chemotherapy were administered. Two interesting observations could be made in this study; (I) there was a significant mOS benefit for the patients receiving bevacizumab of 2.8 months; (II) the mOS in the control arm had increased to 15 months.(20) The latter observation indicates that there may have been a better selection of patients since the SoC reported only a 12-13 months mOS. It remains unclear if this observation is related to the selection for patients fit to receive bevacizumab or that the natural history of the disease has changed in the last 10-15 years. Nowadays, the addition of bevacizumab has been registered as possible new standard of care in some countries.

Table 1: angiogenesis inhibitors

	Mode of action	***dose***	***General outcome***
Axitinib (10)	VEGFR-1-3, PDGFR; c-Kit	5 mg twice daily with CT vs CT	No difference in ORR
Bevacizumab (20-22)	VEGF	15 mg/kg i.v. q 3 wks with CT	mOS 18.8 v.s. 16.1 HR 0.77 (significant)
Cederanib (11,12)	VEGFR1-3; c-KIT; PDGFRb	45mg daily	PR 9-10% significant toxicity
Dovitinib (13)	VEGF; FGF	500mg daily x 5/week	Not active
Nintedanib (19)	VEGFR1-3; PDGFR; FGFR	200mg twice daily with CT	Phase III study PFS: HR 1.01 OS: HR1.12
Sorafenib CALGB 30307 (15,16)	RAS/RAF/MEK; VEGF; c-KIT	400 mg twice daily	PR 6%
Sunitinib (14,17)	VEGF; c-KIT; PDGF	37.5-50 mg daily	PR 3-12% Toxicity when combined with CT
Thalidomide (9)	Inhibits VEGF release and bFGF	200 mg daily	Phase III study OS: HR 1.2
Vatalanib (18)	VEFG; PDGF; c-KIT	1250mg daily after CT	PR 6%

CT: Chemotherapy; HR: Hazard Ratio

Maintenance therapies

The use of maintenance therapy has attracted attention in different tumor types and has been tested in patients with MPM. In the first phase III study reported, thalidomide was tested in a dose of 200 mg orally until progression. As stated above, no difference in median progression free survival (PFS) was noted. The mPFS was 3.5 months in both groups with a HR of 0.99.(9) Pemetrexed has been tested as a maintenance drug in a randomized phase II trial. The data of this study were presented as poster during ASCO 2019. The study suffered from a very slow accrual and with only 49 patients entered, no difference were observed in both mPFS (3.4 vs 3.0 months) and mOS (16.3 vs 11.4 months p= 0.67). The study was stopped for slow accrual.(23)

Recently a randomized phase II has been reported during ESMO 2019 with interesting outcomes. In the maintenance setting, gemcitabine was administered in a dose of 1250 mg/m2 weekly x 2 every 3 weeks. This regimen was compared to BSC and patients could enroll when no signs of progression were noted after 4-6 courses of platinum-pemetrexed. The drug was well tolerated but a number of patients had dose reductions or change in interval due to toxicity. The primary endpoint was met with an improvement of mPFS of 3 months compared to BSC (3.2 vs 6.2 months). The HR of 0.42 (0.28-6.3) and a p< 0.0001 makes this an interesting observation. Eagerly, the mOS data are awaited.(24)

Epigenetic interference

Another cell cycle regulatory pathway which attracted interest and is transcription pathway of DNA. In this process, histone deacetylase (HDAC) regulates the timely transcription of DNA by unfolding parts of DNA from the histones. Vorinostat is a HDAC inhibitor with a small molecular weight (<264 gr/mol) and leads to induction and accumulation of acetylated histones. This results in a reduction of proliferation of cells, especially tumor cells. This oral medication was tested in second- and third-line treatment in one of the largest phase III studies reported. Despite a positive indication of success in the interim analysis, the final results of 661 randomized patients did not show any difference in mPFS or mOS (30.7 vs 27.1 weeks mOS).(25) It was concluded that single agent HDAC inhibition is not an effective strategy and should probably be combined with other targeted approaches.(26)

A more recent development is the observation that the Polycomb Repressor Complex (PRC) is involved in the suppression of tumor suppressor genes in mesothelioma. It was demonstrated that the Enhancer of Zeste Homolog 2 (EZH2) is over-expressed in MPM, and the related PRC-2 is a potential therapeutic target in this tumor. Further studies of

TCGA confirmed an up-regulation of EZH2 in MPM cells.(27) In order to inhibit the EZH2/PCR2 complex, a drug named tazemetostat has been tested. This compound has now been tested in a small series of 74 patients with MPM, but has not resulted in a full publication (NCT02860286).

Single agent immune checkpoint inhibitors

In the past several years multiple promising data on immune checkpoint inhibitors (ICI) have been reported in the second or later lines (summarized in Table 2). Single agent PD-1 ICI have consistent objective response rates of about 20% in mainly phase II trials.(28-33) Single agent CTLA-4 checkpoint inhibitor tremelimumab however did not show any benefit compared to placebo.(34)

At ESMO 2019 meeting the PROMISE-meso trial was presented. An ETOP initiated phase III trial with pembrolizumab versus chemotherapy (gemcitabine or vinorelbin) in further lines. Although a significant difference in ORR was seen (22% versus 6%, p=0.004), it did not result in a difference in PFS or OS. The ORR of 22% is consistent with the earlier phase II trials. Treatment related adverse events of grade 3 or higher were experienced in more patients in the chemotherapy group (19% versus 24%).(35) Whether a small subgroup exists that does have a survival advantage for ICI over chemotherapy is not yet known, neither how to select patients that will have a response. In most of the above-mentioned trials, tumors with PD-L1 expression have a higher response-rate to ICI than tumors without PD-L1 expression. But this is not consistent, and also tumors without PD-L1 expression have responses.

Combination of immune checkpoint inhibitors with chemotherapy

In line with the positive effect of combining chemotherapy and an immune checkpoint inhibitor in NSCLC, different phase II and III trials are ongoing, with different combinations. (NCT02899195, NCT02784171, NCT03762018)

Table 2: Single agent checkpoint inhibitors

Author – trial	Checkpoint inhibitor	Patients (n)	ORR (%)	DCR (%)	PFS (months)	OS (months)
Alley - Keynote028 Phase 1B (28)	Pembrolizumab	25	20	72	5.4	18
Metaxas Phase II (29)	Pembrolizumab	93	18	48	3.1	7.2
Popat – Promise-meso Phase III (35)	Pembrolizumab vs chemotherapy	73 vs 71	22 vs 6		2.5 vs 3.4	10.7 vs 11.7
Quispel - Nivomes Phase II (30)	Nivolumab	34	26	47	2.6	11.8
Okada - Merit Phase II (31)	Nivolumab	34	29	68	6.1	17.3
Scherpereel - MAPS-2 Phase II (32)	Nivolumab	62	17	43	4.0	11.9
Hassan – Javelin Phase 1B (33)	Avelumab	53	9	47	4.1	10.7
Maio – Determine Phase III (34)	Tremelimumab vs placebo	382 vs 189	4.5 vs 1.1	27.7 vs 21.7	2.8 vs 2.7	7.7 vs 7.3

Combination of immune checkpoint inhibitors

In the last 2 years, three separate phase II trials testing a combination of checkpoint inhibitors were published, one combining durvalumab plus tremelimumab (NIBIT-MESO-1) and two with nivolumab plus ipilimumab (MAPS-2 and INITIATE).(32,36,37) These are summarized in table 3. Response rates between 25% and 38% were seen, which seem a bit higher than from single agent PD-1 inhibitors. Whether this will induce a survival benefit is now being tested in a first line phase III trial randomizing between standard chemotherapy and nivolumab plus ipilimumab (Checkmate 743; NCT02899299). Results are being expected next year. The combination of nivolumab plus ipilimumab is already included in the NCCN guidelines. In line with the single agent ICI, selecting patients for the treatment seems crucial; but a biomarker is not yet found.

Table 3: combination checkpoint inhibitors

Author Trial	Checkpoint inhibitors	patients (n)	ORR (%)	DCR (%)	PFS (months)	OS (months)
Calabro Nibit-Meso Phase II (37)	Durvalumab + tremelimumab	40	27	65	5.7	16.6
Disselhorst Initiate Phase II (36)	Nivolumab + ipilimumab	34	38	68	6.2	NR
Scherpereel MAPS-2 Phase II (32)	Nivolumab + ipilimumab	63	24	50	5.6	15.9

Dendritic cell immunotherapy

In dendritic cell immunotherapy autologous monocyte–derived dendritic cells are pulsed with allogenic tumor lysate from five different mesothelioma cell lines and reintroduced into the patient by a vaccination. In the first phase 1 trial 9 patients were treated with this (Mesopher) vaccination, which resulted in a DCR of 100%.(38) This led to a randomized phase II/III trial testing maintenance vaccination versus observation after effective first-line chemotherapy. This European study is currently including patients (NCT03610360).

Mesothelin targeted therapy:

Mesothelin is a cell surface glycoprotein normally expressed on mesothelial cells, and highly expressed in different cancers, especially in epithelioid mesothelioma. Thereby it is an interesting target for therapy, and different approaches are used over the last two decades (figure 1).

One of the approaches is as a chimeric high-affinity monoclonal antibody (amatuximab), potentially this reduces tumor growth by inhibiting mesothelin binding to the extracellular substrate and by antibody-dependent cellular cytotoxicity. But in a multicenter phase II study, amatuximab in combination with pemetrexed and cisplatin failed to show a difference in PFS over historical controls.(39)

Another way to target mesothelin is with immunotoxins. An antibody fragment that targets mesothelin is fused to a bacterial exotoxin payload, and after binding it is internalized by the cell via endocytosis and can induce apoptosis. Two different drugs have been, or are now being tested in clinical trials, SS1P and LMB-100. In SS1P a fragment of Pseudomonas exotoxin A (PE38) is fused. As single agent it has modest efficacy, and problem is induction

of rapidly evolving antibodies which neutralize the drug.(40) The newer drug LMB-100 has a designed PE (PE24) and is designed to be less immunogenic and thereby less toxic; and is now being tested in clinical trials. (NCT03644550, NCT02798536)

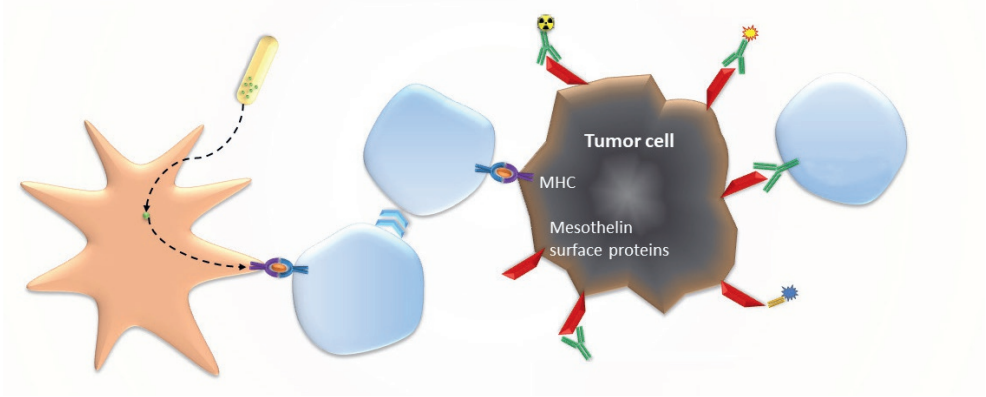

Figure 1. Therapeutics to target mesothelin.

APC: antigen presenting cell. MHC: major histocompatibility complex. PE: Pseudomonas exotoxin. TCR: T cell receptor. Different mechanisms of targeting mesothelin, a suface glycoprotein.

The third approach is via antibody drug conjugates. Anetumab ravtansine, is an anti-mesothelin antibody fused to DM4, a maytansinoid tubulin inhibitor. After internalization it releases the DM4 metabolite in the tumor cell. In a phase II trial, presented at WCLC 2017, anetumab ravtansine had an objective response of 8.4% and was not superior to vinorelbine with respect to PFS.(41) A study randomizing between anetumab ravtansine plus pembrolizumab versus pembrolizumab alone is now recruiting (NCT03126630).

BAY2287411 is a thorium-227-labeled antibody-chelator conjugate, currently being tested in a phase I clinical trial (NCT03507452).

Cancer vaccines are designed to induce a tumor-specific immune response. CRS-207 uses a live-attenuated Listeria monocytogenes strain engineered to express mesothelin. It has been tested in phase I trials, as single agent or in combination with chemotherapy, but although it induces a change in tumor micro-environment and seems to give small benefit, it is no longer tested anymore.(42,43) Another cancer vaccine (JNJ-64041757) is also no longer in development.

Over the last two decades many different trials have been performed, unfortunately most without clear effect.

Anti-mesothelin chimeric antigen receptor (CAR) T cells are modified from autologous patient T cells, to express a mesothelin-binding T-cell receptor, and providing antigen specificity to T-cells against tumor associated antigens on the cell surface. Mesothelin CARs are being tested in several trials. A recent phase I basket trial with CAR-T-cells engineered by lentiviral transduction showed it was well tolerated, but showed limited clinical benefit.(44) Inefficient T cell infiltration and short persistence by systemic delivery are common obstacles for solid tumor CAR-T cell therapy. Other problems are a cytokine release syndrome and neurotoxicity. Different phase I clinical trials are ongoing (NCT03054298, NCT02414269, NCT03638206).

Arginine deprivation

For the subgroup of sarcomatoid mesothelioma only recently the importance of the arginine succinate synthase (ASS) pathway has been identified.(45,46) Using a drug to deplete the body from circulating arginine, the sarcomatoid cells will die due to their inability to endogenously produce arginine. Somewhere during the development of the malignant expression of these cells, there has occurred a loss of the ASS enzyme. In a randomized phase II trial arginine deprivation with ADI-PEG20 improved PFS, but not OS, over BSC, in patients with ASS1 deficient mesothelioma.(47) A phase II/III trial randomizes 386 patients with mixed-type and sarcomatoid mesothelioma, to platinum plus pemetrexed, and either ADI-PEG20 or placebo and is currently recruiting patients (NCT02709512).

Surgery in MPM

The role of surgery in diagnosis and palliation has been well established. In the curative setting, surgery has been performed in patients with MPM for several decades as part of a multi-modality setting. Its primary goal is to eradicate all visible tumor and to allow other modalities to kill the remaining microscopic disease. Different approaches have been investigated, with an Extra-Pleural Pneumonectomy (EPP) being the most radical approach. Several nonrandomized phase II studies showed promising outcomes in highly selected patient groups.(48,49) A small but randomized study in the UK (MARS) indicated that toxicity and morbidity was considerable and did not show any signs of improvement.(50) The study execution, however, was criticized but gave rise to renewed interest in more limited resections: pleurectomy decortication (P/D). Different ways of performing this resection of all visible tumor with leaving the lung intact have been published. The major problem which is currently under investigation is how the different PD interventions can be compared. In the UK, the MARS2 study investigates the impact of

extended pleurectomy/decortication (eP/D) when added to chemotherapy alone.(51) The EORTC 1205 study tests the sequence of chemotherapy and eP/D in a randomized study in 64 patients.(52) Multimodality treatment is recommended only within clinical trials.

Selection of the best therapy for a patient

In the last years many promising studies with systemic agents have been reported but it all comes down to a long-term benefit in only 20-25% of patients. Despite many investigations, we have not been able to find reliable biomarkers to select for any of the new therapies. It is therefore generally accepted that a platinum with anti-folate combination, potentially including bevacizumab, remains the cornerstone of first-line treatment until a new randomized study beats this standard.

As general recommendation, patients can be selected using the EORTC or CALGB prognostic models for certain (combination) surgical approaches (53) until better biomarkers have been identified.

In further lines no standard therapy is available. Possibilities include chemotherapy (pemetrexed retreatment, gemcitabine or vinorelbine) or immune checkpoint inhibitors (PD-1 +/- CTLA-4). Since ICI have a higher ORR and less toxicity than chemotherapy, possibly this is preferred when available. In the next years several trials with combining agents will be published.

The high expression of mesothelin in epithelioid mesothelioma provides a promising way for use of targeted therapy, but there are still some obstacles to overcome.

We need to continue to encourage patients to enroll in studies to identify which combination of modalities is the most promising and has the least toxicity. It is strongly recommended that these clinical investigations all have strong translational programs.

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4

Chapter 4

ESMO handbook of Immuno-Oncology – chapter Mesothelioma

ESMO Handbook of Immuno-oncology
Chapter 2.3.3 Mesothelioma
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Definition

Malignant Pleural Mesothelioma (MPM) has been known for its resistance to a variety of therapies, and has therefore been the focus for new treatment approaches such as immuno-oncology treatment. Although mesothelioma is not a typical immunogenic tumour, in the past it has been observed that some patients with MPM responded well on the instillation of BCG (Bacillus Calmette-Guérin) or after the development of an empyema (1). In the 20th century, some groups observed that immune infiltration in biopsies predicted for a better survival. Mesothelioma is also infiltrated by immune effector cells, cytokines and regulatory T-cells (2,3). This led to the idea that the immune system could play an important role in the biology of MPM.

Predictive and /or prognostic biomarkers of clinical relevance

Mesothelioma has a moderate expression of PD-L1, 20%-40% of patients have an expression of >1%. Non-epithelioid histological subtype has a significant higher number of PD-L1-positive (PD-L1+) patients. The PD-L1-negative (PD-L1-) patients have a significantly better prognosis than the PD-L1+ patients, with a median survival of 16.3 versus 4.8 months respectively. The effect of PD-L1 status on prognosis does not depend on the histology (4,5). Mesotheliomas have a low protein-altering mutation rate. Compared with other cancers it is in the lowest third of the tumour mutational burden landscape (6). There is no significant difference in mutational burden between the histological subtypes of mesothelioma (7). Despite this low mutational burden, in a subgroup of patients with mesothelioma immune-oncologic therapy is beneficial, possibly due to the presence of immune cells in the tumour-microenvironment.

The prognostic significance of immune cells infiltrating the tumour has been investigated in several studies. With more CD4-expressing cells or CD8+ lymphocytes in the mesothelioma there is a tendency to longer survival. High levels of IL-7R are associated with an increased risk of death. CD163+ cells and their ratio to tumour-infiltrating lymphocytes (TILs) [CD8+ T cells and CD20+ B cells] are an independent marker of prognosis in mesothelioma (8).

Clinical results

Unlike the turbulent development in melanoma and lung cancer, the number of studies in MPM has developed at a slow pace. The studies reported in peer-reviewed journals or presented at major meetings are listed in Table 1. Most of these studies focus on the

anti-programmed cell death protein 1 (PD-1) monoclonal antibodies nivolumab and pembrolizumab.

Data emerging from these studies indicate that the overall response rate (ORR) is comparable with the results obtained in lung cancer and other tumours, but there seems to be no clear correlation between PD-L1 expression level and response. In general, the primary endpoint of the second line studies is the disease control rate (DCR) at 12 weeks. Long-term survivors have not yet been reported due to the recent initiation of these studies.

Table 1: Completed studies of immuno-oncology therapy for mesothelioma

Study	Drug(s)a	Phase	# Pts	Outcome
Determine (13)	Tremelimumab vs placebo 2:1	IIB	571	DCR: 28 vs 22% OS: 7.7 vs 7.3 months
NivoMes (11)	Nivolumab	II	33	DCR: 50% ORR: 15%
Javelin (12)	Avelumab	IB	53	DCR: 57% ORR: 9.4% mPFS: 17 weeks
Keynote 028 (9)	Pembrolizumab 10mg/kg 2qw For PD-L1 > 1%	IB	25	DCR: 72% ORR: 20% mPFS: 5.4 months mOS: 18 months
Pembro (10) NCT02399371	Pembrolizumab	II	34	DCR 76% ORR 21% mPFS: 6.2 months mOS: not reached
MAPS 2 (14)	Nivolumab vs Nivolumab + ipilimumab (1:1)	II	125	DCR: 43 vs 52% ORR: 17 vs 26%
INITIATE NCT03048474	Ipilimumab + nivolumab	II	38	DCR: 72% ORR: 28%
DC vaccine (15)	DC-based immunotherapy + cyclophosphamide	I	10	DCR: 80% Reduces regulatory T cells Safe
Antimesothelin immunotoxin (16)	Cisplatinum + pemetrexed + SS1P	I	24	Safe Well tolerated PR: 77%

The number between brackets stands for references
DC, dendritic cell; DCR, disease control rate; mOS, median overall survival; mPFS, median progression-free survival; ORR, overall response rate; OS, overall survival; PD-L1, programmed cell death 1; PR, partial response; Pts, patients; qXw, every X weeks.
[a] Standard dosages of therapy, unless otherwise specified

PD-1 blockade.

One phase Ib study, Keynote 028, examined pembrolizumab in a variety of tumour types. This is the only study that included patients who expressed PD-L1 (defined as > 1%), including a subset of 25 patients with MPM. The ORR for mesothelioma was 20% and DCR was 72%. The clinical benefit (complete response [CR] + partial response [PR] + stable disease [SD]) at 6 months was 40%. Median overall survival was 18 months. Historical data on median overall survival with second-line therapy ranges from 5.7 to 10.9 months.

Five patients (20%) presented treatment-related adverse events (trAEs) of grade ≥ 3, including thrombocytopaenia, dyspnoea, increase in alanine aminotransferase, neutropaenia, decrease in appetite and pyrexia (9).

An interim analysis of a phase II study with single agent pembrolizumab confirmed the DCR and limited toxicity profile (10). In Switzerland, data collected from patients who received pembrolizumab for relapsed MPM were reviewed retrospectively. Response rates and survival outcomes were promising in the unselected population and comparable with clinical trials for patients with Eastern Cooperative Oncology Group (ECOG) 0-1 and 2nd line treatment (as were inclusion criteria for Keynote 028).

Comparable results were reported when nivolumab was used (11).

PD-L1 blockade

Limited studies have been performed with PD-L1 blockers. The JAVELIN solid tumour study, a phase IB trial, tested the use of avelumab in 53 patients. ORR was 9.4% and DCR was 57%. Median PFS was 17 weeks. The toxicity profile was acceptable, four patients (7.5%) had trAEs of grade ≥ 3 (colitis, lymphopenia, increased gammaglutamil transferase (GGT) or creatine phosphokinase (CPK)) (12).

CTLA-4 blockade

One of the largest studies performed in MPM is the use of tremelimumab in second and third line. A total of 571 patients were randomised to receive tremelimumab or placebo (2:1). The preliminary safety profile of tremelimumab was acceptable. This was a negative study, since no difference in the primary end point, overall survival, was noted (13).

Combination checkpoint inhibitors

In the MAPS2 trial 125 patients were included that received either nivolumab or nivolumab plus ipilimumab. Interim analysis for the first 108 patients showed a DCR of 43% at 12 weeks with nivolumab and 52% with nivolumab plus ipilimumab. ORR was 17% with nivolumab alone and 26% with nivolumab plus ipilimumab (14).

An interim analysis of 26 patients in the Dutch INITIATE trial (NCT03048474), a phase II trial in which patients receive nivolumab plus ipilimumab showed comparable results with a DCR of 69% and ORR of 27% at 12 weeks. Toxicity was relatively low.

Potential future developments

In table 2, ongoing studies are reported. For checkpoint inhibitors, two trials explore the toxicity and changes in immunologic micro-environment with immunotherapy as neoadjuvant treatment for surgery. One study investigates the toxicity of pembrolizumab when given after radiotherapy.

A few studies investigate the difference in efficacy for chemotherapy (ChT) versus immunotherapy, some in first line and some in further lines.

Adoptive cell therapy

A few phase I studies are investigating the safety and feasibility of intrapleural or intravenously administered human chimeric antigen receptor (CAR) modified T cells in patients with mesothelin (MSLN)-expressing cancers. No results have been published for mesothelioma.

Anticancer vaccines

Dendritic cells (DCs) have been used in tumour cell vaccinations for mesothelioma. Cornelissen et al described 10 patients in whom dendritic cell vaccination was given after immune modulation of the body with cyclophosphamide. This resulted in radiographic disease control in 8 out of 10 patients. Seven of these 10 patients survived 24 months or more and 2 patients were alive at 50 and 66 months after treatment (15).

This approach is now being investigated in two other trials (see table 2). The European DENIM phase III trial will test DC-based immunotherapy with allogeneic tumour lysate as maintenance treatment after chemotherapy.

Table 2: Ongoing studies of immuno-oncology therapy for mesothelioma

Study	Drug(s)	Phase	# Pts	Primary endpoint	Remarks
Neoadjuvant pembrolizumab NCT02707666	Pembrolizumab before surgery	I	15	Toxicity γ gene expression	University of Chicago
Adjuvant pembrolizumab NCT02959463	RT + adjuvant pembro (+/- surgery or ChT)	I	24	Toxicity	MD Anderson
Durvalumab Tremelimumab + surgery NCT02592551	-Durva + surgery -Durva + tremelimumab + surgery -Control arm + surgery	II	-8 -8 -4	CD8/Treg ratio and ICOS	Single center Houston
Pembrolizumab vs chemo NCT02784171	-Cisplatin + pemetrexed -Cisplatin + pemetrexed + pembro -Pembro alone	II	126	PFS	Canada
Promise NCT02991482	Pembro vs standard of care	III	142	PFS	ETOP study
Durvalumab and tremelimumab NCT03075527	Durva q4w + tremelimumab q4w	II	40	ORR	Dana-Farber Institute
PrE0505 NCT02899195	Durva q4w + ChT	II 1L	55	OS	ECOG study
Checkmate 743 NCT02899299	Nivo + ipi vs Platinum+ pemetrexed	III 1L	600	OS and PFS	Multinational
NIBIT-MESO-1 NCT02588131	Durva + tremelimumab	II 1L,2L	40	ORR	Italian study
Keynote 158 Pembrolizumab NCT02628067	Pembro	II	1350	ORR	Multinational
MesoDec NCT02649829	Autologous DC vaccination	I/II	20	Feasibility and safety	Single centre Antwerp
MesoCancerVac NCT02395679	DCs loaded with allogeneous cell lysate	I	9	Tolerability	Single centre Rotterdam
Oncolytic virus NCT02714374	Neoadjuvant GL-ONC1 vaccinia +/- eculizumab	IB	36	Treatment-related AE	Single centre San Diego
NCT01503177	Intrapleural measles virus	I	36	AE	Mayo clinic

1L, first line; 2L, second line; AE, adverse event; ChT, chemotherapy; DC, dendritic cell; Durva, durvalumab; ECOG, Eastern Cooperative Oncology Group; ETOP, European Thoracic Oncology Platform; ICOS, inducible T cell co-stimulator cells; Ipi, ipilimumab; OS, overall survival; ORR, objective response rate; Nivo, nivolumab; Pembro, pembrolizumab; PFS, progression-free survival; Pts, patients; qXw, every X weeks; RT, radiotherapy; trAE, treatment-related adverse event; Treg, regulatory T cell.

Immunotoxin immunotherapy

Mesothelin (MSLN) is overexpressed in mesothelioma. SS1P is an immunotoxin consisting of an anti-MSLN antibody fragment fused to pseudomonas exotoxin. Hassan showed that SS1P can be administered safely and had an impressive tumour response in mesothelioma. Thirteen out of 24 patients received the maximum tolerated dose, and 77% demonstrated a partial response in combination with ChT (16).

Another MSLN-targeted immunotoxin that is currently being investigated is LMB-100.

Oncolytic viral therapy

For vaccinia immunotherapy, there is still only preclinical research. Two phase I studies are investigating the toxicity of oncolytic viral therapy for mesothelioma (see Table 2, NCT02714374 and NCT01503177).

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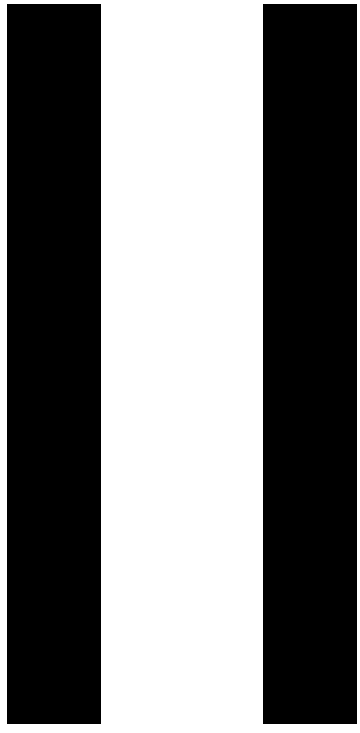

PART II

Clinical research

5

Chapter 5

Ipilimumab and nivolumab in the treatment of recurrent malignant pleural mesothelioma (INITIATE): results of a prospective, single-arm, phase 2 trial

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Abstract

Background

Single-drug checkpoint inhibition has shown activity in patients with recurrent malignant pleural mesothelioma. Here, we assessed the safety and efficacy of the combination of nivolumab, an anti-programmed death receptor 1 antibody, plus ipilimumab, an anti-cytotoxic-T-lymphocyte-associated antigen 4 antibody, in patients with previously treated and relapsed malignant pleural mesothelioma.

Methods

INITIATE was a prospective single-centre, single arm, phase 2 trial. Patients with malignant pleural mesothelioma who progressed after at least one line of platinum-containing chemotherapy were enrolled. Key eligibility criteria were measurable disease according to the modified Response Evaluation Criteria in Solid Tumours for mesotheliomas, Eastern Cooperative Oncology Group performance status 0 or 1, and adequate organ function. Patients received intravenous nivolumab (240 mg every 2 weeks) plus intravenous ipilimumab (1 mg/kg every 6 weeks up to four times). Treatment was continued for up to 2 years or until confirmed progression or unacceptable toxicity. The primary endpoint was disease control at 12 weeks. All patients who received at least one dose of therapy were included in safety analysis and all patients who received one dose of therapy and at least one radiological assessment were included in the primary analysis. This trial is registered at ClinicalTrials.gov, number NCT03048474.

Findings

Between Oct 5, 2016 and Aug 3, 2017, 38 patients were enrolled in the study, of which two patients were excluded because they were not eligible for biopsy. Of 36 eligible patients, one deteriorated before the start of the study so was not included in any analyses and one withdrew consent after one treatment cycle before radiological assessment so was included in the safety population only. 34 patients were evaluable for response assessment at 12 weeks. Of these, ten (29%) patients had a partial response and 13 (38%) patients had stable disease, thus disease control was achieved by 23 (68%, 95% CI: 50 - 83) of 34 patients. Treatment related adverse events were reported in 33 (94%) patients, the most common adverse events were infusion related reactions, skin disorders, and fatigue. Grade 3 treatment-related adverse events were reported in 12 (34%) of 35 patients.

Interpretation

In this single-centre phase 2 trial, the combination of nivolumab plus ipilimumab showed marked efficacy in patients with recurrent malignant pleural mesothelioma. The safety profile was consistent with known data on the combination regimen. Our results warrant further investigation of this combination in a phase 3 trial.

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Research in context

Evidence before this study

Few treatment options are available for patients with malignant pleural mesothelioma after one line of chemotherapy. We searched PubMed from January 1 2010 to June 1, 2018, with the following terms: "mesothelioma" AND "PD-1" OR "PD-L1" OR "CTLA-4" OR "checkpoint". We also searched clinical trial registers (ClinicalTrials.gov and WHO International Clinical Trials Registry Platform). This literature review indicated that there are several studies of monotherapy immune checkpoint inhibitors for malignant pleural mesothelioma and ongoing studies for combination checkpoint inhibitors. In both phase I and II studies monotherapy with a PD-1/PD-L1 antibody has meaningful efficacy and an acceptable safety profile, in contrast to monotherapy CTLA-4, which doesn't have clinical efficacy in a phase IIB study compared to placebo. Combination therapy with durvalumab and tremelimumab showed encouraging results. No phase III trials have been published.

Added value of this study

Results from the phase II INITIATE trial show that the combination of nivolumab plus ipilimumab has significant clinical efficacy for patients with malignant pleural mesothelioma after first line chemotherapy and a safety profile that is consistent with previously reported data.

Implications of all the available evidence

The clinical efficacy shown by our study suggests that combination checkpoint inhibition for malignant pleural mesothelioma should be tested in phase III studies in first and second line malignant pleural mesothelioma. The first-line phase III trial comparing nivolumab plus ipilimumab with platinum plus pemetrexed is ongoing (NCT02899299).

Introduction

Malignant pleural mesothelioma is an aggressive tumour originating from the mesothelial cells of the pleura. Asbestos exposure is the major risk factor for malignant pleural mesothelioma, with latency time from exposure to diagnosis varying from 20 to more than 50 years.1,2

The approved first-line treatment option for patients with malignant pleural mesothelioma who are not eligible for surgery is platinum-based chemotherapy with an antifolate.3,4 This treatment leads to a median overall survival of about 12-16 months, increasing to almost 19 months with the addition of the angiogenesis inhibitor bevacizumab.3,5

No approved second-line therapy exists yet. Responses with chemotherapy vary between 10% and 20% of patients and median overall survival ranges from 5.6 to 10.9 months.6-10

A few studies using a single-agent checkpoint inhibitor for second-line treatment of malignant pleural mesothelioma have been published. The programmed death receptor 1 (PD-1) checkpoint inhibitors pembrolizumab and nivolumab were used in the Keynote-028 and NivoMes trial respectively, with partial responses achieved by five (20%) of 25 patients and nine (26%) of 34 patients, disease control achieved by 18 (72%) of 25 patients and 16 (47%) of 34 patients, and survival at 12 months of 63% (95% CI 40-79) and 50% (36-70).11,12 In the Javelin phase 1b trial with programmed cell death ligand 1 (PD-L1) checkpoint inhibitor avelumab 5 (9.4%)of 53 patients had an overall response.13 But the randomized, double-blind, placebo-controlled phase 2 DETERMINE trial analysing second-line treatment with single-drug cytotoxic-T-lymphocyte-associated antigen 4 (CTLA-4) checkpoint inhibitor tremelimumab in 571 patients with mesothelioma did not show benefit.14

Preclinical data suggest a synergistic effect of CTLA-4 and PD-1 checkpoint inhibitors.15 The ongoing first-line CheckMate 743 phase 3 randomised controlled trial (NCT02899299) in patients with malignant pleural mesothelioma is comparing platinum-based chemotherapy plus pemetrexed with nivolumab plus ipilimumab. In the phase II single-arm NIBIT-MESO-1 trial, patients received a combination of tremelimumab (anti-CTLA-4 antibody) and durvalumab (anti-PD-L1 antibody) in the first or second line setting. 11 (28%) of 40 patients had an immune-related objective response and 25 (63%) achieved disease control. Median progression free survival (PFS) was 5.7 months and median overall survival (OS) 16.6 months.16 The MAPS2 randomised phase II trial by Scherpereel and colleagues assesed nivolumab with or without ipilimumab in patients with relapsed mesothelioma, and showed similar results for the combination treatment and monotherapy, 17 although a formal comparison was not done.

Patients with mesothelioma usually have moderate expression of PD-L1, with 20–40% of tumours expressing PD-L1 in more than 1% of cells. PD-L1 expression is more common in the non-epithelioid histological subtype than in the epithelioid subtype. In cohorts of patients who have not been treated with checkpoint inhibitors, patients with PD-L1-positive tumours have a substantially worse prognosis (median survival 4.8 months) than those with negative tumours (16.3 months), independent of histology (epithelioid or non-epithelioid subtypes). The heterogeneity of tumour biopsy procedures in malignant pleural mesothelioma, and non-uniformity of staining procedures, including differences in cutoff levels, makes comparison between studies difficult.18-21

In line with our previous study on nivolumab monotherapy, we here report the efficacy and safety data of our INITIATE trial assessing the combination of ipilimumab and nivolumab in the treatment of malignant pleural mesothelioma, including results of PD-L1 expression.

Methods

Study design and participants

INITIATE is a prospective single-centre, single arm, phase 2 trial for patients with unresectable malignant pleural mesothelioma who have disease progression or recurrence after at least one line of platinum-containing systemic therapy. The study was approved by the institutional review board and in accordance to the Declaration of Helsinki. All patients provided written informed consent before enrollment.

Patients were aged at least 18 years, had an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1 and could have any subtype of histologically confirmed recurrent malignant pleural mesothelioma. Additional inclusion criteria were measurable disease on CT scan according to the modified Response Evaluation Criteria in Solid Tumours (mRECIST), 22 life expectancy greater than 12 weeks and adequate hematologic and organ function within the 14 days prior to first study treatment.23

Exclusion criteria were previous treatment with any checkpoint inhibitor or current treatment with systemic immunosuppressive medication (use of systemic prednisolone at maximum dosage of 10mg/day or equivalent was allowed), previous malignancy (except adequately treated basal cell, squamous cell skin cancer, superficial or in-situ cancer of the bladder or other cancer for which the patient had been disease-free for at least five years), brain metastases and patients with only peritoneal malignant mesothelioma.

Other exclusion criteria were a history of active autoimmune disease, idiopathic pulmonary fibrosis, severe infections in the 4 weeks before start of study treatment, active

tuberculosis, significant cardiovascular disease, myocardial infarction in the 6 months before enrolment, unstable angina, or unstable arrhythmias, pulmonary or hepatic disease constituting a high risk for investigational treatment as per investigator's judgement, and unresolved (drug-induced) pneumonitis, organizing pneumonia, or active pneumonitis on CT scan. Relevant gastrointestinal disease, prior allogeneic bone marrow or solid organ transplantation or a history of HIV were also exclusion criteria, as well as any major surgical procedures within the 28 days before starting study treatment.

Patients with uncontrolled pleural or peritoneal effusion requiring recurrent (once monthly or more frequently) drainage procedures, and patients with uncontrolled tumour-related pain were excluded. Pain medication had to be on a stable regimen at study entry and lesions amenable to palliative radiotherapy had to be treated prior to enrollment.

Procedures

After giving informed consent, histological tumour biopsies and peripheral blood were collected before treatment administration. Thoracoscopy was the preferred method, but ultrasound or CT-guided transthoracic needle core biopsies (6 x 16 Gauge) were allowed. After six weeks of treatment, a second tumour biopsy was obtained for research purpose. PD-L1 expression on formalin-fixed, paraffin-embedded tissue samples was assessed with immunohistochemistry using the 22C3 pharmDx antibody (Agilent, Santa Clara, CA, USA).

Patients received the PD-1 checkpoint inhibitor nivolumab in combination with CTLA-4 checkpoint inhibitor ipilimumab (Bristol-Myer Squibb, New York, NY, USA). Nivolumab was administered intravenously over at least 30 min at a fixed dose of 240 mg, every 2 weeks. Ipilimumab was administered intravenously in 30 min at a dose of 1 mg/kg, after nivolumab infusion, every 6 weeks for up to four doses, on the basis of results of melanoma trials. Patients received nivolumab therapy for a maximum of 2 years, or until disease progression or unacceptable toxicity. Treatment delay criteria include any grade ≥ 2 non-skin, drug-related adverse event as assessed with Common Terminology Criteria for Adverse Events (CTCAE version 4.03) with a few exceptions as specified in full protocol. Re-treatment could be given when all toxicities had resolved to grade 1, or according to the protocol.

Tumour imaging via CT scan was done in the 28 days before start of therapy and for response assessments every 6 weeks of treatment until disease progression was observed. Evaluation of CT scans was done by one independent reviewer using mRECIST criteria for mesothelioma.23 Laboratory tests were performed every 2 weeks and included a standard hematology and chemistry panel. Thyroid and adrenal function tests were performed every 6 weeks.

After treatment completion patients had follow-up visits every 6 weeks for the first 48 weeks, then every 12 weeks, until progression or death. All patients with progressive disease had follow-up visits every 3 months to assess survival.

Outcomes

The primary outcome of the study was the proportion of patients who achieved disease control at 12 weeks after start of nivolumab plus ipilimumab. Disease control was defined as either complete response, partial response or stable disease according to the modified RECIST criteria for mesothelioma.

Secondary outcomes were safety, objective response (complete or partial response) at 6 months, disease control at 6 months, progression-free survival (time from first treatment to progression or death) and overall survival (time from first treatment to death of any cause) and immunological changes of mesothelioma before and after 6 weeks of treatment. Immunological results will be presented elsewhere.

Statistical analysis

To test the hypothesis that combination treatment of nivolumab plus ipilimumab will improve disease control from 20% to 50%,6 an optimal two-stage design was used with the type I error rate (α) being 0.02 and the power (1−β) being 90%.24 The null hypothesis that 20% of patients will receive a true response, was tested against a one-sided alternative. The planned sample size was 33 patients, with an interim analysis after 12 patients. The study would be stopped for futility if at the time of interim analysis 3 or less out of 12 patients showed disease control at 12 weeks. Treatment with nivolumab plus ipilimumab is deemed successful if the study is not stopped at interim analysis and at least 12 out of 33 patients show disease control at 12 weeks.

Anticipating possible drop-out cases, we included 36 patients, which yielded 34 evaluable patients. To account for this change from the planned population size, the adjusted p-value was calculated as the conditional probability (under the null hypothesis of a DCR of 20%) of finding at least the obtained number of patients with disease control in 34 patients, conditional on at least four patients having disease control among the first 12 patients. This adjusted p-value was then compared against the pre-specified type I error rate of 2%.

All patients who received at least one dose of immunotherapy and at least one radiologic evaluation were considered evaluable. All patients who received at least one dose of immunotherapy and had at least one follow-up visit were included in the safety analysis.

Time-to-event endpoints (ie, progression-free survival and overall survival) were estimated with the Kaplan-Meier method. Treatment outcomes (partial response, stable disease

or progressive disease at 12 weeks, described as an ordinal variable) were compared between PD-L1 positive and negative patients using the linear-by-linear association test. Clinical benefit (partial response or stable disease for > 6 months) was compared between PD-L1 positive and PD-L1 negative patients using the Fisher's exact test. PD-L1 expression in tumour cells was scored as the percentage of all tumour cells that expressed PD-L1. PD-L1 expression in tumour-infiltrating immune cells was categorised into four groups according to the percentage of immune cells (ie, non-tumour cells) that were PD-L1 positive as follows: less than 1% was scored as 0, at least 1% to less than 5% was scored as 1, at least 5% to less than 10% was scored as 2, and at least 10% was scored as 3. All analyses were done in R statistical software (version 3.4.0). The trial was registered with ClinicalTrials.gov, number NCT03048474.

Role of funding source

The funder had a role in study design, but not data collection, analysis, or interpretation or writing of the report. All authors had full access to the raw data. All authors confirmed the accuracy and completeness of the data and made the decision to submit the manuscript.

Results

Patients and treatment

Between Oct 5, 2016 and Aug 3, 2017, 38 patients with progression of malignant pleural mesothelioma after at least one line of chemotherapy gave informed consent. Of these, 36 patients were eligible for inclusion (figure 1). One patient deteriorated quickly and could not begin immunotherapy at the planned start of treatment so was excluded from analyses.

Most patients in the cohort were men (27 [77%] of 35) and most had the epithelioid subtype (30 [86%]); the median age was 65 years (IQR 62–71; range 37–79 years; table 1). All patients had received at least one line of chemotherapy containing a platinum doublet with pemetrexed, 22 cisplatin and 13 carboplatin. Other previous therapies included gemcitabine (five [14%] of 35 patients), vinorelbine (one [3%]), pemetrexed monotherapy (three [9%]), and bevacizumab (one [3%]). Some patients were previously treated in a clinical trial with either anetumab ravtansine (a mesothelin-targeting antibody–drug conjugate), nintedanib, tazemetostat (competitive inhibitor of histone methyl transferase EZH2), or dendritic cell therapy (each in one [3%] patient).

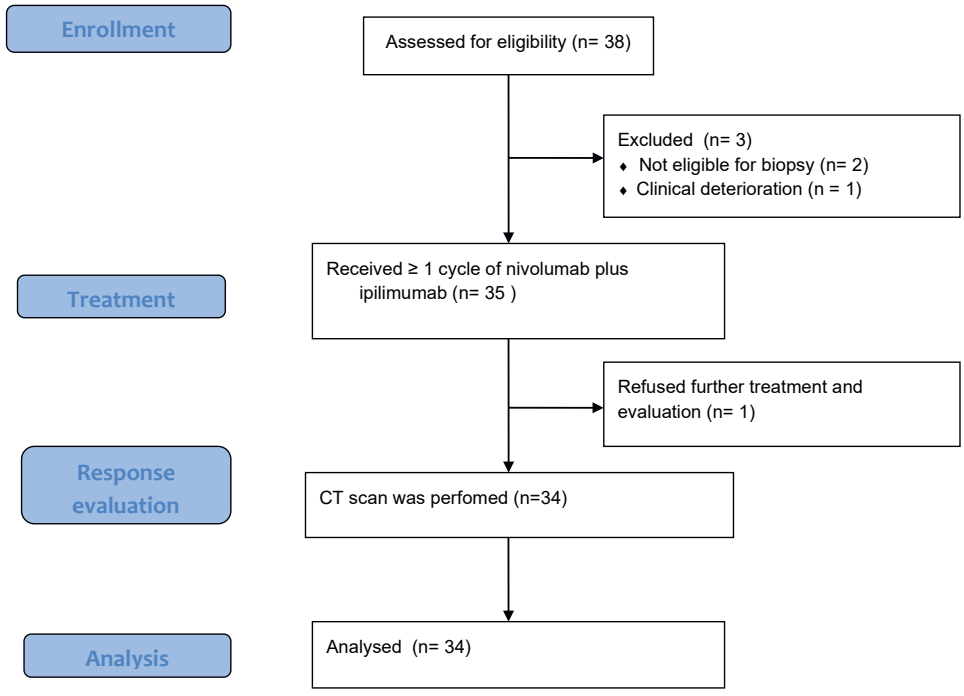

Figure 1 patient flow chart

A large variation existed in time between diagnosis and time of enrollment in study, ranging from 2·2 months until 95·4 months (almost 8 years), with a median of 12 months (IQR 8.8 – 22.7). The median time between the last systemic treatment to enrollment in the study was 6.4 months (range 1 – 61, IQR 3.2 – 20.1).

Of the 36 patients eligible patients one deteriorated quickly and could not begin immunotherapy at the planned start of treatment. Another refused any further treatment or control visits after only 1 cycle of immunotherapy and was not included in the analysis. A total of 34 patients received at least one dose of immunotherapy and a radiologic evaluation and thus were evaluable for response assessment. A total of 35 patients received immunotherapy and had at least one follow-up visit. The first patient started treatment on November 9, 2016 and the last patient on August 28, 2017.

Table 1. Baseline characteristics (n = 35).

Median age (years)	65
range	37-79
Sex	
men	27 (77%)
women	8 (23%)
Histology	
Epithelioid	30 (85%)
Sarcomatoid	3 (9%)
Mixed	2 (6%)
ECOG performance status at registration	
0	10 (29%)
1	25 (71%)
Ethnicity	
Caucasian	34 (97%)
Negroid	1 (3%)
Prior lines of therapy	
1	29 (83%)
2	4 (11%)
3	1 (3%)
4	1 (3%)
Disease stage	
I - III	21 (60%)
IV	14 (40%)
Smoking status	
Never	12 (34%)
Former	17 (49%)
Current	6 (17%)
PD-L1 expression on tumour cells	
Negative (<1%)	19 (54%)
Positive (≥ 1%)	15 (43%)
Not scored	1 (3%)

At time of data cut-off (June 1, 2018) patients who started treatment had a median of 12 doses of nivolumab (range 1–37, IQR 8.3 – 21.8 doses) and a median of 4 doses of ipilimumab (range 1–4, IQR 3-4 doses) administered. In 13 patients (37%) (in 30 cycles) nivolumab was postponed, mainly due to toxicities and/or corticosteroid use for toxicities, but also due to flu (2 patients – 6%) and family circumstances (2 patients – 6%). In five patients (14%) (6 cycles) ipilimumab was delayed because of toxicity.

One patient (3%) decided to stop due to toxicity (malaise grade 2), after receiving all four doses of ipiliumumab. The patient who withdrew consent stopped treatment after one cycle of immunotherapy. Ten (29%) patients were still on treatment at the time of data cutoff. All others with data available (23 patients [66%]) had to stop immunotherapy because of radiological progression.

Efficacy

For the primary endpoint at 12 weeks, 23 (68%; 95% CI 50–83) of 34 patients had achieved disease control (ten [29% had a partial response and 13 [38%] had stable disease; table 2, figure 2). 11 (32%) patients had progressive disease and none had a complete response at 12 weeks. Disease control in 23 (68%) patients was enough to refute the null hypothesis of 20% disease control at the one-sided preplanned 98% confidence level (98% one-sided CI 49–100, accounting for the planned interim analysis after 12 patients). In fact, these numbers exceeded our expectations. The results reject our own alternative hypothesis of 50% disease control with 95% confidence (95% one-sided CI 52–100).

Table 2. Clinical activity.

Radiological response at twelve weeks	
Complete response	0
Partial response	10 (29%)
Stable disease	13 (38%)
Progressive disease	11 (32%)
Disease control rate	23 (68%, 50 – 83) *
Objective response	13 (38%, 22 – 56)
Ongoing response **	11 (32%, 17 – 51)
Median follow up time (months)	14.3 (12.7 – 15.7)
Median duration of response (months) ***	14·3 (6·4 - NR)
Median progression-free survival (months)	6·2 (4·1- NR)
Progression-free survival at 6 months	50% (36-70)
Median overall survival (months)	NR (12·7 - NR)
1 year overall survival	64% (50 – 83)

Data are n (%), n (%; 95% CI), median (95% CI), or % (95% CI). NR=not reached

* confidence interval calculated accounting for the planned interim analysis after 12 patients.

**patients with partial response or stable disease for more than 6 months, on study drugs or at end of treatment.

*** time from start of response to progression

At data cutoff, three more patients had achieved a partial response, two after 18 weeks and one after 24 weeks of treatment, resulting in a total of 13 patients (38%) with a partial response as their best response. The median time to response was 2.6 months (95% CI 2.4–not reached). The median duration of response (time from start of response to progression) was 14.3 months (95% CI 6·4-not reached).

At six months 13 (38%) of 34 patients had a partial response, four (12%) patients had stable disease and 17 (50%) patients had progressive disease; thus, disease control at six months was achieved by 17 (50%) patients (95% CI 32% – 68%;appendix). Objective response at six months was 38% (95% CI 22 - 56).

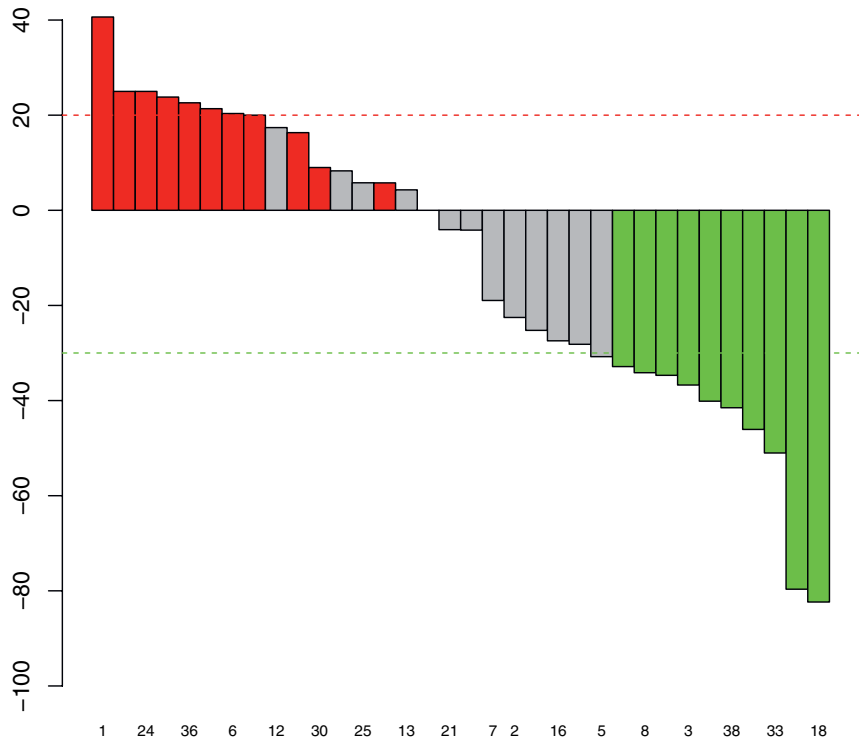

Figure 2: Percentage change in tumour size, baseline to week 12.

Change in sum of target lesions measured according to modified Response Evaluation Criteria in Solid Tumours by independent reviewer at 12 weeks as percentage change from baseline. Horizontal dotted line at 30% decrease shows cutoff for partial response and dotted line at 20% increase shows cutoff for progressive disease. Some patients have progressive disease based on non-target lesions. Orange shows progressive disease; blue shows stable disease; and green shows partial response.

At data cutoff, 10 (29%) patients were still receiving immunotherapy in this study, six of them for more than a year. Median progression free survival was at least 6.2 months (95% CI: 4.1 months – not reached; table 2, figure 3). The proportion of patients achieving progression free survival at six months was 50% (95% CI: 36-70; table 2, figure 3).

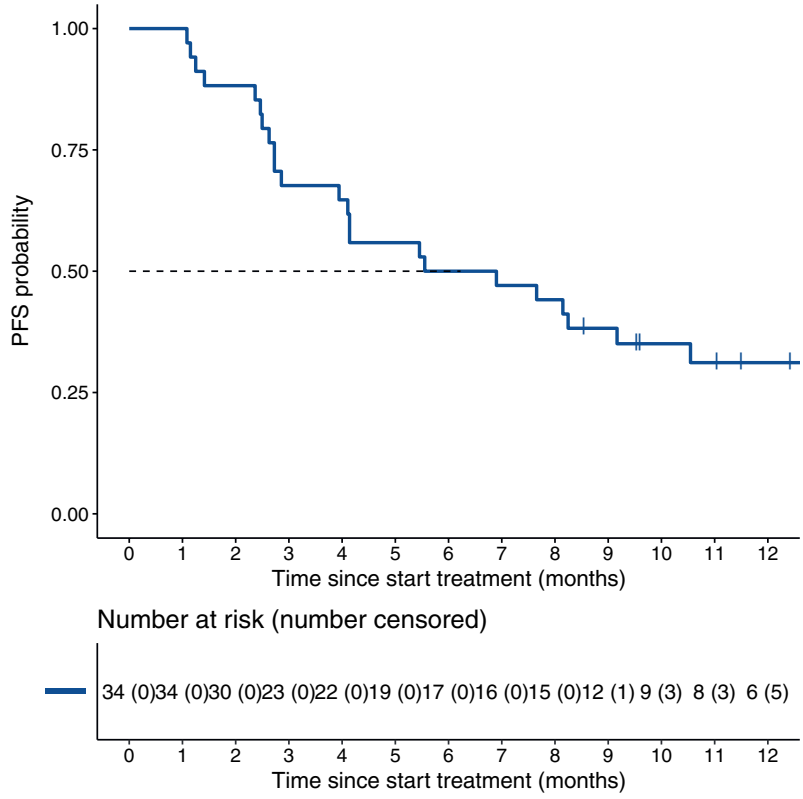

Figure 3: Kaplan-Meier curve of progression-free survival

Median follow-up (since first treatment) was 14.3 months (95% CI 12.7 – 15.7). Median overall survival was not yet attained, since only 13 patients (38%) had died, but with 95% confidence, the median overall survival will be greater than 12.7 months. Overall survival at six months was 85% (95% CI: 74 – 98) and overall survival at twelve months was 64% (95% CI: 50 – 83; table 2).

The small number of tumours with non-epithelioid histology did not allow a meaningful comparison between histological subtypes.

Safety

33 patients (94%) reported any treatment-related adverse event (table 3). The most frequent were infusion related reactions and skin disorders (each in 17 [49%] of 35 patients), including pruritus (11 [31%]) and dry skin (eight [23%]). Other treatment-related adverse events were fatigue (nine [26%] patients), anorexia (seven [20%]), diarrhoea (seven [20%]), nausea (six [17%]), and increased aspartate transaminase (five

[14%]). All other adverse events occurred in four patients or fewer. In the 33 patients, 134 treatment-related adverse events occurred. 12 patients (34%) had one or more grade 3 events related to treatment, including diarrhoea (three patients [9%]), increased alanine aminotransferase, anorexia, increased aspartate transaminase, and pleural effusion (all in two patients [6%]). Only one grade 4 event occurred, an increase in γ-glutamyltransferase, which decreased after a delay of one cycle of nivolumab. No grade 5 adverse events were reported. One patient discontinued treatment because of several toxicities, in particular malaise, but also mucositis, dysgeusia, pruritus, fatigue, hypothyroidism, and arthralgia.

Notably, many patients had infusion-related reactions (49%), grade 1 or 2, starting at first or second nivolumab dose. In those patients, the infusion was interrupted and symptomatic treatment was given (acetaminophen or antihistaminic drug, or both), with a prompt response. At all following immunotherapy cycles, prophylactic treatment (acetaminophen with or without antihistamine drug) was given and the infusion rate of nivolumab was slowed down, preventing further reactions. No patients required a prolonged admission or had to stop treatment because of infusion-related reactions. It was not possible to attribute adverse events to either nivolumab or ipilimumab, with the exception of infusion-related reactions, which seemed to be caused by nivolumab, based on time of onset of reaction. Six treatment-related serious adverse events (all grade 3) occurred in five patients, including pleural effusion (two patients), dyspnoea (in one patient; the same patient as one of the pleural effusion events), asthma cardiale, diarrhoea, and adrenal insufficiency (each in one patient).

Concomitant systemic corticosteroids for treatment of immune-related adverse events were administered in eight (23%) of 35 patients; for adrenal insufficiency (two patients [6%]), arthralgia (two [6%]), colitis (two [6%]), decrease of renal function (two [6%]), and pneumonitis (one [3%]). All these patients were re-treated with immunotherapy, but only when toxicity had decreased to a lower grade and patients were off steroids or on low-dose steroids. Some patients had more than one treatment related toxicity for which they needed steroids at different timepoints. In one patient, treatment stopped because of progressive disease while on systemic corticosteroids for treatment-related toxicity for the second time. Incidence of treatment-related toxicities was compared between those who achieved a partial response and those who had stable disease or progressive disease, but the occurrence of any of these adverse events did not differ between the two groups.

Table 3. Treatment-related adverse events (n=35)

	All grades (1-5)	Grade 1	Grade 2	Grade 3	Grade 4
Adrenal insufficiency	3 (9%)	0	2 (6%)	1 (3%)	0
Alanine aminotranferase (ALT) increase	3 (9%)	1 (3%)	0	2 (6%)	0
Anorexia	7 (20%)	4 (11%)	1 (1%)	2 (6%)	0
Arthralgia	4 (11%)	1 (3%)	3 (9%)	0	0
Aspartate transaminase (AST) increase	5 (14%)	2 (6%)	1 (1%)	2 (6%)	0
Asthma cardiale	1 (3%)	0	0	1 (3%)	0
Diarrhea	7 (20%)	3 (9%)	1 (1%)	3 (9%)	0
Dyspnea	4 (11%)	2 (6%)	1 (3%)	1 (3%)	0
Fatigue	9 (26%)	5 (14%)	4 (11%)	0	0
Gamma-glutamyltransferase (GGT) increase	1 (3%)	0	0	0	1 (3%)
Infusion related reaction	17 (49%)	2 (6%)	15 (43%)	0	0
Malaise *	3 (9%)	0	3 (9%)	0	0
Mucositis oral	1 (3%)	0	0	1 (3%)	0
Myalgia	4 (11%)	2 (6%)	2 (6%)	0	0
Nausea	6 (17%)	0	6 (17%)	0	0
Pleural effusion	2 (6%)	2 (6%)	0	2 (6%)	0
Pleural infection	1 (3%)	0	0	1 (3%)	0
Skin disorder	17 (49%)	10 (29%)	6 (17%)	1 (3%)	0
Pruritus	11 (31%)	10 (29%)	1 (3%)	0	0
Dry skin	8 (23%)	5 (14%)	3 (9%)	0	0
Rash	10 (29%)	5 (14%)	4 (11%)	1 (3%)	0

Data are n (%). For grades 1–2 events, only those that occurred in 10% or more patients are reported. All grade 3 and 4 events are reported. No grade 5 events occurred. *Resulted in treatment discontinuation for one patient.

We did a post-hoc analysis of clinical benefit (partial response or stable disease for more than 6 months) and treatment outcome (partial response, stable disease, or progressive disease at 12 weeks), according to PD-L1 expression status. Pretreatment biopsies of all 34 evaluable patients were scored for PD-L1 expression (22C3 antibody). 15 (44%) samples had PD-L1 expression on at least 1% of tumour cells (table 4), of which 12 (80%) were epithelioid, one (7%) was mixed, and two (13%) were sarcomatoid. Both patients with sarcomatoid subtype had a PD-L1 expression of 50%. Five (15%) patients had PD-L1 expression of at least 50%. Responses at 12 weeks for the 15 PD-L1-positive patients (ie, PD-L1 expression of ≥1%) were partial response in seven (47%), stable disease in six (40%), and progressive disease in two (13%), which were significantly better than responses for the 19 PD-L1-negative patients, which were partial response in three (16%), stable disease in seven (37%), and progressive disease in nine (47%; p=0·018, linear-by-linear association test). PD-L1 positivity (vs negativity) was significantly associated with clinical benefit (ie, partial response or stable disease for >6 months; p=0.037, Fisher's exact test). 11 (73%) of

the 15 PD-L1-positive patients had clinical benefit, whereas only six (32%) of 19 PD-L1-negative patients had clinical benefit (table 4).

PD-L1 expression on immune cells (scored 0–3) was significantly associated with response, with higher expression corresponding to better response (p=0.001, linear-by-linear association test). Most notably, of the 11 patients who progressed at 12 weeks, ten (91%) had PD-L1 expression of less than 1% on immune cells (score 0). Seven (21%) of the total 34 patients had PD-L1 expression of at least 5% (score ≥2), and all had clinical benefit. For the ten patients with both PD-L1 expression on tumour cells and immune cells, nine (90%) had clinical benefit. The hazard ratio of tumour cell PD-L1 expression versus no expression was 0.39 (95% CI 0.17–0.94) for progression-free survival (figure 4A) and 0.16 (0.04–0.73) for overall survival (figure 4B), indicating both clinical and statistical significance. The hazard ratio of immune cell PD-L1 expression versus no expression was significant (0.18; 95% CI 0.04–0.78) for progression-free survival (figure 4C) but non-significant (0.30; 0.08–1.1) for overall survival (figure 4D).

Table 4. clinical benefit by PD-L1 expression

	Tumour cell PD-L1 expression, as a percentage of all tumour cells			Tumour-infiltrating immune cell PD-L1 expression, as a percentage of all non-tumour cells		
	Negative	Positive ≥1%	≥50%	Negative IC 0	Positive IC ≥ 1	IC ≥ 2
pre-treatment biopsy (n=34)						
clinical benefit	6	11	4	5	12	7
no clinical benefit	13	4	1	14	3	0
total	19	15	5	19	15	7
on-treatment biopsy (n=32)						
	Negative	Positive ≥1%	≥50%	Negative IC 0	Positive IC ≥ 1	IC ≥ 2
clinical benefit	3	8	2	1	15	8
no clinical benefit	7	6	0	4	11	6
Total *	12	14	2	5	26	14

Clinical benefit was partial response or long-term stable disease (≥6 months). *Six patients did not have a tumour at the time of on-treatment biopsy, so PD-L1 expression in tumour cells could not be measured; in one patient, tumour-infiltrating immune cell PD-L1 expression could not be scored. PD-L1=programmed cell death ligand 1.

After 6 weeks of treatment, we obtained biopsy samples from 32 patients; in one (3%) patient, no accessible tumour remained and one (3%) patient was not fit for a thoracoscopy. Six on-treatment biopsy samples showed no tumour cells; in five of them a

dense infiltration of immune cells was seen. Of the 19 patients that were PD-L1 negative at baseline, eight (42%) were positive during treatment, of which four (21%) had clinical benefit and the other four (21%) did not. Conversely, of the 15 patients that were PD-L1 positive at baseline, three (20%) were negative during treatment (appendix). When assessing PD-L1 expression on tumour cells in on-treatment samples, an association with response was noted (p=0.053, linear-by-linear association test), but the association was less strong than in the pretreatment samples. The on-treatment samples also showed that PD-L1-positive patients had a better response at 12 weeks (29% partial response, 50% stable disease, and 21% progressive disease) than PD-L1-negative patients (8% partial response, 33% stable disease, and 58% progressive disease). No association was noted when analysing on-treatment PD-L1 expression on immune cells.

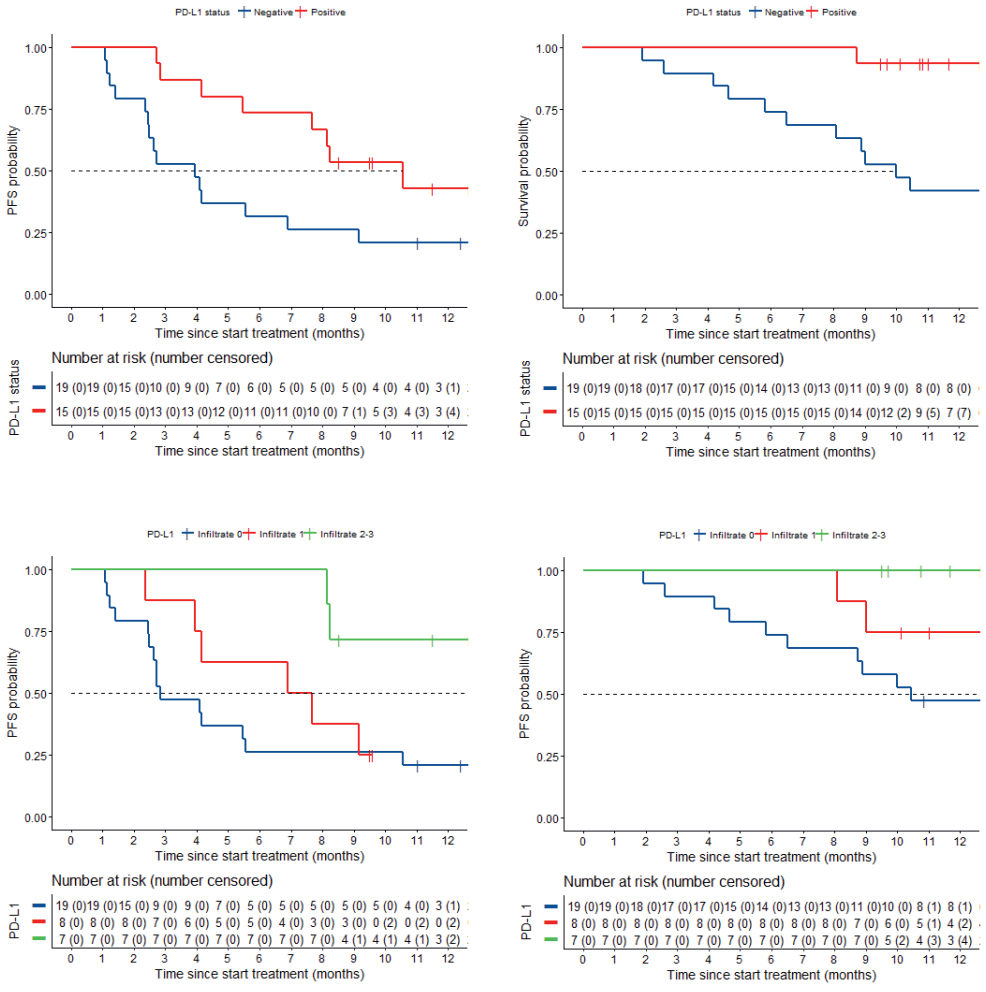

Figure 4: Kaplan-Meier survival curves in patient subgroups

Progression-free survival (A) and overall survival (B) by PD-L1 tumour cell expression level at baseline and progression-free survival (C) and overall survival (D) by PD-L1 expression in immune infiltrate. PD-L1=programmed cell death ligand 1.

Discussion

Our study shows that the combination of nivolumab and ipilimumab has marked clinical activity in previously treated relapsed patients with malignant pleural mesothelioma. The regimen was well tolerated and toxicity was reversible and considered manageable when adhering to protocol guidelines.

Four (31%) of the 13 patients who achieved a partial response did so by the 6-week assessment six (46%) did so by the 12-week assessment, and three (23%) did so after 12 weeks. The median time to response was 2.6 months, which is similar to time to response with nivolumab in another study of patients with malignant pleural mesothelioma (12).

The objective response of 36% is much better than the response reported for second line chemotherapy (10-20%) (6,7,10) or monotherapy with a checkpoint inhibitor (10-20%) for malignant pleural mesothelioma (11-14). However, these studies are difficult to compare because of a potential selection bias, related to the heterogeneity between studies with respect to included patients, inclusion criteria and treatment history.

Regarding the CTLA-4 checkpoint inhibitor tremelimumab, single center phase II studies seemed promising (25,26), but the multicenter randomized phase IIB study was negative, compared to placebo (14). Whether this result was due to selection bias or variations in tumour or patient biology is unclear. No positive phase III studies have been published for checkpoint inhibitors in mesothelioma yet. Whether our results will translate to a survival benefit for patients with mesothelioma needs to be investigated in a phase III trial. Results for the first line multicenter phase III study comparing nivolumab plus ipilimumab with platinum plus pemetrexed (Checkmate 743) are awaited.

The same combination of checkpoint inhibitors nivolumab plus ipilimumab was also analysed in the MAPS-2 trial (17). Our study and the MAPS2 trial showed similar proportions of patients achieving 12-week disease control. Our median progression-free survival of 6.2 months (95% CI 4.1–not reached) is similar to the MAPS2 median progression-free survival of 5.6 months (3.1–8.3) for the combination treatment, as is our overall survival at 12 months of 64% (50–83%) to their result of 58% (46–70%). Another combination of checkpoint inhibitors (anti-PD-L1 durvalumab plus anti-CTLA-4 tremelimumab) as first-line and second-line treatment was tested in the NIBIT-MESO-1 clinical trial and similar efficacy and toxicity results were obtained (16).

Metaxas and colleagues (27) did a real-world analysis of varying regimens of pembrolizumab in patients with malignant pleural mesothelioma. The general observation was that in the unselected population, including patients with a performance status of 2, treatment with a checkpoint inhibitor was feasible. However, as described in a comment by De Gooijer and Baas (28), there were many limitations of the analysis, including the absence of a control group and the large proportion of patients with a high performance status.

Although all patients but one experienced any treatment-related adverse event, only 12 (34%) patients had a grade 3 or 4 adverse event. Most treatment-related AEs were reversible and considered manageable when adhering to protocol guidelines. Only one patient

discontinued treatment due to toxicities. Of all 577 planned cycles of immunotherapy, 32 cycles (6%) were not given due to treatment-related AEs.

The combination of CTLA-4 and PD-1 inhibitors increased toxicity in our study compared to with other monotherapy trials, we mainly attributed the high numbers of toxicities to the CTLA-4 inhibitor (11,12,14,16). Many patients had grade 1 or 2 toxicities, and these did not delay treatment and were considered manageable with standard protocols.

The reason for the many infusion-related reactions (IRR) to nivolumab (in 49% of all patients) is not clear. For nivolumab monotherapy in malignant pleural mesothelioma (Nivomes trial) two (6%) IRR were described (12), although conditions were similar to our study (240mg infused over 30 min). In the Keynote-028 trial assessing pembrolizumab monotherapy only one (4%) patient had an infusion-related reaction (11). In Checkmate-057, which assessed nivolumab monotherapy (3 mg/kg infused over 60 min) for patients with non-small-cell lung carcinoma, 3% of patients had infusion related reactions.29 In other studies, with combination treatment of nivolumab plus ipilimumab in melanoma (1mg/kg nivolumab over 60 min and 3 mg/kg ipilimumab over 90 min) grade 1 or 2 infusion related reactions occurred in 3% of patients (30). The discrepancies with our study might be related to the combination therapy plus the differences in infusion rate (30 min in our study), even though safety studies for shorter infusion rates of combined nivolumab and ipilimumab and other monoclonal antibodies showed acceptable safety (30,31). We also observed a variety of skin-related toxicity (50%), including pruritus, dry skin, and rash. This toxicities responded well to symptomatic local treatment.

Limitations of this study include the small sample size and single-arm setting. Despite recruiting almost all patients that were referred to our hospital, a limited selection of participants were enrolled. The median time from diagnosis to start of study in our trial was 12 months and greater than 4 years in two patients, whereas the mean overall survival for mesothelioma is only 12–16 months (3,5). Because few patients with mesothelioma have a performance status of 0–1 after one or more lines of therapy, our cohort does not resemble the general population of patients with malignant pleural mesothelioma who have relapsed after treatment; our patients progressed more slowly or were more sensitive to treatment.

In a few clinical trials of checkpoint inhibitors, PD-L1 expression was measured with variable response results. One of the inclusion criteria for the Keynote-028 study was PD-L1 expression in more than 1% of tumor cells, assessed by the 22C3 antibody. Whether a higher expression resulted in a better or longer response was not reported (11). In the Javelin trial with avelumab for malignant pleural mesothelioma, 43 patients were evaluable for PD-L1 expression, with a cutoff for positivity of more than 5% of tumour

cells. Objective response was achieved by three (19%) of 16 PD-L1-positive patients and two (7%) of 27 PD-L1-negative patients (13). In the Nivomes trial (12) assessing nivolumab in patients with malignant pleural mesothelioma, PD-L1 expression of more than 1% (assessed with 28-8 antibody) was measured in 27% of patients, with no clear association with clinical benefit. Baseline tumour PD-L1 expression (SP-263 assay) in the NIBIT-MESO-1 trial16 did not correlate with response or survival. In the MAPS-2 trial, PD-L1 expression of at least 1% significantly correlated with objective response, and high PD-L1 expression (≥25%) was correlated with both objective response and disease control. In our study PD-L1 expression on tumour cells was significantly correlated with response. But like in other studies, not all patients with PD-L1 expression achieved a response and some who were PD-L1 negative did respond. We noted a change in PD-L1 expression between pre- and on-treatment biopsies (appendix), this might be due to the (known) heterogeneity of malignant pleural mesothelioma (32), or the effect of therapy (33).

We noted a significant association of immune cell PD-L1 expression with outcome, in line with research in other types of cancer (34). These immune cells might be of different subtypes, which could be the reason for the better outcome. This will be focus of our ongoing translational research.

PD-L1 expression on both tumour cells and immune cells at baseline might serve as a prognostic biomarker for the effect of checkpoint inhibitors in patients with malignant pleural mesothelioma. But both are insufficient for prediction of response. Patient characteristics and other biomarkers need to be studied prospectively to establish which subgroup of patients will benefit from checkpoint inhibitors.

In conclusion, in this single-centre phase II study, the combination of nivolumab and ipilimumab has marked clinical efficacy in patients with malignant pleural mesothelioma. The safety profile is consistent with previously reported data of combination checkpoint inhibitors. Our results add to the growing evidence that immunotherapy is a promising treatment, warranting further research in a phase 3 trial.

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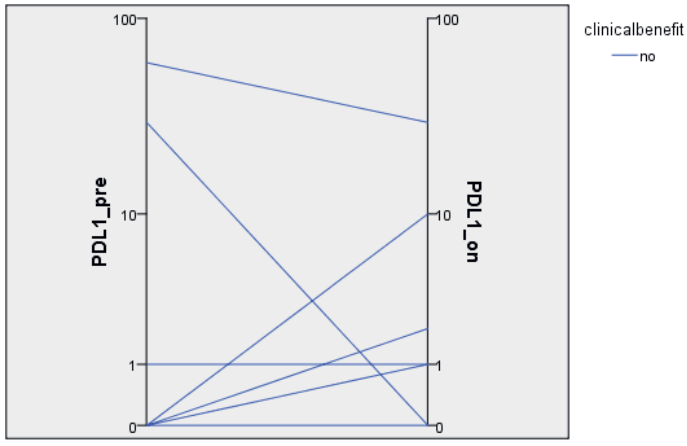
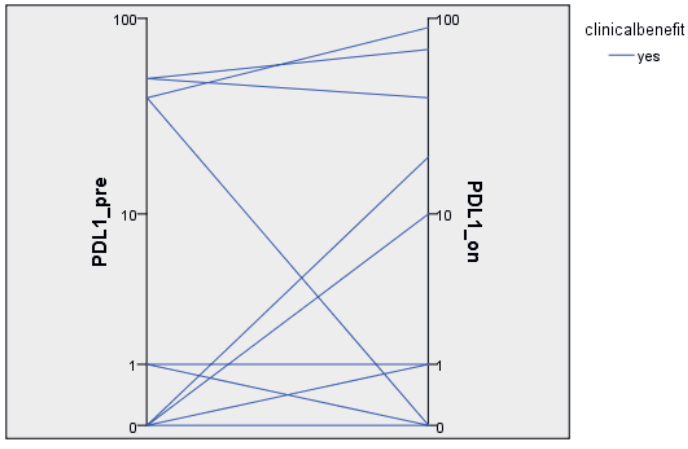
Supplementary data

Supplementary figure 1: change in PD-L1 expression during treatment.

A:

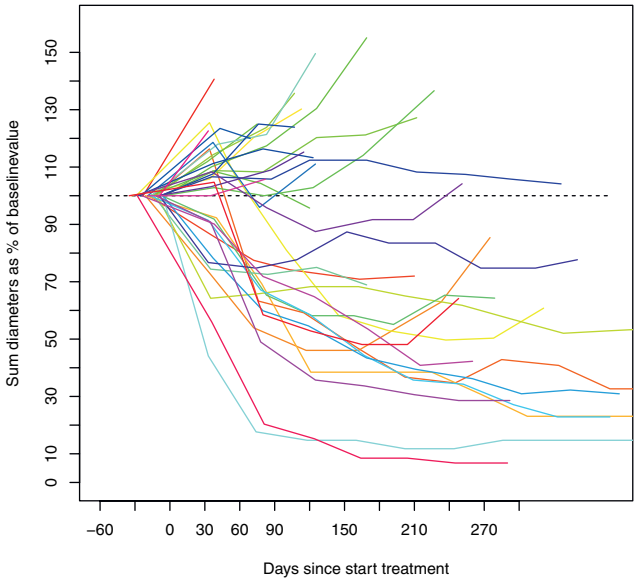


B:



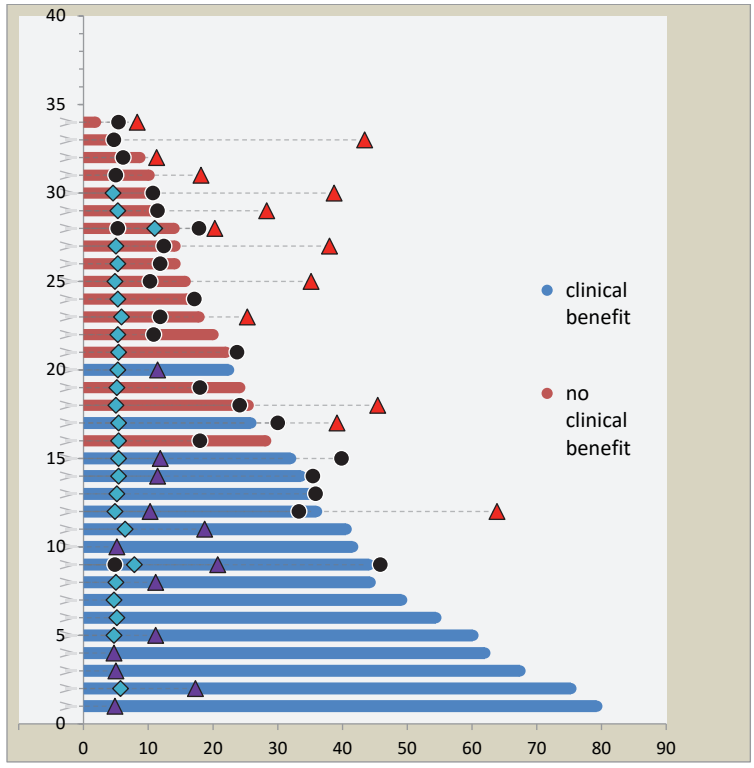
Change in PD-L1 expression on tumor cells during treatment in patients without clinical benefit (A) and with clinical benefit (B). Left y-axis is PD-L1 expression in pre-treatment biopsies and right y-axis is PD-L1 expression in on-treatment biopsies. Both as a percentage of all tumor cells on a logarithmic scale.
In A: nine patients do not have change in expression from 0.

Supplementary figure 2. plot representing the change in sum of target lesions from baseline over time in days (%).

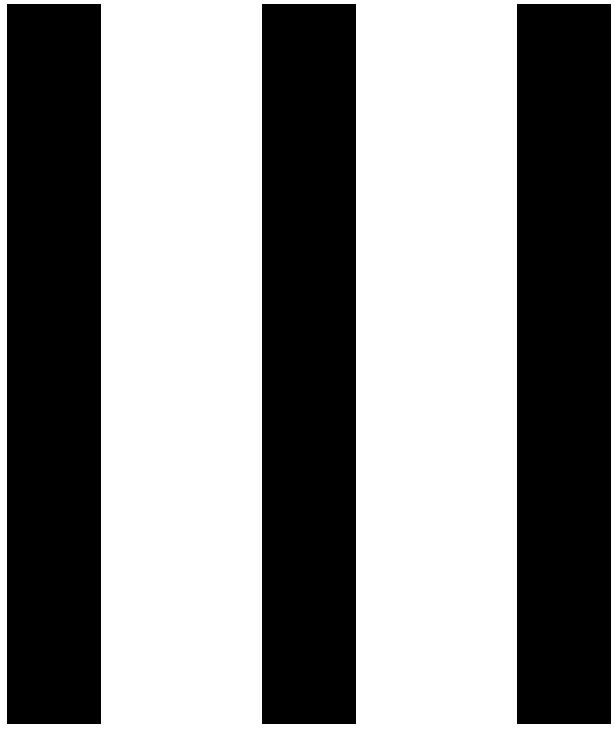


Percentage change in sum of target lesions from baseline over time in days. Positive change indicates tumour growth and negative change indicates tumour reduction. N = 34

Figure 3. Swimmer plot: treatment exposure and response duration in weeks.



The length of each bar corresponds with treatment duration in weeks. Response symbols represent the time when first reported (and not best response). We defined clinical benefit as partial response or stable disease for more than 6 months.



PART III

Translational research

6

Chapter 6

Efficacy of nivolumab and ipilimumab in patients with malignant pleural mesothelioma is related to a subtype of effector memory cytotoxic T cells: translational evidence from two clinical trials.

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Abstract

Background

Combined immune checkpoint inhibitor (ICI) treatment targeting PD-1 and CTLA-4 was suggested to yield clinical benefit over chemotherapy in malignant pleural mesothelioma (MPM), whereas aPD-1 monotherapy failed to provide benefit in phase-III trials. Success of ICI depends on the presence and activation of tumor-specific T cells. Therefore, we investigated whether T-cell characteristics are underlying clinical efficacy of ICI treatment in MPM.

Methods

Comprehensive immune cell profiling was performed on screening and on treatment peripheral blood samples of mesothelioma patients treated with nivolumab (aPD-1) monotherapy (NCT02497508), or a combination of nivolumab and ipilimumab (aCTLA-4) (NCT03048474).

Findings

aPD-1/aCTLA-4 combination treatment induced a profound increase in proliferation and activation of T cells, which was not observed upon aPD-1 monotherapy. Moreover, patients that responded to combination treatment had low frequencies of naive CD8 T cells and high frequencies of effector memory CD8 T cells that re-expressed RA (TEMRA) at screening. The frequency of Granzyme-B and Interferon-g producing TEMRAs was also higher in responding patients.

Interpretation

High proportions of TEMRAs and cytokine production by TEMRAs before treatment, was associated with a better clinical outcome. TEMRAs, which likely comprise tumor-specific T cells, tend to require blockage of both aPD-1 and aCTLA-4 to be reactivated. In conclusion, peripheral blood TEMRAs can play a key role in explaining and predicting clinical benefit upon aPD-1/aCTLA-4 combination treatment.

Funding

Bristol-Myers Squibb sponsored NivoMes and INITIATE clinical trials and provided study drugs. No external funding was applicable for the flow cytometric analyses of peripheral blood samples described in this manuscript.

Research in context

Evidence before this study

Immune monitoring, the assessment of peripheral blood immune cell subsets, yielded valuable insight into peripheral blood T-cell responses to immune checkpoint inhibitors (ICI) in non-small cell lung cancer (NSCLC) and melanoma patients. We searched Pubmed for scientific literature published between Jan 1st 2010 and June 15th 2020 with the following terms: "mesothelioma" AND ("PD-1" OR "PD-L1" OR "CTLA-4" OR "checkpoint") AND ("peripheral blood" OR "immune monitoring"). No previous studies have assessed the peripheral blood immune cell compartment upon ICI treatment in malignant pleural mesothelioma (MPM).

Added value of this study

To our knowledge, we are the first to perform extensive immune monitoring in MPM patients treated with both aPD-1 monotherapy and aPD-1/aCTLA-4 combination therapy. Recently, promising results of Checkmate-743 (NCT02899299) demonstrated that treatment of MPM patients with nivolumab and ipilimumab yielded a statistically significant and clinically meaningful improvement in overall survival, compared to platinum-based chemotherapy plus pemetrexed. These results are in contrast to the lack of benefit seen earlier in the PROMISE-meso trial (NCT02991482) that investigated nivolumab monotherapy as compared to chemotherapy in MPM. We here provide a rationale for the benefit observed upon aPD-1/aCTLA-4 combination treatment in MPM by indicating differences in the peripheral blood T-cell compartment in two phase II clinical trials that assessed aPD-1 monotherapy and aPD-1/aCTLA-4 combination therapy.

Implications of all the available evidence

Combination checkpoint inhibition appears to be more effective than their use alone in MPM, which was already shown in the MAPS2 phase II randomized trial. Preliminary results of the Checkmate-743 support this statement. These findings, combined with our peripheral blood analyses, warrant further research into aPD-1/aCTLA-4 combination in MPM with in-depth peripheral blood and intratumoral T-cell characterization.

Introduction

Malignant pleural mesothelioma (MPM) is a malignancy arising from the mesothelial cells in the pleural cavity, primarily caused by asbestos exposure. Treatment options for MPM are very limited, as platinum-based chemotherapy combined with an antifolate and the optional addition of bevacizumab, are the only approved first-line treatment for MPM. This treatment leads to a median overall survival OS) of 12 -16 months (1,2). Currently, no registered second-line treatments are available, illustrating the urgent need for new treatment options.

Immunotherapies aim for activation of the immune system, leading to efficient tumor-specific immune responses. In current clinical practice, these therapies include monoclonal antibodies that block inhibitory checkpoint receptors, i.e. programmed death 1 (PD-1), programmed death ligand 1 (PD-L1) and cytotoxic T lymphocyte associated antigen 4 (CTLA-4), thereby reinvigorating anti-tumor immune responses (3). So-called immune checkpoint inhibitor (ICI) treatments have transformed the treatment landscape for various malignancies, such as non-small cell lung cancer (NSCLC) and melanoma (4,5).

Unfortunately, ICI treatments are less effective in MPM as compared to other malignancies. The DETERMINE trial showed no survival benefit of ipilimumab (anti-CTLA-4, aCTLA-4) monotherapy over placebo (6) and pembrolizumab and nivolumab, both anti-PD-1 (aPD-1) monotherapies, demonstrated objective response rates (ORR) of 21% and 26% in the KEYNOTE-028 and NivoMes trials respectively (7,8). Recently, the PROMISE-meso phase III randomized trial (NCT02991482) failed to show improvement in PFS (progression-free survival) and OS upon second line aPD-1 treatment (pembrolizumab), as compared to single agent chemotherapy (institutional choice of gemcitabine or vinorelbine) (9). The lack of effective ICI treatment in MPM is thought to be dependent on the small number of tumor-infiltrating lymphocytes (TILs) in MPM (10,11) and the immunosuppressive tumor microenvironment (12,13).

Combining aPD-1 and aCTLA-4 therapy has been shown to induce synergistic effects in both preclinical and clinical studies (14,15). Phase II trials in MPM also suggest improved clinical responses upon combination ICI treatment, as the MAPS2 trial (nivolumab plus ipilimumab), the NIBIT-MESO trial (durvalumab (aPD-L1) plus tremelimumab (aCTLA-4)) and the INITIATE trial (nivolumab plus ipilimumab) reported better clinical responses upon combination ICI treatment than reported by trials that investigated monotherapy (nivolumab or pembrolizumab) (16 18). Recently, the first positive results were announced for the Checkmate-743 (19), a phase III trial that combined aPD-1 (nivolumab) with aCTLA-4 (ipilimumab) treatment in previously untreated MPM patients. These results are very promising, although the magnitude of the benefit is still awaited.

Success of aPD-1 treatment in NSCLC and melanoma is thought to depend on pre-existing T-cell infiltration of the tumor (20), proliferation of peripheral PD-1-expressing CD8 T cells (21) and the ratio between T-cell reinvigoration and tumor burden (22). It remains unclear whether the enhanced efficacy observed in ICI combination treatment trials is due to an additive effect of the respective therapies or truly depends on a novel immunological mechanism that is engaged by targeting both PD-1 and CTLA-4 (23).

In order to dissect the immunological mechanisms responsible for the clinical benefit from aPD-1 and aCTLA-4 therapy in MPM, we aimed to investigate the characteristics of lymphocytes present in peripheral blood of MPM patients treated with aPD-1 monotherapy (nivolumab) in the NivoMes trial (8) and aPD-1 and aCTLA-4 combination therapy (nivolumab/ipilimumab) in the INITIATE trial (16). We specifically aimed to evaluate the T- and NK-cell compartment of the peripheral blood, since prior studies established the value of this compartment in the context of aPD-1 and aCTLA-4 treatment (21,22,24).

Methods

Study population

Patients in this study were enrolled in either the NivoMes study (NCT02497508) or the INITIATE study (NCT03048474). Both studies were approved by the institutional review board of the Netherlands Cancer Institute and in accordance with the Declaration of Helsinki. All patients provided written informed consent before enrolment. Collection and analysis of immune cell subsets in peripheral blood were planned a priori as part of the two trials. Clinical results of the NivoMes and INITIATE were previously published (8,16). In summary, in the NivoMes trial, 34 MPM patients progressing after at least one cycle of platinum based chemotherapy, were treated with nivolumab 3 mg/kg every 2 weeks. In the INITIATE trial, 35 MPM patients progressing after at least one cycle of platinum based chemotherapy were treated with nivolumab (240 mg flat dose every 2 weeks) plus ipilimumab (1 mg/kg every 6 weeks up to four times). Peripheral blood was collected from patients on the day of the first ICI treatment and after six weeks of treatment. These samples correspond to the 'screening' and 'on treatment' time points. Response to treatment was assessed according to modified RECIST criteria for mesothelioma (25). For comparison purposes, we decided to define responding patients as having a complete response (CR), partial response (PR) or stable disease (SD) at six months of follow up and non-responding patients as having progressive disease (PD) at six months of follow up. All patients in the 'responder' group experienced a PFS of six months or longer and all patients in in the 'non-responder' group progressed within six months.

Processing of peripheral blood

Fifty milliliters of blood was drawn at screening and on treatment time points in EDTA tubes and processed. Peripheral blood mononuclear cells (PBMC) were isolated via standard density-gradient centrifugation using Ficoll-Hypaque (GE Healthcare, Chicago, IL, USA). Cells were cryopreserved in 10% dimethylsulfoxide (Sigma-Aldrich, Saint Louis, MO, USA), 40% FCS (Gibco, ThermoFisher, Waltham, MA, USA) and RPMI (Invitrogen, ThermoFisher, Waltham, MA, USA) until further use.

Flow cytometry

Flow cytometry staining was performed on the cryopreserved PBMC samples. After thawing of the PBMCs, cells were stimulated for 4 hours with phorbol 12-myristate 13-acetate and ionomycin (both from Sigma-Aldrich, Saint Louis, MO, USA) and GolgiStop (BD Biosciences, Franklin Lakes, NJ, USA), prior to continuation of the cytokine staining. Supplementary table 1 lists the antibodies used for the different stainings. First, extracellular markers were stained for 30 min at 4 °C. Secondly, the cells were stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, ThermoFisher, Waltham, MA, USA) for 10 min at 4 °C in order to identify dead cells. Next, FoxP3 transcription factor fixation/permeabilisation mix (eBioscience, ThermoFisher, Waltham, MA, USA) was used to fixate the cells. Subsequently, intracellular markers were stained for 60 min at 4 °C. Data were acquired using an LSR II flow cytometer equipped with three lasers. We used FlowJo v10 (BD Biosciences, Franklin Lakes, NJ, USA) to analyze the data. Fig. 1A, C, D, F and H show the gating strategy. Specific maturation subsets of T cells were identified by the cell surface markers CD45RA and CCR7. Fractions of CD45RA+CCR7+ naive (N) T cells, CD45RA CCR7+ central memory (CM) T cells, CD45RA CCR7 effector memory (EM) T cells and CD45RA+CCR7 effector memory re-expressing RA (EMRA) T cells were identified in both the CD4 and CD8 T-cell compartments.

Statistical analysis

Statistical analyses were performed in R version 4.0.2 and GraphPad V8.0 (GraphPad, San Diego, CA, USA). $P < 0.05$ was considered statistically significant. Significant differences between the groups were determined with Mann Whitney U tests (non-parametric, non-paired data) and Wilcoxon signed rank tests (non-parametric, paired data). P values were corrected for multiple testing, using the Benjamini and Hochberg False Discovery Rate (26). Log rank test was used to compare Kaplan-Meier curves for PFS and OS. To stratify PFS and OS for proportions of T-cell subsets, the median was used as a cut off for high vs low proportions.

Role of funding sources

Bristol-Myers Squibb sponsored the clinical studies and provided the study drugs in both the NivoMes and INITIATE clinical trials. The analyses of peripheral blood mononuclear

cells (PBMCs) by flow cytometry, described in this manuscript, were not sponsored by any external funding.

Table 1 Characteristics of patients included in translational analysis.

		Nivomes	Initiate
Patients screened		38	38
Included, received at least 1 cycle of treatment		34	35
At least 1 CT for response evaluation available		33	34
At least 1 PBMC sample for FCM available at screening or on-treatment time point		31	38
PBMC sample at screening time point available		24	38
PBMC sample at screening time point and response evaluation available		23	32
Baseline characteristics			
N		23	32
Age (years) (range)		67 (62 - 73)	65 (62 - 72)
Gender (%)	Male	19 (82.6%)	24 (75%)
	Female	4 (17.4%)	8 (25%)
Histological subtype (%)	Epithelioid	21 (91.3%)	28 (87.5%)
	Sarcomatoid	2 (8.7%)	2 (6.2%)
	Mixed	0 (0%)	2 (6.2%)
WHO (%)	0	10 (43.5%)	11 (34.4%)
	1-2	13 (56.5%)	21 (65.6%)
6 months response (%)	CR	0 (0%)	0 (0%)
	PR	6 (26.1%)	12 (37.5%)
	Epithelioid	6 (100%)	11 (91.7%)
	Sarcomatoid	0 (0%)	1 (8.3%)
	Mixed	0 (0%)	0 (0%)
	SD	1 (4.3%)	4 (12.5%)
	Epithelioid	1 (100%)	2 (50%)
	Sarcomatoid	0 (0%)	1 (25%)
	Mixed	0 (0%)	1 (25%)
	PD	16 (69.6%)	16 (50%)
	Epithelioid	14 (87.5%)	15 (93.8%)
	Sarcomatoid	2 (12.5%)	0 (0%)
	Mixed	0 (0%)	1 (6.2%)
PFS (months) (95% CI)		2.44 (1.3 - 10.0)	6.25 (4.1 - 11.0)
OS (months) (95% CI)		11.5 (5.1 - 21.6)	23.0 (12.5-not reached)

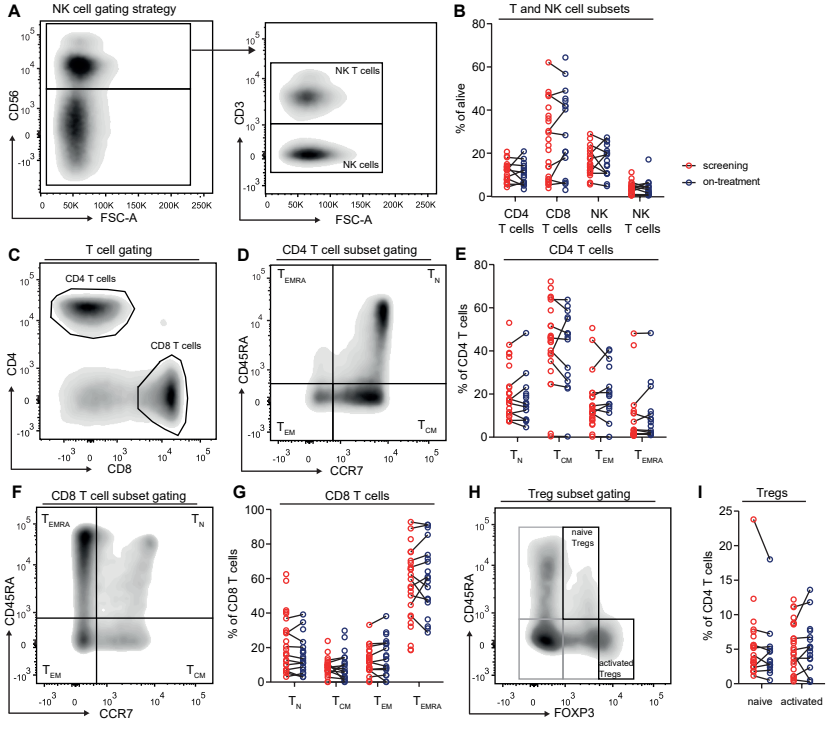
Results

Patient characteristics

Table 1 demonstrates the numbers of peripheral blood samples available from the two clinical trials. Baseline characteristics are shown for the patients of whom PBMCs were collected at screening and at least 1 CT-scan for response evaluation was available.

Monotherapy with aPD-1 treatment does not induce T-cell proliferation

In both NSCLC and melanoma, it was shown that aPD-1 treatment increased proliferation of CD8 T cells in peripheral blood, and the majority of these proliferating CD8 T cells were PD-1 positive (21,22). We therefore analyzed whether aPD-1 monotherapy induced similar changes in T- or NK cell subsets of MPM patients. No significant differences were observed in the frequencies of T cells (Fig. 1B), T-cell subsets (Fig. 1E, G, I), NK cells and NK T cells (Fig. 1B) between screening and 6 weeks after start of treatment. Surprisingly, aPD-1 monotherapy also induced no increase in proliferation of T-cell subsets, as assessed by Ki-67 expression, a cell cycle marker expressed by cycling or recently divided cells (Fig. 1J-L).



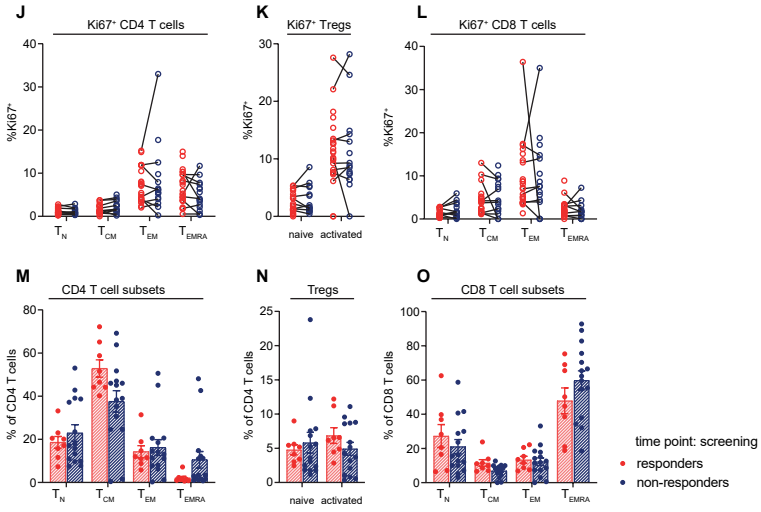


Fig. 1. T- and NK-cell characteristics before and during aPD-1 monotherapy (a, c, d, f, h). Gating strategy for NK-cells (a), T-cells (c), CD4 T-cells subsets (d), CD8 T-cells subsets (f) and Treg subsets (h) respectively. (b, e, g, i) Percentage of T-and NK-cell subsets (b), CD4 T-cell subsets (e), CD8 T-cells subsets (g) and Treg subsets (I) respectively, at screening and on-treatment time points. (j, k, l) Percentage of Ki67+ CD 4 T-cell subsets (j), Tregs subsets (k) and CD8 T-cell subsets (l) respectively, at screening and on-treatment time points. (m, n, o). Paired samples are shown connected by black lines. Percentage of CD4 T-cell subsets (m), Treg subsets (n) and CD8 T-cell subsets (o) respectively, at the screening time point in responding and non-responding patients. Bars depict mean values with standard error of the mean.

Next, we examined whether differences in the frequencies and phenotype of T cells prior to treatment, could help identify patients that responded to aPD-1 monotherapy. We found that MPM patients with a response upon aPD-1 had slightly higher frequency of CM CD4 T cells, whereas all other T-cell frequencies were similar between responding and non-responding MPM patients (Fig. 1M - O). No changes were found in the proportions of proliferating T- and NK cells, assessed by Ki67 expression (data not shown).

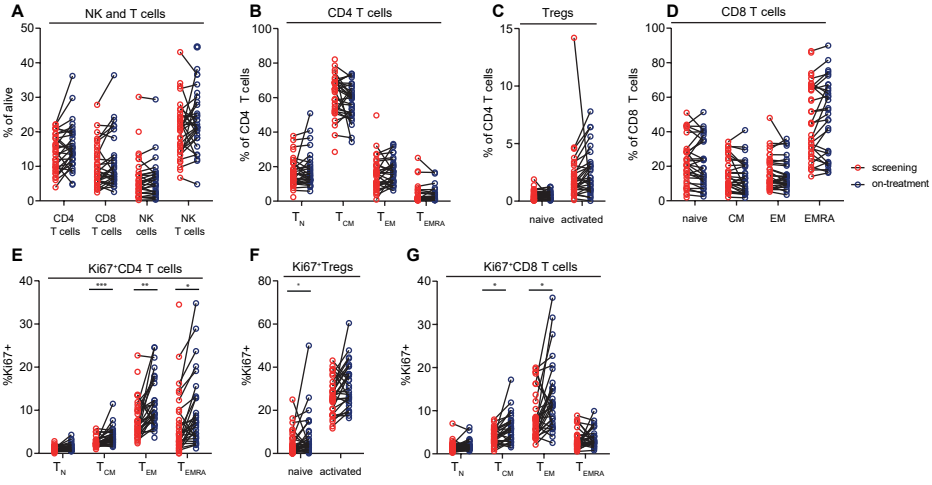
Furthermore, no changes in the frequencies of PD-1, CD28, 4-1BB, HLA-DR, inducible T-cell costimulator (ICOS), CD39, lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) and CTLA-4 expressing T-cell subsets induced by aPD-1 treatment or between responding and non-responding patients were observed (data not shown).

In conclusion, aPD-1 treatment did not induce changes in the proportion and proliferation of T-cell and NK cell subsets in MPM patients. No major differences were found between responding and non-responding patients prior to treatment.

aPD-1 and aCTLA-4 combination therapy promotes proliferation of memory T-cell subsets

Secondly, we examined whether aPD-1 and aCTLA-4 combination treatment induced proliferation and activation of T cells. We found that combination treatment increased the proliferation of CM, EM and EMRA CD4 T-cells and in naive and CM CD8 T-cells (Fig. 2E - G). This increase in proliferation was independent of clinical response (Fig. 2 H - J). Furthermore, the frequency of CM, EM and EMRA CD4 T-cell subsets, and CM and EM CD8 T cells that expressed ICOS increased upon combination therapy, indicating that combination therapy induced T-cell activation (Fig. 3A - C). In the CD4 T-cell compartment, this activation was most prominent in non-responding patients (Fig. 3D). Combination treatment did not induce differences in the frequency of the activation and inhibitory markers CD28, 4-1BB, HLA-DR, PD-1, LAG-3, TIM-3, CD39 and CTLA-4 in both CD4 and CD8 T-cell subsets (data not shown).

In conclusion, combining aPD-1 and aCTLA-4 treatment induced proliferation and activation of memory T-cell subsets, however, this proliferation was independent of clinical response.



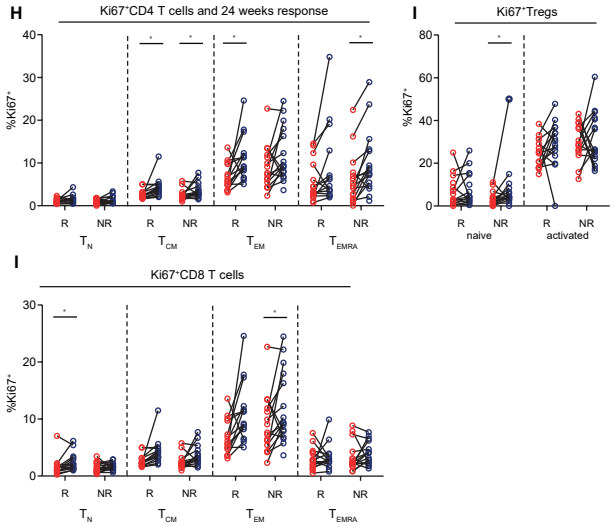


Fig. 2. T- and NK-cell characteristics before and during aPD-1/CTLA-4 combination therapy(a, b, c, d) Percentage of T-and NK-cell subsets (a), CD4 T-cell subsets (b), Treg subsets (c) and CD8 T-cells subsets (d) respectively, at screening and on-treatment time points. (e, f, g) Percentage of Ki67+ CD 4 T-cell subsets, (TCM p = 0.003, TEM p = 0.007, TEMRA p = 0.028) (e), Tregs subsets (f) and CD8 T-cell subsets (TN p = 0.036, TCM p = 0.03,) (g) respectively, at screening and on-treatment time points. (h, i, j) Comparison between responding (R) and non-responding (NR) patients for the percentage of Ki67+ CD 4 T-cell subsets (TCM R p = 0.01, TCM NR p = 0.04, TEM R p = 0.01) (h), Tregs subsets (i) and CD8 T-cell subsets (j) respectively, at screening and on-treatment time points. Paired samples are shown connected by black lines in each graph. Significance (Wilcoxon signed-rank test for paired analysis of screening and on-treatment samples and Mann-Whitney U test for comparison of response groups) is shown in each graph, with * $p < 0.05$ and ** $p < 0.01$. P values were corrected for multiple testing, using the Benjamini and Hochberg False Discovery Rate.

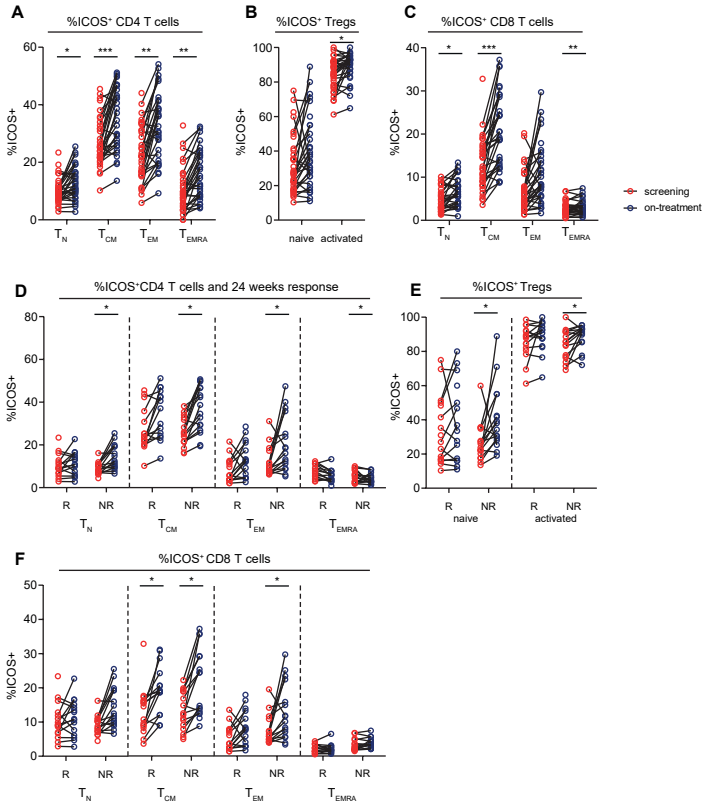


Fig. 3. Percentage of ICOS+ T cell subsets before and during aPD-1/CTLA-4 combination therapy (a, b, c) Percentage of ICOS+ CD 4 T-cell subsets (TCM p = 0.002, TEM p = 0.003, TEMRA p = 0.004) (a), Tregs subsets (b) and CD8 T-cell subsets (TCM p = 0.003, TEM p = 0.012) (c) respectively, at screening and on-treatment time points. (d, e, f) Comparison between responding (R) and non-responding (NR) patients for the percentage of ICOS+ CD 4 T-cell subsets (TN NR p = 0.01, TCM NR p = 0.02, TEM NR p = 0.03, TEMRA NR p = 0.01 (d), Tregs subsets (nTreg NR p = 0.01) (e) and CD8 T-cell subsets (TCM R p = 0.03) (f) respectively, at screening and on-treatment time points. Paired samples are shown connected by black lines in each graph. Significance (Wilcoxon signed-rank test) is shown in each graph, with *p < 0.05 and ** p < 0.01. P values were corrected for multiple testing, using the Benjamini and Hochberg False Discovery Rate

MPM patients responding to combined aPD-1 and aCTLA-4 treatment showed an altered distribution of CD8 T-cell subsets prior to treatment

We investigated whether the frequency or phenotype of T-cell subsets was different prior to treatment in patients that responded, compared to patients that did not respond to aPD-1 and aCTLA-4 combination treatment. MPM patients that responded had a different distribution of their T-cell compartment prior to treatment, with significantly lower

frequencies of naive and CM CD8 T cells and a higher frequency of EMRA CD8 T cells (Fig. 4A - C). Log rank test revealed that patients with a high EMRA CD8 T-cell proportion (cut-off based on the median proportion) at screening, had a significantly longer PFS upon combination treatment (median PFS of 13.1 vs 3.5 months, p = 0.045). Although the OS curves also appeared to differ (median OS of 25.9 vs 10.2 months), this difference was not statistically significant (Fig. 4D and E). Upon further characterization of these EMRA CD8 T cells, we found that the frequency of Granzyme-B and IFNg-expressing EMRA CD8 T cells was increased in responding patients (Fig. 5A and B). Increased cytokine expression was also observed in CM CD8 T cells and EM CD8 T cells (Fig. 5A and B). High or low proportion of Granzyme-B positive EMRA CD8 T cells (cut-off based on the median proportion) prior to treatment was used to stratify PFS and OS. Median PFS was 10.8 months vs 3.5 months for the high vs low groups and median OS was 32.6 vs 10.2 months. Log rank test did not reveal any significant differences between the two curves for both PFS and OS, although a clear trend was seen in the OS curves.

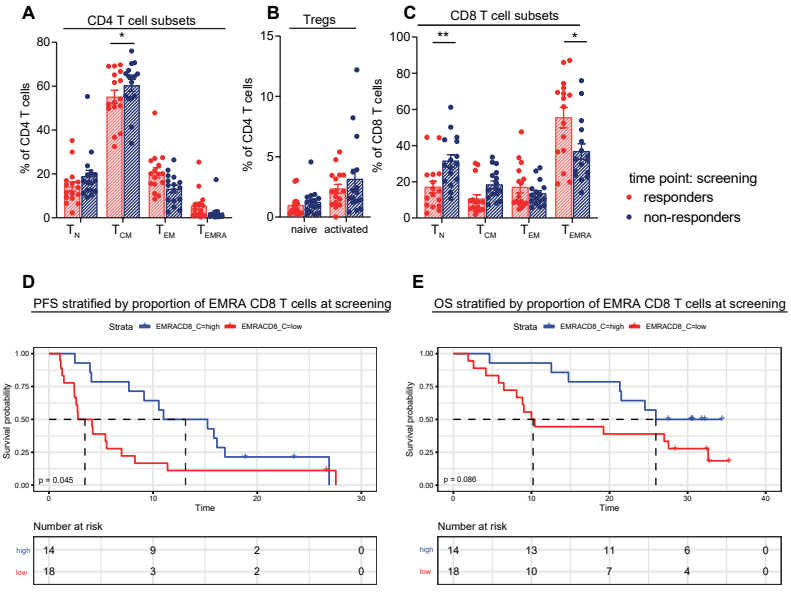


Fig. 4. Comparison of T-cell characteristics before aPD-1/CTLA-4 combination therapy in responding and non-responding patients (a, b, c) Percentage of CD4 T-cell subsets (a), Treg subsets (b) and CD8 T-cell subsets (TN p = 0.017, TCM p = 0.008, TEMRA p = 0.028) (c) respectively, at the screening time point in responding and non-responding patients. Bars depict mean values with standard error of the mean. Significance (MannWhitney U test) is shown in each graph, with * p < 0.05 and ** p < 0.01. P values were corrected for multiple testing, using the Benjamini and Hochberg False Discovery Rate. (d, e) EMRA CD8 T-cells proportions prior to treatment were used to stratify progression-free survival (PFS) (d) and overall survival (OS) (e). Median proportion of EMRA CD8 T cells was used as a cut off between the 'high' vs 'low' group. Statistical significance of the difference between the two

KaplanMeier curves was tested by log rank test with p = 0.045 for PFS (median PFS of 3.5 vs 13.1 months) and p = 0.086 for OS (median OS of 10.2 vs 25.9 months).

In conclusion, patients that responded to combined treatment with aPD-1 and aCTLA-4 had a different T-cell distribution, in particular more EMRA CD8 T cells and less naive CD8 T cells, prior to treatment. The frequency of cytokine-expressing memory CD8 T cells was increased in responding patients, indicating that these memory CD8 T cells are more functionally active.

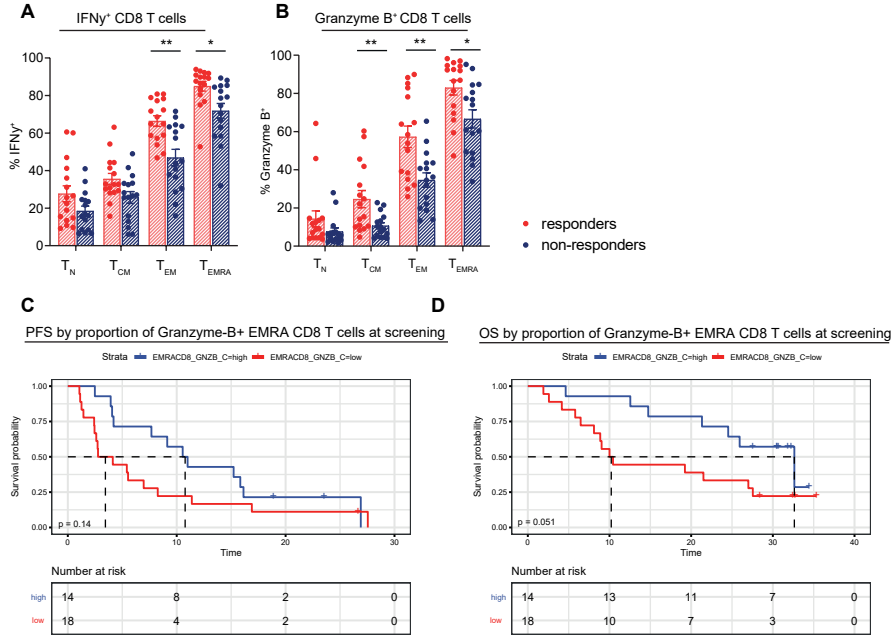


Fig. 5. Comparison of cytokine frequencies in CD8 T-cell subsets before aPD-1/CTLA-4 combination therapy in responding and non-responding patients (a, b) Percentage of IFNg+ CD8 T-cell subsets (TEM p = 0.008, TEMRA p = 0.006) (a) and Granzyme-B+ CD8 T-cell subsets (TN p = 0.02, TCM p = 0.032, TEMRA p = 0.02) (b) respectively, at the screening time point in responding and non-responding patients. Bars depict mean values with standard error of the mean. Significance (Mann-Whitney U test) is shown in each graph, with * $p < 0.05$ and ** $p < 0.01$. P values were corrected for multiple testing, using the Benjamini and Hochberg False Discovery Rate. (c, d) Proportions of Granzyme-B+ EMRA CD8 T-cells prior to treatment were used to stratify progression-free survival (PFS) (d) and overall survival (OS) (e). Median proportion of Granzyme-B+ EMRA CD 8 T cells was used as a cut off between the 'high' vs 'low' group. Statistical significance of the difference between the two KaplanMeier curves was tested by log rank test with p = 0.14 for PFS (not significant, median PFS of 3.5 vs 10.8 months) and p = 0.051 for OS (not significant, median OS of 10.2 vs 32.6 months).

Discussion

Recently, the first positive results were announced for the Checkmate-743 trial, demonstrating that combining aPD-1 and aCTLA-4 therapy led to improved OS in MPM, as compared to chemotherapy (19). In contrast, aPD-1 monotherapy failed to improve PFS and OS (9). Understanding the immunological mechanisms explaining why combination therapy of aPD-1 and aCTLA-4 is effective and monotherapy is not, is thus vital to select effective treatment options for MPM. To the best of our knowledge, we are the first to investigate T-cell characteristics of MPM patients treated with either aPD-1 monotherapy or aPD-1/aCTLA-4 combination therapy, treated during two ICI trials (8,16).

Using comprehensive immune monitoring, we demonstrate that combining aPD-1 with aCTLA-4 treatment strongly induces memory T-cell proliferation and activation of both CD4 and CD8 T cells. Higher frequencies of ICOS-expressing CD4 T cells were only observed in the combination therapy. Since this proliferation and activation was irrespective of clinical response, these results could indicate that aPD-1/ aCTLA-4 treatment induces proliferation and activation of bystander, non-tumor specific T cells, which lack the ability to respond to tumor antigens and do not result in a successful anti-tumor immune response. However, the distribution of T-cell subsets prior to treatment was different in MPM patients with a clinical response to combined aPD-1 and aCTLA-4 treatment. Herein, we found increased frequencies of EMRA CD8 T cells (TEMRAs) at the cost of naive CD8 T cells. Survival analysis also showed that PFS was significantly longer in patients with high frequencies of TEMRAs prior to treatment. Furthermore, in responding patients, we found higher frequencies of TEMRAs expressing Granzyme-B and IFNg. Thus, combined aPD-1/ aCTLA-4 treatment was associated with the activation and proliferation of memory T cells, but only MPM patients with high frequencies of TEMRAs prior to start of treatment, did benefit. The beneficial presence of TEMRAs could indicate that TEMRAs in particular comprise tumor-specific memory T cells that can be reinvigorated by combination treatment, but not by aPD-1 monotherapy, as these associations were not found in the aPD-1 monotherapy study. Our results are supported by several studies investigating memory CD8 T-cell biology, both in general and in relation to ICI treatment. Characterization of TILs in melanoma patients treated with combined aPD-1/ aCTLA-4 therapy revealed that tumors of responding patients harbored an effector memory T-cell population (CD8+ EOMES+CD69+CD45RO+) that was less abundant in non-responding patients (27). Wei et al. revealed that dual blockade of PD-1 and CTLA-4 engages biological pathways partly different from aPD-1 monotherapy (28). Combined aCTLA-1/ aPD-1 treatment increased the frequencies of a terminally differentiated TBET+EOMES+ CD8 T-cell subset in peripheral blood of melanoma patients, whereas aPD-1 monotherapy did not. Therefore, the authors speculated that combination therapy may be sufficient to attenuate or even reverse T-cell exhaustion. Both studies demonstrated that the combination of aPD-1/aCTLA-4 has a

distinct effect on borderline terminally differentiated memory T-cells, which was not observed upon aPD-1 monotherapy.

Our findings indicate that combination ICI treatment, in contrast to aPD-1 monotherapy, is able to reactivate these crucial TEMRA cells. Further research should provide mechanistic insight in how combined aPD-1 and aCTLA-4 treatment reactivates TEMRAs and should indicate their specificity.

In contrast to the observations of others in NSCLC and melanoma patients, we did not observe increases in T-cell proliferation upon aPD-1 monotherapy in MPM patients. These studies reported that the increase in proliferation peaked 3 weeks after start of treatment, and declined afterwards (21,22). As we evaluated immunological differences 6 weeks after start of treatment, we were most likely too late to assess the effects of aPD-1 monotherapy. However, these differences could also be dependent on tumor type, as aPD-1 therapy depends on pre-existing tumor-specific PD-1-expressing cells, which could be more frequent in NSCLC and melanoma as compared to MPM. Moreover, it has been described earlier that aPD-1 and aCTLA-4 therapy induced longer lasting transcriptional alterations as compared to aPD-1 monotherapy (29), potentially enabling us to detect changes in T-cell characteristics in combination ICI treatment in peripheral blood at a later point in time.

It is important to highlight that the immunological differences found in the two treatment modalities, although they clearly seem to fit response observations, could still be of a phenomenological nature. Thus, our results do not warrant any general conclusions on differences in ICI monotherapy and combination therapy in tumor types other than MPM. Given the limited number of patients analyzed in these studies and the limited number of responding patients, especially in the aPD-1 monotherapy study, our findings need to be validated in a larger and independent MPM patient cohort. Investigating the immunological changes induced by ICI treatment on multiple time points after start of treatment will also provide insight into the duration of these immunological changes upon different ICI treatments, and enable the comparison between MPM and other malignancies. Furthermore, it is not known whether changes in peripheral T-cell subsets reflect changes in the tumor microenvironment (TME) in MPM, and whether tumor specific T cells migrated from the peripheral blood into the TME or vice versa. We are also aware of the fact that nivolumab was administered in a weight dependent dose of 3 mg/kg every 3 weeks in NivoMes, thus modestly differing from the fixed dose of 240 mg/kg every 3 weeks that was administered in INITIATE. However, since Selby et al. (15) demonstrated that no significant alterations in lymphocyte subsets were seen upon different dosing regimens of nivolumab in macaques, we believe that the immune cell alterations described in this manuscript are most likely not caused by dosing differences. At last, it is important to

keep in mind that the presumed similarity between pembrolizumab and nivolumab is subject to an ongoing debate in MPM, especially since several studies in non-Caucasian populations demonstrated ORRs to nivolumab that appear to be higher than what was seen in studies performed in Europe and the United States (30,31).

In conclusion, the combined treatment of aPD-1 and aCTLA-4 induced a robust T-cell proliferation and activation in MPM patients, whereas aPD-1 monotherapy did not. The absence of a correlation to clinical response could indicate that these are bystander T-cells, unable to react to tumorantigens. High proportions of TEMRAs that expressed cytokines, prior to treatment, were associated with a better clinical outcome to combination therapy, likely because TEMRAs comprise tumor-specific T cells. This also suggests that TEMRAs can only be reactivated upon combined blockade of both aPD-1 and aCTLA-4. These findings have important implications for future clinical trial design. First, it provides an explanation for the discouraging results of aPD-1 mono-therapy in MPM, since aPD-1 monotherapy appears unable to reinvigorate tumor-specific terminally differentiated memory CD8 T cells in MPM. Second, it grants directions for future research, since aPD-1/aCTLA-4 appears to be a promising treatment modality for MPM, especially now that we are able to select patients up front that are likely to respond. And, finally, it provides a rationale for studying the efficacy of combining these treatments with vaccination strategies like dendritic cell vaccines in non-responding patients, since these vaccines have been shown to induce tumor specific T cells (32).

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Supplementary Data

Supplementary Table S1. Antibodies used for flow cytometry staining.

Antibody	Fluorochrome	Intracellular/ extracellular	Manufacturer	Clone	CAT number
T/NK cell staining with costimulatory markers					
Ki67	FITC	Intracellular	Ebioscience	20Raj1	11-5699-42
FOXP3	PE	Intracellular	Ebioscience	236A/E7	12-4777-42
CD45RA	PE Texas Red	Extracellular	Ebioscience	MEM-56	MHCD45RA17
CD28	PE Cy7	Extracellular	Biolegend	CD28.2	302926
CD137 (4-1BB)	PerCP Cy5.5	Extracellular	BD	4B4-1	309813
PD-1	APC	Extracellular	Biolegend	EH12.2H7	329907/329908
CD3	APC Cy7	Extracellular	Thermofisher (Invitrogen)	UCHT1	557832
CD8	AF700	Extracellular	Biolegend	SK1	344724
CCR7	BV421	Extracellular	Biolegend	G043H7	353208
CD56	BV605	Extracellular	BD	NCAM16.2	562780
ICOS	BV650	Extracellular	BD	DX29	563832
HLA-DR	BV711	Extracellular	BD	G46-6	563696
CD4	BV786	Extracellular	BD	SK3	563877
LIVE/DEAD stain	BV510	Extracellular	Thermofisher (Invitrogen)	-	L34966
T/NK cell staining with coinhibitory markers					
Ki67	FITC	Intracellular	Ebioscience	20Raj1	11-5699-42
FOXP3	PE	Intracellular	Ebioscience	236A/E7	12-4777-42
CD45RA	PE Texas Red	Extracellular	Ebioscience	MEM-56	MHCD45RA17
LAG-3	PE Cy7	Extracellular	Biolegend	11C3C65	369309
CTLA-4	PerCP Cy5.5	Extracellular	Thermofisher (Invitrogen)	14D3	14-1529-82
PD-1	APC	Extracellular	Biolegend	EH12.2H7	329907/329908
CD3	APC Cy7	Extracellular	Thermofisher (Invitrogen)	UCHT1	557832
CD8	AF700	Extracellular	Biolegend	SK1	344724
CCR7	BV421	Extracellular	Biolegend	G043H7	353208
CD56	BV605	Extracellular	BD	NCAM16.2	562780
TIM-3	BV650	Extracellular	BD	7D3	565565
CD39	BV711	Extracellular	BD	TU66	563680
CD4	BV786	Extracellular	BD	SK3	563877
LIVE/DEAD stain	BV510	Extracellular	Thermofisher (Invitrogen)	-	L34966
T/NK cell staining with intracellular cytokine markers					
Granzyme-B	FITC	Intracellular	Biolegend	QA16A02	372206
FOXP3	PE	Intracellular	Ebioscience	236A/E7	12-4777-42
CD45RA	PE Texas Red	Extracellular	Ebioscience	MEM-56	MHCD45RA17
IL-10	PE Cy7	Intracellular	Biolegend	JES3-9D7	501420
TNFα	PerCP Cy5.5	Intracellular	eBioscience	MAb11	560679
PD-1	APC	Extracellular	Biolegend	EH12.2H7	329907/329908
CD3	APC Cy7	Extracellular	Thermofisher (Invitrogen)	UCHT1	557832
CD8	AF700	Extracellular	Biolegend	SK1	344724
CCR7	BV412	Extracellular	Biolegend	G043H7	353208
CD56	BV605	Extracellular	BD	NCAM16.2	562780
IL-2	BV650	Intracellular	BD	5344.111	563467
IFNγ	BV711	Intracellular	BD	B27	564039
CD4	BV786	Extracellular	BD	SK3	563877
LIVE/DEAD stain	BV510	Extracellular	Thermofisher (Invitrogen)	-	L34966

7

Chapter 7

eNose in malignant mesothelioma – prediction of response to immune checkpoint inhibitor treatment

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Abstract

Introduction

Recent clinical trials with immune checkpoint inhibitors (ICI) have shown that a subgroup of patients with malignant pleural mesothelioma (MPM) could benefit from these agents. However, there are no accurate biomarkers to predict who will respond. The aim of this study was to assess the accuracy of exhaled breath analysis using electronic technology (eNose) for discriminating between responders to ICI and non-responders.

Methods

This proof of concept prospective observational study was part of an intervention study (INITIATE) in patients with recurrent MPM who were treated with nivolumab (anti-PD-1) plus ipilimumab (anti-CTLA-4). At baseline and after six weeks of treatment breath profiles were collected by an eNose. Modified Response Evaluation Criteria in Solid Tumors (RECIST) were used to assess efficacy at six months follow up. For data processing and statistics, we used independent t-test analyses followed by linear discriminant and receiver operating characteristic (ROC) analysis.

Results

Exhaled breath data of 31 MPM patients who received nivolumab plus ipilimumab were available at baseline. There were 16 with and 15 without a response after six months of treatment. At baseline breath profiles significantly differed between responders and non-responders, with a cross validation value of 71%. The ROC-AUC after internal cross-validation was 0.90 (CI: 0.80-1.00)

Conclusion

An eNose is able to discriminate at baseline between responders and non-responders to nivolumab plus ipilimumab in MPM, thereby potentially identifying a subgroup of patients that will benefit from ICI treatment.

Introduction

Malignant pleural mesothelioma (MPM) is a rare disease, mainly caused by exposure to asbestos, with a latency time of 30 to 50 years (1). Since 2004, the first-line treatment consists of a platinum compound plus pemetrexed with a median overall survival (OS) of 12-16 months. The addition of bevacizumab is reported to increase the OS to 18 months in a selected group of patients (2,3).

Immune checkpoint inhibitors (ICI), both as single agent and combination therapy, have shown promising anticancer activity against mesothelioma in single arm phase II clinical trials. For single agent anti-programmed cell death 1 antibody (anti-PD-1) ICI treatment, the overall response (ORR) is about 20% and progression free survival (PFS) between 2.5 and 6 months (4-7). For combination treatment with anti-PD-1 plus anti-CTLA-4, the ORR is around 27% and mPFS 6 months (7-9). The phase III PROMISE-meso trial, comparing the efficacy of pembrolizumab, an anti-PD-1 antibody, versus chemotherapy in recurrent mesothelioma shows that the ORR, is nearly four times higher with pembrolizumab (22% vs 6%). Unfortunately, median PFS, OS and duration of response (DOR) are similar for both treatment arms. However, long-term responders to pembrolizumab are observed.(10) Results from the recently presented phase III Checkmate 743 study show a significant OS benefit for first-line nivolumab (anti-PD-1 ICI) plus ipilimumab (anti-CTLA-4 ICI) compared to platinum plus pemetrexed chemotherapy (18.1 versus 14.1 months, HR 0.74 (95% CI 0.61–0.89; P=0.002). (11) These results are expected to change practice guidelines for mesothelioma.

As in other cancers, not all mesothelioma patients will benefit from ICI treatment.(12) Upfront identification of the subgroup that will benefit (or will not) could ultimately lead to improved outcomes. Unfortunately, relevant biomarkers have not been identified yet (13,14).

Over the last decades, exhaled breath analysis has shown potential as a non-invasive and easy-to-use technology for diagnosis and phenotyping of a wide range of diseases including mesothelioma and lung cancer (15-19). Exhaled breath consists of up to thousands of volatile organic compounds (VOCs) that are produced by both physiological and pathophysiological processes in the body and respiratory tract (20). Among the different available techniques, electronic nose (eNose) technology can be applied for pattern recognition of the complete mixture of VOCs using multiple cross-reactive sensors. Combined sensor signals produce a characteristic "breath profile" that is unique for each person (21,22). Recently de Vries *et al.* have shown that eNose technology allows for upfront discrimination between responders and non-responders to pembrolizumab or nivolumab in patients with advanced non-small cell lung cancer (NSCLC) with an accuracy as high as 90% (23). The results were confirmed in a separate validation set of patients, suggesting that this technology can be used upfront to predict the efficacy or failure of ICI therapy in these patients (23).

Therefore, in the current study, we aimed to assess as proof of concept whether the eNose was able to discriminate at baseline between mesothelioma patients with and without clinical response to anti-PD-1 plus anti-CTLA-4 therapy. Next, we explored the changes in breath profiles of responders and non-responders from baseline after 6 weeks of treatment with ICI.

Methods

Study design and population

This is a prospective observational study linked to a prospective single-center, single arm, phase II trial (the INITIATE trial) in patients with recurrent MPM who were eligible for treatment with nivolumab (anti-PD-1) plus ipilimumab (anti-CTLA-4). Details of the INITIATE trial have been published elsewhere.(8) In short, patients were treated with nivolumab 240mg every two weeks plus ipilimumab 1mg/kg every 6 weeks for a maximum of 4 times. In the INITIATE trial pulmonary function tests were performed at baseline and after six weeks of treatment. All patients provided written informed consent before enrolment in the INITIATE trial. Exclusion criteria for participating in the present study were the recent (<12hours) intake of alcohol (which affects eNose signals) or if patients were not willing or able to participate. In order to increase the applicability in clinical practice, there were no further restrictions. Patients completed a short survey about factors relevant for exhaled breath analysis, such as smoking history and food intake in the last two hours.

Definition of Treatment Response

Response to therapy was monitored by computed tomography (CT) scans performed every 6 weeks, using the modified Response Evaluation Criteria in Solid Tumors (mRECIST) for mesothelioma (24,25). The outcome of complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD) was recorded. Since our aim was to differentiate between responders and non-responders, we grouped patients with CR, PR and SD for more than 6 months as responders and patients with PD at 6 months as non-responders.

Measurements

Exhaled breath analysis was performed at baseline and after six weeks of treatment using a cloud-connected eNose, the so-called SpiroNose (23). This SpiroNose is an integration between eNose technology and routine spirometry and has been technically and clinically validated (23,26). It has 7 different cross-reactive metal-oxide semiconductor sensors. These sensors are present in duplicate on both the inside (to measure VOCs in exhaled breath) and on the outside of the SpiroNose (to measure VOCs in ambient air). During the measurement, patients were instructed to perform five tidal breaths followed by a single inspiratory capacity maneuver up to total lung capacity, a five second breath-hold and slow

(<0.4 L/s) maximal expiration towards residual volume. The exhaled breath measurement was performed in duplicate for each patient. The sensor signals were uploaded in real-time to the online analysis platform, BreathBase, for signal processing and analysis. From each sensor two variables were determined, 1) the highest sensor peak, normalized to the most stable sensor (sensor 2), to minimize inter-array differences; and 2) the ratio between the sensor peak and the breath hold (BH) point. A detailed description of the SpiroNose and the processing of data is available in the supplementary material.

Signal processing

The processing of the SpiroNose sensor signals included filtering, detrending, ambient correction and peak detection as was previously published (21,22). The signal processing resulted in a .csv file containing the selected parameters (sensor peak- and peak/BH ratios) serving as the source document for statistical analysis.

Statistical analysis

SPSS (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.) and MatLab (2019B, MathWorks, Natick, MA) were used for data analysis. Descriptive statistics were expressed as mean ±SD if data were normally distributed and as median (interquartile range) for non-normally distributed data. Between-group comparisons were carried out using Mann–Whitney U tests, two-sample unpaired t-tests or chi-squared tests.

Exhaled Breath Analysis

The normalized sensor peaks and peak/BH ratios were compared between groups using independent sample t-tests. The variables that discriminated ($P<0.05$) between responders and non-responders were selected for further analysis. Independent t-tests were internally validated by 1000 iterations of bootstrap. Subsequently, linear discriminant analysis was carried out using the selected variables. A discriminant function was calculated that best distinguished between the two groups. The accuracy of this model was defined as the percentage correctly classified patients. Cross-validation using the leave-one-out method was used to calculate the cross-validated accuracy value (CVV, %). The discriminant scores were used to construct receiver operating characteristics (ROC) curves. Finally, mean baseline and follow-up sensor values were compared using independent sample t-tests.

Results

Response to ICI treatment

In the INITIATE trial, 35 patients with MPM were included (8). ENose data were available for 31 (89%) patients and they were included in this observational study. From the other

4 patients we only have measurements after start of treatment (1; 3%), was no response evaluation available (1; 3%) or were not scheduled for measurements at all (2; 5%). Baseline characteristics of these patients are shown in table 1. As in most mesothelioma trials, the mean age was 65 years, most patients were male (74%) and the majority had epithelioid subtype (88%). After 6 months of treatment, 16 patients (52%) had a response (PR 39% plus SD 13%) and 15 patients (48%) were non-responders.

There were no significant differences between responders and non-responders regarding their demographic data and baseline characteristics.

Table 1: Baseline characteristics and radiological response data

	All	Responder	Non-responder	P value
N (%)	31	16 (52)	15 (48)	
Age, years (range)	65 (37-79)	67	63	0.25
Gender, n (%)				0.35
Male	23 (74)	13 (81)	10 (67)	
Female	8 (26)	3 (19)	5 (33)	
Ethnic background, n (%)				0.32
White	30 (97)	15 (94)	15 (100)	
Black	1 (3)	1 (6)		
WHO PS, n (%)				0.81
0	10 (32)	6 (37)	5 (33)	
1	21 (68)	10 (63)	10 (67)	
Smoking, n(%)				0.096
current smoker	5 (16)	1 (6)	4 (27)	
ex-smoker	14 (45)	10 (63)	4 (27)	
never smoker	12 (39)	5 (31)	7 (46)	
BMI ($kg.m^{-2}$) *	25.5	25.2	25.8	0.69
FEV1 (L) *	2.20	2.20	2.20	0.96
FEV1 (% predicted) *	71	74	67	0.34
Histologic subtype, n(%)				0.37
Epithelioid	27 (88)	13 (81)	14 (93)	
Sarcomatoid	2 (6)	2 (13)	0	
Mixed	2 (6)	1 (6)	1 (7)	
Line of treatment, n(%)				0.68
2	26 (84)	13 (81)	13 (87)	
>2	5 (16)	3 (19)	2 (13)	
Radiological response, n (%)				0.000
Complete response	0	0	0	
Partial response	12 (39)	12 (75)	0	
Stable disease	4 (13)	4 (25)	0	
Progressive disease	15 (48)	0	15 (100)	

WHO, world health organization; PS, performance status; BMI, body mass index; FEV1, forced expiratory volume in one second.
*data of one non-responder missing.

Exhaled breath analysis at baseline

Results of the independent t-test analysis showed that at baseline, sensor 3 (p=0.034), sensor 5 (p=0.04) and sensor 6_BH (p=0.017) were significantly different between patients with (n=16) and without response (n=15). Sensor 3 and 5 indicate the normalized sensor peak and sensor 6_BH the ratio between the highest sensor peak and the breath hold point. Linear discriminant analysis showed a cross-validated value of 71%. The ROC-Area Under the Curve (AUC) after internal cross-validation was 0.90 (95%CI: 0.80-1.00) (Figure 1).

A: **B:**

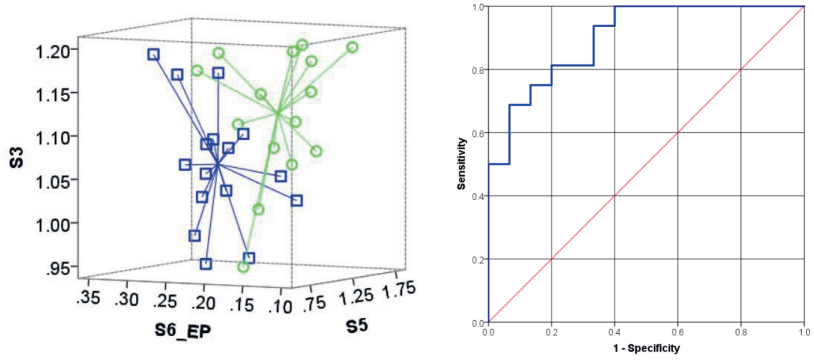

Figure 1. A: Three-dimensional scatter plot showing discrimination of exhaled breath profiles between responders (blue) and non-responders (green) along discriminative variables. The x and y axes represent normalized sensor values. B. ROC-curve showing sensitivity and specificity for the identification of non-responders (ROC-AUC: 0.90 (CI: 0.80-1.00))

Exhaled breath analysis after six weeks of treatment

In 25 patients (81%) of this cohort, follow-up exhaled breath measurements were performed after 6 weeks of treatment. In patients with a partial response (n=11), normalized sensor peak of sensor 3 and sensor 5 at follow-up were significantly (p<0.01) different from baseline measurements (Figure 2). In patients with progressive disease (n=10), a significant difference (p<0.01) between follow-up and baseline parameters was seen from sensor 3 and sensor 5 (Figure 3). In patients with a partial response, an increase in normalized sensor peak values was noted while in patients with progressive disease, a decrease in parameters was found (Figure 2 & 3). In patients with long-term stable disease, no significant changes in sensor values were seen during treatment. However, follow up measurements were only available for 4 patients (data not shown).

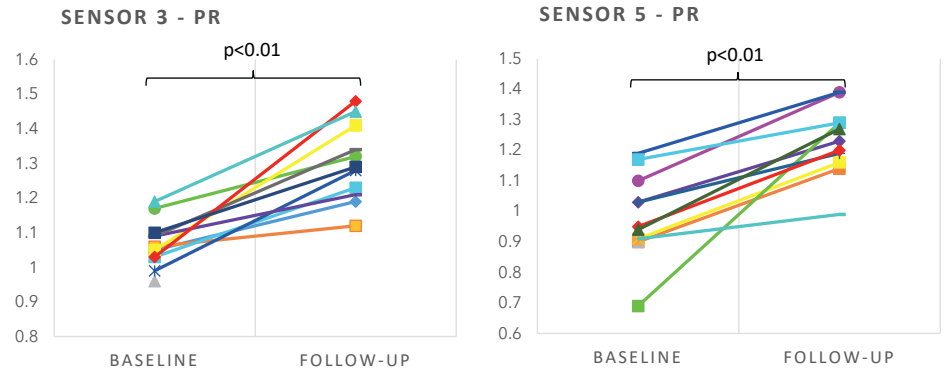

Figure 2. Change between baseline and follow up at week six in two significantly different sensors (sensor 3 and sensor 5) in patients with a partial response (PR). Y-axis in both figures correspond to the highest sensor peak normalized to the most stable sensor (sensor 2).

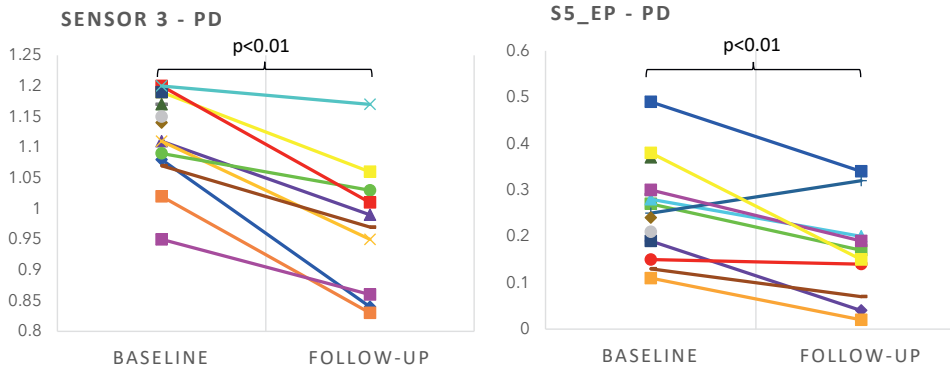

Figure 3. Change between baseline and follow up at week six in two significant different sensors (sensor 3 and sensor 5_BH) in non-responding patients. Left, Y-axis from sensor 3 corresponds to the highest sensor peak normalized to the most stable sensor (sensor 2). Right, Y-axis from sensor 5_BH corresponds to ratio of breath hold point and highest sensor peak.

Discussion

In this study, we showed that exhaled breath analysis by eNose at baseline allows for discrimination between mesothelioma patients with and without clinical response to nivolumab plus ipilimumab. The eNose could become a tool for prediction of response.

We also assessed within-patient changes in breath profiles during 6 weeks of treatment with ICI. We observed a significant change in sensor values from baseline both in those with partial response and progressive disease, though in opposite directions. Although some questions have to be addressed concerning the effect of changes in tumor size, and thereby tumor metabolites and/or changes in inflammatory response on VOCs,(27) this however suggests that the eNose may also be suitable as a monitoring tool to assess prognosis or effect of therapy of MPM. Especially since radiological response measurements are difficult and often inaccurate in MPM due to the unique nonradial pleural rind, eNose could discriminate between responders and non-responding patients.

Results from several clinical trials suggest that there is a subgroup of MPM patients that benefit from ICI therapy (7,8,10). Identifying those has been difficult, however, this is of utmost importance. Particularly, since in the near feature, many patients with MPM will be treated with nivolumab plus ipilimumab in first line (11). In NSCLC similar results for eNose analysis were reported in a cross-sectional study of 143 patients (training: 92, validation: 51) who received ICI therapy. De Vries *et al.* demonstrated that the eNose was able to prevent ineffective anti-PD-1 therapy in 24% of patients with NSCLC, without withholding anyone effective treatment. The study also showed that the eNose outperformed the currently used biomarker PD-L1 in NSCLC (90% vs 66% accuracy) (23). In the INITIATE study, PD-L1 expression at baseline on both tumor and immune cells correlated with response, but both proved insufficient for prediction of response (8).

To the best of our knowledge, this is the first trial to study the use of exhaled breath analysis by eNose to assess clinical responsiveness to anti-PD-1 plus anti-CTLA-4 therapy among patients with MPM. Most patients in the INITIATE trial were measured, and the patients included in this trial adequately represent the normal mesothelioma population since inclusion criteria and baseline characteristics are comparable to those in other MPM trials. Another strength is the eNose data are comparable to NSCLC. However, since nivolumab plus ipilimumab is not (yet) standard therapy for MPM, we could not include a higher number of patients, or validate our results in a separate cohort. Therefore, despite these encouraging results, the main limitation of the study is the lack of external validation. Since nivolumab plus ipilimumab will soon be standard of care in first line, these results can then be validated in an independent set of patients.

Both characteristics of the host and characteristics of the tumor microenvironment such as infiltration of lymphocytes, extracellular matrix, cytokine expression and tumor mutation burden, are known to have a significant influence on response to immunotherapy (28,29). Evidence shows high inter-patient and intra-tumor heterogeneity in the mesothelioma microenvironment, which can further complicate the prediction of response to anti-PD-1 therapy (30,31). Considering the complex and dynamic nature of the tumor

microenvironment, it is not surprising that a single marker such as PD-L1 is not able to provide sufficient information to predict response.

ENoses contain an array of cross-reactive sensors, each interacting with overlapping groups of VOCs without the identification of individual compounds (20). The technology applies pattern recognition algorithms and artificial intelligence for the discovery of multi-dimensional and composite biomarkers that are considered to be more informative than single markers (32). Thus, the high accuracies in these studies are not unexpected. Whether the associations between VOCs and treatment response are a direct effect of metabolite production by the tumor cells, or the immunological or inflammatory host responses remains to be determined (33,34). However, this does not influence the clinical utility of a breath test for the prediction of response to anti-PD-1 therapy.

Interestingly, in both MPM and NSCLC (23) normalized sensor peaks of sensors 3 and 5 were significantly different between responders and non-responders. This suggests that VOC compositions that differentiate between responders and non-responders may be similar in both diseases. This could reflect overlapping mechanisms within the tumor microenvironment or host that influence responsiveness to anti-PD-1 therapy (35). Sensor 3 has the highest sensitivity to hydrocarbons such as natural gas and methane. Di Gilio *et al.* have recently shown that 10 VOCs including hydrocarbons, ketones and alkanes can discriminate between MPM patients and healthy controls (36). Similarly, *in-vitro* studies also report hydrocarbons to differentiate between lung cancer and normal lung cell lines (33,37). Studies with analytical chemistry technologies such as Gas Chromatography-Mass Spectrometry (GC-MS) can provide more insight into individual VOCs involved in these processes (22). In order to unravel the underlying mechanisms, other high-throughput technologies like (epi)genomics, proteomics and transcriptomics may be more appropriate (38).

In conclusion, eNose technology has the potential to become a novel tool for predicting response to nivolumab plus ipilimumab among patients with MPM. In first line, many patients with mesothelioma will be treated with nivolumab plus ipilimumab in the near future (11), but this will not be effective for all patients. Therefore, eNose might be of importance to identify those patients who are at risk of failure or those who are candidates for continuation of treatment with ICI's when the CT scan is indiscriminative. Further validation of the results in a larger prospective multi-center study may lead to the use of eNose technology as a rapid and non-invasive tool at the point-of-care.

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Supplementary data

Exhaled breath analysis

SpiroNose measurement setup

The eNose measurement setup used in this study included a mouthpiece, nose clamp, viral/bacterial filter (Lemon Medical GmbH) attached to a Masterscreen™ pulmonary function testing system (Masterscreen, Jaeger, CareFusion) and the SpiroNose (Figure S1, left) (26,39). The SpiroNose consists of 8 separate sensor arrays, 4 reference sensor arrays to monitor environmental air and 4 sensor arrays used to monitor the VOCs in exhaled breath (Figure S1, right). The SpiroNose contains 7 different metal oxide semiconductor sensors (Table S1) and each sensor is present in duplicate in both the reference and breath-monitoring sensor arrays (in total 28 sensors). The sensor stability was verified, as previously described, using the standard test gas for pulmonary diffusion capacity measurements as quality control gas every morning before patient measurements (26,39).

Patients were not allowed to have used alcohol in the 12 hours before the breath test. All patients rinsed their mouth thoroughly 3 times with water. Patients were instructed to perform five tidal breaths followed by a single inspiratory capacity manoeuvre up to total lung capacity, a five second breath hold and slow (<0.4 L/S) maximal expiration towards residual volume (26,39). Exhaled breath was real-time measured (<1 minute) by the SpiroNose, which is connected to an Ethernet cable for immediate secured data transmission to an online server for further automated analysis. From each sensor two variables are determined, first the highest sensor peak, normalized to the most stable sensor (sensor 2), to minimize inter-array differences; and second the ratio between the sensor peak and the breath hold (BH) point (Figure S2).

The normalized sensor peaks and ratios are compared between groups by independent sample t-tests. The variables that discriminated ($p<0.05$) between responders and non-responders to ICI treatment were selected for further analysis. The t-tests were internally validated by 1000 iterations of bootstrap. Linear discriminant analysis was performed using the selected variables. A discriminant function was calculated that distinguished between patients with and without clinical benefit. This was used to construct receiver operating characteristics (ROC) curves.

Table S1 Sensors of the SpiroNose. ppm: parts per million, VOCs: volatile organic compounds

	Type	Highest sensitivity for:	Range (ppm)
Sensor 1	TGS 2602	VOCs (e.g. toluene) and odorous gases (e.g. ammonia and hydrogen sulphide)	1 - 30
Sensor 2	TGS 2610	butane and propane	500 - 10.000
Sensor 3	TGS 2611-COO	methane and natural gas	500 - 10.000
Sensor 4	TGS 2600	air contaminants (e.g. hydrogen, carbon monoxide and ethanol)	1 - 30
Sensor 5	TGS 2603	air contaminants (e.g. trimethylamine, methyl mercaptan)	1 - 30
Sensor 6	TGS 2620	alcohol and solvent vapors	50 - 5.000
Sensor 7	TGS 2612	methane, propane and iso-butane	500 - 10.000

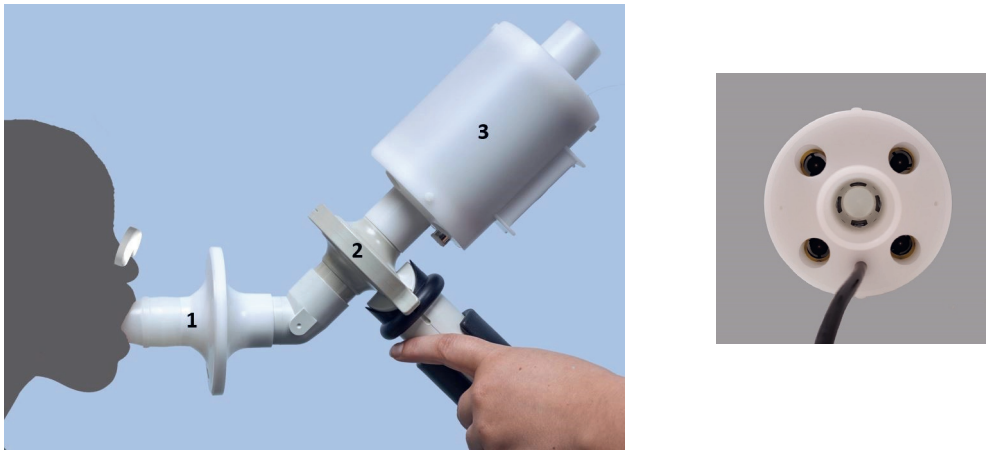

Figure S1. Left: SpiroNose measurement setup: (1) Mouthpiece, nose clamp and bacteria filter, (2) Spirometer, (3) SpiroNose. Right: Front view of the SpiroNose and the positioning of the sensor arrays. Yellow arrow: four sensor arrays monitoring exhaled breath. Red arrow: four reference sensor arrays monitoring ambient VOCs.

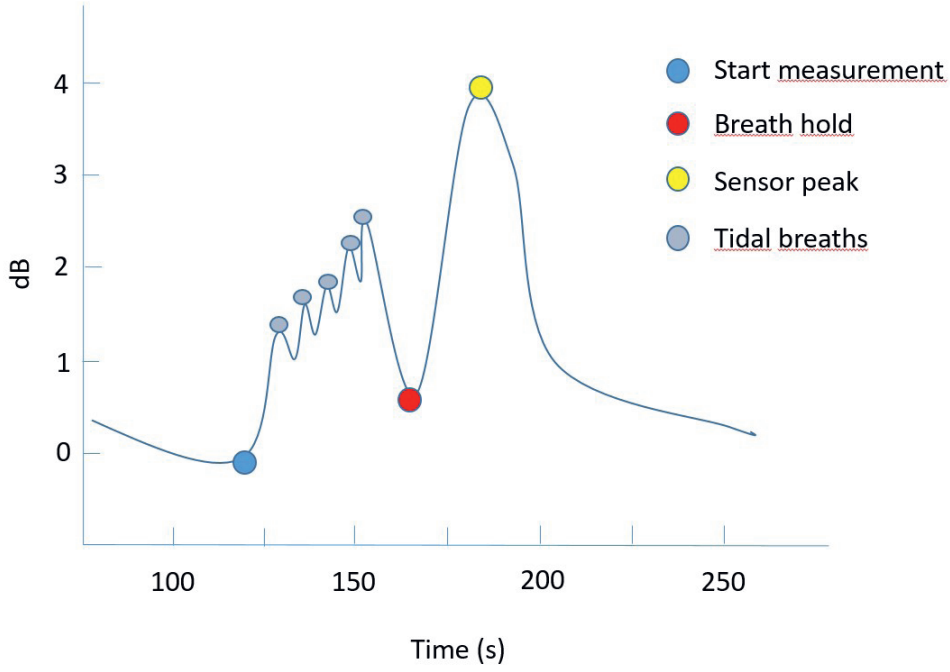

Figure S2. Data analysis SpiroNose.

8

Chapter 8

Immune cells in mesothelioma microenvironment simplistic marker of response to nivolumab plus ipilimumab?

Short communication

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Abstract

Introduction

Malignant pleural mesothelioma (MPM) is a malignant disease of the pleura which recently can be treated with immune checkpoint inhibitors (ICI). To optimize this treatment, a better understanding of the tumor micro environment is needed. We investigated subgroups of immune cells in subsequent tumor biopsies of patients treated with ICI.

Methods

Biopsies from MPM patients included in two clinical ICI trials (nivolumab alone and an ipilimumab/nivolumab combination) were examined. At baseline and after 6 weeks of treatment, pleural biopsies were taken to examine the tumor microenvironment (CD20+, CD4+, CD8+, FoxP3+ and PD-1+). Cell density was defined as the number of marker positive cells per mm^2. Radiological responses were evaluated as partial response, stable disease or progressive disease according to modified RECIST criteria.

Results

Thirty-four and 36 patients were included in the nivolumab and ipiliumumab/nivolumab trial respectively. In the nivolumab trial, no significant differences in cell densities were seen in baseline biopsies of patients with partial response versus progressive disease. In contrast, in the ipilimumab/nivolumab trial, a higher cell density of CD4+, CD8+, FoxP3+ and PD-1+ cells at baseline was significantly correlated with partial responses. On-treatment biopsies of both trials did not show significant changes when compared to baseline biopsies.

Conclusion

Biopsies from patients responding to nivolumab plus ipilimumab treatment show a significant higher cell density of CD4+, CD8+, FoxP3+ and PD-1+ cells, without a change after 6 weeks of treatment. This observation is a first step in exploring the tumor microenvironment as predictor of response in ICI treatment in MPM.

Introduction

Malignant pleural mesothelioma (MPM) is a rare malignant tumor arising from the mesothelial cells of the pleura. It is mainly caused by exposure to asbestos, with a latency time between exposure and diagnosis of 30 to 50 years.(1)

For decades, standard systemic treatment for MPM was combination chemotherapy consisting of platinum plus pemetrexed. But recently immune checkpoint inhibitor (ICI) treatment with nivolumab (anti-PD-1 antibody) and ipilimumab (anti-CTLA-4-antibody) was approved as first line therapy, following the results of the phase III Checkmate 743 trial. This study showed a survival benefit of combination ICI treatment over standard chemotherapy (18.1 versus 14.1 months, HR 0.74 (96.6% CI 0.60-0.91, p=0.002)).(2)

Unfortunately, ICI treatment is not effective in all patients and may lead to side effects. A better understanding of MPM and its microenvironment is needed to select the proper patients for ICI treatment. The tumor micro-environment (TME) plays an important role in the response to ICI therapy. The TME in MPM is composed of stromal, endothelial and immune cells and has a heterogenous distribution in the pleural cavity.

We investigated the possible impact of subgroups of immune cells in subsequent tumor biopsies of patients treated with ICI.

Materials and methods

Patients from the Nivomes (NCT02497508) (3) and Initiate (NCT03048474) (4) clinical trials were included in this analysis. In these two single center phase II trials, patients with recurrent MPM were treated with nivolumab monotherapy (Nivomes) or nivolumab plus ipilimumab (Initiate). In both trials pleural biopsies were taken at baseline and after 6 weeks of treatment and stored formalin-fixed paraffin-embedded (FFPE).

For nivolumab treated patients, two multiplex immunofluorescence panels were used. Panel 1 included antibodies against CD4, FOXP3, CD68, CD163, pancytokeratin (panCK) and DAPI to identify all nucleated cells. Panel 2 included antibodies against CD8, PD-1, CD20, panCK and DAPI. Macrophage markers (CD68 and CD163) of panel 1 could not be validated and evaluated. For the nivolumab plus ipilimumab trial, immunohistochemistry staining was performed for CD4, CD8, FoxP3 and PD-1.

The stained slides were annotated and analyzed using HALO software for counting and calculating the percentage of all nucleated cells. Cell density was defined as the number

of marker positive cells per mm2. Details about stainings and HALO software are provided in the supplementary methods.

In both trials, PD-L1 staining was performed. In the nivolumab trial, the PD-L1 expression on tumor cells (TCs) and tumor infiltrating immune cells (ICs) was assessed using the 28-8 antibody (EnVisio, Agilent Dako, Santa Clara, Ca). In the nivolumab plus ipilimumab trial, PD-L1 expression was assessed using the 22C3 antibody (pharmDx Agilent Technologies, Santa Clara, CA). In both trials, expression on TCs and ICs was scored as negative (<1% PD-L1 positive cells) or positive (≥1% PD-L1 positive cells) and as a percentage. Readers were blinded to patient outcomes.

Responses were monitored via computed tomography (CT) scans and evaluated according to modified Response Evaluation Criteria in Solid Tumors (mRECIST) for mesothelioma (5) and reported as partial response (PR), stable disease (SD) and progressive disease (PD). Responses were evaluated at 24 weeks. Patients were monitored every six weeks thereafter (every eight weeks after 24 weeks of treatment) to calculate the median progression free survival (mPFS) and median overall survival (mOS).

A Wilcoxon rank-sum test was used to test for response group similarity based on cell densities, where a rejection region of $p < 0.05$ was regarded significant. Multiple testing correction was performed on all P-values where applicable, using the Bonferroni correction.

Results

Thirty-four patients treated with nivolumab and thirty-six treated with nivolumab plus ipilimumab, were included. At the time of analysis, median follow-up time for the Nivomes trial was 58.6 months. The updated results show a mPFS of 2.6 months (95% CI: 2.2 – 5.5) and a mOS of 11.8 months (95% CI: 9.7 – 15.7). Median follow-up time for the Initiate patients was 46 months (95% CI 44.2 – 46.4 months). The updated results show a mPFS of 6.2 months (95% CI 4.2 – 11.0) and a median OS of 22.9 months (95% CI 12.6 – 32.6).

At baseline, pleural biopsies were obtained from all patients. After 6 weeks of treatment, 31 and 32 on-treatment biopsies were taken from respectively nivolumab and nivolumab plus ipilimumab treated patients. Not all on-treatment biopsies were evaluable: some only contained muscle tissue, others only fibrotic connective tissue or necrosis.

Baseline biopsies

At baseline, in the nivolumab alone group, no significant differences in cell densities of CD20+, CD4+, CD8+ and FoxP3+ were seen in biopsies of patients with partial response versus progressive disease. (suppl fig 1)

In contrast, in the nivolumab plus ipilimumab trial, a significant higher cell density of CD4+ (p=0.002), CD8+ (p=0.001), FoxP3+ (p=0.001) and PD-1+ (p=0.012) cells was observed in patients achieving a partial response compared to those with progressive disease. (fig 1)

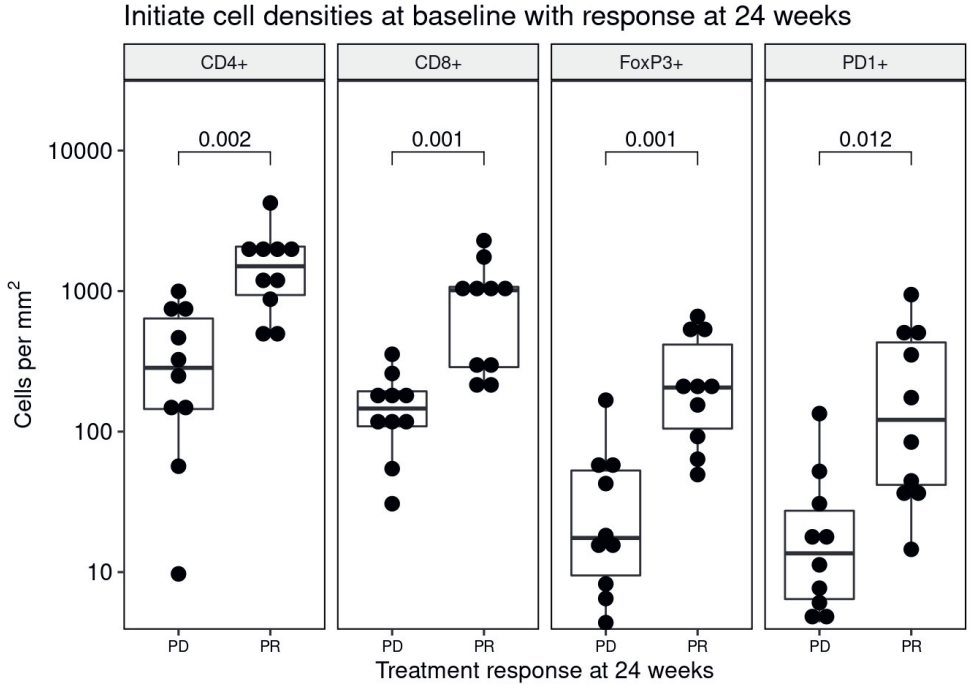

Figure 1. number of CD4+, CD8+, FoxP3+ and PD-1+ cells per mm2 at baseline in the nivolumab plus ipilimumab trial, comparing patients with progressive disease (PD) with partial response (PR) at 24 weeks.

On-treatment biopsies

Cell densities of CD20+, CD4+, CD8+ and FoxP3+ in the nivolumab trial showed no significant change nor difference after six weeks of treatment, not for all responses taken together, nor for partial response and progressive disease separately. On-treatment biopsies in nivolumab trial showed no difference between patients having PR or PD (data not shown).

In the nivolumab plus ipilimumab trial no significant change was seen in cell density of CD4+, CD8+, FoxP3+ and PD-1+ cells in patients having progressive disease or partial response (fig 2).

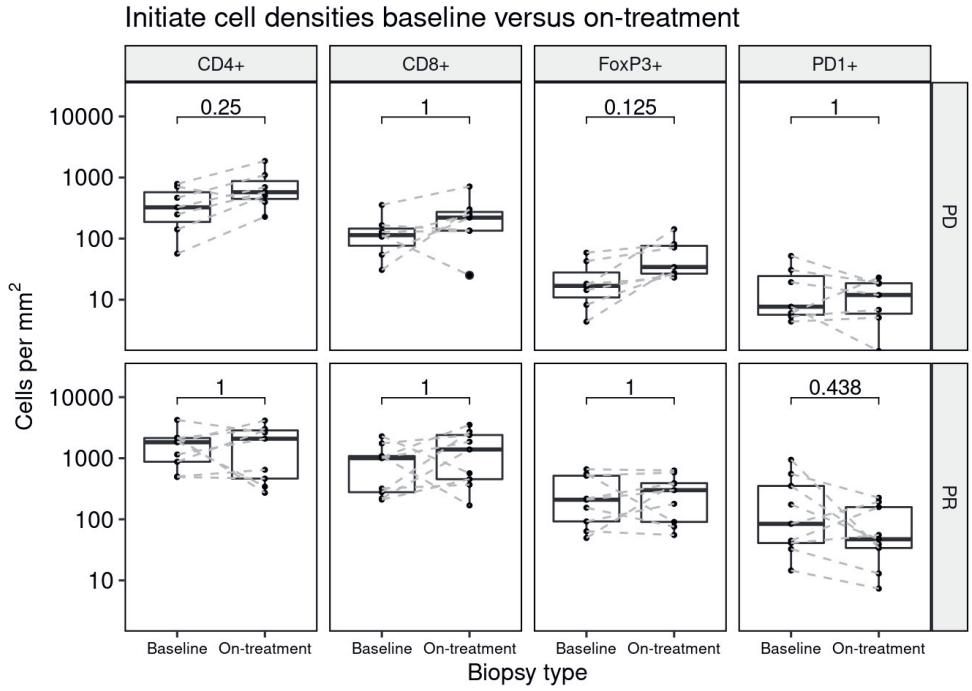

Figure 2. The number of CD4+, CD8+, FoxP3+ and PD-1+ positive cells per mm2 in the nivolumab plus ipilimumab trial, comparing baseline with 6 weeks on-treatment biopsies in patients with progressive disease (PD)(upper plots) and partial response (PR) (lower plots) at 24 weeks.

PD-L1 expression

PD-L1 expression on tumor cells or immune cells was not significantly correlated with PFS or OS (data not shown). Positive PD-L1 expression (≥1 %) on immune cells was correlated with a higher cell density of CD4+, CD8+ and FoxP3+ positive cells in both nivolumab as nivolumab plus ipilimumab group. (suppl Fig 2) This correlation was not observed when looking at PD-L1 expression on tumor cells.

Discussion

In the nivolumab plus ipilimumab study, biopsies of patients with a partial response have a higher cell density of CD4+, CD8+, FoxP3+ and PD-1+ cells, as compared to biopsies from patients having progressive disease. This is not seen in patients treated with nivolumab alone.

Immune cells in the TME can influence tumor growth and mediate response to therapy. In different tumor types it is shown that the density of tumor-infiltrating lymphocytes (TILs) is associated with response to anti-PD-1 treatment.(6) Not only cell density itself, but also the type of immune cells is important; for example, infiltration of CD8+ cytotoxic T cells is associated with higher ORR, longer disease free and overall survival in NSCLC.(7)

The TME in MPM is known to be highly immune suppressive, with the presence of a large amount of tumor associated macrophages, myeloid derived suppressor cells and regulatory T cells. Conflicting data on T cell subsets exists. Some studies in MPM suggest that higher levels of CD8+ T cells have a favourable prognostic impact while others found that higher levels are associated with a lower survival.(8,9) Higher levels of CD4+ and CD20+ cells and lower levels of FoxP3+ cells are linked to a better outcome, irrespective of therapy.(8,10) Until now, no prospective study has been performed with analysis of biopsies in MPM patients treated with ICI.

In our trial we hoped to identify changes in the TME but no significant change in immune cell subsets was observed after 6 weeks of treatment with nivolumab (plus or minus ipilimumab). Therefore, on- treatment biopsies of mesothelioma do not seem to add information on prediction of effect of ICI treatment, in contrast to melanoma, where adaptive immune signatures in early treatment biopsies are predictive of response to ICIs. (11)

In this study we focused on the extreme responses, progressive disease and partial response, to find a signal in studies with a relatively low number of patients. We deliberately excluded patients with stable disease since response analysis in patients with MPM is notoriously difficult. Mesothelioma spreads around the pleura in a circular way making treatment response difficult to determine with unidimensional measurements via modified RECIST criteria.

In our Initiate trial, PD-L1 expression on tumor cells and immune cells was predictive of response to nivolumab plus ipilimumab (4), but did not correlate with PFS or OS. In larger phase III trials, PD-L1 expression on tumor cells was not predictive of response to ICI treatment in MPM.(2,12) Expression on immune cells was, however, not reported. We demonstrated that positive PD-L1 expression (≥1 %) on immune cells but not on tumor cells is correlated with a higher cell density of CD4+, CD8+ and FoxP3+ positive cells in both the nivolumab and in the nivolumab plus ipilimumab group, pointing to a more inflamed environment. Which of the immune-cells co-expressed PD-L1 is not known from our studies.

The prognostic or predictive value of TIL infiltration or specific T cell subsets alone may be a too simple reflection of reality; integrating expression of proliferation markers, inhibitory receptors, cytokines, sequencing or gene expression data is needed to provide more detailed information on the TME and effect of ICI treatment.

Limitations of this study may be the sample size and limited number of representative on-treatment biopsies. In some patients having a partial response, it was not possible to take a biopsy anymore, or only necrosis was found. Also the timing of the biopsy after 6 weeks of treatment could have influenced the effect. In peripheral blood of lung cancer patients, changes in CD8 subsets are already seen within 4 weeks of PD-1 treatment.(13)

Although comparable patient groups were included in both ICI trials, they were not designed to be compared with each other. Besides that, different staining techniques were used for the biopsies. The immunofluorescence technique in the nivolumab trial was performed many years ago and was hard to validate, and not all markers (that is CD68 and CD163) could be used. Therefore, for the successive nivolumab plus ipilimumab trial, immunohistochemistry was used. This makes it difficult to compare both trials.

Based on recent publications, it would be interesting to focus on the non-epithelioid subgroup, since that has a different micro-environment (9) and a larger benefit of ICI treatment compared to the epithelioid subgroup.(2) Brockwell found high proportions of T lymphocytes and CD45RO+ cells in sarcomatoid MPM having prolonged progression free and overall survival to ICI treatment.(14) In our study there were not enough biopsies available to draw any conclusions on the subgroup of non-epithelioid MPM.

In conclusion, biopsies from patients responding to nivolumab plus ipilimumab treatment show a significant higher cell density of CD4+, CD8+, FoxP3+ and PD-1+ cells at baseline, but no specific changes after 6 weeks of treatment. This observation is a first step in exploring the TME as a predictor of response to guide ICI treatment in MPM. Larger studies are needed, with more detailed analyses of the TME.

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Supplementary data

Supplementary methods

Prior to multiplex staining 3µm slides were cut on DAKO Flex IHC slides. Slides were then dried overnight and stored in +4°C. Before a run was started slides were baked for 30 minutes at 70°C in an oven.

Staining was performed on a Ventana Discovery Ultra automated stainer, using the Opal 7-Color Manual IHC Kit (50 slides kit, Perkin Elmer, cat NEL81101KT). Protocol starts with baking for 28 minutes at 75°C, followed by dewaxing with Discovery Wash using the standard setting of 3 cycles of 8 minutes at 69°C. Pretreatment was performed with Discovery CC1 buffer for 32 minutes at 95°C, after which Discovery Inhibitor was applied for 8 minutes to block endogenous peroxidase activity. Specific markers were detected consecutively on the same slide with the following antibodies, Anti-CD68 (Clone KP1, Cat M0814, Dako, 1/500 dilution 1 hour at RT), Anti-CD8 (Clone C8/144B, Cat M7103, Dako, 1/250 dilution, 1 hour at RT), anti-FoxP3 (Clone 236A/47, Cat AB20034, AbCam, 1/50 dilution, 2 hours at RT), Anti-CD163 (Clone 10D6, Cat NCL-CD163, Leica, 1/500 dilution, 1 hour at RT), Anti-CD4 (Clone SP35, Cat 104R-15, Cell Marque, 1/50 dilution, 2 hours at RT), Anti-PanCytoKeratin (Clone AE1AE3, Cat MS-343-P, Thermo Scientific, 1/1000 dilution, 1 hour at RT), Anti-CD3 (Clone SP7, Cat M3074, Spring Bioscience, 1/400 dilution 1 hour at RT), Anti-PD1 (Clone NAT105, Abcam, 1/100 dilution 1 hour at RT), Anti-CD20 (L26, DAKO, 1/1600 dilution 32 minutes at RT), Anti-PDL1 (Clone E1L3N, Cell signaling Technologies, 1 hour at RT). Each staining cycle was composed of four steps: Primary Antibody incubation, Opal polymer HRP Ms+Rb secondary antibody incubated for 32 minutes at RT, OPAL dye incubation (OPAL520, OPAL540, OPAL570, OPAL620, OPAL650, OPAL690, 1/50 or 1/75 dilution as appropriate for 32 minutes at RT) and an antibody denaturation step using CC2 buffer for 20minutes at 95°C. Cycles were repeated for each new antibody to be stained. At the end of the protocol slides were incubated with DAPI (1/25 dilution in Reaction Buffer) for 12 minutes.

After the run was finished slides were washed with demi water and mounted with Fluoromount-G (SouthernBiotech, cat 0100-01) mounting medium.

For the Initiate trial, immunohistochemistry of the FFPE tumor samples was performed on a BenchMark Ultra autostainer or Discovery Ultra autostainer (CD3-CD56 double staining). Briefly, paraffin sections were cut at 3 um, heated at 75°C for 28 minutes and deparaffinised in the instrument with EZ prep solution (Ventana Medical Systems). Heat-induced antigen retrieval was carried out using Cell Conditioning 1 (CC1, Ventana Medical Systems) for 32 minutes at 950C (CD4, CD8,PD1) or 64 minutes at 950C (FOXP3). CD4 was detected using clone SP35 (1/25 dilution, 32 minutes at 370C, Cell Marque), CD8 clone C8/144B (DAKO /

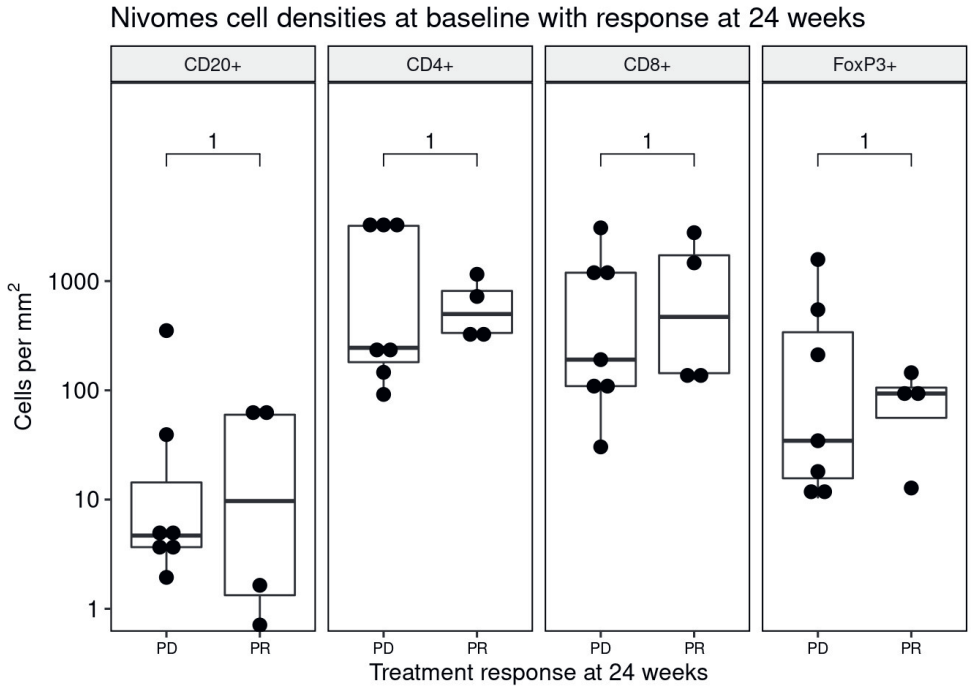
Agilent) using 1/200 dilution 32 minutes at 370C, FOXP3 using clone 236A/E7 (Abcam) at 1/200 dilution for 2 hours at RT, PD1 clone NAT105 (1/1600 dilution, 32 minutes at 370C, Abcam). Bound antibody was detected using the OptiView DAB Detection Kit (Ventana Medical Systems). Slides were counterstained with Hematoxylin and Bluing Reagent (Ventana Medical Systems).

The stained slides were annotated and analyzed using HALO software (V3.0.311.346, Indica Labs). A pathologist marked the regions of interest (ROI), consisting of (residual) tumor area and immune cells. To prevent variations in size and annotated area, consecutive slides were superimposed using the image registration tool with synchronized navigation. ROI were annotated using the brush and flood annotation tools. The Indica Labs Multiplex IHC v2.0.3 analysis algorithm was used as a template, with adjusted settings mentioned in suppl. table 1. All annotation layers were analyzed and both the summary data and object data were exported in comma separated value files using the export manager in HALO.

S1 Algorithm settings for analysis on DAB-stained slides. Indica Labs Multiplex IHC v2.0.3 was used as template, with the following adjustments.

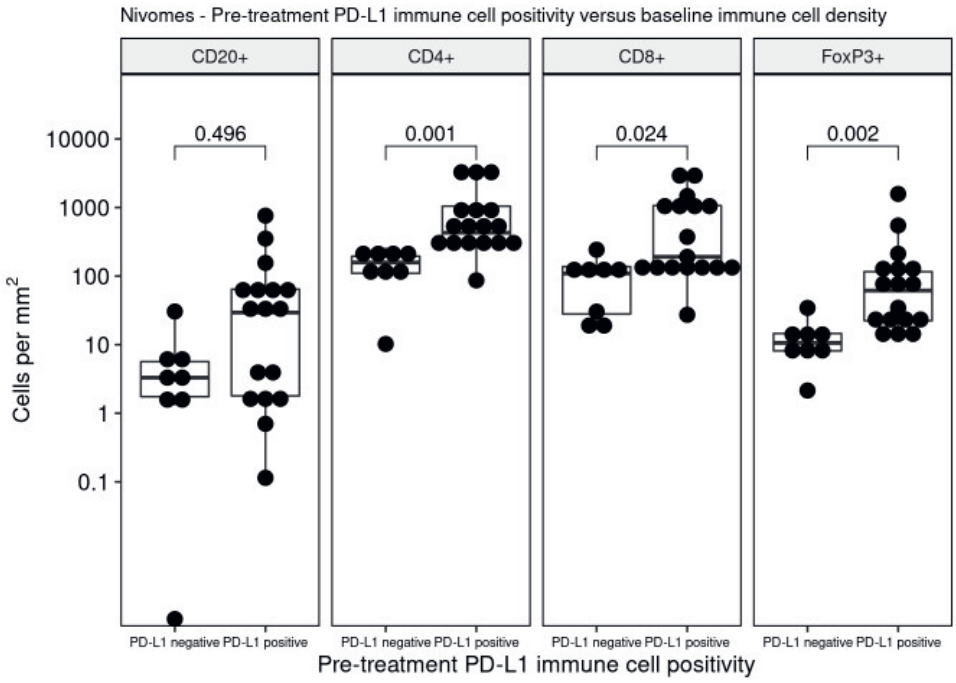
Analysis Magnification	**1**
Hematoxylin Nuclear detection weight	1.1
DAB Nuclear detection weight	1.5
Nuclear contract threshold	0.52
Minimum nuclear optical density	0.347
Nuclear size	10,118.93
Nuclear segmentation aggressiveness	0.6
Fill nuclear holes	False
Hematoxylin Markup color	124,137,180
Hematoxylin nucleus positive threshold	0.15
DAB markup color	62,39,35
DAB nucleus positive threshold	0.185
Minimum tissue OD	0.037

Supplementary figures

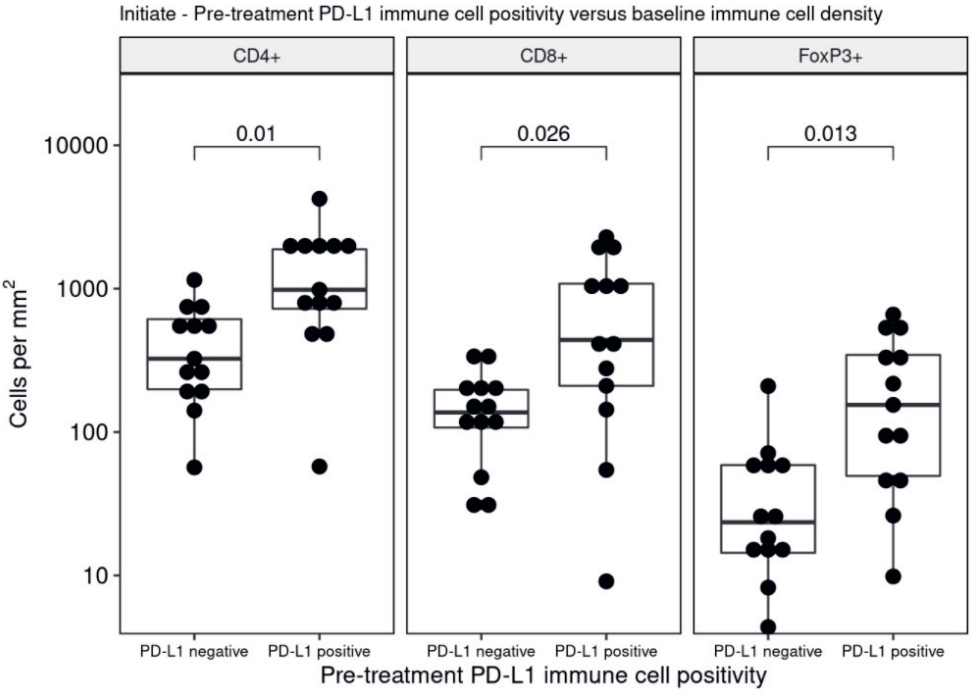


Supplementary figure 1. number of CD20+, CD4+, CD8+ and FoxP3+ cells per mm2 at baseline in nivolumab trial, comparing patients with progressive disease (PD) with partial response (PR) at 24 weeks.

A


Nivomes - Pre-treatment PD-L1 immune cell positivity versus baseline immune cell density
CD20+
CD4+
CD8+
FoxP3+
0.496
0.001
0.024
0.002
10000
1000
100
10
1
0.1
Cells per mm2
PD-L1 negative PD-L1 positive
Pre-treatment PD-L1 immune cell positivity

B


Initiate - Pre-treatment PD-L1 immune cell positivity versus baseline immune cell density
CD4+
CD8+
FoxP3+
0.01
0.026
0.013
10000
1000
100
10
Cells per mm2
PD-L1 negative
PD-L1 positive
Pre-treatment PD-L1 immune cell positivity

Supplementary figure 2: number of CD4+, CD8+ and FoxP3+ cells per mm2 in PD-L1 positive versus PD-L1 negative immune cells at baseline in nivolumab (A) and nivolumab plus ipilimumab (B) trial.

9

Chapter 9

Tumor junction burden and antigen presentation as predictors of survival in mesothelioma treated with immune checkpoint inhibitors

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Abstract

Introduction

The favorable outcomes with immunotherapy for mesothelioma were somewhat unexpected since this tumor has a low tumor mutation burden which has been associated with benefit in other cancers. Since chromosomal rearrangements are common in mesothelioma and have neoantigenic potential, we sought to determine whether they are associated with survival in patients treated with immunotherapy.

Methods

Pleural biopsies of mesothelioma after at least one line of therapy were obtained from patients (n=44) prior to treatment with nivolumab alone (NCT29908324) or in combination with ipilimumab (NCT30660511). RNA and whole genome sequencing were performed to identify the junctions resulting from chromosomal rearrangements, and antigen processing and presentation gene set expression. Associations with overall survival were estimated using cox models. An overall survival cutoff of 1.5 years was used to distinguish patients with and without durable benefit for use in receiving operating characteristic (ROC) curves.

Results

While tumor junction burdens were not predictive of overall survival, we identified significant interactions between the junction burdens and multiple antigen processing and presentation gene sets. The "regulation of antigen processing and presentation of peptide antigen" gene set demonstrated an interaction with tumor junction burden and was predictive of overall survival. This interaction also predicted 1.5-year or greater survival with an area under the ROC of 0.83. This interaction was not predictive of survival in a separate cohort of patients with mesothelioma who did not receive immune checkpoint inhibitors.

Conclusions

Analysis of structural variants and antigen presentation gene set expression may facilitate patient selection for immune checkpoint inhibitors.

Introduction

Given the mixed results observed with immune checkpoint inhibitors for the treatment of mesothelioma, it is more important than ever to identify biomarkers that may predict outcomes and guide the use of these therapies. Unlike other tumor types with high tumor mutations burdens where clear survival benefits have been demonstrated with immune checkpoint inhibitors, mesothelioma has a very low mutation burden. Mesothelioma primarily arises as a result of the exposure to the carcinogen asbestos, although some cases develop after therapeutic radiation, or are inherited due to loss of function mutations in BRCA1 Associated Protein 1 (*BAP1*) (1). Recent studies reported very low tumor mutation burdens (TMB) using next-generation sequencing (NGS) to evaluate mesothelioma (2,3). This finding was unexpected because other tumors associated with carcinogenic exposures such as malignant melanoma, small cell and non-small cell lung cancer typically have a high TMB from ultraviolet radiation and tobacco exposure, respectively (4). High TMBs are thought to be a surrogate for an increase in neoantigens that can be recognized by the adaptive immune system and facilitate tumor elimination. Despite the reportedly low TMB in mesothelioma, the combination of the PD-1 inhibitor nivolumab and the CTLA-4 inhibitor ipilimumab was shown to be superior to treatment with cisplatin and pemetrexed chemotherapy in patients with unresectable mesothelioma, and is now approved by the United States Food and Drug Administration for frontline use (5).

Current clinically available NGS approaches do not fully characterize the genomic complexity of tumors. Cytogenetic studies have identified recurrent, structural chromosomal abnormalities in mesothelioma (6,7), yet these events are not commonly reported in more recent NGS studies (2,3). For this reason, in prior work, we used a sequencing approach that tiles the whole genome with large DNA fragments (2-5 kb compared to standard 200-500 bp) to improve the detection of structural variants such as insertions, deletions and translocations. Chromosomal rearrangements disrupt gene regions generating truncations or fusion transcripts reading into normally distal gene regions or noncoding DNA. We previously found multiple chromosomal rearrangements that resulted in discordant DNA junctions with the potential for novel fusions in mesothelioma (8). Many of these events fit a pattern of chromoanagenesis such as chromothripsis or chromoplexy (9). Since structural abnormalities like insertions, deletions, and chromosomal translocations have neoantigenic potential (8,10,11), we sought to determine their role in predicting outcomes in patients with mesothelioma treated with immune checkpoint inhibitors.

Materials and methods

Patients and specimens: Biopsies were obtained from patients just prior to treatment with nivolumab (NCT02497508) (12) or nivolumab with ipilimumab (NCT03048474) (13), after previous treatment with platinum-based chemotherapy. DNA and RNA were purified using the AllPrep DNA/RNA/miRNA Universal kit (Qiagen, #80224) following the instructions provided by the manufacturer. The buffer included β-mercaptoethanol for the specimens obtained from NCT02497508, and dithiothreitol for the ones obtained from NCT03048474. Otherwise, there were no differences in the handling of the specimens or nucleic acid purification. The clinical trials and translational studies were approved by the local institutional ethics committees. Characteristics of the patients included in our analysis were compared to those of patients who were excluded due to insufficient materials using the Fisher's exact test for categorical variables and the Mann Whitney U test for continuous variables. Survival between these groups was compared using the R packages "survival" and "survminer".

Determination of tumor junction burdens: Chromosomal rearrangements were reported by sequencing DNA prepared according to the mate-pair whole-genome library protocol (Nextera Library Prep Protocol). Sequencing results were mapped by BIMA, and the junctions of the chromosomal rearrangements were called by SVAtools. BIMA and SVAtools are Mayo Clinic in-house informatic pipelines (14,15). The junctions of the chromosomal rearrangements were annotated with 1) the position of the junction with a resolution of 200-500bp, 2) direction of the chromosomal rearrangement and 3) genes at the junction using NCBI RefSeq genes for GRCh38. The number of chromosomal rearrangements per sample was assessed by counting the number of unique genes hit by all junctions in the sample. All specimens had 60X or greater bridged coverage for the detection of junctions, except one which had 40X bridged coverage. Chromosomal rearrangements may refer to insertions, deletions, translocations, and inversions. Junctions are the locations of the breaks of these chromosomal rearrangements. There may be one junction (deletion, insertion, translocation), two junctions (inversion, balanced translocations) or multiple junctions (three-way, four-way etc. translocation) involved with each chromosomal rearrangement.

RNA-seq analyses: Mapping of the RNA-seq data and estimations of gene expression counts in each sample were performed by MAP-RSeq pipeline developed previously by the Mayo Bioinformatics Core (16). Raw "count" files were processed by the "edgeR" package to generate log 2 normalized gene expression values.

Antigen processing and presentation (APP): The Biological Processes Gene Ontology dataset in the Molecular Signature database was searched for gene-sets with names

that included "antigen" and "presentation." Of the 21 found hits, nine were eliminated for processes involving lipid, polysaccharide, exogenous antigens or processes representing dendritic cell or T-cell antigen processing and presentation. Single sample enrichment scores in the remaining 12 gene-sets were calculated by using the "ssGSEA" (single sample gene set enrichment analysis) algorithm in the "GSVA" package.

Survival and immune checkpoint inhibitor survival analyses: A statistical interaction is present when the association between two variables depends on a third variable. In our case, we hypothesized that the associations between tumor junction burden and survival (in terms of either hazard or odds ratio in cox or logistic regression models, respectively) depended on the APP capabilities of tumors. Therefore, we tested the statistical significance of APP and tumor junction burden interactions in predicting OS or $S_{1.5yr}$. Associations of interactions between gene-sets and log2 transformed junction burden (APP * log2[junction burden]) with overall survival (OS) were found by using the "coxph" (cox proportional hazard) program in the "survival" package. Associations of these interactions with response to immune checkpoint inhibitors in terms of survivals at 1.5-year ($S_{1.5yr}$) were calculated by logistic regression (LR) using the "glm" (generalized linear model) package. APP and junction burden interactions were considered significant when either or both of the following conditions were met: (i) log-rank p-values and the interaction terms in the OS models were significant ($p < 0.05$), or (ii) the interaction terms in LR analysis was significant and the LR model had an accuracy based on area under the curve (AUC) greater than 0.7. To create the Kaplan Meir plot representing an individual gene-set interaction with junction burden, samples were categorized as either "High" or "Low" by using the median multiplication product of gene-set scores and log2[junction burden] as the threshold. Reported p-values in the plot are associations of the interaction and the model (log rank test) with overall survival by "coxph" program.

Forest plots: Median enrichment scores in each of the APP gene-sets were used to group samples into high and low APP categories. In each category, hazard ratios representing associations between junction burdens and overall survival were calculated by "coxph" and plotted using the "forestplot" package.

Examination of existing models: Immunotherapy response models described elsewhere (17) were examined for predicting significant benefit (SB) and no significant benefit (NSB). Log2 transformed gene expression data were normalized in each row by subtracting average values across all samples according to the authors instructions. Normalized expression values were input to the python program "tidepy" to estimate individual tumor scores in 14 models. Logistic regression analyses were then used to estimate the accuracy of models with the CD8 model having been found as the best performer. Finally, "pROC" program was used to plot the ROC curves for TIDE, IFNG, PD-L1, and CD8 models.

Immune deconvolution: The immunedeconv package in R was used to assess the tumor microenvironment. immunedeconv contains six approaches (quantiseq, timer, cibersort_abs (and first generation cibersort), mcp_counter, xCell, and epic) to estimate the abundance scores of multiple cell types, including adaptive and innate immune cells, based on ssGSEA data. Statistical significance of differential cell type enrichment between cohorts of patients with high or low "REGULATION OF APP OF PEPTIDE ANTIGEN" gene set expression was compared the t test.

Results

Sixty-eight patients with pleural mesothelioma were treated with the PD-1 inhibitor nivolumab alone or in combination with the CTLA-4 inhibitor ipilimumab on the NivoMes (n=34) and INITIATE (n=34) clinical trials, respectively (12,14) (Supplementary Table 1). These patients had received at least one prior line of platinum-containing therapy. Biopsies were obtained on 65 of these patients just prior to the start of treatment with an immune checkpoint inhibitor(s), and 44 of these specimens had sufficient DNA and RNA content for analysis. There were no significant differences between the characteristics of the patients included in this analysis and those excluded based on sample insufficiency including sex, trial treatment, performance status, line of therapy, age, or overall survival. Despite the historic median survivals of less than six months with second or later line therapy in mesothelioma,(18) there was a separation in overall survival at 1.5 years ($S_{1.5yr}$) from start of treatment on trial which we selected to group patients into categories of significant benefit (SB, > $S_{1.5yr}$) and no significant benefit (NSB, ≤ $S_{1.5yr}$)(Figure 1A).

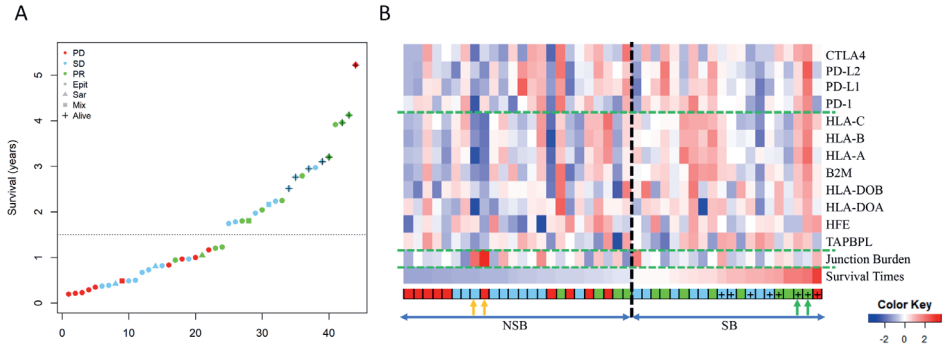

Figure 1: (A) Survival times of the study cohort. Red, blue, and green represent the best responses of progression of disease (PD), stable disease (SD), and partial response (PR), respectively. Circles, triangles, and squares represent epithelioid (Epit), sarcomatoid (Sar, including mesenchymal), and mixed (Mix) histology, respectively. "+" designates alive at the last follow-up. (B) Heatmap representing survival times, junction burden, antigen processing and presentation, and immune

checkpoint markers. The lower bar represents best responses with PD, SD, and PR as per Figure 1A. Orange arrows point to two cases with high junction burdens, short survival times, and low expression in genes involving antigen processing and presentation (APP). On the contrary, green arrows point to two cases with moderate junction burdens, long survival times, and robust APP expression.

There were no differences in overall survival between those who receive nivolumab with or without ipilimumab (Supplementary Figure 1). The biopsies obtained just prior to treatment were analyzed by mate-pair DNA sequencing and RNA-seq. There were many chromosomal rearrangements in each specimen (median 130 junctions, range 23-348), and a fraction of these involved unique genes (median 18, range 1-68). We selected the chromosomal rearrangements involving unique genes in each tumor for our analysis given their potential to be expressed and refer to them as the tumor junction burden from hereon.

Given our prior findings of the neoantigenic potential of chromosomal rearrangements, we sought to determine whether tumor junction burdens were associated with survival in patients with mesothelioma treated with immune checkpoint inhibitors. We did not find an association between tumor junction burden and overall survival (Cox model log rank $p > 0.5$)(Supplementary Figure 2A). Notably, two patients with the highest tumor junction burdens had very short survival times, whereas two other patients with moderate tumor junction burdens had a durable survival benefit (Figure 1B). The two patients with the highest tumor junction burdens and poor survival had low expression of genes involved in antigen processing and presentation (APP). On the other hand, patients with moderate tumor junction burdens and more durable survival had very robust expression of APP associated genes.

	P-IA-cox	*p-Log Rank*	*P-IA-lr*	*AUC*
REGULATION_OF_AP&P_OF_PEPTIDE_ANTIGEN	**0.0026**	**0.0031**	**0.0221**	**0.831**
AP&P_OF_ENDOGENOUS_PEPTIDE_ANTIGEN	**0.021**	**0.041**	**0.040**	**0.724**
AP&P_OF_PEPTIDE_ANTIGEN_VIA_MHC_CLASS_IB	**0.048**	**0.019**	**0.071**	**0.759**
AP&P_OF_ENDOGENOUS_PEPTIDE_ANTIGEN_VIA_MHC_CLASS_I_VIA_ER_PATHWAY	**0.049**	**0.010**	**0.072**	**0.811**
AP&P_OF_ENDOGENOUS_ANTIGEN	0.061	0.079	0.025	0.748
AP&P_VIA_MHC_CLASS_IB	0.154	0.045	0.023	0.800
NEGATIVE_REGULATION_OF_AP&P	0.034	0.145	0.072	0.702
REGULATION_OF_AP&P	0.065	0.253	0.079	0.697
AP&P_OF_PEPTIDE_ANTIGEN_VIA_MHC_CLASS_I	0.080	0.318	0.240	0.610
POSITIVE_REGULATION_OF_AP&P	0.197	0.382	0.100	0.660
AP&P	0.201	0.597	0.289	0.542
AP&P_OF_PEPTIDE_ANTIGEN	0.242	0.673	0.519	0.559

Table 1 legend. The statistical significance of interactions (P-IA-cox) and log-rank (p-Log Rank) in cox models, interactions (P-IA-lr) and area under the curve (AUC) in logistic regression models are listed. The gene sets with significant interactions are in bold. APP and junction burden interactions were considered significant when either or both of the following conditions were met: (i) log-rank p-values and the interaction terms in the OS models were significant ($p < 0.05$), or (ii) the interaction terms in LR analysis was significant and the LR model had an accuracy based on area under the curve (AUC) greater than 0.7.

Since the impact of tumor junction burdens appeared to be modulated by APP, we hypothesized that the neoantigenic potential of chromosomal rearrangements was dependent upon the capability of cancer cells to present neo-antigens to the immune system. To examine whether there was an interaction between APP gene sets and tumor junction burdens that impacted outcomes, we selected 12 APP gene sets from the Gene Ontology - Biological Processes dataset in the Molecular Signature Database and calculated their enrichment scores (Supplementary Table 2). We then used these scores to test for interactions between APP gene sets and junction burdens on survival and found significant interactions with six APP gene sets (Table 1). With these six APP gene sets, the hazard ratios representing associations between tumor junction burdens and overall survival favored patients with high APP scores (all hazard ratios <1) more so than patients with low APP scores (all hazard ratios >1)(Figure 2). There were no differences in survival between patients with high or low APP scores (Supplementary Figure 2B). In patients with low APP scores, those with a high tumor junction burden were at increased risk of death compared with patients with low tumor junction burdens (Supplementary Figure 2C). On the other hand, in patients with high APP scores, those with high tumor junction burdens were at reduced risk of death compared with patients with low tumor junction burdens (Supplementary Figure 2D).

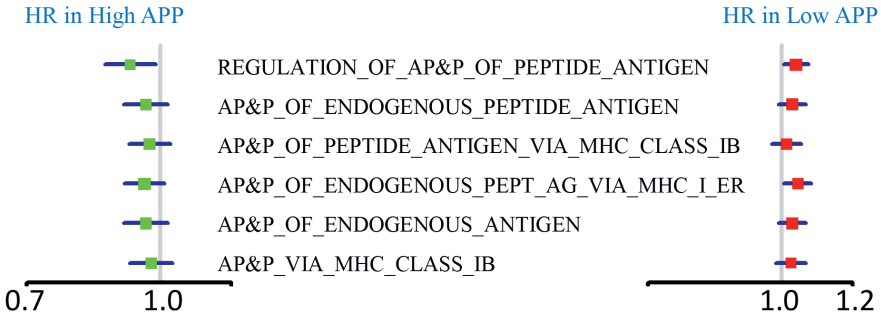

Figure 2: Forest plots displaying the hazard ratios for junction burdens and overall survival associations in samples with high and low APP gene set expression, respectively in gene sets identified as significant.

We further examined the interaction models that included the "REGULATION OF APP OF PEPTIDE ANTIGEN" gene set which included 6 genes (PYCARD, HFE, HLA-DOA, HLA-DOB, TREM2, and TAPBPL). Both the interaction parameter between this gene set and the tumor junction burden, and the survival model were highly significant (Table 1 and Figure 3A). Furthermore, this interaction was highly predictive of $S_{1.5yr}$ with an AUC of 0.831 (Figure 3B). For comparison, we tested several available gene models previously reported to associate with response to immune checkpoint inhibitors including TIDE, IFNG, PD-L1, CD8, and others.(17) In our cohort, none of these other models performed as well as the interaction of APP gene sets with tumor junction burdens in predicting $S_{1.5yr}$, but the most accurate of these gene models was the CD8 model with an AUC of 0.683 (Figure 3C). Based on this observation, and to account for the role of antitumor lymphocytes in survival with immune checkpoint inhibition, we included CD8A in our prediction model. This addition increased the accuracy of the model from 0.831 to 0.890 (Figure 3D).

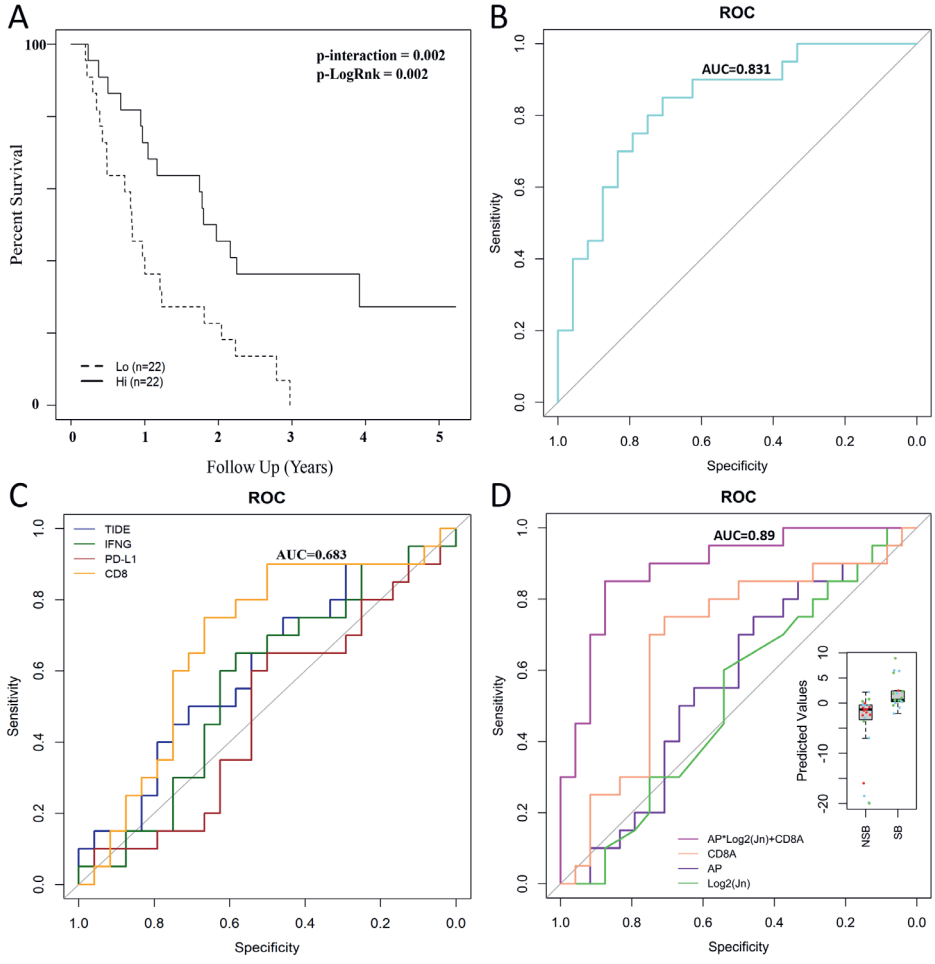

Figure 3: (A) The Kaplan Meier curve representing a survival model based on the interactions between "REGULATION OF APP OF PEPTIDE ANTIGEN" gene-set and junction burdens is shown. Both the interaction terms and the log-rank test were significant. (B) The ROC curve representing APP and log2[junction burden] interactions (cyan) in predicting NSB and SB is shown for the REGULATION_OF_AP&P_OF_PEPTIDE_ANTIGEN gene set. (C) ROC curves representing the accuracy of TIDE (blue), IFNG (green), PD-L1 (dark red), and CD8 (orange) models in predicting NSB and SB. (D) ROC curves representing APP (purple), log2[junction burden] (lime green), CD8A (light salmon), and the final model including APP / log2[junction burden] interactions and CD8A (magenta). The inlet is a boxplot and individual patient prediction values by the final model in NSB and SB categories. Colors represent radiologic responses as defined in Figure 1

We sought to determine if the interaction models were predictive of patient overall survival irrespective of treatment approach. To the best of our knowledge, the only available mesothelioma dataset that includes both chromosomal rearrangements from whole genome sequencing, and RNA-seq, is from our previous study of patients (n=24) who provided biopsy or surgical specimens prior to any cytotoxic systemic therapy (Mayo_2019 cohort) (8). The patients in the Mayo_2019 cohort did not receive immune checkpoint inhibitors as these therapies were not available during their lifetimes. There was a break in overall survival at 1.5 years from diagnosis in this cohort that was used as the threshold for categorizing patients as NSB and SB (Supplementary Figure 3). The Mayo_2019 cohort performed similar to other historic mesothelioma cohorts as a previously established mesothelioma survival signature gene set (19) had very high prognostic significance for both overall survival and $S_{1.5yr}$ (Supplementary Figure 4). We did not find an interaction between the tumor junction burdens and any of the 12 APP gene sets on overall survival to be statistically significant (Supplementary Table 3). In further analysis, we noted that the tumors in the Mayo_2019 cohort had fewer junctions than the current cohort (Supplementary Figure 5) which may have affected the predictive values of the interaction models.

Finally, we used RNAseq for computational immune deconvolution to compare the tumor microenvironment (TME) in mesotheliomas with low and high expression of the "REGULATION OF APP OF PEPTIDE ANTIGEN" gene set. The "immunedeconv" package used for our analyses provides results from 6 different computational approaches (see Methods). In all approaches, we observed a lower concentration of immune cells suggesting a "cold" TME in tumors with low compared to high APP gene set expression (Supplementary Figures 6-9). We found higher TME and immune scores (by xCell) and cytotoxicity score (by MCP-counter), and an enrichment of lymphocytes that are often associated with anti-tumor immunity such as B, T, and NK cells and M1 macrophages in tumors with high APP.

Discussion

Genomic structural variants are common in mesothelioma. In the current analysis the tumor junction burdens resulting from chromosomal rearrangements were associated with improved survival outcomes in patients treated with immune checkpoint inhibitors in the presence of antigen processing and presentation gene set expression. In contrast, tumor junction burdens in the absence of antigen processing and presentation gene set expression were associated with reduced survival despite treatment with immune checkpoint inhibitors. Our model was further improved by the inclusion of CD8A, a marker of cytotoxic lymphocytes. We interpreted these observations to be consistent

with our understanding of the mechanisms of adaptive anti-tumor immunity where antigen-specific T cell responses that are restored or generated by PD-1 and CTLA-4 inhibition require tumor cell presentation of neo-antigens. Since the interaction signature between the tumor junction burdens and APP gene sets did not favorably impact overall survival in a separate cohort of patients who did not receive immune checkpoint inhibitors, this signature is not likely to be predictive in settings outside of treatment with immunotherapy. Chromothripsis represents a complex pattern of multiple chromosomal rearrangements typically on a single chromosome. We previously identified that higher numbers of chromothripsis-like patterns detected from copy number segmentation data were a negative prognostic factor in mesothelioma (8), and others have suggested that chromothripsis is a negative prognostic marker across multiple tumor types (20). Despite the negative prognostic significance that has been attributed to increases in these complex patterns of chromosomal rearrangements in mesothelioma and other tumors, tumor junction burdens were associated with improved survival in the context of antigen processing and presentation gene set expression in this cohort of patients with mesothelioma treated with immune checkpoint inhibitors.

Given the marked differences between the TME in tumors with and without high APP gene set expression, we speculate that methods to manipulate the TME might be beneficial for these patients. Recently it was shown that low-dose radiotherapy in murine models promotes T cell infiltration, enabling response to combination immunotherapy (21). A clinical trial has recently activated to test this approach in mesothelioma (NCT04926948). Other work has suggested that oncolytic virotherapy may reprogram the TME to enable responses to immune checkpoint inhibitors (22). It is a major initiative across tumor types to identify means of converting tumors to be responsive to immune checkpoint inhibitors.

There have been inconsistent results with the use of immune checkpoint inhibitors for the treatment of mesothelioma. Based on the Checkmate 743 trial, the frontline use of ipilimumab and nivolumab clearly benefits patients with non-epithelioid mesothelioma, partially because chemotherapy is so ineffective for this group (5). The same degree of benefit was not observed in the epithelioid group, as chemotherapy is more effective for patients with that variant of disease. Since the survival analysis of all randomized patients was positive in the Checkmate 743 trial with a stratified HR of 0.74 (96·6% CI 0.60 0.91; p=0.0020), ipilimumab and nivolumab were approved by the United States FDA for frontline treatment of unresectable pleural mesothelioma regardless of histologic subtype. In second or later lines of treatment, single agent PD-1 inhibitors have been demonstrated to be superior to placebo in the CONFIRM (23) trial, but not superior to gemcitabine or vinorelbine in the PROMISE-meso trial (24); however, these studies both reported that there are responses with immune checkpoint inhibitors in patients with epithelioid disease. Surprisingly, the overall response rate with the PD-1 inhibitor

pembrolizumab was higher than that observed with chemotherapy (22% v. 6%) in the PROMISE-meso trial, although this difference did not translate into a survival benefit. PD-L1 expression was not able to discriminate benefit in the CONFIRM (23) or PROMISE trials (24), or in our cohort. Given the discrepancies with survival outcomes between these clinical trials, it is critical to develop better predictive biomarkers, especially for patients with epithelioid disease where benefit with immune checkpoint inhibitors is less certain.

There have been multiple efforts to identify predictors of benefit with immune checkpoint inhibitors (25). Mismatch repair deficiency is strongly associated with response to treatment across tumor types (26). TMB has also been proposed as a surrogate of neoantigens that can be recognized by the adaptive immune system for elimination. Recently, a PD-1 inhibitor has been approved for solid tumors with a TMB ≥10 mutations/Mb (27); however, these findings have been challenged by others who have failed to identify benefit across tumor types with this cutoff (28). There is significant heterogeneity in the approaches used to determine TMB, and use of population germline variant databases to filter calls can inflate scores and introduce racial bias (29,30). TMBs frequently do not assess or include structural variants or junction burdens. Also, TMB fails to incorporate the full complexity of an adaptive, anti-tumor immune response.

Immunograms may provide better predictors of response to immune checkpoint inhibitors as these would incorporate tumor foreignness (using comprehensive mutation burdens), the ability of tumors to present neoantigens with MHC proteins (antigen processing and presentation), lymphocytes and their ability to traffic to tumors, and the expression of immune checkpoints and other regulatory signals (31). Our findings represent one step towards adopting an immunogram to predict survival with immune checkpoint inhibitors in mesothelioma by incorporating antigen processing and presentation gene set expression in our analysis. These results also suggest that genomic approaches that identify and incorporate junction burdens can improve the determination of TMB, especially in tumors like mesothelioma that have relatively few single nucleotide mutations.

We tested the tumors of patients who had received prior platinum-based chemotherapy. Since the numbers of junctions were slightly higher in the current cohort than a separate cohort of patients who had not received prior platinum-based chemotherapy, it is possible that cytotoxic therapy introduced structural variants. Along these lines, our findings will need to be validated in a cohort of treatment-naïve patients. Also, given the DNA sample requirements to perform our analysis of structural variants, we did not have sufficient materials to perform traditional sequencing approaches to assess single nucleotide mutations. Given the reportedly low TMB in mesothelioma and our prior findings of large, complex rearrangements in this malignancy, we felt it was reasonable to focus our efforts on these structural variants. Finally, efforts are underway to develop chemoimmunotherapy

regimens for mesothelioma. We are not certain whether structural variants would retain their association with survival outcomes in the setting of combination cytotoxic and immunotherapy.

In conclusion, in the context of antigen processing and presentation gene set expression, tumor junction burdens were associated with improved survival in patients with mesothelioma treated with immune checkpoint inhibitors. In contrast, in the absence of antigen processing and presentation, tumor junction burdens were associated with poor survival. The inclusion of genomic approaches that can detect structural variants, and transcriptomics to assess antigen processing and presentation, may help refine the selection of patients to receive immune checkpoint inhibitors, especially for patients with mesothelioma.

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Supplementary data

Supplementary Table 1: Patient characteristics

Characteristics	Patients (n=44)
Sex, n (%)	
Male	37 (84%)
Female	7 (16%)
Histology, n (%)	
Epithelioid	38 (86%)
Biphasic	3 (7%)
Sarcomatoid	3 (7%)
Treatment, n (%)	
Nivolumab	24 (55%)
Nivolumab, Ipilimumab	20 (45%)
Line of therapy, n (%)	
2	38 (86%)
≥3	6 (14%)
Age, median (range)	66 (47-81)

Supplementary Table 2

Gene set Name	Included Genes
GO_REGULATION_OF_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_PEPTIDE_ANTIGEN	PYCARD, HFE, HLA-DOA, HLA-DOB, TREM2, TAPBPL
GO_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_ENDOGENOUS_PEPTIDE_ANTIGEN_VIA_MHC_CLASS_I_VIA_ER_PATHWAY	HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, HLA-G, HLA-H, AZGP1
GO_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_PEPTIDE_ANTIGEN_VIA_MHC_CLASS_IB	HLA-E, HLA-F, HLA-G, HLA-H, AZGP1, B2M, TAP2
GO_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_ENDOGENOUS_PEPTIDE_ANTIGEN	ABCB9, HFE, HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, HLA-G, HLA-H, IDE, ERAP1, AZGP1, B2M, ERAP2, TAP1, TAP2, TAPBP
GO_ANTIGEN_PROCESSING_AND_PRESENTATION_VIA_MHC_CLASS_IB	HLA-E, HLA-F, HLA-G, HLA-H, AZGP1, B2M, TAP2, AP3B1, AP3D1, CD1A, CD1B, CD1C, CD1D, CD1E
GO_ANTIGEN_PROCESSING_AND_PRESENTATION_OF_ENDOGENOUS_ANTIGEN	ABCB9, HFE, HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, HLA-G, HLA-H, IDE, ERAP1, AZGP1, B2M, ERAP2, TAP1, TAP2, TAPBP, CD1A, CD1B, CD1C, CD1D, CD1E, ATG5, CD74

Supplementary Table 3: Antigen processing and presentation gene set analysis in Mayo_2019 cohort

	P-IA-cox	p-Log Rank	P-IA-lr
AP&P_OF_PEPTIDE_ANTIGEN_VIA_MHC_CLASS_IB	0.24	0.44	0.08
AP&P_OF_PEPTIDE_ANTIGEN_VIA_MHC_CLASS_I	0.18	0.37	0.20
AP&P_VIA_MHC_CLASS_IB	0.25	0.48	0.20
AP&P_OF_ENDOGENOUS_PEPTIDE_ANTIGEN	0.13	0.31	0.10
AP&P_OF_ENDOGENOUS_PEPTIDE_ANTIGEN_VIA_MHC_CLASS_I_VIA_ER_PATHWAY	0.24	0.39	0.06
REGULATION_OF_AP&P	0.37	0.52	0.38
NEGATIVE_REGULATION_OF_AP&P	0.46	0.54	0.36
POSITIVE_REGULATION_OF_AP&P	0.33	0.51	0.44
REGULATION_OF_AP&P_OF_PEPTIDE_ANTIGEN	0.88	0.41	0.24
AP&P	0.16	0.28	0.19
AP&P_OF_ENDOGENOUS_ANTIGEN	0.09	0.20	0.10
AP&P_OF_PEPTIDE_ANTIGEN	0.17	0.31	0.17

The statistical significance of interactions (P-IA-cox) and log-rank (p-Log Rank) in cox models, interactions (P-IA-lr) and area under the curve (AUC) in logistic regression models are listed for the Mayo_2019 cohort.

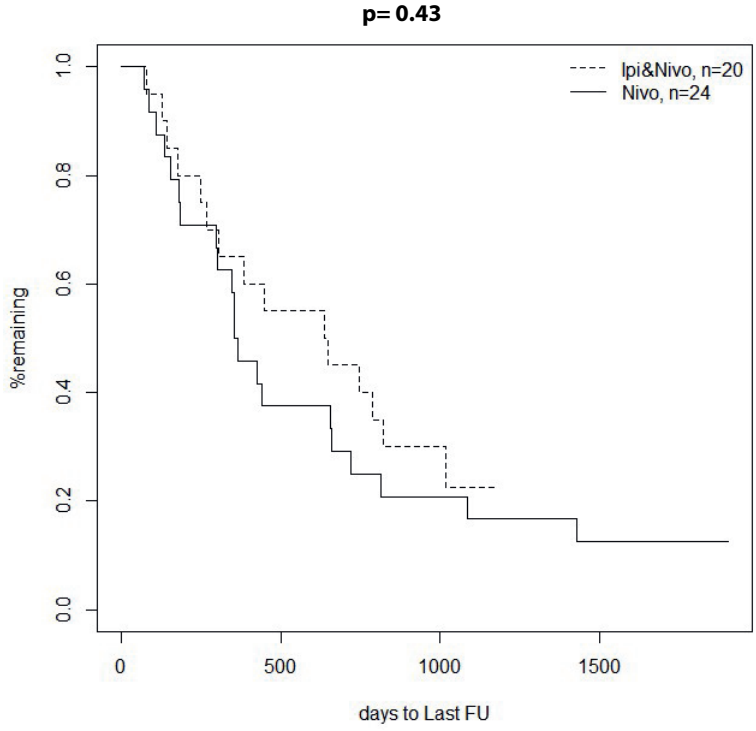

Figure S1: Kaplan-Meier plot of overall survival of patients with MPM treated with nivolumab with or without ipilimumab.

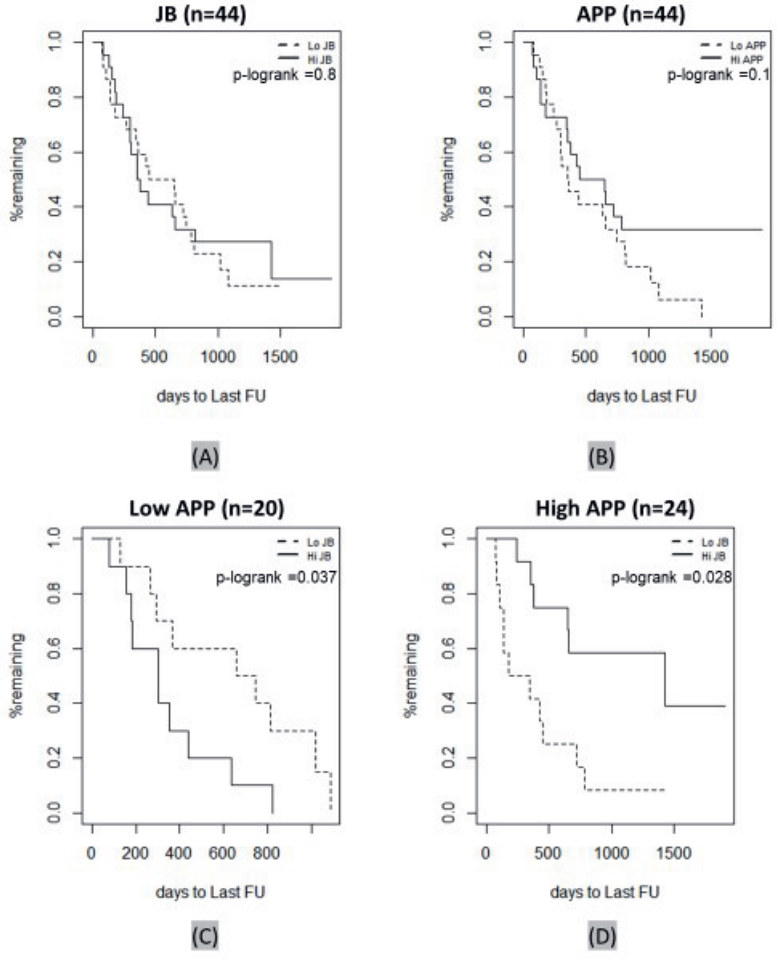

Figure S2: Kaplan-Meier plots of overall survival of patients with MPM are shown based on tumor junction burden (JB) categorizations (A) and "regulation of AP&P of peptide antigen" gene set expression categorizations (B). Similarly, the overall survival of patients with MPM with low (C) and high (D) "regulation of AP&P of peptide antigen" gene set expression based on tumor junction burdens is plotted.

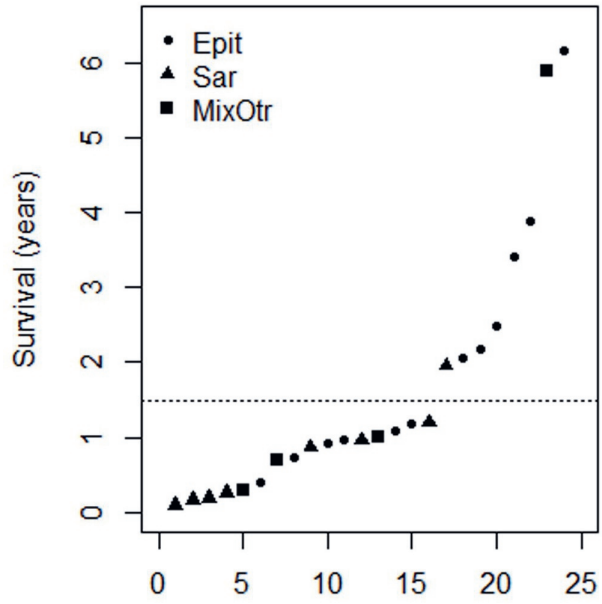

Figure S3: Overall survival times of patients with MPM from the Mayo_2019 cohort (8) are shown. Circles, triangles, and squares represent epithelioid (Epit), sarcomatoid (Sar, including desmoplastic), and mixed or other (MixOtr) subtypes.

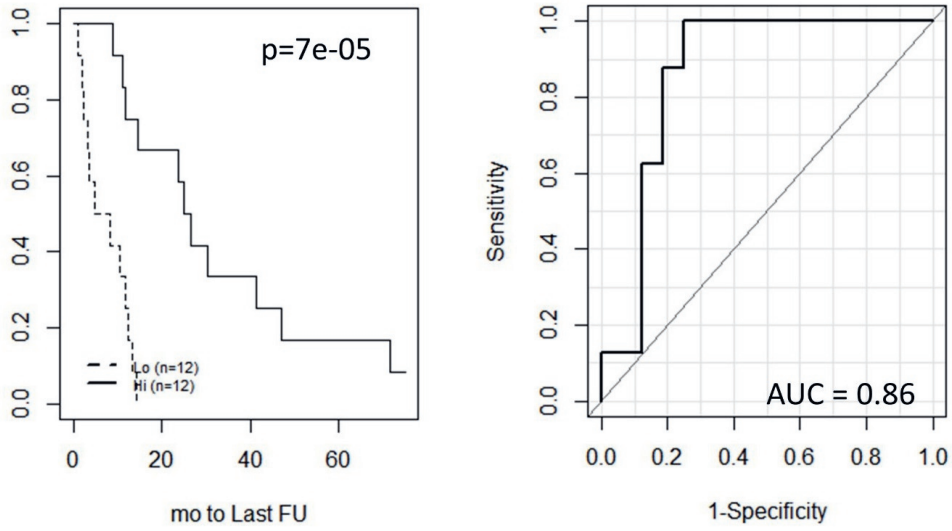

Figure S4: (A) Kaplan Meier plot representing associations between the gene signature "MESOTHELIOMA SURVIVAL OVERALL UP" from Lopez et al (19) and overall survival in the Mayo_2019 cohort are shown (8). (B) The ROC curve based on logistic regression predicting non-significant benefit (NSB) and significant benefit (SB) from the Mayo_2019 cohort is shown.

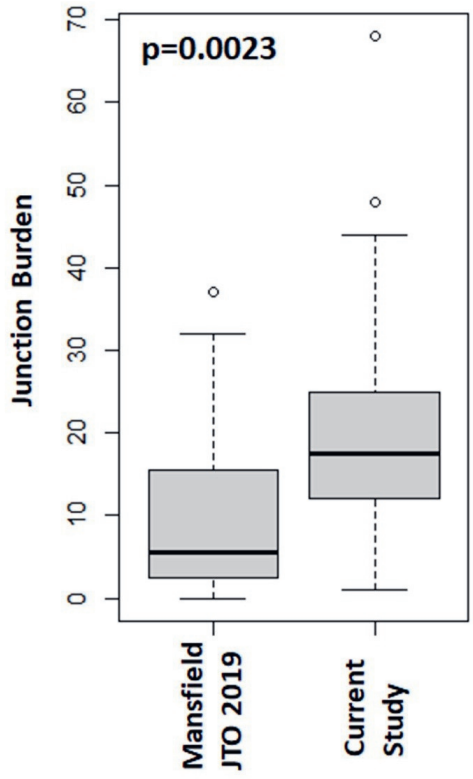

Figure S5: The number of junctions from the Mayo_2019 cohort (8) and the current study are presented.

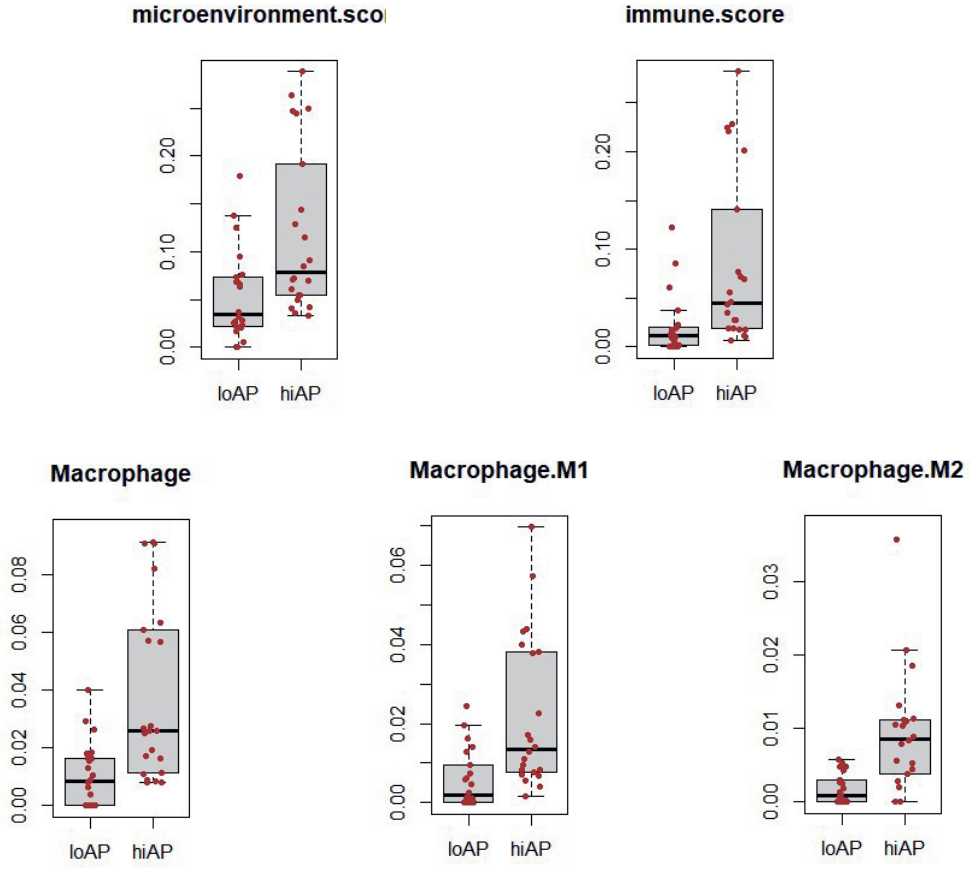

Figure S6: Immune profiling by xCell demonstrating higher TME scores, immune scores and macrophages and their subsets in tumors with high antigen processing and presentation gene set expression based on "REGULATION OF APP OF PEPTIDE ANTIGEN" gene set. The box plots represent the medians with the bars, the interquartile ranges with the boxes, and the ranges with the whiskers.

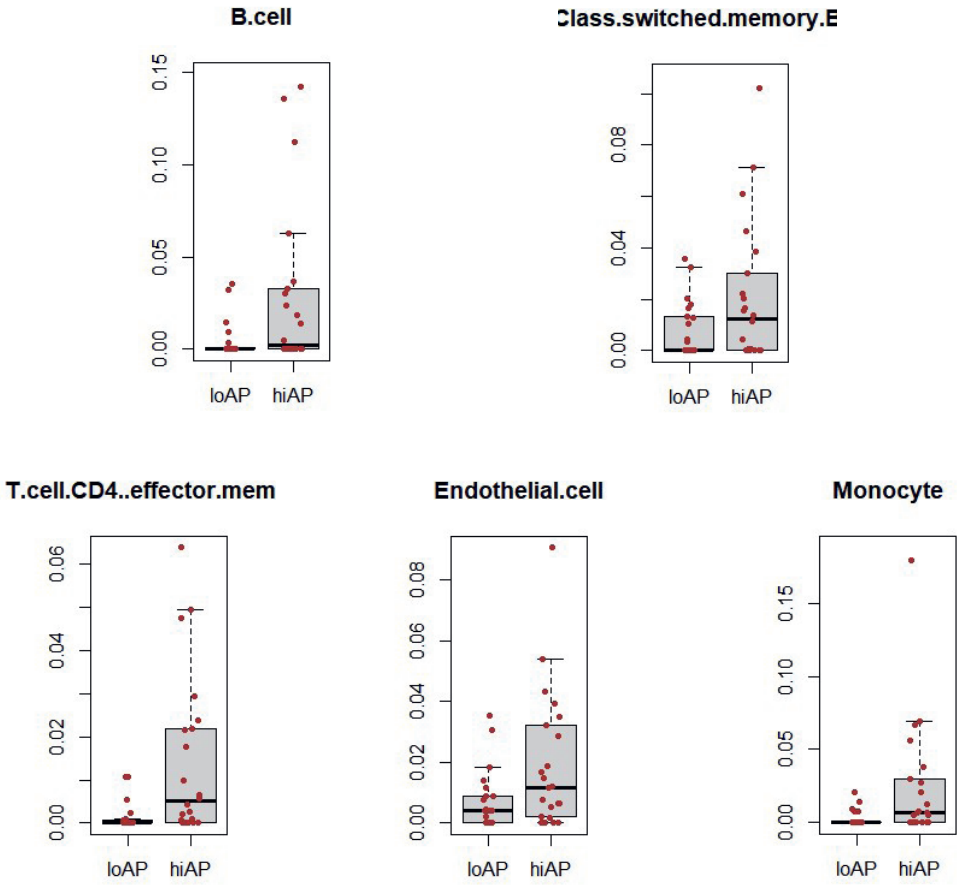

Figure S7: Immune profiling by xCell demonstrating higher B cells, T cells, endothelial cells and monocytes in tumors with high antigen processing and presentation gene set expression based on "REGULATION OF APP OF PEPTIDE ANTIGEN" gene set. The box plots represent the medians with the bars, the interquartile ranges with the boxes, and the ranges with the whiskers.

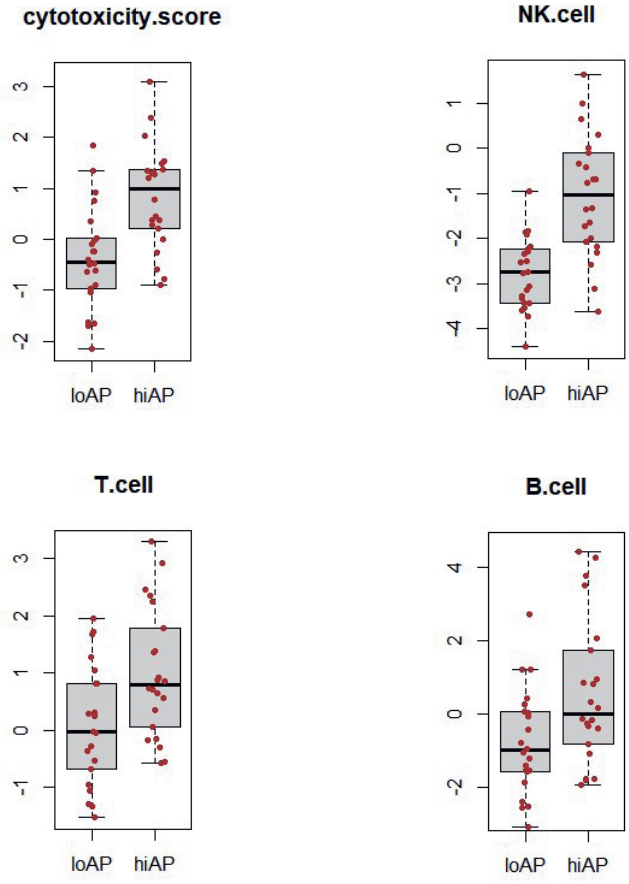

Figure S8: Immune profiling by MCP Counter demonstrating higher cytotoxicity scores, B cells, T cells and NK cells in tumors with high antigen processing and presentation gene set expression based on "REGULATION OF APP OF PEPTIDE ANTIGEN" gene set. The box plots represent the medians with the bars, the interquartile ranges with the boxes, and the ranges with the whiskers.

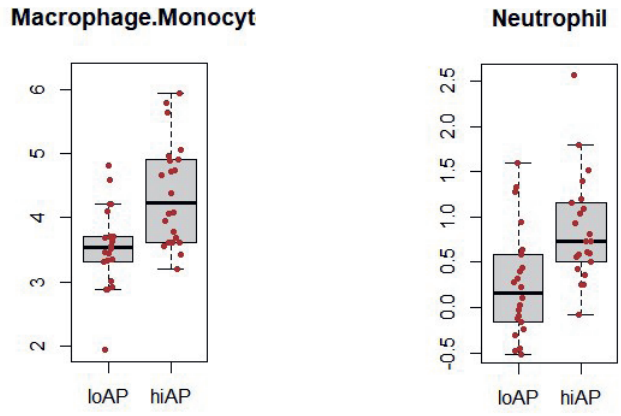

Figure S9: Immune profiling by MCP Counter demonstrating higher macrophages and monocytes, and neutrophils in tumors with high antigen processing and presentation gene set expression based on "REGULATION OF APP OF PEPTIDE ANTIGEN" gene set. The box plots represent the medians with the bars, the interquartile ranges with the boxes, and the ranges with the whiskers.

IV

PART IV

10

Chapter 10

Discussion

Discussion and future perspective

After almost 20 years of chemotherapy as standard of care chemotherapy for malignant pleural mesothelioma (MPM), the treatment changed to immune checkpoint inhibitors (ICI) administration during the course of this thesis. After the INITIATE phase II trial, described in this thesis (chapter 3), the randomized phase 3 trial of nivolumab plus ipilimumab versus platinum plus pemetrexed chemotherapy demonstrated an impressive overall survival benefit for ICI treatment (mOS 18.1 months vs 14.1 months; HR 0.74; p=0.0020) (1). Now, nivolumab plus ipilimumab is considered the standard of care for treatment-naïve unresectable MPM. Thereby, the INITIATE paper in this thesis contributed to a better treatment for mesothelioma.

Although CheckMate 743 showed a survival benefit, unfortunately some people performed worse with ICI treatment. A similar PFS between the two treatment arms exists (median 6.8 months versus 7.2 months; HR 1.00 (95% CI 0.82–1.21), but a clear inferior PFS for ICI treatment in the first 6 months is seen, with marked crossing of PFS curves. Less clear, but this crossing is also seen in OS curves. A proportion of patients have progressive disease when treated with ICIs compared to chemotherapy. Finally, the benefit of ICI treatment is mainly seen in non-epithelioid subtype (1).

Patients failing the ICI therapy may have primary resistant tumors for ICI treatment, have rapidly progressive disease which requires a fast(er) working agent (added) or have hyperprogression. Therefore it is of utmost importance to select patients that will benefit. This is actually an unmet need in all cancers treated with ICIs, so far a useful predictive biomarker has not been established.

A prognostic biomarker is a clinical or biological characteristic that provides information about the patients overall cancer outcome, regardless of therapy. On the other hand, a predictive biomarker provides information on the probability of response to a particular therapy. Usually this biomarker is measured before start of treatment, but sometimes a biomarker can be measured early during treatment. A predictive biomarker can be a target for therapy, for example as seen in EGFR mutated lung cancer.

In reality, all biomarkers will have some degree of prognostic value, and some degree of predictive value. For example PD-L1 expression on tumor cells in lung cancer, but also in MPM, does have prognostic value; it has been associated with poor prognosis (2-7). PD-L1 suppresses T cell activation and as a result, the tumor is able to escape anti-tumor immune response. But PD-L1 expression is also predictive of response to PD-1 checkpoint inhibitors in NSCLC. Tumors with higher PD-L1 expression usually respond better to IO treatment (8-10).

In MPM a few phase II studies have been performed on evaluating predictive effect of PD-L1 expression on antiPD-(L)1 treatment, with conflicting results(11-14). In the larger phase III trial evaluating nivolumab, PD-L1 expression was not predictive of response for either overall survival or progression free survival (15).

Our INITIATE trial with nivolumab plus ipilimumab showed that PD-L1 expression was significantly associated with clinical benefit (i.e. partial response or stable disease for > 6 months, p = 0.037). But in the larger phase III Checkmate743 trial PD-L1 expression did not show a correlation with outcome (1).

All studies used different antibodies (22C3, SP-263, 28-8, E1L3N) for the PD-L1 staining; used different cut-off points (> 1%, > 5%, > 50%) and had different timing of examining the PD-L1 expression (archival, before chemotherapy, pre-treatment). Even when taking this into account, PD-L1 expression alone can probably not be used as a predictive biomarker.

Dissecting complete mechanisms by which tumor-infiltrating immune cells participate in the development of a systemic antitumor response are still under exploration. The relationship between the tumor, tumor-infiltrating immune cells, the host, and the antitumor effects of ICI is complicated. Comprehensive studies with large sample size are needed. However, so far, this type of research is still lacking, in many tumor types but especially in mesothelioma. Over the last years lots of interesting research has been published on this subject, gaining more and more knowledge.

Mesothelioma research is difficult for a few reasons. Mesothelioma is relatively rare, only about 500 patients in the Netherlands are diagnosed every year. MPM is a heterogenous type of cancer, epithelioid and non-epithelioid subtypes have their own different clinical behavior and response to therapy; making the possible groups for research even smaller. And the larger epithelioid subgroup itself is very heterogeneous. Furthermore patients with MPM are often diagnosed at late stage, which affects the physical condition of the patients negatively; this reduces the number of patients eligible for clinical trials even further.

Another difficulty in clinical trials in MPM is response measurements. Modified RECIST criteria for mesothelioma are used in clinical trials (16, 17), but this is not completely representative for the whole pleural enlargement. Uni-dimensional measurements of tumor thickness perpendicular to the chest wall are measured in 2 sites at 3 different levels on CT scans. Even with a small increase in one diameter, volume increases much more. In the future artificial intelligence techniques for measuring volume of pleural rim could be used. Since MPM is usually slow-growing, radiological stable disease does not always tell something about treatment effect.

Earlier phase II clinical trials, including the INITIATE trial, used disease control (complete response, partial response and stable disease) as primary endpoint. Since it includes stable disease, apparently it does not necessarily measures treatment effect. Different trials showed promising effects in phase II measured by DCR, which did not translate into survival benefit in phase III clinical trials (18-24). Primary endpoint of phase II trials could better be ORR of PFS.

MPM typically has a low tumor mutational burden (TMB), leading to a low neo-antigen burden(25). Based on that, one would not expect a favorable outcome with ICI treatment, since most tumor types having low TMB do not have clinical benefit (26). A possible explanation why responses to ICI treatment are observed may be related to chromosomal rearrangements which serve as neo-antigens. It is hypothesized by Mansfield et al. that the number of alterations actually targeted by T cells, may have a stronger association with ICI response than does TMB. This is based on a mechanism called chromothripsis, a mutational process by which chromosomal rearrangements occur within one or between chromosomes (27). In chapter 9 of this thesis, part of the research in collaboration with Mansfield is published, to determine whether these chromosomal rearrangements are associated with survival benefit in patients from nivolumab and nivolumab plus ipilimumab trial. Junction burden alone was not predictive of overall survival but a significant interaction was seen between junction burden and multiple antigen processing and presentation gene sets. A specific gene set in combination with junction burden was predictive of survival.

Several studies identified a link between T cell infiltration and outcome in patients with mesothelioma, however it is not established that this is a predictor of response to immunotherapy (28-31). Our trial found a correlation of higher numbers of CD4+, CD8+ and FoxP3+ cells in responding patients to therapy. But this was only in a small group of patients.

Gene-expression profiling signatures that identify tumors with a T cell inflamed phenotype show some promising results predicting response to ICI treatment (32). Based on immune gene expression profiles, some trials classified MPMs into different subtypes, different from the histological subtypes. For example, in 3 groups based on immune cell gene expression, forty percent of cases were classified in group 1 (immune desert), the rest were classified in group 2 (higher B-cell and antigen presentation-related gene expression) and group 3 (higher T-cell related gene expression), suggesting that a significant number of MPMs are inflamed tumors (33). In a cohort of 516 MPM patients, groups were analyzed based on presence of T-helper 2 and cytotoxic T-cells. The group with low T-helper 2 cells and high cytotoxic T-cell levels (8.5% of the total group) had the best survival, and on a transcriptional level, upregulation of immune pathways was observed in this group (34).

Another study showed two tumor types based on tumor microenvironment of MPM, the good molecular signature (only 5 patients) had a good radiological response to ICI treatment (35). This suggests an immune based signature in some of the mesotheliomas, with possible clinical relevance. But it needs to be validated in larger cohorts.

On-treatment biopsies and PBMCs samples in Nivomes and INITIATE trial were performed at 6 weeks of treatment. At the time of writing the Methods sections for both trials, less was known about tumor microenvironment of mesothelioma and effect of ICI treatment. With current knowledge we would have taken blood samples also earlier on treatment and at time of progression or after a certain time of response. A peak in proliferating cells in other tumor types is seen at 3 weeks of antiPD-1 therapy. It would be interesting to see whether the changes seen in peripheral blood are long lasting or not.

A different timing of the biopsies could have influenced results. In some patients pseudoprogression or hyperprogression to ICI treatment is observed (in 9% in Nivomes trial and in 6% in INITIATE trial). Also it would be informative to take biopsies at progression and beyond, to examine the early changes and duration of these in time. Many patients allowed us to take extra biopsies only for research purposes, but taking even more invasive biopsies would be difficult.

In patients with advanced non-small cell lung cancer (NSCLC), the addition of chemotherapy to immunotherapy in the first-line setting has avoided the crossover of survival curves, thereby reducing the risk of (hyper)progressive disease (36). And evidence exists that chemotherapy can deplete circulating and MPM-infiltrating MDSCs to lift their protumorigenic effect (37, 38). In line with that, combination of chemotherapy plus ICI for MPM showed promising data in two single arm phase 2 trials. In DREAM trial, treatment with durvalumab plus platinum plus pemetrexed in first line demonstrated a PFS at 6 months of 57% and mOS of 18.4 months (39). In PrE0505 trial, presented at ASCO 2020, using the same regimen showed a PFS at 6 months of 69% and mOS of 20.4 months (40). Phase 3 trials combining chemotherapy with ICIs are underway, DREAM3R with the same regimen as DREAM trial (NCT04334759), IND227 trial combining pembrolizumab, platinum and pemetrexed (NCT02784171), and BEAT-Meso trial, randomizing between carboplatin-pemetrexed-bevacizumab and carboplatin-pemetrexed-bevacizumab-atezolizumab (NCT03762018).

Anti-angiogenic agents have been used in different clinical trials in MPM, since angiogenesis plays an important role in MPM. But most trials showed disappointing results in MPM, either being not effective or too toxic. Newer strategies focus on the potential synergistic effects of antiangionesis and immunotherapy. Anti-VEGF has been shown to modulate T cell proliferation, migration and activation (41) and the combination

is now evaluated in clinical trials. The phase III BEAT-meso trial of the ETOP is assessing the combined treatment with atezolizumab, bevacizumab (anti-VEGF antibody) and chemotherapy (NCT03762018). The comparator is not the standard treatment but bevacizumab plus chemotherapy. The combination nivolumab and ramucirumab (anti-VEGFR2 antibody) (NCT03502746) and the combination pembrolizumab and lenvatinib (multikinase inhibitor against VEGF) (NCT04287829) are under investigation in two phase II trials in patients with relapsed mesothelioma.

Other innovative ways to manipulate the immune system are being explored. Genetically engineered T cells called chimeric antigen receptor T (CAR-T) cells have been designed to target mesothelin, an antigen seen in mesothelioma cells. In the first phase I clinical trial these CAR-T cells were delivered intrapleurally in combination with an immune checkpoint inhibitor. A disease control of 60% in 19 patients was seen (42).

Anti-tumor vaccines are in development and are under early phase investigation in combination with checkpoint inhibition (NCT04040231).

Of course many more clinical trials have or are being performed with different kinds of agents, including anti-angiogenic agents, anti-mesothelin, arginine deprivation, cell cycle inhibitors, CAR-T cell, dendritic cell therapy; but it is beyond the scope of this thesis.

In conclusion, malignant pleural mesothelioma is a heterogeneous disease that is almost always lethal. Over the last years progress was made in unraveling the tumor and its microenvironment, in respect to tumor cells, immune cells and its inhibitory receptors, cytokines, genetics and sequencing data. In the (near) future, further steps will be made to improve treatment, probable in a personalized way.

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11

Chapter 11

Summary

Malignant pleural mesothelioma is an aggressive tumor originating from the mesothelial cells of the pleural cavity. It has a causal relation with (occupational) asbestos exposure. The latency time between asbestos exposure and diagnosis of mesothelioma is 20 to 50 years. Despite the fact that the use of asbestos has been banned for almost 30 years, approximately 500 patients in the Netherlands are diagnosed with mesothelioma every year. Patients are typically men and older than 65 years. The tumor spreads along the pleura and can result in pleural thickening and fluid in the pleural cavity. Leading to symptoms of shortness of breath, chest pain, night sweats, fatigue and weight loss. Upon diagnosis, usually there is no treatment with curative intent, and systemic treatment is given.

This dissertation focuses on the treatment of malignant mesothelioma, with the aim of improving patient outcomes.

Chapter 1 provides a general introduction to mesothelioma, focusing on treatment options, tumor microenvironment, and possible biomarkers.

Part I – Treatment of mesothelioma

This part of the thesis focuses on what is already known about treatment of mesothelioma.

In *Chapters 2 and 3* we provide an overview of systemic treatment of malignant pleural mesothelioma. Standard of care has long been platinum containing chemotherapy plus an antifolate, leading to a median survival of 12 to 16 months. Several trials have been published on the addition of an anti-angiogenesis inhibitor to chemotherapy. Bevacizumab gives a survival gain compared to the control group, but this has not led to a change in the standard approach, especially given the side effects. Other angiogenesis inhibitors do not give any gain over chemotherapy.

Maintenance therapy with pemetrexed, thalidomide or defactinib did not show benefit. In the NVALT19 trial, better progression-free survival is seen with switch maintenance gemcitabine. No single chemotherapy regimen did prove clinical benefit as a second- or third-line systemic treatment over best supportive care.

Other potential treatment options in mesothelioma are mesothelin-targeted therapy. Mesothelin is a tumor antigen that is strongly expressed in mesothelioma. Several agents, for example anti-body drug conjugates, anti-mesothelin immunotoxins and chimeric antigen receptor T cell therapies are being tested in clinical trials, with varying degrees of success.

BRCA1-associated protein-1 (BAP1) has a role in DNA repair. It is inactivated in around 25% of tumors and could be a potential target. This is also being tested in clinical trials.

Immune checkpoint inhibitor treatment, or immunotherapy for short, has emerged as an effective treatment for certain types of cancer. In normal cells, immune checkpoints ensure immune tolerance and prevent autoimmune diseases. In tumor cells, the expression of these inhibitory checkpoints can be dysregulated, causing the cells to become immune resistant and not recognized by the immune system. Binding of, for example, inhibitory programmed death-ligand 1 (PD-L1) on tumor cells to programmed death 1 (PD-1) on immune cells activates an inhibitory signal. Immune cells are thereby inactivated and the tumor can evade the immune system. Immune checkpoint inhibitors can block these inhibitory checkpoints, thereby restoring immune system function and evoking an anti-cancer immune response. In mesotheliomas PD-L1 is expressed in about 25%, and as in other tumor types, is associated with worse survival.

Another inhibitory checkpoint is Cytotoxic T-Lymphocyte-associated protein 4 (CTLA-4). Anti CTLA-4 antibodies impact the lymphoid compartment, increasing the immune T cell response.

In *Chapter 4* a part of the "ESMO handbook immuno-oncology" is published, namely the chapter mesothelioma. It describes several promising phase I and II trials that test PD-(L)1 inhibitors in mesothelioma. The disease control rate (complete response plus partial response plus stable disease, i.e. that is no growth of the tumor) in these trials ranged from 50 to 76%. The overall response rate ranged from 9 to 21%. CTLA-4 inhibitor tremelimumab failed to show a survival benefit in a phase IIB trial.

Combining PD-(L)1 and CTLA-4 inhibitors has been shown to induce synergistic effects in preclinical and clinical trials.

Part II – Clinical research

Chapter 5 describes the INITIATE study, in which this combination is given to investigate whether this is also effective in mesothelioma. It is a prospective phase 2 study with one arm, conducted in the NKI-AvL. Patients should have been treated with platinum-containing chemotherapy at least earlier, and then had progression. They received intravenous nivolumab (anti-PD-1) every two weeks plus intravenous ipilimumab (anti-CTLA-4) every six weeks, the latter being given up to 4 times. Treatment was continued for as long as it was effective, or until serious side effects occurred, and for up to 2 years.

Primary endpoint was disease control rate at 12 weeks, as measured by modified response evaluation criteria in solid tumors (RECIST) for mesothelioma.

35 patients were included in the safety analysis and 34 were evaluable for response assessment at 12 weeks. Of these, ten (29%) patients had a partial response and 13 (38%) patients had stable disease, so disease control was achieved in 23 (68%) of the 34 patients. Safety was similar to known data on this combination treatment, with the exception of infusion-related reactions, which were more common in this study (49%).

In conclusion, the study shows that treatment with combination immunotherapy appears to be effective and well tolerated, that toxicity is largely reversible and considered manageable.

Part III – translational research

In the INITIATE study, all patients did give permission to take biopsies of the pleura prior to treatment and during treatment (after 6 weeks), extra blood was also taken at these times and lung function tests have been performed. Various translational studies have been carried out with all these materials and data.

Some of these studies also used material from the Nivomes study. This is also a prospective phase 2 single arm study, conducted in the NKI-AvL, with the same inclusion and exclusion criteria as the INITIATE study. Patients in this study received intravenous nivolumab only every two weeks, without ipilimumab.

The success of immune checkpoint inhibitors depends on presence and activation of tumor-specific T cells. In *Chapter 6* comprehensive immune cell profiling was performed on pre-treatment and on-treatment peripheral blood samples of patients treated with nivolumab monotherapy (from the Nivomes trial) and patients treated with combination of nivolumab plus ipilimumab (from the INITIATE trial). Characteristics and quantities of the different immune cells can be assessed with this. Combination immunotherapy has been shown to induce a profound increase in proliferation and activation of T cells, which is not seen in nivolumab monotherapy. In addition, in patients that responded to combination treatment had low frequencies of naive CD8 T cells and high frequencies of effector memory CD8 T cells that re-expressed RA (TEMRA) in the pre-treatment blood samples. These TEMRAs also produce cytokines more often. These TEMRAs probably comprise tumor-specific T cells, and need blocking of both PD-1 and CTLA-4 to be reactivated.

In *Chapter 7*, exhaled breath is analyzed. This is a non-invasive and easy-to-use technology for diagnosing and phenotyping a wide range of diseases, for example asthma, lung cancer and mesothelioma. Exhaled breath consists of up to thousands of volatile organic compounds (VOCs) that are produced by (patho)physiological processes of the body.

The electronic nose (eNose) technology can be used for pattern recognition of the mixture of VOCs. Combined sensor signals produce a characteristic breath profile. It is shown that an electronic nose can be used to discriminate upfront between responders and non-responders to pembrolizumab or nivolumab in patients with stage IV NSCLC with an accuracy of 90%.

Here, the eNose (in this case the SpiroNose) is used to predict the response to nivolumab plus ipilimumab. For 31 patients of the INITIATE trial eNose data were available, for 16 responders (including complete response, partial response and stable disease for 6 months) and 15 non-responders (progressive disease and stable disease for less than 6 months). At baseline, the breath profiles differed significantly between responders and non-responders to treatment. The eNose could become a tool for prediction of response to immune checkpoint inhibitors, although this needs to be evaluated in larger trials.

We also assessed changes in breath profiles during the first 6 weeks of treatment with nivolumab plus ipilimumab. We observed a significant change in sensor values from baseline in patients with partial response and progressive disease, though in opposite directions. This suggests the eNose may be used as a monitoring tool to asses prognosis or effect of therapy in mesothelioma. Although this also should to be evaluated in larger trials.

Chapter 8 describes translational data from the biopsies in patients treated with nivolumab from the Nivomes study and nivolumab plus ipilimumab from the INITIATE study. Staining has been done with different markers to be able to characterize the cells. The marker-positive cells are counted using software, the cell density is defined as the amount of positive cells per mm2. Prior to combination treatment, in patients with a partial response at 24 weeks, there are higher cell densities of CD4+, CD8+, FoxP3+ and PD-1+ cells, compared to patients with progressive disease after 24 weeks of treatment. This difference is not seen in patients receiving nivolumab monotherapy.

After six weeks of treatment, there are no significant changes compared to baseline biopsies. Not in number and not in type of marker.

A single marker may not be specific enough to be able to say anything about the tumor microenvironment and thus the effect of treatment with immunotherapy.

Chapter 9 describes the sequencing data on the biopsies of the Nivomes and INTIATE study. Genetic analysis was performed on the freshly frozen samples using RNA and whole genome sequencing. In mesothelioma, structural chromosomal changes are found, which can result in neoantigens. These junctions alone are not predictive of survival after immunotherapy. Different gene sets were also looked at and the gene set 'regulation of antigen processing and presentation of peptide antigen' showed an interaction with the amount of junctions and the combination is predictive of survival in patients treated with immunotherapy.

Finally, *Chapter 10* provides a brief summary of recent developments in the systemic treatment of malignant pleural mesothelioma.

12

Chapter 12

Nederlandse samenvatting

Maligne pleuraal mesothelioom is een agressieve tumor die ontstaat uit de mesotheliale cellen van de pleura, het borstvlies. Het wordt veroorzaakt door (beroeps)blootstelling aan asbest. De latentietijd tussen blootstelling aan asbest en diagnose van mesothelioom is 20 tot 50 jaar. Ondanks dat het gebruik van asbest al bijna 30 jaar verboden is, worden in Nederland jaarlijks ongeveer 500 patiënten gediagnosticeerd met mesothelioom. Patiënten zijn meestal mannen en ouder dan 65 jaar. De tumor verspreidt zich langs de pleura en kan resulteren in pleurale verdikkingen en vocht in de pleuraholte. Leidend tot symptomen van onder meer kortademigheid, pijn op de borst, nachtelijk zweten, vermoeidheid en gewichtsverlies. Bij diagnose is er meestal geen curatieve behandeling mogelijk. En wordt er systemische behandeling gegeven.

Dit proefschrift richt zich op de behandeling van maligne mesothelioom, met als doel de uitkomsten voor patiënten te verbeteren.

In *hoofdstuk 1* wordt een algemene inleiding gegeven over mesothelioom, met de nadruk op behandelingsopties, tumormicro-omgeving en mogelijke biomarkers.

Deel I – Behandeling mesothelioom

Dit deel van het proefschrift richt zich op wat er al bekend is over de behandeling van mesothelioom.

In *hoofdstuk 2 en 3* geven we een overzicht over systemische behandeling van maligne pleuraal mesothelioom. Standaard behandeling is al lange tijd platina bevattende chemotherapie plus een antifolaat geweest, wat leidt tot een mediane overleving van 12 tot 16 maanden. Er zijn verschillende studies gepubliceerd over het toevoegen van een anti-angiogeneseremmer aan chemotherapie. Bevacizumab geeft een overlevingswinst ten opzichte van de controlegroep, maar dit heeft niet tot een verandering van de standaardbenadering geleid, met name gezien de bijwerkingen. Andere angiogeneseremmers geven geen winst boven chemotherapie.

Onderhoudstherapie met pemetrexed, thalidomide of defactinib na chemotherapie toont geen voordeel aan. In de NVALT19-studie wordt een betere progressie-vrije overleving gezien bij switch maintenance gemcitabine. Geen enkel chemotherapieregime laat klinisch voordeel zien als tweede- of derdelijns behandeling ten opzichte van de beste ondersteunende zorg.

Andere mogelijke behandelingsopties bij mesothelioom zijn mesotheline-gerichte therapie. Mesotheline is een tumorantigeen dat sterk tot expressie komt in mesothelioom.

Verschillende middelen, bijvoorbeeld anti-body drug conjugaten, anti-mesotheline immunotoxinen en chimere antigeen receptor T cel therapieën worden getest in klinische studies, met wisselend succes.

BRCA1-geassocieerd eiwit-1 (BAP1) heeft een rol bij DNA-reparatie. Het is geïnactiveerd in ongeveer 25% van de mesotheliomen en zou een potentieel doelwit kunnen zijn. Dit wordt ook getest in klinische onderzoeken.

Immuun checkpoint inhibitor behandeling, kortweg immuuntherapie, blijkt een effectieve behandeling voor bepaalde typen kanker te zijn. In normale cellen zorgen immune checkpoints voor immuun-tolerantie en voorkomen auto-immuunziekten. In tumorcellen kan de expressie van deze remmende checkpoints worden ontregeld, waardoor de cellen immuunresistent worden en niet worden herkend door het immuunsysteem. Binding van bijvoorbeeld inhibitory programmed death-ligand 1 (PD-L1) op tumorcellen aan programmed death 1 (PD-1) op immuuncellen activeert een remmend signaal. Immuuncellen worden daardoor geïnactiveerd en de tumor kan het immuunsysteem ontwijken. Immuun checkpoint inhibitors kunnen deze remmende checkpoints blokkeren, waardoor de functie van het immuunsysteem wordt hersteld en een anti-kanker immuunrespons wordt opgeroepen. In mesotheliomen komt bij ongeveer 25% PD-L1 tot expressie, en zoals in andere tumortypen, is dit geassocieerd met een slechtere overleving.

Een ander remmend checkpoint is cytotoxisch T-lymfocyten-geassocieerd eiwit 4 (CTLA-4). Anti CTLA-4-antilichamen beïnvloeden het lymfoïde compartiment, waardoor de T-cel respons toeneemt.

In *hoofdstuk 4* wordt een deel uit het "ESMO handboek immuno-oncologie" gepubliceerd, namelijk het hoofdstuk mesothelioom. Hierin worden verschillende veelbelovende fase I- en II-onderzoeken die PD-(L)1-remmers in mesothelioom testen beschreven. De "disease control rate", dat wil zeggen complete afname, plus gedeeltelijke afname plus stabiele ziekte (dus dat er geen groei is van de tumor) varieerde in onderzoeken met monotherapie van 50 tot 76%. Het totale responspercentage (een afname van de dikte van het mesothelioom met 30% of meer) varieerde van 9 tot 21%. Monotherapie met CTLA-4-remmer tremelimumab toonde geen overlevingsvoordeel in een fase IIB-studie.

Deel II – Klinisch onderzoek

Het combineren van PD-(L)1- en CTLA-4-remmers heeft aangetoond dat het synergetische effecten induceert in preklinische en klinische onderzoeken.

In *hoofdstuk 5* wordt de INITIATE studie beschreven, waarbij deze combinatie is gegeven om te onderzoeken of dit ook effectief is bij mesothelioom. Het is een prospectieve fase 2-studie met één arm, uitgevoerd in het NKI-AvL. Patiënten moesten ten minste eerder zijn behandeld met platinabevattende chemotherapie, en daarna progressie hebben. Ze kregen elke twee weken intraveneus nivolumab (anti-PD-1) plus elke zes weken intraveneus ipilimumab (anti-CTLA-4), dit laatste werd maximaal 4 keer gegeven. De behandeling werd gecontinueerd zolang als het effectief was, of tot er ernstige bijwerkingen optraden, en maximaal 2 jaar. Primair eindpunt was disease control rate na 12 weken, zoals gemeten aan de hand van gemodificeerde responsevaluatiecriteria in solide tumoren (RECIST) voor mesothelioom.

Er werden 35 patiënten geïncludeerd in de veiligheidsanalyse en 34 waren evalueerbaar voor responsbeoordeling na 12 weken. Hiervan hadden tien (29%) patiënten een partiele respons en 13 (38%) patiënten hadden stabiele ziekte, dus disease control werd bereikt in 23 (68%) van de 34 patiënten. De veiligheid was vergelijkbaar met bekende gegevens over deze combinatiebehandeling, met uitzondering van infusie-gerelateerde reacties, welke vaker voorkwamen in deze studie (49%).

Concluderend toont de studie aan dat de behandeling met combinatie immuuntherapie effectief lijkt en goed wordt verdragen, dat de toxiciteit grotendeels reversibel iss en als beheersbaar wordt beschouwd.

Deel III – translationeel onderzoek

In de INITIATE studie hebben alle patiënten toestemming gegeven om biopten van de pleura af te nemen voorafgaand aan de behandeling en tijdens behandeling (na 6 weken), er is op deze momenten ook extra bloed afgenomen en er zijn longfunctietesten gedaan. Met al deze materialen en gegevens zijn verschillende translationeel onderzoeken verricht.

In sommige van deze onderzoeken is ook uit materiaal van de Nivomes-studie gebruikt. Dit is ook een prospectieve fase 2-studie met één arm, uitgevoerd in het NKI-AvL, met dezelfde in- en exclusiecriteria als de INITIATE-studie. Patiënten in deze studie kregen alleen om de twee weken intraveneus nivolumab, zonder ipilimumab.

Het succes van immuuncheckpointremmers hangt af van de aanwezigheid en activering van tumorspecifieke T-cellen. In *hoofdstuk 6* wordt uitgebreide immuuncelprofilering uitgevoerd op perifere bloedmonsters voor en tijdens de behandeling van patiënten die werden behandeld met nivolumab monotherapie (uit de Nivomes studie) en patiënten die

werden behandeld met een combinatie van nivolumab plus ipilimumab (uit de INITIATE studie). Karakteristieken en hoeveelheden van de verschillende immuuncellen kunnen hiermee worden beoordeeld. Er wordt aangetoond dat combinatie immuuntherapie een aanzienlijke toename van proliferatie en activering van T-cellen veroorzaakte, dit wordt niet gezien bij nivolumab monotherapie. Bovendien zitten er bij patiënten die effect hebben van combinatiebehandeling lage frequenties van naïeve CD8 T-cellen en hoge frequenties van effectorgeheugen CD8 T-cellen die RA (TEMRA) opnieuw tot expressie brengen, in het bloed voorafgaand aan behandeling. Deze TEMRA's produceren ook vaker cytokines. Deze TEMRA's bestaan waarschijnlijk uit tumorspecifieke T-cellen en er moet zowel PD-1 als CTLA-4 worden geblokkeerd om ze te reactiveren.

In hoofdstuk 7 wordt uitademings lucht geanalyseerd. Dit is een niet-invasieve en eenvoudig te gebruiken technologie voor het diagnosticeren en fenotyperen van een breed scala aan ziekten, bijvoorbeeld astma, longkanker en mesothelioom. Uitgeademde lucht bestaat uit maximaal duizenden vluchtige organische stoffen (VOCs) die worden geproduceerd door (patho)fysiologische processen van het lichaam.

De elektronische neus (eNose) technologie kan worden gebruikt voor patroonherkenning van het mengsel van VOC's. Gecombineerde sensorsignalen produceren een karakteristiek ademprofiel. Het is eerder aangetoond dat een elektronische neus kan worden gebruikt om vooraf onderscheid te maken tussen responders en non-responders op pembrolizumab of nivolumab bij patiënten met stadium IV NSCLC met een nauwkeurigheid van 90%.

Hier wordt de eNose (in dit geval de SpiroNose) gebruikt voor het voorspellen van de respons op nivolumab plus ipilimumab. Voor 31 patiënten van de INITIATE-studie waren eNose-gegevens beschikbaar, voor 16 responders (waaronder volledige respons, partiële respons en stabiele ziekte gedurende 6 maanden) en 15 non-responders (progressieve ziekte en stabiele ziekte gedurende minder dan 6 maanden). Bij baseline verschilden de ademprofielen significant tussen responders en non-responders op de behandeling. De eNose zou een hulpmiddel kunnen worden voor het voorspellen van de respons op immuuncheckpoint remmers, hoewel dit in grotere onderzoeken moet worden geëvalueerd.

De verandering van de ademprofielen tijdens de eerste 6 weken van de behandeling met nivolumab plus ipilimumab is ook geanalyseerd. Er wordt een significante verandering in sensorwaarden ten opzichte van baseline bij patiënten met partiële respons en progressieve ziekte gezien, in tegengestelde richtingen. Dit suggereert dat de eNose kan worden gebruikt als een monitoringinstrument om de prognose of het effect van therapie bij mesothelioom te beoordelen. Hoewel dit ook in grotere onderzoeken moet worden geëvalueerd.

In *hoofdstuk 8* wordt translationele data beschreven van de biopten bij patiënten die werden behandeld met nivolumab uit de Nivomes-studie en nivolumab plus ipilimumab uit de INITIATE studie. Er zijn kleuringen gedaan met verschillende markers om de cellen te kunnen typeren. De marker-positieve cellen zijn geteld met behulp van software, de celdichtheid is gedefinieerd als de hoeveelheid positieve cellen per mm^2. Voorafgaand aan combinatie behandeling zijn er bij patiënten met een partiële respons na 24 weken, hogere celdichtheden van CD4+, CD8+, FoxP3+ en PD-1+ cellen, in vergelijking met patiënten met progressieve ziekte na 24 weken behandeling. Dit verschil wordt niet gezien bij patiënten die nivolumab monotherapie kregen.

Na zes weken behandeling zijn er geen significante veranderingen in vergelijking met de baseline biopten. Niet in aantal en niet in type marker.

Mogelijk is een enkele marker niet specifiek genoeg om iets te kunnen zeggen over de tumor micro-omgeving en daarmee het effect van behandeling met immuuntherapie.

In *hoofdstuk 9* wordt de sequencing data beschreven op de biopten van de Nivomes en INITIATE studie. Op de vers ingevroren monsters is genetisch analyse verricht middels RNA en whole genome sequencing. In mesothelioom worden structurele chromosomale veranderingen gevonden, deze kunnen resulteren in neoantigen. Deze junctions alleen zijn niet voorspellend voor de overleving na immuuntherapie. Er is ook gekeken naar verschillende genensets en de genenset 'regulatie van antigeenverwerking en presentatie van peptide-antigeen' toonde een interactie met de hoeveelheid junctions en de combinatie is voorspellend voor de overleving bij patienten die behandeld zijn met immuuntherapie.

Ten slotte geeft *hoofdstuk 10* een korte samenvatting van de recente ontwikkelingen in de systemische behandeling van maligne mesothelioom van de pleura.

List of publications

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Curriculum vitae

Maria Johanna Disselhorst was born on June 27th 1981 in Castricum, The Netherlands. She graduated from the gymnasium in Castricum in 1999. After one year of health sciences in Maastricht she started medical school at the VU medical center in Amsterdam in 2000. In 2007 she graduated as a medical doctor.

After a few years working as a resident in internal medicine, cardiology, pulmonary medicine, emergency room and intensive care unit, she started her training as a chest physician at the VU medical center. After finishing this in 2017 she started working as a fellow thoracic oncology at the Antoni van Leeuwenhoek – Nederlands Kanker Instituut, combining clinical work with a PhD trajectory.

In 2020 she started as a chest physician in NoordWest Ziekenhuis groep in Alkmaar, with a focus on thoracic oncology.