ADVANCING IMMUNOTHERAPY STRATEGIES FOR PLEURAL MESOTHELIOMA

Harnessing dendritic cells to turn the tide

Bob Belderbos

Advancing Immunotherapy Strategies for Pleural Mesothelioma

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Advancing Immunotherapy Strategies for Pleural Mesothelioma

Harnessing dendritic cells to turn the tide

Innoveren van de immunotherapeutische behandeling van het pleuraal mesothelioom

Het tij keren met dendritische cel therapie

Proefschrift

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Upside Down ♫
Jack Johnson

“There’s no stopping curiosity”

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1

General introduction

General Introduction

Pleural mesothelioma (PM) is an incurable cancer with limited treatment options, which originates from the mesothelium that lines the pleural cavity and lungs. The median age at diagnosis is 71 and 87% of patients is male. [1] Median overall survival (mOS) varies from 5.7-18.4 months depending on treatment and stage of disease at time of diagnosis.[1,2]

Mesothelium

The mesothelium is derived from the mesoderm, which is one of 3 layers (ectoderm, mesoderm, endoderm) that make up the gastrula during embryonic development.[3] The mesothelium consists of squamous-like cells that form a thin membrane that line several body cavities and organs, such as the pleural cavity and lungs (pleura), abdominopelvic cavity and omenta (peritoneum), heart (pericardium) and testis (tunica vaginalis).[3] The function of the mesothelium is to produce lubricating fluid that enables smooth movement between the cavity and its organs (intracoelomic movement), e.g. the pleural cavity and lungs during breathing.[4] PM makes up for 80-90% of all mesothelioma cases.[5-7]

Etiology

Asbestos

Asbestos is a naturally occurring mineral composed of strong, heat and chemical resistant fibers and there are six types of asbestos, which can be divided into two main categories: amphiboles (straight, long fibers) e.g. crocidolite (blue asbestos) and serpentine (short, curly fibers) e.g. christolyte (white asbestos).[8] Historically, asbestos was widely used in products varying from construction products to fake snow in movies. However, its use became controversial as evidence emerged linking asbestos exposure to significant health risks. Since the 1920's the correlation between asbestos exposure and asbestosis, a form of diffuse pulmonary fibrosis, has been established.[9,10] The first scientific publication showing an association between asbestos exposure and PM dates from 1960.[11] In the Netherlands, dr. Stumphius related asbestos exposure to PM in shipyard working class people in 1969.[12,13] The amount of asbestos exposure correlates positively to the risk of developing PM,[14] but even minimal and brief exposure leads to an increased risk of PM.[15] Exposure to amphiboles induces the highest risk for PM. [16] Occupational asbestos exposure is correlated to PM in 85% of patients but only 4% of people with occupational exposure develop PM. Furthermore, a small proportion of PM is not related to asbestos exposure.[17] Although asbestos exposure does not lead

to PM in most individuals, it increases the risk of developing lung cancer by tenfold and with hundredfold when combined with smoking.[18]

Other causes of PM

Besides the causal relationship between exposure to asbestos and PM, other factors can contribute to PM pathogenesis. One such factor is BRCA1-associated protein 1 (BAP1), a tumor suppressor protein implicated in critical processes, including cell cycle regulation, gene transcription and DNA repair. Germline mutations in BAP1 are associated with the BAP1 cancer syndrome, which confers an elevated risk for PM, uveal melanoma and various other malignancies.[19] Despite the increased risk of developing PM, patients with this mutation tend to have better prognosis, with up to 7 times longer OS.[20] Radiation exposure, including radiotherapy, is known to be carcinogenic. Research has shown that patients with breast cancer who were treated with radiotherapy had an increased risk of developing PM.[21] Finally, exposure to naturally occurring asbestos-like fibers, such as erionite and antigorite, have also been linked to PM.[22] Erionite is a fibrous mineral that is highly present in Cappadocia, a region in Turkey, and is present in the stones used to build houses. In this region the prevalence of malignant pleural mesothelioma is up to 70 times higher than in other regions in Turkey.[23]

Regulations and incidence of asbestos

Despite knowledge on the potential harm of producing and using asbestos since the 1960s, western European countries only started regulating and banning asbestos in the 1980s. In the Netherlands the first regulatory measures were taken in 1977, but only in 1993 a total ban of asbestos was welcomed.[24,25] From 1917 till 1993 770.000 tons of asbestos fibers were imported and produced within the Netherlands, with a peak from 1955 till 1978.[26,27]

Strikingly, the current incidence worldwide is approximately 30.000 per year and not yet declining.[28] This could be due to the fact that many countries (i.e. China, Kazakhstan, Russia) asbestos is still being produced in high numbers.[29,30] Moreover, exposure to naturally occurring asbestos, such as erionite in Turkey, is also a contributing factor to the PM incidence worldwide.[22] Use of asbestos declined since 1977 in the Netherlands as a consequence of regulations called “Het Asbestbesluit” and the median latency time for PM is 40 years. “Het Asbestbesluit” in combination with the latency time would logically result in a decline in the incidence of PM in the Netherlands. However, no decline was reported in the Netherlands so far[27] and environmental exposure is hypothesized as a contributing factor.[22]

The pathogenesis of PM is multifactorial, complex and not fully understood. However, it is known that inhaled asbestos fibers migrate to the pleura via the efferent lymphatic

system. The fibers irritate the pleura with each breath during intracoelomic movement, leading to cycles of tissue damage and repair.[31] The long straight fibers resist clearance by the mesothelial cells, prompting the release of chemokines (e.g. CCL2) to attract macrophages.[32] The macrophages are unable to phagocytize asbestos fibers longer than 5 μm[33], resulting in frustrated phagocytosis and the subsequent formation on an inflammasome, driven by the release of inflammatory cytokines.[34-36] The fiber-induced inflammation causes macrophages to generate reactive oxygen species and reactive nitrogen species that lead to DNA damage, which facilitates malignant transformation. Moreover, the macrophages start producing the cytokine tumor necrosis factor alpha (TNF-α), which inhibits apoptosis and stimulates proliferation of mesothelial cells contributing to tumor development.[37]

Pathology

The current histopathological classification of PM comprises three subtypes: epithelioid consisting of polygonal cells; sarcomatoid consisting of spindle-shaped cells; and biphasic, which is a combination of both epithelioid and sarcomatoid (Fig. 1).[38] Epithelioid PM accounts for approximately 60% of cases and has the most favorable prognosis. In contrast, sarcomatoid PM, accounting for around 20% of cases is linked to the poorest prognosis.[39] Biphasic PM, also contributing to 20% of cases presents an intermediate prognosis, reflecting its mixed histological composition.[1,40]

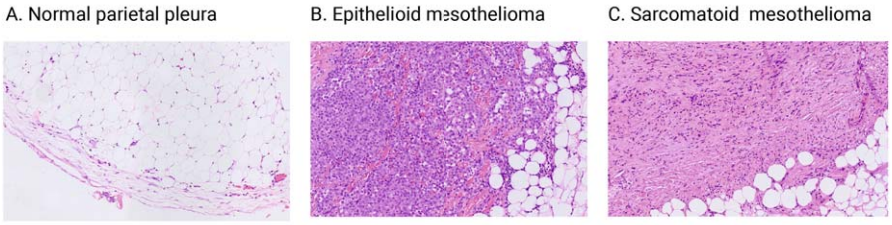

Figure 1. Images of heamatoxylin and eosin (H&E) stained normal pleura (A), epithelioid (B) and sarcomatoid (C) mesothelioma subtypes. *Created in https://BioRender.com*

Immunology in cancer

Since Rudolf Virchow's discovery of leukocytes within tumor tissue in the 19th century, inflammation and carcinogenesis have been linked to each other.[41] The interplay between the immune system and tumors is currently conceptualized as a continuous process of tumor death and immune cell activation, called the cancer immunity cycle (Fig 2.)[42]

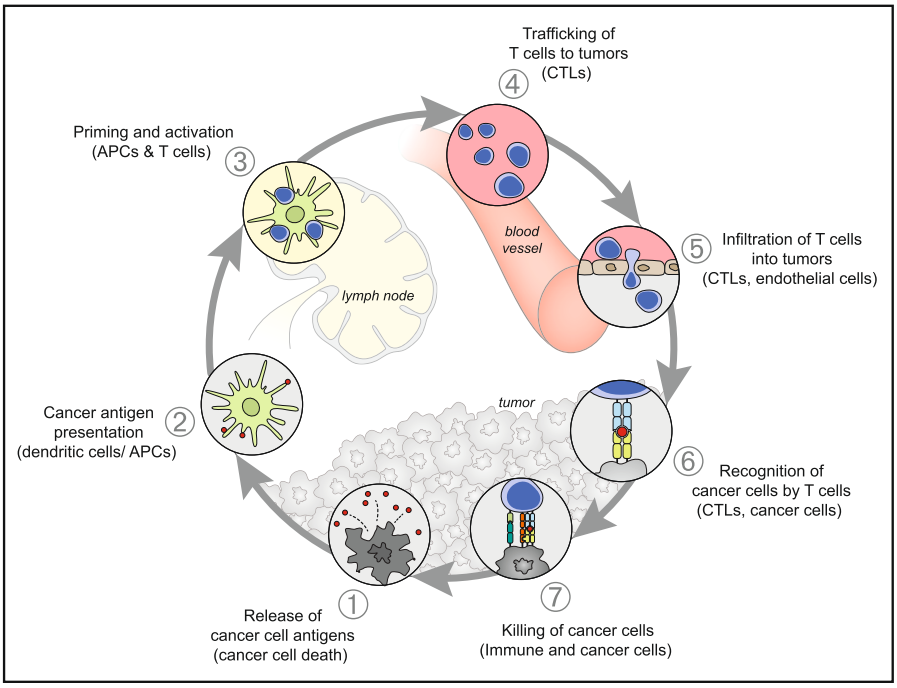

Figure 2. Cancer immunity cycle. *Chen et al.*

During carcinogenesis numerous genetic mutations disrupt normal cellular metabolic processes, which are essential for tumor development. These mutations cause upregulation of tumor-associated antigens (TAAs), including neo-antigens, differentiation antigens or cancer testis antigens on the cell surface. The amount of TAAs determines the tumor's antigenicity and subsequently the potential of an anti-tumor response. The initiation of the cancer immunity cycle starts with immunogenic cell death (ICD) of tumor cells, resulting in the release of tumor-specific antigens (step 1).[43] ICD causes secretion of ATP and high mobility group box 1 (HGMB-1), a nuclear protein that acts as a danger associated protein (DAMP), which induces migration of antigen-presenting cells (APCs), specifically dendritic cells (DCs) towards the tumor[44,45] The apoptotic cancer cells express calreticulin, which works as an engulfment signal for DCs.[46] The DCs will

engulf the TAAs and cross present antigen in major histocompatibility complex class I and II (MHC I and II) molecules (step 2).[47] Next, DCs migrate towards the lymph node and activate naïve CD4+ and CD8+ T cells that recognize antigen presented in MHC II and I, respectively (step 3).[42] For effective T-cell activation, DCs must deliver three critical signals: antigen presentation (signal 1), co-stimulation through surface molecules such as CD40, CD80, CD86 (signal 2) and secretion of pro-inflammatory cytokines like interferon-□ (IFN- □) and IL-12 (signal 3) (Fig. 3).[48]

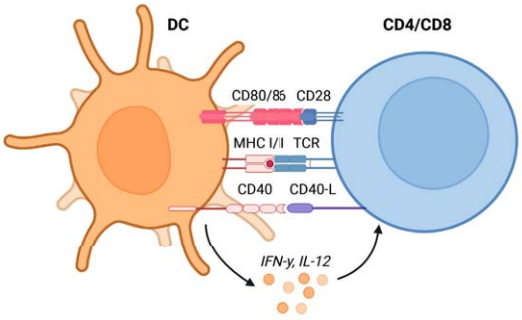

Figure 3. T cell priming. *Created in https://BioRender.com*

Activated T cells will then traffic towards the tumor (step 4), infiltrate (step 5), and execute their cytotoxic functions by producing cytokines such as IFN- □ upon recognizing the TAAs, ultimately leading to the destruction of tumor cells. (step 6,7).[42]

Hampering immune responses

Tumor cells or immunosuppressive immune cells can hamper the anti-tumor immune response during every step of the cancer immunity cycle. Tumors with a low tumor mutational burden (TMB) have a lower antigenicity, leading to a weaker anti-tumor immune response (step 1).[49] In the next two steps (step 2,3), antigen presentation without co-stimulatory signals will result in tolerance[50,51] and stromal barriers can reduce T cell infiltration (step 5).[52] Moreover, immune checkpoint signaling (text box) can reduce T cell activity in the tumor and lymph node (step 3,7).[53,54] Finally, tumors can escape anti-tumor immunity through Human Leukocyte Antigen - Loss of Heterozygosity (HLA-LOH), which is a process where tumor cells lose one or more of their HLA alleles (step 6).[55] HLA molecules are responsible for presenting tumor antigens on the cell surface, allowing T cells to recognize and destroy tumor cells. However, when tumor cells lose their HLA alleles through HLA-LOH, their visibility to tumor-specific T cells reduces.

Box 1.

Immune checkpoints, such as programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4), are molecules expressed on the surface of T cells that regulate the immune response.[56] When these immune checkpoints interact with their respective ligands, such as PD-L1 (programmed cell death ligand 1) expressed on tumor cells and antigen-presenting cells (APCs), they suppress T cell activity, leading to immune evasion by cancer cells. Checkpoint inhibitors (CIs), including antibodies targeting PD-1 and CTLA-4, disrupt these inhibitory interactions, thereby releasing the brakes on the immune system and reinvigorating anti-tumor immunity (Fig. 4). This results in enhanced activation and proliferation of tumor-specific CD8+ T cells, leading to improved tumor control. CI therapy has shown remarkable success in several malignancies, particularly melanoma, renal cell carcinoma, and non-small cell lung cancer (NSCLC), dramatically altering the prognosis of previously considered "untreatable" tumors.

B cell mediated anti-tumor immunity

T cells are known to have an important role in anti-tumor immunity as shown by the paradigm of the cancer immunity cycle. Over the last decade, B cells have emerged as important players in the tumor microenvironment (TME), particularly in the context of tertiary lymphoid structures (TLS).[57-61] In general, B cells can exert anti-tumor immunity through several mechanisms, such as enhancing T cell function, complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity.[62] However, the phenotype and function of B cells is determined by the maturity – presence of germinal centers - of TLS. Immature TLS induce regulatory B cells with immunosuppressive capacities, such as IL-10 production. B cells within mature TLS differentiate into plasma cells and secrete antibodies.[63] The presence of TLS correlates with response to CI therapy in several cancers, including NSCLC.[61] Specifically, memory B cells and plasma cells present in the tumor were found to be correlated with favorable outcome to CI therapy in melanoma and NSCLC.[57,64] In peripheral blood, the number of B cells was also predictive of response to CI therapy in several tumors.[65] In PM, data on B cells and response to CI therapy is scarce.

The immune system in mesothelioma

Step 1: Low TMB and antigenicity potentially impairing anti-tumor immune responses

PM is considered a tumor with a low TMB, based on next generation sequencing (NGS) data. Median TMB in PM was 1.7 mut/Mb compared to 8.8-9.7 mut/Mb in NSCLC, which is considered a high TMB tumor.[66,67] The low TMB can result in low expression of neoantigens[68] and thus low antigenicity and immunogenicity of the tumor, leading to poor anti-tumor immunity. In contrast, analysis of the TMB with more detailed sequencing (mate-pair sequencing) instead of NGS showed a relative high occurrence of inter- and intra- chromosomal rearrangements.[69,70] However, neoantigen formation from these genomic alterations has not yet been proven.[19] In conclusion, the TMB of PM might be higher than previously described, but it is still unclear if this leads to higher antigenicity with a suitable anti-tumor immune response.

Step 2,3: Impaired function of DCs

Similar to the immunogenicity of tumors, DCs are crucial for the induction of an anti-tumor immune response. All major DC subtypes (type 1 conventional DC (cDC1), cDC2 and plasmacytoid DC (pDC)) in PM are lower in numbers compared to healthy controls, which could hamper the anti-tumor immune response.[71]

Step 6,7: Function of anti-tumor T cells and evasion by tumor cells

Tumor-infiltrating lymphocytes (TILs) are crucial for anti-tumor immunity and thus response to CI therapy. However, reports on the correlation between the number of TILs and response to CI therapy have been inconsistent in PM. This variability may be attributed to the inter-[70,72,73] and intra-tumoral[74] heterogeneity in both the extent of immune cell infiltration and the composition of the immune cell infiltrate. The heterogeneity is illustrated by the reported percentage of immune cell infiltration ranging from 17.6% to 99.8%, with the proportion of T cells within this infiltrate varying between 5.2% and 81.2%.[73]

The lack of correlation between response to CI therapy and the number of TILs may also be due impaired functionality of TILs. Cytotoxic CD8+ T cells can enter a state of exhaustion, characterized by reduced effector functions and sustained expression of inhibitory receptors, such as PD-1, TIM-3, and LAG-3. This exhaustion, driven by persistent antigen exposure, chronic inflammation, or factors derived from the tumor microenvironment, impairs the ability of these T cells to mount effective anti-tumor responses.[75,76] Exhausted CD8+ T cells are more abundant in PM tissue as compared to tissue from patients with pleuritis.[77] Moreover, T cells develop an exhausted phenotype during tumor progression in mesothelioma mouse models and subsequently produce less cytokines, such as IFN-□.[78] The importance of functionality of T cells is supported

by the fact that the presence of highly functioning T cells (effector memory CD8+ T cells) correlates with response to CI combination therapy (anti-PD-1 and anti-CTLA-4).[79]

Tumor cells can evade T-cell cytotoxicity either through upregulation of PD-L1 and/or through HLA-LOH. PD-L1 expression in PM varies from approximately 20-80%[70] and is increased in tumors with high numbers of T cells.[73,80] Furthermore, HLA-LOH is more frequent in PM tumors with high CD8+ T-cell infiltration and subclonal antigen load, causing worse survival.[81] Thus, even when functional CD8+ TILs are present, mechanisms such as PD-L1 upregulation and HLA-LOH enable PM tumor cells to escape anti-tumor immunity (Fig. 4).

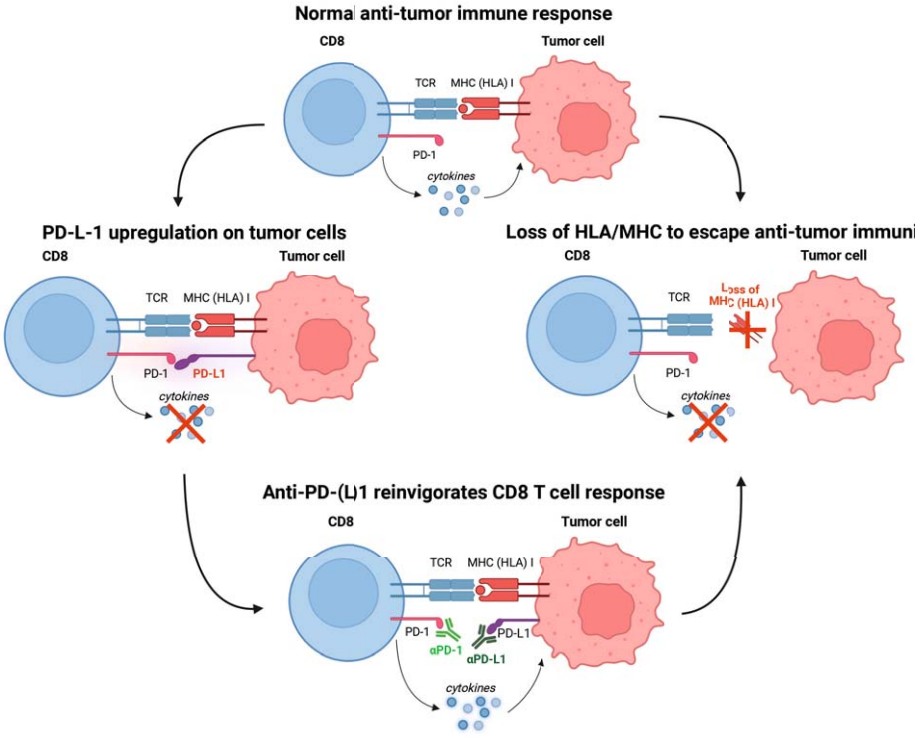

Figure 4. Escape mechanisms in PM and working mechanism of anti-PD-(L)1. *Created in https://BioRender.com*

Immune cells in the tumor micro environment that hamper anti-tumor immunity

Tumor-associated macrophages (TAMs)

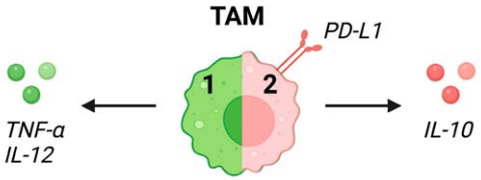

Figure 5. Tumor associated macrophages can be divided into type 1 (pro-inflammatory, secreting TNF-α and IL-12) and type 2 (immunosuppressive, secreting IL-10 and PD-L1 inhibitory signaling) macrophages. *Created in https://BioRender.com*

Tumor-associated macrophages (TAMs) represent 20-30% of the immune infiltrate in the TME. [72] Classically, TAMs are categorized into two subtypes: pro-inflammatory M1 TAMs and immunosuppressive M2 TAMs (Fig. 5). [70] Although recent studies indicate that some TAMs can simultaneously produce both pro-inflammatory cytokines (TNF-α) and immunosuppressive cytokines (IL-10), the dichotomy between pro- and anti-inflammatory TAMs is often used for clarity.[82] M2 TAMs enable tumor growth through secretion of IL-10 and TGF-b. In addition, 95% of TAMs express PD-L1[72] allowing them to inhibit T-cell cytotoxicity. High numbers of M2 TAMs are correlated to poor OS in epithelioid PM. Moreover, preclinical data shows that depletion of TAMs leads to increased OS and less TIL exhaustion.[83]

Regulatory T cells (Treg)

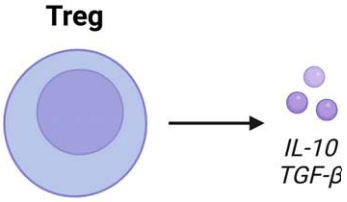

Figure 6. Regulatory T cells can hamper anti-tumor immunity through secretion of immunosuppressive cytokines, such as IL-10 and TGF-β. *Created in https://BioRender.com*

In PM tumors 12.8% of CD4+ T cells are regulatory T cells (Tregs), whereas the fraction of Tregs in donor lung tissue was only 2.2%.[72] Just like TAMs, Tregs can impede anti-tumor immunity through secretion of IL-10 and TGF-β or through CI signaling (CTLA-4) (Fig. 6).[84] Tregs can even suppress DCs and thereby hamper induction of an anti-tumor immune response.[84,85] Moreover, the amount of Tregs is negatively correlated

to IFN-g production by TILs.[72] It has been demonstrated that Treg gene expression is higher in neo-antigen rich, immunogenic tumors, which have generally more CD8+ T-cell infiltration.[86] Furthermore, intra-tumoral abundance of Tregs is correlated to worse OS,[77,87,88] and depleting Tregs leads to reduced tumor growth in preclinical PM models.[89,90]

Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells with immunosuppressive capacities. Besides TAMs, MDSCs are one of the most abundant immune cells in the TME. MDSCs comprise two subsets: monocytic MDSC (mMDSC) and polymorphonuclear MDSC (pMDSC).[91] Monocytic MDSCs tend to be more immunosuppressive than pMDSCs, as they are capable of both antigen-dependent and antigen-independent inhibition of T cell responses.[92] Via enzymatic processes, MDSCs can ultimately cause T cell depletion and inhibition of T cell function.[93] Moreover, MDSCs can attract Tregs through chemokine signaling (CCL4 and CCL5), secretion of IL-10[94] and upregulation of PD-L1 on their cell surface (Fig. 7).[95] Both pMDSCs and mMDSCs are correlated to worse OS and progression-free survival in PM.[77]

Myeloid derived suppressor cells (MDSC)

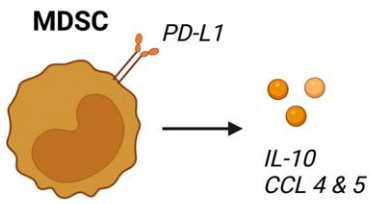

Figure 7. Myeloid-derived suppressor cells can hamper anti-tumor immunity through secretion of immunosuppressive cytokines, such as IL-10 and CCL4 and CCL5, and inhibitory signaling via PD-L1. *Created in https://BioRender.com*

Current therapies

This section will highlight the historical development of FDA-approved treatments, specifically chemotherapy and immunotherapy, and will also address the current role of surgical intervention in the management of PM. Although extensive research is being conducted on emerging therapeutic modalities for PM, including targeted therapies, T-cell therapy, vaccine therapies, and virotherapies, the scope of this thesis will be limited to the exploration of DC therapy as a developmental treatment option for PM.

Systemic therapy

For over 20 years combination chemotherapy consisting of platinum and pemetrexed, has been the first-line treatment for PM, improving survival to 12 months from start

of therapy.[96] Since CI therapy revolutionized treatment outcomes in NSCLC and melanoma,[97-99] many trials have been performed with either anti-CTLA-4 or anti-PD-1 antibodies.[100-107] All phase III trials with CI monotherapy have failed to show efficacy in PM,[100,108,109] although a very small amount of patients did seem to benefit from CI therapy.[110] Results from the Checkmate 743 study did show efficacy of combining anti-CTLA-4 and anti-PD-1 antibodies in PM, especially in non-epithelioid PM where response to chemotherapy is notoriously bad.[2] Median OS for patients receiving combination CI therapy was 18.1 months, whereas OS for patients receiving chemotherapy was 14.1 months.[2] These results let to the approval of combination CI therapy as first-line therapy for PM.

Surgery

Approximately 10-15% of PM patients with early-stage PM are eligible for surgery, but "radical" surgery does not necessarily offer curative potential and there is a high rate of disease recurrence. Therefore, the role of surgery in early-stage PM is a matter of controversy. Combining systemic treatment with surgery improves recurrence-free survival compared to surgery alone and therefore current consensus in Europe is that surgical treatment should only be performed as part of a multimodality approach in a clinical trial setting.[111]

Two surgical techniques for complete macroscopic resection of the visceral and parietal pleura are available: extrapleural pneumonectomy (EPP) and extended pleurectomy/decortication (eP/D) (Fig (8).

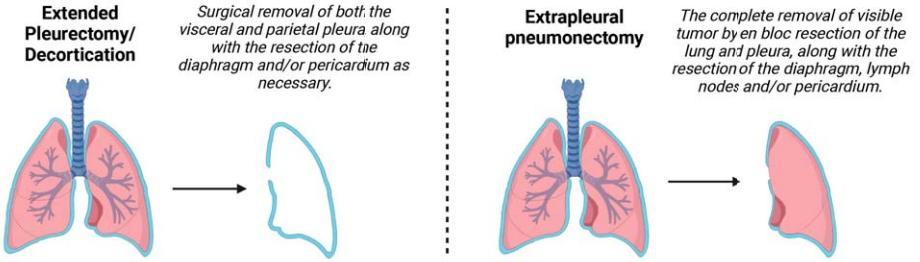

Figure 8. Showing the difference in two different surgical treatment modalities in PM; Extended pleurectomy/decortication (left), Extrapleural pneumonectomy (right). *Created in https://BioRender.com*

Previously, EPP was considered the standard choice for eligible patients, but the procedure carries a high risk of perioperative complications and mortality.[112] In addition, there is no significant survival difference between EPP and eP/D, leading most mesothelioma centers to shift towards eP/D.[113-115] The mOS for eP/D ranges from 10.4 to 32 months, surpassing historical data with systemic therapy alone.[112] However, this comparison is probably biased as patients are selected on high-performance status,

low tumor stage, and limited comorbidities for surgical treatment. In conclusion, eP/D is currently the preferred type of surgery for PM and should be used in a multimodality approach. However, the potential for suboptimal outcomes and negative impact on quality of life[116] necessitates the identification of patients who would derive optimal benefits from surgery.

Extensive research has identified prognostic factors for PM that include histology, sex, age, TNM stage, performance score, weight loss, and peripheral blood values (e.g., albumin, CRP). Prognostic score models such as the European Organization for Research and Treatment of Cancer (EORTC) score and modified Glasgow Prognostic score (mGPS) are validated for PM.[117-119] While the EORTC score has been validated in surgically treated PM patients, it has not reliably identified patients who would benefit from surgery. Tumor-related parameters like nuclear atypia, mitotic rate, necrosis, BAP1 loss, and Ki67 expression also have prognostic value.[120-127] Ki67, a marker of proliferation, is used in peritoneal mesothelioma to predict outcomes and guide treatment decisions. [128] However, its role as a predictive marker in surgery for PM patients remains unvalidated.

DC therapy

DCs are the most potent APCs and are capable of activating tumor-specific CD4+ and CD8+ T cells that can potentially infiltrate the TME. As discussed previously, DCs are low in numbers and impaired in function in PM.[71] With DC therapy, monocyte-derived DCs (moDCs) can be activated and loaded with TAAs *in vitro*, circumventing this potentially hampered process *in vivo.*[129]

After preclinical studies proved the efficacy of DC therapy in mesothelioma mouse models, clinical studies were done. In the initial phase 1 clinical trial (MM01) evaluating autologous tumor lysate loaded moDC therapy, 10 patients with PM received at least three biweekly vaccinations.[129,131] The response rate in this trial was 33% and the mOS from the time of diagnosis was 19 months.

In the subsequent trial (MM02), low-dose cyclophosphamide was incorporated to deplete Tregs and enhance the effectiveness of DC therapy.[130,133] Again 10 patients were treated and 3 patients received additional eP/D. Of the remaining 7 patients, 1 patient achieved a complete response (CR), 4 had stable disease (SD), and 2 exhibited progressive disease (PD) as best overall response (BOR). Furthermore, cyclophosphamide treatment effectively depleted Tregs, During the MM01/MM02 trials no treatment-related grade III/IV toxicities were observed.

Box 2.

It takes 10 days to produce activated and TAA-loaded moDC from monocytes. Monocytes are collected from a patient during leukapheresis. On day 2, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL-4) are added to the culture to induce differentiation into immature moDCs. MoDCs are then loaded with tumor lysate on day 5 and are activated and matured with IL-1β, IL-6, TNF-α and prostaglandin E2. On day 10 the matured, activated and TAA-loaded moDCs can be harvested. Two phase I studies on DC therapy in PM used autologous tumor lysate, which was derived from patient's tumor material or pleural fluid.130,131 In the third trial, allogeneic tumor lysate was used, for this is more feasible and consistent.132 Five PM patients with varying histological subtypes and TAAs were selected for the generation of 5 tumor cell lines. The allogeneic tumor lysate (PheraLys) is created from 4 of these cell lines and moDC therapy created with PheraLys is called MesoPher. One dose of MesoPher roughly consists of 25 million moDCs of which 2/3 is injected intravenously and 1/3 is injected intradermally.

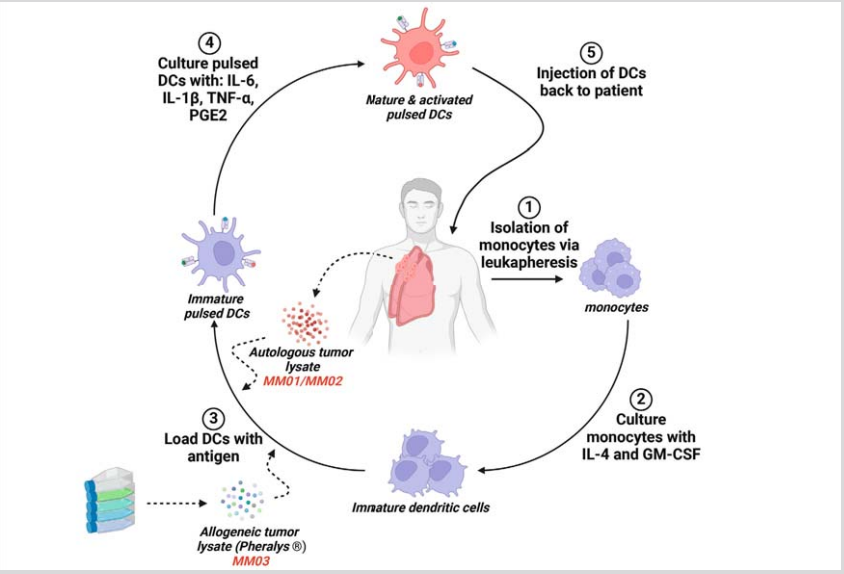

Figure 9. Production process of MesoPher, dendritic cell therapy. *Created in https://BioRender.com*

Regrettably, the utilization of autologous tumor material for generating tumor lysate poses challenges in large-scale phase II/III trials due to the inconsistent quality and limited availability of tumor samples. Loading moDCs with allogeneic tumor lysate, serving as an 'of-the-shelf' source for antigen-loading material, was compared to

autologous tumor lysate-loaded moDC therapy in mice, and induced similar protection against tumor outgrowth.

In the following trial (MM03), the efficacy of allogeneic tumor lysate-loaded moDCs (MesoPher) was assessed in 9 PM patients.[132] Among these patients, two individuals achieved a partial response (PR), and two patients have demonstrated remarkable long-term survival, with a duration of four years since the initiation of treatment. Notably, the administration of MesoPher did not result in any grade III/IV toxicities. Table 1 shows a comprehensive overview of the three phase I trials.[134]

Table 1. overview of phase 1 trials with dendritic cell therapy

Tumor lysate	Additional therapy	n	Outcome	Reference
Autologous	None	10	3PR, 1SD, 6PD mOS 15 months	Hegmans et al. 2010
Autologous	Ctx	10	1CR, 4SD, 2 PD* mOS 26 months	Cornelissen et al. 2016
Allogeneic	None	9	2PR, 7SD mOS 26.7 months	Aerts et al. 2019

*Three patients underwent eP/D and therefore evaluating radiological response to DC therapy was not possible.

Aims and Outline of this thesis

Given that the latency period for PM is approximately 40 years, asbestos regulations introduced in the Netherlands starting from 1977 may eventually reduce the incidence of PM. However, the most recent report on PM incidence in the Netherlands has not yet shown a decline. In **Chapter 2**, we analyzed the latest data on PM treatment, survival, and incidence, and discuss the impact that asbestos regulations have had in the Netherlands.

The main aim of this thesis entails the improvement of immunotherapy in PM with a focus on DC therapy. Immunotherapy, particularly CI therapy, has revolutionized treatment for NSCLC. However, CI monotherapy has not proven effective for PM in phase III trials, despite promising results in phase I/II trials. In **Chapter 3**, we analyzed the effect of nivolumab (anti-PD-1) in PM within a real-world setting, and investigated potential biomarkers for response, as some patients appear to derive significant clinical benefit from CI therapy. Given the ongoing need to understand the response to CI therapy and the emerging role of B cells in anti-tumor immunity, we looked into different B cell subsets in the peripheral blood of NSCLC and PM patients using flow cytometry (**Chapter 4**). This data clarifies whether specific B cell subsets are associated with a

response to CI therapy, potentially enhancing our understanding of the mechanisms behind the lack of response to CI therapy.

The results of an international, randomized phase III trial (DENIM) comparing DC therapy (MesoPher) as maintenance therapy to best supportive care (BSC) after first-line chemotherapy in PM are presented in **Chapter 5**. The primary endpoint of this study was mOS and peripheral blood immune monitoring is conducted to evaluate the immunological effects of DC therapy, potentially correlating with survival outcomes.

In **Chapter 6**, we explored the mechanisms through which the tumor microenvironment (TME) may impede the effectiveness of DC therapy, as well as how other conventional treatments might synergize with DC therapy. One such potentially synergistic combination is DC therapy with CI therapy. In **Chapter 7**, we analyzed this combination in a preclinical mouse model, incorporating extensive immunomonitoring of blood, tumor, and spleen, and translated these findings through a retrospective analysis of patients treated with DC therapy followed by CI therapy.

In preclinical models, low tumor load prior to DC therapy enhances survival, and reduction of tumor load can increase the feasibility of surgery. MesoPher has demonstrated the ability to induce a T cell response and reduce tumor burden in PM patients. Therefore, we initiated a phase I trial combining (neo)adjuvant DC therapy with extended pleurectomy/decortication (eP/D) in resectable PM. Tumor material will be collected before and after DC therapy, allowing for the evaluation of DC therapy-induced changes within the tumor. The results of the first patient in this trial are presented in **Chapter 8**.

Unfortunately, recurrence of disease is quite frequent in patients undergoing eP/D, In addition surgery can decrease quality of life and even negatively impact clinical outcome, which necessitates the identification of patients that do benefit from surgery. In **Chapter 9**, we retrospectively analyzed data from 27 PM patients treated with eP/D at Erasmus MC to identify biomarkers associated with clinical benefit.

Finally, We discussed the results of this thesis in the context of recent literature and suggests potential directions for future research in **Chapter 10**.

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Incidence, treatment, and survival of malignant pleural and peritoneal mesothelioma: A population-based study

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Abstract

Introduction
Malignant mesothelioma (MM) is an aggressive cancer that primarily arises from the pleura (MPM) or peritoneum (MPeM), mostly due to asbestos exposure. This study reviewed the Dutch population-based incidence, treatment, and survival since the national ban on asbestos in 1993.

Materials and methods
Patients with MPM or MPeM diagnosed from 1993-2018 were selected from the Dutch cancer registry. Annual percentage change (APCs) was calculated for (age- and sex-specific) Revised European Standardized incidence Rates (RESR). Treatment pattern- and Kaplan-Meyer overall survival analyses were performed.

Results
In total, 12,168 patients were included in the study. For male patients younger than 80 years, MM incidence significantly decreased in the last decade (APC ranging between -9.4% and -1.8%, $p<0.01$). Among both male and female patients aged over 80 years, incidence significantly increased during the entire study period (APC 3.3% and 4.6% respectively, $p<0.01$). From 2003 onwards, the use of systemic chemotherapy increased especially for MPM (from 9.3% to 39.4%). Overall, 62.2% of patients received no anti-tumour treatment. The most common reasons for not undergoing anti-tumour treatment were patients' preference (42%) and performance status (25.6%). Median overall survival improved from 7.3 (1993-2003) to 8.9 (2004-2011) and 9.3 months from 2012-2018 ($p<0.001$).

Conclusion
The peak of MM incidence was reached around 2010 in the Netherlands, and currently incidence is declining in most age groups. The use of systemic chemotherapy increased from 2003, which likely resulted in improved overall survival over time. The majority of patients do not receive treatment, though, and prognosis is still poor.

Key messages

What is the key question?
How did the 1993 national ban on asbestos affect malignant mesothelioma incidence, and how did therapeutic advances affect mesothelioma prognosis in the Netherlands?

What is the bottom line?
Malignant mesothelioma incidence has peaked about ten years earlier than predicted, after the Dutch national ban on asbestos. While treatment advances have led to somewhat better survival, prognosis is still dismal.

Why read on?
These findings show that asbestos regulation leads to a decreasing mesothelioma incidence sooner than earlier predicted, thereby supporting the notion that malignant mesothelioma cases can for the most part be effectively prevented. The treatment patterns and survival outcomes we observed in over 12,000 mesothelioma patients, highlight the persistent need for better mesothelioma therapeutics.

Introduction

Malignant Mesothelioma (MM) is a highly lethal tumour that primarily arises from the pleura and, to a lesser extent, from the peritoneum. In sporadic cases, it originates from the pericardium or tunica vaginalis testis.[1] Malignant pleural mesothelioma (MPM) represents over 90% of all MM cases. MPM prognosis is very poor, with a median overall survival (OS) of approximately one year when treated with chemotherapy.[2] Recently, combination checkpoint inhibition (CI) therapy, consisting of nivolumab (anti-programmed death-ligand 1 (PD-1)) and ipilimumab (anti-Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA4)), has been shown to increase overall survival in MPM patients compared to standard first-line chemotherapy (median OS 18.1 vs. 14.1 months).[3] As a result, the FDA approved the combination of nivolumab and ipilimumab as first-line treatment for unresectable MPM.[4] For malignant peritoneal mesothelioma (MPeM), the median overall survival is about 6 months.[5] For a long time, treatment options were identical to those for MPM. Since 2009, several studies have demonstrated that long-term survival can be achieved in selected MPeM patients treated with cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC).[6-8]

The main risk factor for MPM development is exposure to asbestos.[9,10] This correlation is less prominent in MPeM. The definitive relation between asbestos exposure and MM was proven in 1960 by Wagner et al.[11] Despite this discovery, production of asbestos peaked worldwide between 1970 and 1980 with a production of more than 4,000,000 metric tons per year. The use of asbestos in the Netherlands also peaked in these years. Its use declined in the following decade due to new regulations, but the ban on asbestos in the Netherlands was finally realized in 1993. Countries, such as Russia, Kazakhstan, and China, are still mass producers of asbestos to this day, and others have only recently banned its use.[12,13] Predicting the future incidence of MM is difficult due to the considerable variation in latency time (i.e. the time between asbestos exposure and MM diagnosis) that has been reported, varying between 10 years and over 50 years.[12,13] In the Netherlands, a peak in MM mortality was expected around 2020.[14,15] The current population-based study reviews national MM incidence 25 years after the nationwide asbestos ban. This could aid others in predicting MM incidence after the implementation of asbestos regulations. Concurrently, it aims to identify patterns in incidence, MM characteristics, treatment, and survival for both MPM and MPeM.

Methods

Data collection

Patients diagnosed with MM between 1993 and 2018 were identified in the Netherlands Cancer Registry (NCR) by searching for cases with ICD-O codes 8000-8005, 9050-9053, and 9990 located in the pleura (C38.4) or peritoneum (C48.2) (International

Classification of Diseases for Oncology, 3rd edition, 1st revision). Incidence rates were available from 1989 to 2018. Data were extracted after the approval of the NCR Monitoring Committee and the NCR Scientific Committee on Pulmonary Oncology. Data were handled in accordance with the latest European privacy regulations (General Data Protection Regulation (GDPR), EU 2016/679). Data on all patients diagnosed with cancer in the Netherlands are collected by the NCR. De Boer et al. previously described their methods.[5] In short: the NCR identifies cancer patients by using the Dutch Pathological Anatomical National Automated Archive (PALGA) and the National Registry of Hospital Discharge Diagnoses. Specialized personnel collect information on diagnosis, stage of the disease, and treatment from medical records. Information on vital status is updated annually through the National Municipal Personal Records Database. Vital status was updated to February 1st, 2020. Data were extracted and provided to the investigators by trained personnel that were not part of the study team.

The tumour stage was registered for all patients. For MPM, the TNM classification current at the moment of diagnosis was used.[16] For MPeM, the Extent of Disease (EOD) coding, according to the Surveillance Epidemiology and End Results (SEER), was used.[17] The EOD coding stratifies the tumour stage into local, regional, and distant progression. It is used by the NCR when no prevailing staging system is available. The location of metastases was specified from 2008 onward. The cause of death was unavailable due to privacy regulations.

Incidence Analyses

Incidence rates from 1989 until 2018 were analysed. Age and sex-specific rates were calculated using the revised European standard rate (RESR) (Eurostat 2013, ISSN 1977-0375). The Surveillance Epidemiology and End Results (SEER) 'Joinpoint Regression Program' (version 4.8.0.1, April 2020) (IMS, Inc. under contract for the National Cancer Institute, Bethesda, MD, USA) was used to identify trends in incidence.[18] This software fits the simplest regression model to incidence rates, thereby identifying trend-breaks or so-called 'joinpoints.' The number of joinpoints is determined by the use of the Monte-Carlo permutation test. Annual percentage changes (APCs) were calculated by the software by fitting a log-linear regression model to the data, using the natural logarithm of the incidence rates with the year of diagnosis as independent variable. APCs were calculated over the segment between two joinpoints or over the entire period when the number of joinpoints was zero.

Treatment Pattern Analyses

Treatment was stratified into four main categories for trend analyses: systemic chemotherapy, surgery, radiotherapy, and best supportive care (BSC). Surgery included several procedures, such as debulking, cytoreductive surgery, decortication, and extra-pleural pneumonectomy. Other treatment categories were targeted therapy and immunotherapy, including the angiogenesis inhibitor bevacizumab, tyrosine kinase

inhibitors (not further specified in the data), and checkpoint inhibitors comprising the anti-PD(L)-1 and anti-CTLA-4 agents nivolumab, pembrolizumab, and ipilimumab. Treatment strategies were reviewed per year for MPM and per five years for MPeM. From 2015 onward, the reason for not undergoing anti-tumour therapy was recorded.

Survival Analyses

To define survival trends, data were stratified into three time periods. The first period included cases diagnosed from 1993 until 2003, the second period reached from 2004 until 2011, and the third period comprised cases diagnosed from 2012 up to 2018. The year 2003 was deliberately chosen, as Vogelzang et al. published the results of their phase III trial on combination chemotherapy with cisplatin and pemetrexed in that year.[2] The following years were divided into two equal periods to assess if survival had improved since. To compare survival between MPM patients diagnosed at different stages of disease, only data from 2008-2018 were used, as the new and improved 7th edition of the TNM staging manual was published in 2007. Stage of disease was not compared for MPeM, as there is no widely used staging system. Kaplan-Meyer overall survival curves were also drafted for different treatment modalities (i.e. chemotherapy, surgery and 'best supportive care') to illustrate survival for these different groups, but were deliberately not compared statistically as these outcomes are heavily influenced by (selection) bias.

Statistical analyses

Continuous variables are shown as median with interquartile range (IQR) and were compared with the independent samples median test. Categorical variables are given as numbers with percentages and were compared with the chi-squared test. Survival analyses were performed by use of the Kaplan Meyer method. Survival between groups was compared with the log-rank method in case of proportional hazards or generalized Wilcoxon in case of non-proportional hazards. Two-sided p-values smaller than 0.05 were considered statistically significant. Statistical analyses were performed with 'Statistical Package for Social Sciences' (SPSS), version 25.0.0.1 (IBM Corporation, Armonk, NY, USA) and R version 4.0.2 (http://www.r-project.org). Incidence rates were calculated with SAS version 9.4.

Results

Patient and Tumour Characteristics

There were 12,168 MM patients identified in the NCR between 1993 and 2018. This comprised 11,539 (94.8%) cases of MPM and 629 (5.2%) cases of MPeM. A comprehensive overview of patient and tumour characteristics at the time of diagnosis is provided in table 1. Generally, patients with MPeM were younger (median age 69 [61 – 76] versus 71 [64 – 77] years, p=0.004). The MPM group comprised more male patients (87.4%

versus 72.3%, p<0.001). Furthermore, there were more cases with epithelioid subtype among MPeM patients (88% versus 76.2%, p<0.001). An attempt to detect trends in the occurrence of histological subtypes over time failed due to the lack of pathological registration data. It did reveal, though, that subtypes have been increasingly specified by pathologists over time, from 46.7% of cases in the first ten years of the study period to 83% of cases in the last ten years (supplementary figure 1). Different staging systems were used for both tumour types and could therefore not be compared between MPM and MPeM. Also, an attempt to determine trends for stage of disease at diagnosis failed due to the large number of cases with unknown stage. This analysis revealed that the use of staging has slightly increased during the study period, but still for about one-third of patients no stage is recorded (supplementary figure 2).

Table 1. Patient and Tumour Characteristics

	MPM (n=11,539)	**MPeM** (n=629)	**Total** (n=12,168)	**p-value**
Age (median, IQR)	71 [64 – 77]	69 [61 – 76]	71 [64 – 77]	0.004
Male (n, %)	10,085 (87.4)	458 (72.3)	10,543 (86.6)	<0.001
Histology (n, %)*				
Epithelioid	6,061 (76.2)	368 (88)	6,429 (76.8)	<0.001*
Sarcomatoid	1,149 (14.5)	18 (4.3)	1,167 (13.9)	
Biphasic	739 (9.3)	32 (7.7)	771 (9.2)	
Unknown	3,590	211	3,801	
Side (n, %)*				
Left	4,499 (40.4)	-	-	
Right	6,631 (59.6)	-	-	
Unknown	409	-	-	
Stage (TNM) (N, %)*				
I	2,840 (37.2)	-	-	
II	918 (12)	-	-	
III	1,971 (25.8)	-	-	
IV	1,897 (24.9)	-	-	
Unknown	3,913	-	-	
Stage (EOD) (N, %)*				
1. In situ	-	0	-	
2. Local Disease	-	190 (40.8)	-	
3. Contiguous/invasive	-	123 (26.4)	-	
4. Regional lymph nodes	-	22 (4.7)	-	
5. Regional progression	-	12 (2.6)	-	
6. Distant progression	-	119 (25.5)	-	
Unknown	-	163	-	
Metastases (n, %)**	610 (10.6)	54 (18.6)	664 (11)	<0.001
Multiple metastatic sites	149 (2.6)	11 (3.8)	160 (2.6)	0.021

Table 1. Patient and Tumour Characteristics. (continued)

	MPM (n=11,539)	**MPeM** (n=629)	**Total** (n=12,168)	**p-value**
Most common metastatic sites (n,%)**				
Lung	163 (2.8)	7 (2.4)	170 (2.8)	
Lymph node	159 (2.8)	11 (3.8)	170 (2.8)	
Bone	135 (2.3)	-	135 (2.2)	
Liver	73 (1.3)	13 (4.5)	86 (1.4)	
Peritoneum	79 (1.4)	5 (1.7)	84 (1.4)	
Soft tissue	59 (1)	3 (1)	62 (1)	
Adrenal glands	52 (0.9)	1 (0.3)	53 (0.9)	
Pleura	12 (0.2)	15 (5.2)	27 (0.4)	
Cutaneous	12 (0.2)	-	12 (0.2)	
Brain	15 (0.3)	-	15 (0.2)	

MPM= Malignant Pleural Mesothelioma, MPeM= Malignant Peritoneal Mesothelioma, TNM= Tumour, Node, Metastases classification. EOD= Extent Of Disease classification. *Percentage and p-value based on known cases. **Metastases were registered nationwide from 2008 onward, percentages based on 6,038 cases (MPM n=5,757, MPeM n=291).

Incidence

During the study period, MM incidence for both sexes combined ranged between 2.6 – 4.1 cases per 100,000 person-years (RESR). Incidence significantly increased with 1.6% annually (95%CI 1 to 2.1) up to 2010, after which a non-significant decrease, or plateau, was observed of -1.7% annually (95%CI -3.9 to 0.6).

For MPM, the incidence was increasing with a rate of 1.8% per year (95%CI 1.1 to 2.5) from 1989 to 2007, thereby ranging between 2.4 and 3.5 cases per 100,000 person-years (RESR). From 2007 onward, a non-significant decrease was observed (APC= -1% (95%CI -2.5 to 0.4)), with rates ranging between 3.3 and 3.8 cases per 100,000 person-years (RESR) (figure 1).

For MPeM, about 0.15 – 0.25 cases per 100,000 person-years (RESR) were reported annually during the entire study period (figure 1), no significant trend was observed APC= -0.5 (95%CI= -1.4 to 0.4). Male incidence ranged between 0.2-0.5 cases per 100,000 person-years (RESR), whereas female incidence ranged between 0-0.2 cases per 100,000 person-years (RESR).

Analyses of sex- and age-specific incidence rates were performed for both MPM and MPeM combined, as separate age-specific rates were not available for MPeM only. For males, significantly decreasing MM incidence rates were observed for all age groups except for patients older than 80 years, for whom incidence increased during the entire study period, with an average APC of 3.3% (95%CI 2.5 to 4.1) (figure 2). For males between 0-54 years, there was an average decrease of 6.2% per year between

1989-2018 (95%CI -7.2 to -5.2). For males between 55-64 years, significantly declining incidence was observed between 2009-2018 (APC=-9.4% (95%CI -13.1 to -5.4)). For males aged 65-79, there was a significant increase between 1989-2006 (APC=3.4% (95%CI 2.3 to 4.5)), after which a significant decrease was observed between 2006-2018 (APC=-1.8% (95%CI -3.5 to -0.1)). For females younger than 65 years, no significant trends in MM incidence were observed. For females aged 65 years or older, a significant increase in incidence was observed over the entire study period (APC for 65-79 years= 1.8% (95%CI 0.9 to 2.6), APC for 80 and older=4.6% (95%CI 2.2 to 6.9).

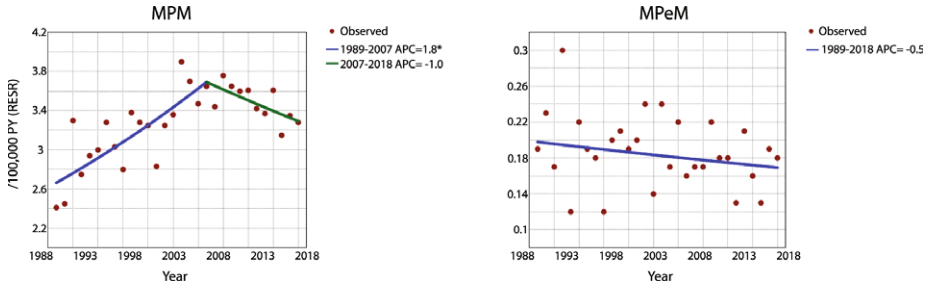

Figure 1. Malignant pleural and peritoneal mesothelioma incidence per 100.000 person years (PY) (RESR) between 1989 and 2018 in the Netherlands. APC=Annual Percentage Change. *Indicates that the APC is significantly different from zero at the alpha=0.05 level.

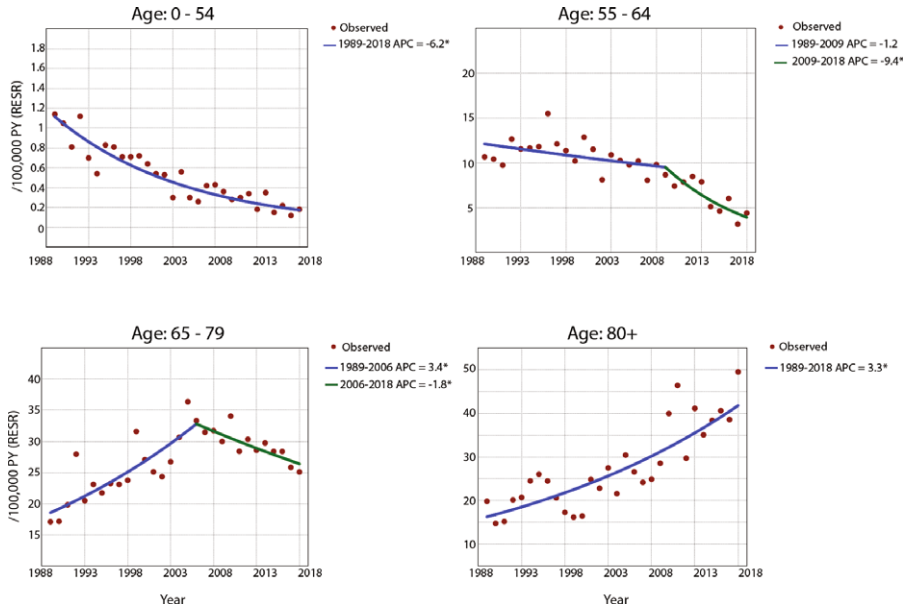

Figure 2. Age specific incidence rates for male patients with malignant mesothelioma (both pleural and peritoneal mesothelioma), per 100,000 person years (PY) (RESR). APC=Annual Percentage Change. *Indicates that the APC is significantly different from zero at the alpha=0.05 level.

Treatment

A comprehensive overview of treatment modalities is provided in table 2. From 1993 until 2018, the majority of MM patients (62.1%) received 'best supportive care' (BSC). From 2015, the reason for not undergoing anti-tumour treatment was registered for 898 patients. For most cases, this was due to the patient's preference (42%) or because of performance status (25.6%). Chemotherapy was given to 28.1% of MM patients, radiotherapy was given in 8.8% of patients, and 3.4% of patients received a type of surgery. No information was registered whether a macroscopically complete resection was the intended goal of the surgery or whether this was achieved. In total, 4.2% of MM patients were treated with a multimodality approach. Targeted therapy was used in 74 cases (0.6%) and immunotherapy in 55 (0.5%).

Table 2. Treatment

	MPM (n=11,539)	**MPeM** (n=629)	**Total** (n=12,168)
All (n, %)*			
Chemotherapy	3,273 (28.4)	142 (22.6)	3,415 (28.1)
Surgery	343 (3.0)	65 (10.3)	408 (3.4)
Radiotherapy	1,072 (9.3)	4 (0.6)	1,076 (8.8)
Immunotherapy	52 (0.5)	3 (0.5)	55 (0.5)
Targeted therapy	72 (0.6)	2 (0.3)	74 (0.6)
Best Supportive Care	7,132 (61.8)	423 (67.2)	7,555 (62.1)
Unknown	144 (1.2)	7 (1.1)	151 (1.2)
Multimodal treatment (n, %)			
Chemotherapy and Surgery	92 (0.8)	10 (1.6)	102 (0.8)
Chemotherapy and Radiotherapy	252 (2.2)	1 (0.2)	253 (2.1)
Chemotherapy and Targeted therapy	44 (0.4)	1 (0.1)	45 (0.4)
Chemotherapy and Immunotherapy	11 (0.1)	-	11 (0.1)
Surgery and Radiotherapy	47 (0.4)	2 (0.3)	49 (0.4)
Surgery and Chemotherapy and Radiotherapy	40 (0.3)	-	40 (0.3)
Other	9 (0.1)	3 (0.5)	12 (0.1)
Total	495 (4.3)	17 (2.7)	512 (4.2)
Reason no treatment (i.e. BSC) (n, %)**			
Comorbidity	18 (2.1)	3 (5.9)	21 (2.3)
Performance status	214 (25.3)	16 (31.4)	230 (25.6)
Age	32 (3.8)	1 (2)	33 (3.7)
Patient preference	363 (42.9)	14 (27.5)	377 (42)
Disease too extensive	55 (6.5)	8 (15.7)	63 (7)
Deceased before start treatment	30 (3.5)	0 (0)	30 (3.3)
Other/Unknown	135 (15.9)	9 (17.6)	144 (16)

MPM= Malignant Pleural Mesothelioma, MPeM= Malignant Peritoneal Mesothelioma.
*Total percentage >100% due to 4.2% of patients receiving multimodal treatment.
**Registered for 898 patients (847 pleural, 51 peritoneal), percentages based on registered cases.

MPM patients were treated more frequently with chemotherapy (28.4% vs. 22.6%) and radiotherapy (9.3% vs. 0.6%) than MPeM patients. On the other hand, surgery was more often applied in patients with MPeM (10%) compared to MPM (3%). Figure 3A shows an increase in the use of systemic chemotherapy between 2002 and 2006 for MPM from approximately 10% to 40% yearly. After 2006, the use of chemotherapy for the treatment of MPM remained stable. Radiotherapy was used in about 10 to 15% of cases up to 2008, after which its use decreased to 4-5% of cases yearly. As can be seen in figure 3B, MPeM patients were most commonly treated with BSC. Its use ranges from about 75% between 1993 and 1997 to about 65% from 2013 to 2018. The use of chemotherapy increased from around 2003 onward and peaked between 2008 and 2012, after which its use decreased again. The role of surgery remained limited during the study period and varied between 4 and 13% per five years.

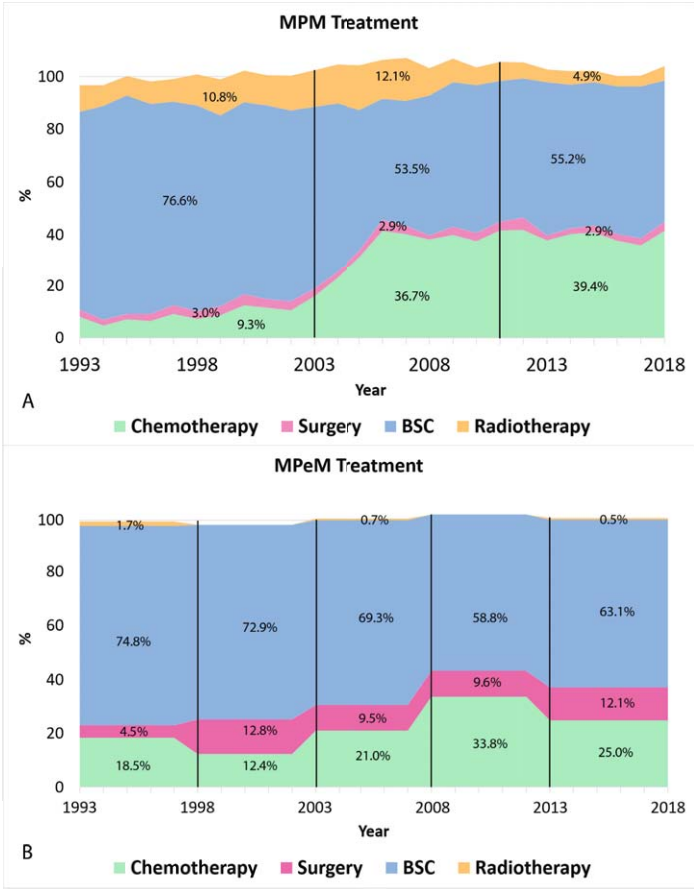

Figure 3A-B. Treatment patterns for malignant pleural mesothelioma (MPM) per year (A) from 1993-2018. Treatment patterns per five years for malignant peritoneal mesothelioma (MPeM) from 1993-2018 (B). Stacked areas do not add up to 100% due to patients receiving multimodal treatment, or patients receiving other treatment.

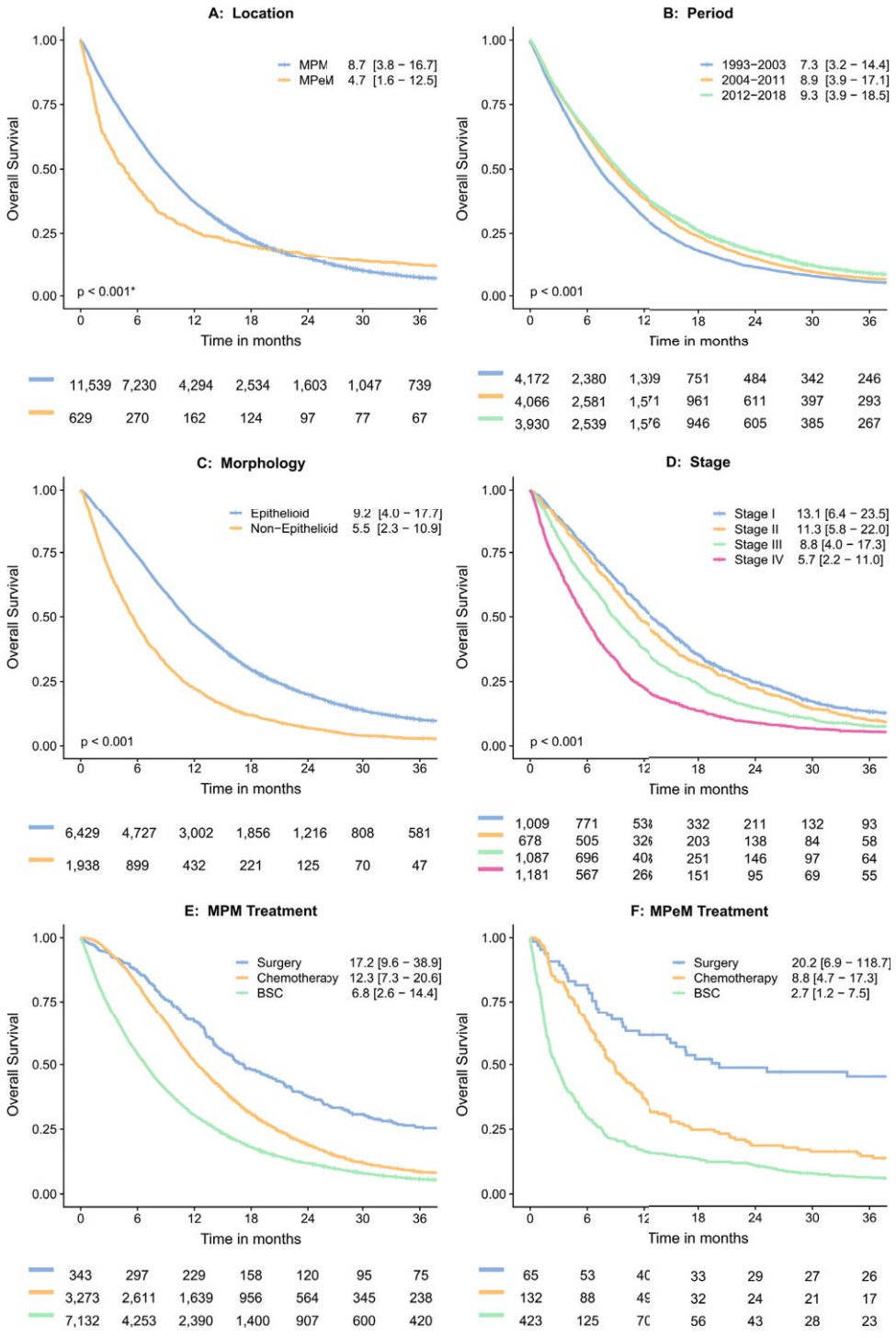
A: Location
MPM 8.7 [3.8 – 16.7]
MPeM 4.7 [1.6 – 12.5]
p < 0.001*
Overall Survival
Time in months
11,539 7,230 4,294 2,534 1,603 1,047 739
629 270 162 124 97 77 67
B: Period
1993–2003 7.3 [3.2 – 14.4]
2004–2011 8.9 [3.9 – 17.1]
2012–2018 9.3 [3.9 – 18.5]
p < 0.001
Time in months
4,172 2,380 1,399 751 484 342 246
4,066 2,581 1,571 961 611 397 293
3,930 2,539 1,576 946 605 385 267
C: Morphology
Epithelioid 9.2 [4.0 – 17.7]
Non-Epithelioid 5.5 [2.3 – 10.9]
p < 0.001
Time in months
6,429 4,727 3,002 1,856 1,216 808 581
1,938 899 432 221 125 70 47
D: Stage
Stage I 13.1 [6.4 – 23.5]
Stage II 11.3 [5.8 – 22.0]
Stage III 8.8 [4.0 – 17.3]
Stage IV 5.7 [2.2 – 11.0]
p < 0.001
Time in months
1,009 771 538 332 211 132 93
678 505 328 203 138 84 58
1,087 696 408 251 146 97 64
1,181 567 266 151 95 69 55
E: MPM Treatment
Surgery 17.2 [9.6 – 38.9]
Chemotherapy 12.3 [7.3 – 20.6]
BSC 6.8 [2.6 – 14.4]
Time in months
343 297 229 158 120 95 75
3,273 2,611 1,639 956 564 345 238
7,132 4,253 2,390 1,400 907 600 420
F: MPeM Treatment
Surgery 20.2 [6.9 – 118.7]
Chemotherapy 8.8 [4.7 – 17.3]
BSC 2.7 [1.2 – 7.5]
Time in months
65 53 40 33 29 27 26
132 88 49 32 24 21 17
423 125 70 56 43 28 23

Figure 4. Kaplan Meyer actuarial overall survival curves with median OS [interquartile range] in months and numbers at risk. MPM=malignant pleural mesothelioma. MPeM=malignant peritoneal mesothelioma. *Breslow (Generalized Wilcoxon) p-value, calculated for non-proportional hazards. For figure 4D, only data from 2008-2018 were used as the 7th edition of the TNM staging manual was published in 2007.

Survival

Median OS for the entire cohort was 8.4 months [interquartile range: 3.6 – 16.6 months]. Kaplan Meyer curves with the median OS for different subgroups are provided in figure 4. MPeM patients had worse median survival compared to MPM (4.7 versus 8.7 months, p<0.001), also when treated with systemic chemotherapy (8.9 versus 12.7 months, p<0.001, data not shown). Survival of the entire cohort significantly improved from about 2003, likely due to the introduction of combination-chemotherapy, together with the increased use of this treatment. Between 2012 and 2018, the median OS for all patients was 9.3 months, compared to 7.3 months between 1993-2003. Patients with non-epithelioid morphology (i.e. biphasic and sarcomatoid histology) had a significantly worse prognosis (5.5 versus 9.2 months, p<0.001), also when treated with systemic chemotherapy (8.9 versus 13.6 months, data not shown). For MPM, overall survival significantly differed for different stages of disease (data used from 2008-2018). Patients diagnosed with stage I pleural mesothelioma had a median survival of 13.1 months (IQR 6.4 – 23.5), compared to 5.7 months (IQR 2.2 – 11.0) for patients with stage IV disease. Figures 4 E and F illustrate the survival for chemotherapy, surgery and 'best supportive care' for MPM and MPeM respectively. These outcomes were not statistically compared because they are subjected to selection bias.

Discussion

This study shows that MM incidence among most age groups is currently declining in the Netherlands. The peak of MM incidence appears to have been passed around 2010, and currently there is a strong trend towards declining incidence for the whole population. Historically, the Netherlands has been among countries with relatively high MM incidence rates. [9] In recent decades, the rapid increase in MM incidence has been monitored with some apprehension. [14 19 20] Earlier studies have tried to predict the impact and mortality of mesothelioma in the Netherlands.[14 15] Now, for the first time, with updated incidence numbers, this study observed decreasing MM incidence among most age groups in the Netherlands. Similar results have earlier been reported for Sweden, the US, and Australia.[21-23] For other countries with high MM incidence that have banned the use of asbestos, such as the UK, these outcomes can further aid in predicting future MM incidence.

These findings are remarkable, as it was predicted that incidence would peak in the Netherlands around the year 2020.[14] However, this peak has already been reached,

approximately around 2010. This indicates that MM incidence is not only associated with the complete ban on asbestos but also with measures that were implemented before 1993. The thesis of J. Stumphius (an occupational physician at a Dutch shipyard) that was published in 1969 unmistakably related asbestos exposure to mesothelioma in the Netherlands.[24] This thesis led to additional research, which finally resulted in governmental regulation in 1978, resulting in a decrease of approximately 75% in the amount of asbestos that was processed in the Netherlands in the 1980s compared to the 1970s. As the processing and use of asbestos plummeted from 1980 onwards and the latency time for mesothelioma is 30 years on average, the decline of incidence from 2010 onwards could have been expected. This also explains why we observed that incidence among patients younger than 55 years was already declining before the complete ban on asbestos in 1993.

The age-specific incidence rates that were analysed in this study indicate that the MPM incidence among most age-groups is currently declining, with the exception of male patients aged over 80 years and female patients aged over 65 years. These observations were to be expected, considering the latency time between asbestos exposure and MM development.[13,25,26] Patients that are currently older than 80 years have likely been heavily (occupationally) exposed to asbestos in the past. However, the current decline in incidence among all other male age groups indicates that the number of new MM cases will further diminish in the near future and the peak incidence lies behind us. The group of patients that have been heavily exposed to asbestos, will become smaller each year due to the ban on asbestos in 1993, and other earlier measures. The 80+ age group has relatively high incidence rates, but the group size is decreasing each year. Therefore, the weight of this age group on the total incidence rates diminishes. Combined with declining incidence rates among all other age groups, this causes the current decline of the total MM incidence.

Age-specific incidence rates were unavailable for MPeM due to the small number of patients. For the general MPeM population, however, the incidence rate remained more or less stable over time. This could imply that the link between asbestos exposure and MPeM is less prevailing than it is in MPM.[27] This also suggests that there are other causes for MPeM development that are more dominant, such as previous radiation therapy or germline mutations.[27,28] The proportion of MPeM in this cohort was 5.2%. This is lower than expected based on literature, where rates between 10-30% have been reported.[27,29] It has been observed though that MPeM is often misdiagnosed, which can explain the small proportion of observed cases in this cohort.[27]

With regard to treatment, between 2002 and 2006, there was an evident increase in the use of systemic chemotherapy, especially for MPM. In 2003, Vogelzang et al. showed that platinum-pemetrexed treatment increased median overall survival by three months, compared to cisplatin alone in MPM.[2] The increased treatment rate,

together with the fact that combination treatment prolongs survival, likely resulted in improved survival at a population level from 2003 onward.

The majority of patients however, did not undergo anti-tumour treatment. For MPM patients that did not receive anti-tumour treatment, over 40% was due to patients' preference. Perhaps, patients are reluctant to receive toxic therapy, especially when the benefit is limited. Similar patterns were seen in Belgium and England.[30] For MPeM patients, it was more often due to poor performance status (31.4%) or extensive disease (15.7%). MPeM patients are known to be often diagnosed at an advanced stage of the disease, likely due to the rareness of their condition and non-specific presentation.[31,32]

The use of radiotherapy for MPM was common but decreased around 2009. The updated 2008 ESMO guideline can explain this. It stated that the use of radiotherapy should be limited to local palliative use and pain control, because of the potentially severe side effects, while its benefit in local disease control is controversial.[33] In 2016 and 2019, the lack of benefit from prophylactic tract irradiation was confirmed in randomized trials.[34,35] These results might lead to a further decrease in its use.

In the current cohort, the use and thus the impact of immunotherapy and targeted therapy was minimal with less than one percent of patients treated with either modality. This is due to the fact that Checkpoint Inhibition (CI) therapy is not yet registered as a treatment option, and there have been no major advances regarding targeted therapy.[36,37] Recently, the results of the phase III CheckMate743 study showed that combination CI therapy for MPM patients in first-line increased OS by four months compared to standard first-line chemotherapy.[3] For the non-epithelioid subtype, the survival benefit was even greater. For this group, estimated survival at two years was 38% in the immunotherapy group, compared to 8% in the chemotherapy group. As a result, the FDA has approved the combination of nivolumab plus ipilimumab for the first-line treatment of MM.[4] Monotherapy for MM should not be written off entirely, though, as there are selected patients that might benefit from its use.[38] Nonetheless, novel treatment options are still urgently required to improve survival in patients. An overview of new developments is given by Yap et al.[39] Within our group, we are working on dendritic cell therapy both in pleural and peritoneal mesothelioma.[40,41]

The role of surgery for MM remains controversial. Its use was minimal in the current cohort, with only three percent of MPM patients and about ten percent of MPeM patients receiving surgical treatment. Especially for MPeM patients, this could be a missed opportunity. Several series have been published on the use of CRS-HIPEC for MPeM.[6-8,42] A large series of Yan et al., for example, observed a median OS of 53 months with three and five-year survival rates of 60% and 47%, respectively.[6] This survival can partly be explained by patient selection. However, studies on the use of

systemic chemotherapy are also subjected to patient selection, but rarely show long-term survivors.[43-45]

Strengths and limitations

The strengths of our study are the number of patients included, and the length of time in which the data were collected. There are also some limitations that need to be discussed. As with most population-based registries, the overarching theme of the limitations lies in the details of the data. For example: the stage of disease for MPM was noted according to the TNM stage that was used at time of diagnosis. As there were different TNM stages used between 1993 and 2018, it was hard to compare stage of disease at time of diagnosis throughout time. Moreover, for about one third of patients there was no stage recorded. This is likely due to the minimal use of surgery for MPM in the Netherlands. Therefore the pTNM stage is mostly lacking, and the cTNM stage has no consequences for treatment as systemic chemotherapy is considered the golden standard. Patient selection for systemic chemotherapy is mostly based on performance status and patient preference, rather than stage of disease. Also, details regarding treatment were often missing. With regard to survival analyses, the cause of death is not registered by the NCR due to privacy regulations. Thus cancer-specific survival was unavailable. Nonetheless, given the very poor prognosis of MM, it is very unlikely that this affected our outcomes, even for patients at advanced age. Although these limitations complicated in depth additional analysis on survival between different stages and treatment modalities, they did not influence the main conclusion of our data on the decreasing incidence in the Netherlands from 2010 onwards, nor did they influence the trends in treatment throughout time.

Conclusion

This study shows that MM incidence has reached a peak in the Netherlands around 2010, following the national ban on asbestos in 1993. In most age groups MM incidence is currently declining, with the exception of males aged over 80 years and females aged over 65 years. The number of patients receiving treatment has increased since 2003, although most patients did not receive any anti-tumour therapy. In this period, survival improved slightly, but the prognosis is still poor. Recent advances in therapy might change this perception.

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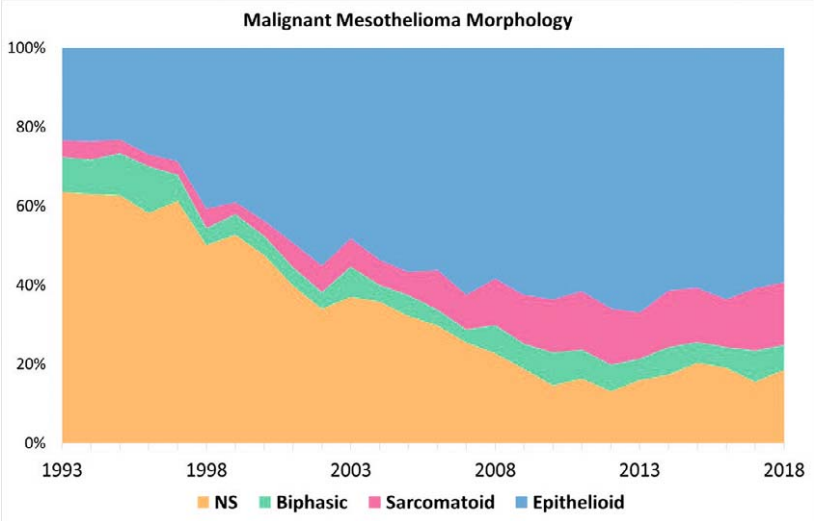
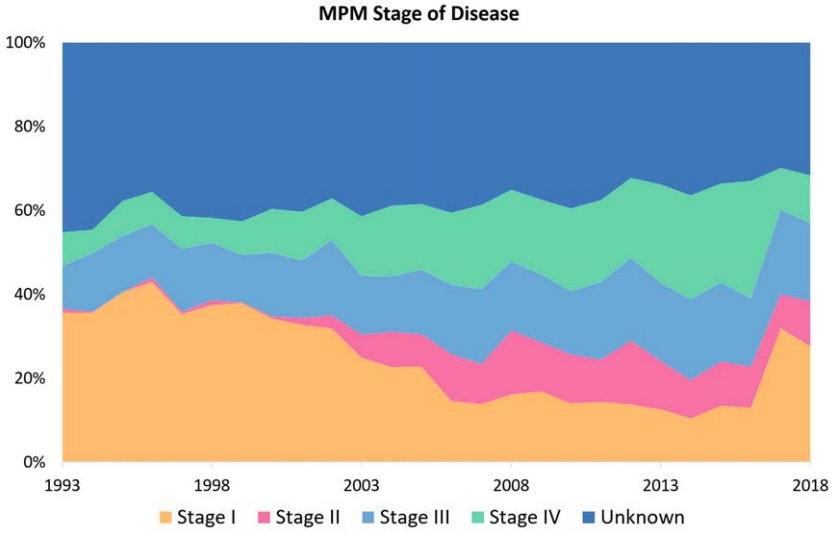
Supplementary



Supplementary Figure 1. Histological subtype trend analysis. NS=Not Specified



Supplementary Figure 2. Stage at diagnosis trend analysis.

3

Nivolumab in pre-treated Malignant Pleural Mesothelioma: real-world data from the Dutch Expanded Access Program

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Abstract

Background
Randomized phase III trials are ongoing to investigate the efficacy of nivolumab in malignant pleural mesothelioma (MPM), but real-world data are still scarce. In this real-world study, we investigated the clinical outcomes of nivolumab treatment in pre-treated MPM patients.

Methods
Data from 107 nivolumab treated MPM patients within the Dutch expanded access program were retrospectively analyzed. Treatment was independent of programmed death ligand 1 (PD-L1) expression on tumor samples. Univariable and multivariable analyses were performed to evaluate the relationship between clinically important factors, baseline peripheral blood parameters and survival. The landmark method was used to compare the outcome of patients according to their radiological response.

Results
In the full cohort, the median progression-free survival (mPFS) was 2.3 months (95% CI: 1.6-2.9) and the median overall survival (mOS) was 6.7 months (95% CI: 6.2-10.0). After 12 weeks, the disease control rate (DCR) was 37% and the objective response rate (ORR) was 10%. PD-L1 status was determined in 33 patients (30%) and PD-L1 positivity (≥1%) was associated with an improved ORR (36% vs 9%, p-value 0.05), but not with PFS or OS. Low albumin was associated with worse OS (p-value 0.002). Median OS was significantly longer for patients who had partial response to treatment (p-value 0.0002).

Conclusions
In this real-world analysis, ORR and mOS were lower compared to those obtained in phase II trials. However, exceptional survival rates were observed in patients who had a radiological response. Although we cannot determine whether prognostic or predictive, PD-L1 expression and albumin were associated with greater response rate and may represent useful biomarkers for nivolumab treatment in MPM.

Introduction

Malignant pleural mesothelioma (MPM) is an uncommon but aggressive neoplasm with low survival rates.[1,2] Current first-line treatment consists of combination chemotherapy with platinum and anti-folate agents,[1,3] with the possible addition of bevacizumab.[2] Historically, no therapeutic agent has shown strong activity against mesothelioma in second or third-line treatment.[4] The breakthrough of checkpoint inhibitors (CIs) in solid tumors has led to their investigation in MPM patients as well. Despite promising results in phase I/II trails with CIs, phase III trials investigating both single agent anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and anti-programmed cell death 1 (PD-1) treatments failed to show efficacy.[5,6] Recently, the PROMISE-meso, a phase III randomized clinical trial (RCTs), comparing the PD-1 CI pembrolizumab to chemotherapy (gemcitabine or vinorelbine) as second-line treatment, failed to show superiority of the anti-PD-1 treatment for the primary endpoint progression free survival (PFS).[6] The objective response rate (ORR) was significantly higher in the pembrolizumab arm (22%) than in the chemotherapy arm (6%), but duration of response (DoR) and overall survival (OS) were equal. Nivolumab, another PD-1 inhibitor, showed promising results in phase II trials in pre-treated MPM patients (with ORR up to 29%)[7-10] and is currently being tested in the context of phase III RCTs (NCT03063450, NCT02899299).

Only one study has reported real-world data on second or third-line PD-1 inhibition (pembrolizumab) in MPM.[11] In this study, both PFS and OS did not match phase II trial results which could be explained by the use of strict inclusion criteria in the clinical trials.[8-10] Outside of clinical trials, there are no reports on the role of nivolumab in pre-treated MPM patients. Most probably, as already observed in phase II/III trials, a small group of MPM patients might benefit from CI treatment.

Relevant biomarkers for response have not yet been determined in this specific setting of MPM. Programmed death-ligand 1 (PD-L1) expression on tumor cells has a controversial role in predicting outcome in MPM.[7,11] The low predictive value of PD-L1 expression in MPM has been explained by intra-patient heterogeneity, different cut-off points for PD-L1 positivity and the use of different immunohistochemistry (IHC) markers.[7,11] Likewise different cancer types,[12,13] other tumor and patient characteristics, as well as peripheral blood values should then be investigated in MPM patients treated with nivolumab, to identify biomarkers for response.

Since February 2018, nivolumab has been provided to MPM patients in the Netherlands through an expanded access program (EAP). This program has offered the unique opportunity to conduct a real-world analysis to investigate the outcome of nivolumab in a population of MPM patients pre-treated with antifolate and platinum-based chemotherapy. Furthermore, we extensively analyzed the correlation between clinically important factors, baseline peripheral blood parameters and clinical outcomes. The

impact of radiological response on outcome was also investigated. We present the following article in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Guidelines. Reporting Checklist (available at http://dx.doi.org/10.21037/tlcr-19-686).

Methods

Patients

We retrospectively reviewed data from all 135 MPM patients enrolled at the Erasmus Medical Center (Rotterdam, NL) and The Netherlands Cancer Institute (Amsterdam, NL) in the EAP for nivolumab. Patients had a cytological and/or histological proven MPM and progression after at least one previous line of chemotherapy. Inclusion in the program was independent of PD-L1 expression on tumor samples, which was assessed by IHC using the Ventana SP263 or the Dako 22C3 assays. A recent tumor biopsy was not mandatory. Patients were excluded if they had received any immunotherapy as first-line or maintenance treatment. Patients with a follow-up shorter than 3 months were also excluded from the analysis, unless they progressed or died earlier. Nivolumab was given intravenously at a dose of 3 mg/kg every 2 weeks. Radiological tumor assessment was carried out 6 weeks (±1) after start of treatment and every 6 weeks (±1) until progression depending on previous computed tomography (CT) evaluation.

Data collection

Patient and tumor characteristics, as well as radiological response data and blood count parameters within 14 days before the initiation of nivolumab treatment were collected from the digital patient register. The following variables were collected and investigated in statistical analyses: age, gender (male vs female), histologic subtype (non-epithelioid vs epithelioid), Eastern Cooperative Oncology Group (ECOG) Performance Status (PS) at start of nivolumab (0 vs ≥1), clinical TNM stage (stage III/IV vs I/II [VIII edition]) (14), line of treatment (later-lines vs second-line), PD-L1 status (considered as positive if tumor cell expression levels were ≥1%, negative if <1%), time to progression (TTP) to previous line of chemotherapy (<6 months vs ≥6 months), time interval (TI) from diagnosis to start of nivolumab, body mass index (BMI). Albumin values (as continuous variable), platelet count (as continuous variable), and absolute counts for neutrophils, monocytes, eosinophils and lymphocytes were also collected.

Tumor response was assessed using a combination of modified Response Evaluation Criteria In Solid Tumors (mRECIST) for mesothelioma version 1.0 and RECIST modified for immunotherapeutic agents (iRECIST) (15,16). Per iRECIST, if tumor imaging shows initial progression of disease (PD), tumor assessment should be repeated 4 to 8 weeks later in order to confirm PD with the option of continuing treatment if the patient is clinically stable. Patients who had confirmed disease progression by iRECIST

discontinued treatment, and the date of the initial CT scan was taken as the time of progression. OS was defined as the time from first CI administration to death from any cause, censored at the last tumor assessment date for patients who were alive at the time of data cutoff. PFS was measured from the time of nivolumab initiation to clinical or radiological progression or death from any cause. ORR was defined as the proportion of patients who had a partial (PR) or complete response (CR) to therapy and DCR as the percentage of patients who achieved complete response, partial response and stable disease (SD). A cut-off of 12 weeks (±2) was selected for both ORR and DCR, according to the majority of RCTs investigating CIs in MPM. DoR was defined as the time from documentation of tumor response to disease progression.

Statistical analysis

Patient and disease characteristics were reported using count and percentage for categorical variables, median and range for continuous variables. Median PFS and OS were estimated by the Kaplan–Meier method. Differences in probability of surviving between the strata were evaluated by log-rank (Mantel-Cox) test and Bonferroni's correction was used for comparison between more than two groups. The landmark method was used for handling immortal time bias when comparing the outcome of patients according to their radiological response (17). For this specific analysis, all the patients who died before 12 weeks were excluded. A landmark of 12 weeks was chosen because at that time ORR was also calculated.

The hazard ratios (HR) of progression and death, the odds ratios (OR) of response and their associated 95% confidence intervals (95% CI) for clinically important factors (including PS, histology, stage, gender, age, line of treatment, TTP to previous line of chemotherapy, PD-L1 status) were calculated using a univariable Cox proportional hazard model or a univariable logistic regression.

Missing data in blood-derived parameters analyzed in the multivariable analysis were imputed ten times. In order to determine a subset of variables with the strongest impact on PFS, OS and ORR, blood-derived biomarkers (including albumin, platelets, absolute neutrophils, monocytes, eosinophils and lymphocytes) were combined with clinically important factors and a Cox multivariable proportional hazard regression model or a multivariable logistic regression were performed on the imputed datasets. Since the number of candidate variables exceeded the number of events divided by 10, a ridge version of the models was used for variable selection. Variables were selected in the final model if they were included 5 times or more in the models on the imputed data sets. The final model was fitted on the imputed data sets and the results were pooled using Rubin's rules.[18] As a sensitivity analysis, the final model was also estimated on the complete case data (without imputed data).

Associations between categorical variables were assessed by Pearson's Chi-Square or Fisher exact tests.

A significance level of 0.05 was chosen to assess the statistical significance. All reported p-values were two sided. Statistical analyses were performed using R 3.6.0 (R Foundation for Statistical Computing). Multiple imputation was performed using the "smcfcs" package and pooling was conducted.

Results

Patient characteristics

At the data cut-off of November 2019, 135 patients were treated with at least one cycle of Nivolumab. Among them, 107 patients were eligible for the analysis (**Supplementary Figure S1**). Eighty-eight patients (93%) had a PS of 0 or 1 at start of treatment. Ninety-seven (90%) were treated in second-line. PD-L1 expression was determined in 33 patients: 22 biopsies (66%) were PD-L1 negative and 11 (33%) were PD-L1 positive. PD-L1 positive status was associated with non-epithelioid histology (Fisher's exact test p-value 0.004). The majority of patients (69%) had an advanced clinical stage of disease (stage III/IV). Other baseline patient characteristics are summarized in **Table 1**.

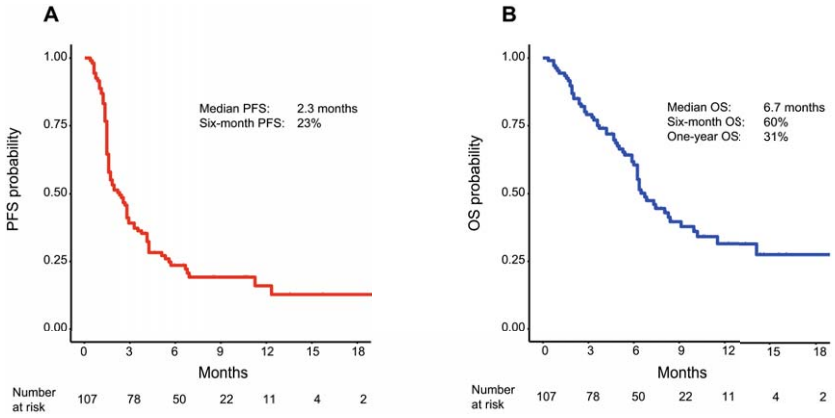

Figure 1. Kaplan-Meier curves of survival in the entire cohort of nivolumab treated MPM patients (median follow-up time of 10.1 months). (A) Overall survival in the entire cohort. (B) Progression-free survival in the entire cohort. PFS, progression-free survival; OS, overall survival.

At a median follow-up time of 10.1 months, 85 patients had progression of disease of whom 59 died. The median PFS (mPFS) was 2.3 months (95% CI: 1.6-2.9) and median OS (mOS) was 6.7 months (95% CI: 6.2-10.0) (**Figure 1A and 1B**). The disease control rate (DCR) was 37% (40 out of 107) after 12 weeks and 11 patients (10%) had an objective radiological response (all partial responders, no complete responses were registered).

The 6-month PFS rate was 23% (95% CI: 16%–33%). The 6-month OS rate was 60% (95% CI: 51%–71%) and the 1-year OS rate was 31% (95% CI: 22%–45%).

Table 1. Patient baseline characteristics

Characteristics	N (total = 107)	%
Median age	69	Range: 34-84
Gender, male	95	87
ECOG PS at start of nivolumab		
0	20	19
1	68	64
2	6	5
Unknown	13	12
Histological subtype		
Epithelioid	78	73
Mixed/Sarcomatoid	22	20
Unknown	7	7
Best response to previous platinum-based chemotherapy		
PD	28	26
SD	46	43
PR	28	26
CR	1	1
Unknown (not reported)	4	4
Line of treatment		
2	97	91
≥3	10	9
Stage at start of nivolumab		
I/II	32	30
III/IV	70	65
Unknown	5	5
PD-L1 status		
Negative	22	20
Positive	11	10
Unknown	74	70

Data are presented as absolute number with according percentages, unless stated otherwise. ECOG PS, Eastern Cooperative Oncology Group Performance Status; PD, progressive disease; SD, stable disease; PR, partial response; CR, complete response; PD-L1, programmed death ligand 1.

Association of clinically important factors with survival outcomes

Univariable Cox proportional hazard regression analysis of clinically important factors revealed that patients with advanced clinical stage (stage III/IV) had a shorter PFS (mPFS 1.6 vs 3.6 months [HR 1.82, 95% CI 1.11-3.01, log-rank p-value 0.02, **Figure 2A**]) but similar OS (mOS 6.5 vs 6.8 months [HR 1.27, 95% CI 0.71-2.28, log-rank p-value 0.40], **Figure 2B**) compared to those with early stage (I/II). All other clinical factors were not significantly associated with PFS or OS (**Table 2**).

In particular, PS was not significantly correlated with PFS or OS, although patients with a PS of 0 had a trend towards a longer mOS compared to patients with PS ≥1 (mPFS 2.9 vs 1.8 months [HR 0.64, 95% CI: 0.36-1.16, log-rank p-value 0.14]; mOS 10.2 vs 6.2 months [HR 0.51, 95% CI: 0.25-1.05, log-rank p-value 0.06]). PFS was also similar among patients with non-epithelioid and epithelioid histology (log-rank p-value 0.89, **Figure 2C**), yet patients with non-epithelioid histology had a non-significant trend towards worse OS (mOS 4.8 vs 7.4 months [HR 1.71, 95% CI: 0.92-3.16, log-rank p-value 0.08], **Figure 2D**). Patients with positive PD-L1 status showed a longer, albeit non-significant, mPFS (4.2 vs 1.7 months [HR 0.52, 95% CI: 0.23-1.20, log-rank p-value 0.11], **Figure 2E**) while no difference in terms of OS was observed (mOS 5.4 vs 6.1 months [HR 0.67, 95% CI 0.27-1.64, log-rank p-value 0.39], **Figure 2F**).

Table 2. Univariable analysis of PFS, OS and ORR for clinically important factors

Parameter	PFS			OS			ORR		
	HR	95% CI	p-value	HR	95% CI	p-value	OR	95% CI	p-value
ECOG PS (0 vs ≥1)	0.64	0.30-1.16	0.14	0.51	0.24-1.05	0.06	1.13	0.97-1.31	0.12
Histologic subtype (non-epithelioid vs epithelioid)	1.02	0.60-1.76	0.91	1.71	0.92-3.16	0.08	1.03	0.89-1.20	0.65
PD-L1 status (positive vs negative)	0.52	0.23-1.20	0.12	0.67	0.27-1.64	0.39	1.31	1.00-1.72	0.05
Age	1.00	0.97-1.02	0.90	1.00	0.97-1.03	0.76	0.99	0.98-1.00	0.25
Gender (male vs female)	1.45	0.70-3.01	0.31	1.56	0.62-3.92	0.33	0.93	0.77-1.11	0.44
Clinical stage (stage III/IV vs I/II)	1.82	1.11-3.01	0.02	1.27	0.71-2.28	0.41	0.93	0.81-1.06	0.29
Line of treatment	0.76	0.35-1.66	0.49	0.89	0.35-2.23	0.80	1.11	0.91-1.35	0.29
TTP to first-line chemotherapy (<6 months vs ≥6 months)	1.42	0.91-2.19	0.11	1.57	0.93-2.62	0.09	1.06	0.94-1.20	0.29
TI from diagnosis to nivolumab	0.98	0.95-1.02	0.32	0.98	0.94-1.03	0.44	1.00	0.99-1.01	0.58
BMI	0.99	0.93-1.07	0.99	0.96	0.89-1.03	0.31	1.00	0.99-1.01	0.29

The univariable Cox proportional hazard model was used to calculate the HRs of progression or death and the univariable logistic regression was used to calculate the ORs of response.

PFS, progression-free survival; OS, overall survival; ORR, objective response rate; HR, hazard ratio; OR, odds ratio; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group Performance Status; PD-L1, programmed death ligand 1; TTP, time to progression; TI time interval; BMI, body mass index.

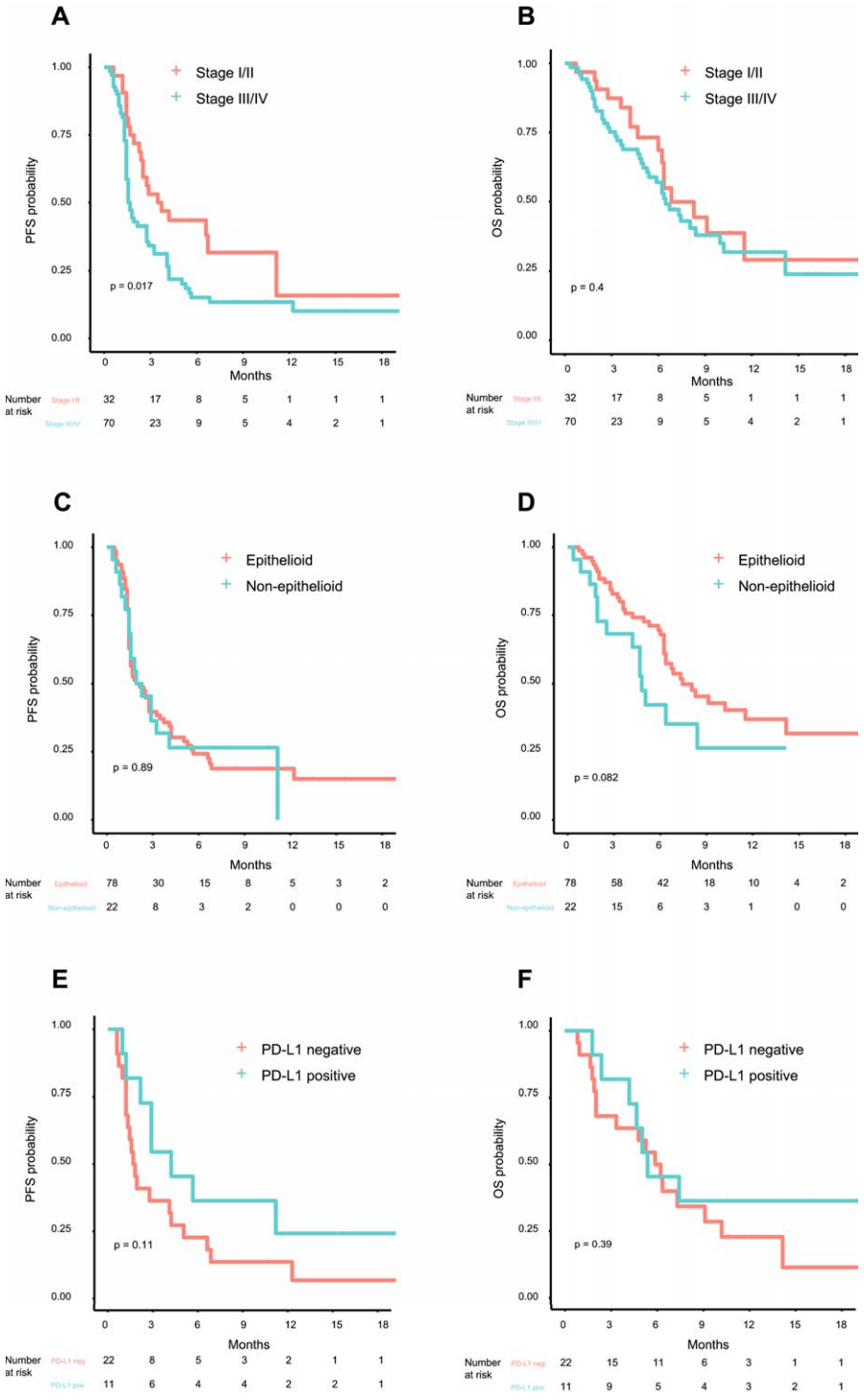
A
PFS probability
1.00
0.75
0.50
0.25
0.00
Stage I/II
Stage III/IV
p = 0.017
0 3 6 9 12 15 18
Months
Number at risk
Stage I/II 32 17 8 5 1 1 1
Stage III/IV 70 23 9 5 4 2 1
B
OS probability
1.00
0.75
0.50
0.25
0.00
Stage I/II
Stage III/IV
p = 0.4
0 3 6 9 12 15 18
Months
Number at risk
Stage I/II 32 17 8 5 1 1 1
Stage III/IV 70 23 9 5 4 2 1
C
PFS probability
1.00
0.75
0.50
0.25
0.00
Epithelioid
Non-epithelioid
p = 0.89
0 3 6 9 12 15 18
Months
Number at risk
Epithelioid 78 30 15 8 5 3 2
Non-epithelioid 22 8 3 2 0 0 0
D
OS probability
1.00
0.75
0.50
0.25
0.00
Epithelioid
Non-epithelioid
p = 0.082
0 3 6 9 12 15 18
Months
Number at risk
Epithelioid 78 58 42 18 10 4 2
Non-epithelioid 22 15 6 3 1 0 0
E
PFS probability
1.00
0.75
0.50
0.25
0.00
PD-L1 negative
PD-L1 positive
p = 0.11
0 3 6 9 12 15 18
Months
Number at risk
PD-L1 neg 22 8 5 3 2 1 1
PD-L1 pos 11 6 4 4 2 2 1
F
OS probability
1.00
0.75
0.50
0.25
0.00
PD-L1 negative
PD-L1 positive
p = 0.39
0 3 6 9 12 15 18
Months
Number at risk
PD-L1 neg 22 15 11 6 3 1 1
PD-L1 pos 11 9 5 4 3 2 1

Figure 2. Kaplan-Meier curves of survival of subgroups based on stage of disease, histological subtype and programmed death ligand 1 (PD-L1) status. (A) Progression-free survival and overall survival by stage of disease as determined by IASLC 8th edition of TNM for pleural mesothelioma. (B) Progression-free survival and overall survival by histology. (C) Progression-free survival and overall survival in patients with a PD-L1 expression ≥1% versus in those with a PD-L1 expression <1%. PFS, progression-free survival; OS, overall survival; PD-L1, programmed death ligand 1.

Impact of radiological response to nivolumab on outcome and association of clinically important factors with response

To better elucidate the importance of response to nivolumab, we compared PFS and OS of patients according to ORR. To avoid an immortal time bias, only patients who were still alive at 12 weeks and underwent radiological assessment at that time point were taken into account for the analysis. Remarkably, with a median follow up of 14.1 months in the group of patients with PR, no deaths were reported and only 2 patients progressed (median DoR not reached, **Figure 3A**). Median OS was not reached for patients with a PR. Median OS was 10.2 months for patients with SD and 6.4 months for those with PD (log-rank p-value 0.0002, **Figure 3B**).

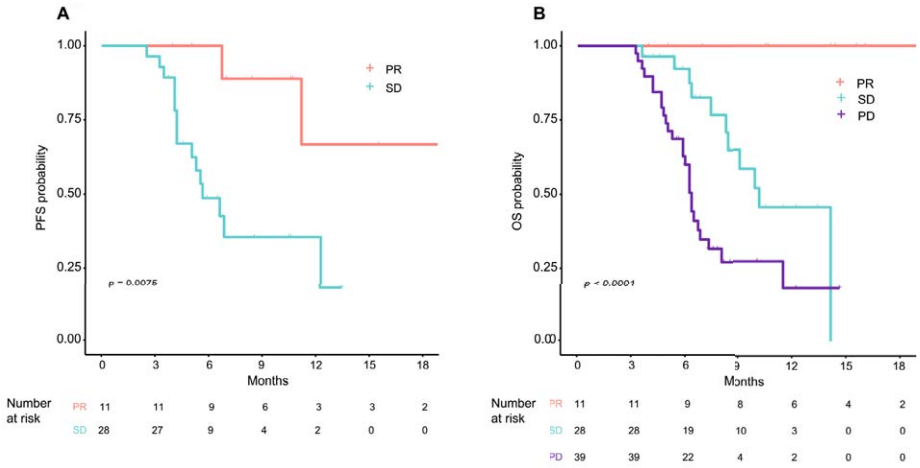

Figure 3. Kaplan-Meier curves of survival according to best overall radiological response. (A) Progression-free survival in patients with a partial response and stable disease as objective response to nivolumab treatment (B) Overall survival in patients with a partial response, stable disease and progressive disease as objective response to nivolumab treatment. PFS, progression-free survival; OS, overall survival; PR, partial response; SD, stable disease; PD, progressive disease.

Among the clinically relevant factors, the only one which seemed to predict ORR in univariable logistic regression was PD-L1 status (**Table 2**). To note, data about PD-L1 expression were only available in 6/11 PR, 8/29 SD and 19/67 PD patients (**Figure 4**). Four of the responders had PD-L1 positive tumors and two had PD-L1 negative tumors

(**Figure 4**). ORR was 36% in the PD-L1 positive group vs 9% in the PD-L1 negative group (OR 1.31, 95% CI 1.00-1.72, p-value 0.05, **Table 2**).

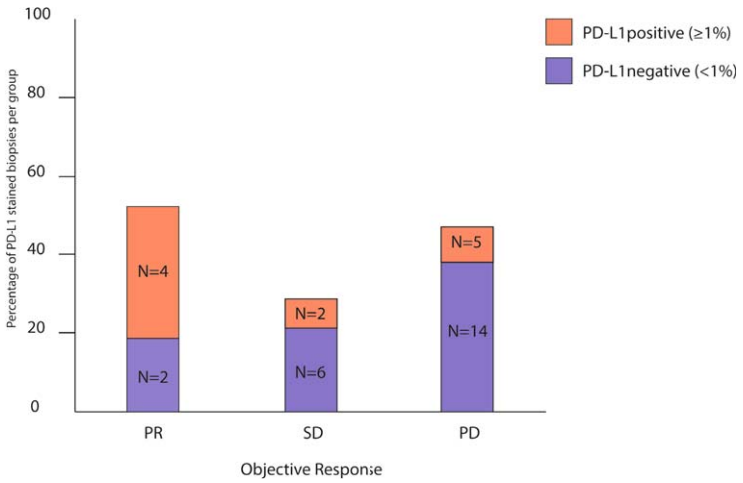

Figure 4. Expression of programmed death ligand 1 (PD-L1) according to objective response to nivolumab treatment. PR, partial response; SD, stable disease; PD, progressive disease; PD-L1, programmed death ligand 1.

Association of peripheral blood biomarkers with survival outcomes and response to nivolumab

After imputation for missing values (refer to **Supplementary Figure S1** for the number of available blood samples at baseline), peripheral blood-derived parameters (albumin, platelets, absolute neutrophils, monocytes, eosinophils and lymphocytes) and clinically important factors (including PS, histology, clinical stage, gender and age) were used as covariates in multivariable analysis to identify independent factors related to the efficacy of nivolumab in terms of PFS and OS. Regarding PFS, only high absolute monocyte count was significantly associated with worse PFS after ridge regression (HR 3.16, 95% CI: 1.56-6.37, p-value 0.001, **Table 3**). The role of monocytes was confirmed also by using non-imputed data (HR 3.78, 95% CI: 1.84-7.76, p-value 0.0002).

The ridge regression for OS showed that albumin, thrombocytes, neutrophils had the strongest association with OS. Subsequent multivariable Cox proportional hazard regression analysis with these variables (**Table 3**) showed that only albumin retained its prognostic value revealing that patients with a high albumin had a lower change of dying (HR 0.87, 95% CI 0.81-0.95, p-value 0.002). The role of albumin was confirmed by the sensitivity analysis with non-imputed data (HR 0.88, 95% CI: 0.80-0.96, p-value 0.005).

Table 3. Multivariable analysis of PFS and OS for peripheral blood derived parameters

Parameter	PFS			OS		
	HR	95% CI	p-value	HR	95% CI	p-value
Monocytes (/μL)	3.16	1.56-6.37	0.001			
Albumin (mg/dL)				0.87	0.81-0.95	0.002
Platelet count (/μL)				1.00	0.99-1.01	0.07
Neutrophils (/μL)				0.86	0.73-1.02	0.10

Only variables that came out more than five times from the ridge regression in the imputed data set were included in this final model. The final model was fitted on the imputed data sets and the results were pooled using Rubin's rules. Co-variables for ridge regression included PS, histology, stage, gender, age, eosinophils and lymphocytes.
PFS, progression-free survival; OS, overall survival, HR, hazard ratio; CI, confidence interval.

A multivariable analysis for ORR with peripheral blood-derived parameters was not performed because of the low number of events (only 11 responder patients). At univariable analysis with imputed data, again only albumin resulted significantly associated with ORR (OR 1.02, 95% CI 1.00-1.03, p-value 0.03, **Supplementary Table S1**).

Since albumin was the only significant prognostic factor for OS and was also associated with ORR in univariable analysis, patients were further divided in quartiles according to their baseline albumin values and their outcomes were analyzed. Patients in the lower quartile (< 38 mg/dL) revealed a significantly shorter OS compared to patients in the other quartiles (HR 3.76, 95% CI: 1.93-7.31, log-rank p-value 0.003 with Bonferroni's correction, **Figure 5**). The median OS for patient with baseline albumin levels below 38 was 2.5 months (95% CI: 1.9 - not reached) compared to 8.0 months (95% CI: 6.4-not reached) for patients with albumin levels above 38. Six-month OS rates were 34% (95% CI: 18%-65%) and 74% (95% CI: 62%-86%), respectively. In addition, 4 out of 20 (20%) patients in the higher quartile (> 43 mg/dL) had a partial response, compared to 3/65 (4%) in the other three quartiles, with a 16% increase in the chance of getting a response to nivolumab (OR 1.16, 95% CI: 1.02-1.33, p-value 0.02).

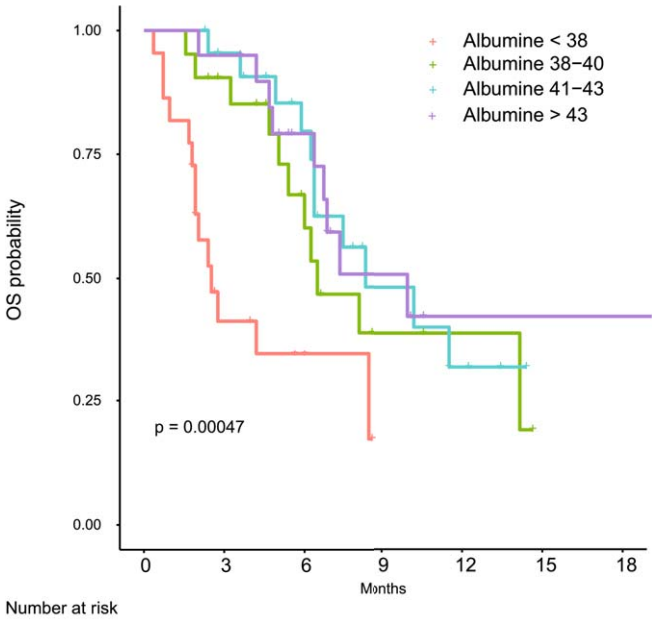

Figure 5. Kaplan-Meier curves of survival in patient groups per quartile of albumin level. OS, overall survival.

Discussion

This is the largest real-world analysis of nivolumab treatment in pre-treated MPM patients. We observed an ORR of 10%, a mPFS of 2.3 months and a mOS of 6.7 months. The PFS and OS did not significantly differ per histological subtype or PD-L1 expression. Patients with PD-L1 positive tumors had a higher ORR than patients with PD-L1 negative tumors. We did not observe an association between time from diagnosis or response to chemotherapy and response to nivolumab. Strikingly, there seemed to be an incremental impact on OS for patients with a PR to nivolumab as we did not observe any deaths in these patients during a median follow-up time of 14.1 months.

By comparing our data with the real-world study of MPM patients treated with pembrolizumab, we observed a similar OS but a worse PFS, which could be explained by the type of radiological assessment used. In the study of Metaxas et al.,[11] the type of radiological assessment was not described. In our study, we retrospectively analyzed all CT scans according to a combination of mRECIST for mesothelioma and iRECIST.[15,16] Per iRECIST, tumor assessment had to be repeated 4 to 8 weeks after first evidence of PD with the option of continuing treatment if the patient was clinically stable. In

case of confirmed progression, the date of the initial CT scan was taken as the time of progression.

By comparing our data with those of clinical trials,[6-10] our ORR and mOS were inferior, which could be explained by the fact that there were no strict inclusion criteria in our analysis, leading to a less selected patient population. In the PROMISE-meso trial an ORR of 22% was reported for the pembrolizumab group and an ORR of 6% for the second-line chemotherapy treated patients. However, this difference in ORR was not translated into a difference in mPFS (pembrolizumab: 2.5 months vs chemotherapy: 3.4 months) or mOS (pembrolizumab: 10.7 months vs chemotherapy: 11.7 months).[6] Conversely, long survival for patients with a PR in our analysis does suggest a clinical benefit that is correlated with ORR. The lack of significant benefit in terms of mPFS and mOS, despite a higher ORR, in the pembrolizumab arm of the PROMISE-meso might be due to the low ORR combined with the short time to progression in patients where therapy is not effective. For example, if only a minority of patients (10-20%) respond to therapy, mPFS and mOS will not be influenced, because more than 50% of the patients will progress or die earlier according to the natural course of disease. Six-months PFS and one-year OS might be more reliable endpoints for (immune) therapies with low response rates. Analysis of those patients who achieved a PR to pembrolizumab in the PROMISE-meso study has not yet been published but could be explanatory.

Since retrospective data may be biased by underreporting of adverse events and misleading, we decided not to report safety data. Nevertheless, to avoid a potentially harmful treatment, identifying a subgroup of MPM patients that benefit from nivolumab becomes crucial. This patient selection should probably be based on multiple parameters.

MPM patients with epithelioid histology have usually a better natural disease course than patients with non-epithelioid tumors.[19] However, in our retrospective analysis we did not see any significant difference in mPFS and mOS according to histological subtypes, suggesting that nivolumab might have had an impact on prognosis of non-epithelioid patients. Moreover, PD-L1 expression was associated with non-epithelioid histology and higher ORR in our study. These results are consistent with the exploratory analysis of the MAPS2 trial, where PD-L1 expression of ≥1% was found to be significantly associated with objective response to immunotherapy.[7] Unfortunately, our analysis on PD-L1 expression was limited because only 30% of biopsies were stained for PD-L1. Another limitation is that PD-L1 expression was often determined on the biopsy from diagnosis, because in most cases there was no biopsy taken prior to nivolumab treatment.

Looking at the role of baseline peripheral blood biomarkers, our study showed that baseline albumin was the only significant prognostic factor for mOS. In addition, patients

with an albumin level higher than 43 mg/dL had a 16% higher chance of responding to therapy than patients with albumin levels below 38 mg/dL. Albumine is known to reflect the nutritional status of cachectic patients and is described as a prognostic factor for many cancer types, including mesothelioma.[20-22] Due to the lack of a control group, we cannot draw definitive conclusions about the predictive role of albumin from our analysis. However, we showed that low levels of albumin might identify patients who are unlikely to benefit from the treatment.

Our analysis also showed that baseline absolute monocyte count represents an optimal predictor of PFS in MPM patients (HR 3.16, 95% CI: 1.56-6.37, p-value 0.001). This negative association between the number of monocytes and outcome in MPM is consistent with previous studies.[23,24] Burt et al. reported that pre-operative peripheral absolute monocyte count was associated with poor OS in patients with MPM, regardless of tumor histology (HR 3.98, 95% CI: 2.64-5.93, p-value <0.0001).[24]

Conclusions

In conclusion, our study showed that ORR and mOS were lower in our real-world database compared to those of clinical trials, which could be due to a less selected population. However, we identified a subgroup of MPM patients with a radiological response to nivolumab that had a significant benefit in terms of PFS and OS compared to patients without a radiological response to nivolumab treatment. We also showed that PD-L1 expression and albumin were associated with higher response rate, yet the retrospective nature of our study and the lack of a control group prevent us from drawing definitive conclusions on their role as potential predictive biomarkers. Future phase III RCTs on CI treatment in MPM should not be conducted without an extensive exploratory analysis plan based on the evaluation of peripheral blood parameters and tumor samples in order to deeply characterize the small group of patients that benefit from CI treatment.

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Supplementary material

Table S1. Univariable analysis of ORR for peripheral blood derived parameters

Parameter	ORR		
	OR	95% CI	p-value
Albumin (mg/dL)	1.02	1.00-1.03	0.03
Platelet count (/µL)	0.99	0.99-1.00	0.42
Neutrophils (/µL)	0.98	0.96-1.00	0.28
Lymphocytes (/µL)	1.00	0.93-1.06	0.99
Monocytes (/µL)	0.98	0.78-1.22	0.89
Eosinophils (/µL)	0.75	0.44-1.28	0.30

The univariable logistic regression was used to calculate the ORs of response for peripheral blood derived parameters (with imputed data).
ORR, objective response rate; OR, odds ratio; CI, confidence interval.

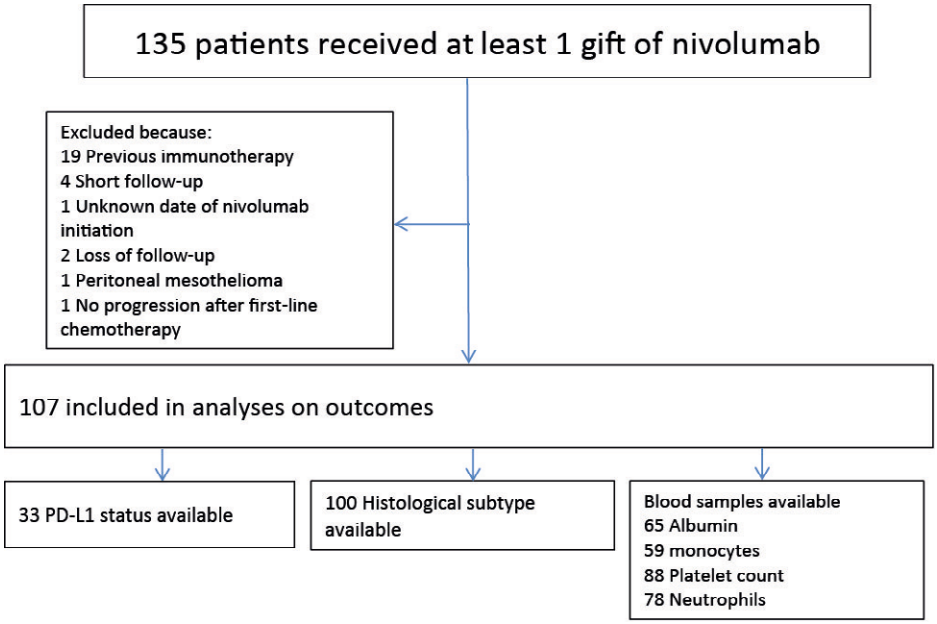

Figure S1. Flow diagram of study population. PD-L1, programmed death ligand 1.

4

Atypical B cells (CD21-CD27-IgD-) correlate with lack of response to checkpoint inhibitor therapy in NSCLC

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Highlights

- Low number of B cells are correlated with lack of response to CI therapy in NSCLC.
- An abundance of Atypical B cells is associated with lack of response to CIs.
- These results are validated in patients with Pleural Mesothelioma.
- Phenotypically these cells resemble exhausted B cells seen in chronic infection.
- Chronic antigen exposure could induce Atypical B cells.

Abstract

Introduction

Checkpoint inhibitor (CI) therapy has revolutionized treatment for non-small cell lung cancer (NSCLC). However, a proportion of patients do not respond to CI therapy for unknown reasons. Although the current paradigm in anti-tumor immunity evolves around T cells, the presence of tertiary lymphoid structures and memory B cells has been positively correlated with response to CI therapy in NSCLC. In addition, double negative (DN) ($CD27^-$ IgD^-) B cells have been shown to be abundant in NSCLC compared to healthy lung tissue and inversely correlate with the intratumoral presence of memory B cells. Nonetheless, no study has correlated DN B cells to survival in NSCLC.

Methods

In this study, we evaluated the presence and phenotype of B cells in peripheral blood with flow cytometry of patients with NSCLC and mesothelioma before receiving CI therapy and correlated these with clinical outcome.

Results

Non-responding patients showed decreased frequencies of B cells, yet increased frequencies of antigen-experienced CD21- DN (Atypical) B cells compared to responding patients and HC, which was confirmed in patients with mesothelioma treated with CI therapy.

Conclusions

These data show that the frequency of CD21- DN B cells correlates with lack of response to CI therapy in thoracic malignancies. The mechanism by which CD21- DN B cells hamper CI therapy remains unknown. Our findings support the hypothesis that CD21- DN B cells resemble phenotypically identical exhausted B cells that are seen in chronic infection or function as antigen presenting cells that induce regulatory T cells.

Keywords: Atypical B cells, B lymphocytes, Immune checkpoint inhibitors, NSCLC, MPM

Introduction

Checkpoint inhibitor (CI) therapy has revolutionized treatment of non-small cell lung cancer (NSCLC) and has led to improved overall survival (OS).[1] However, there are patients that do not benefit from CI therapy. A better understanding of the mechanisms hampering response to CI is needed. The role of T cells in anti-tumor immunity has been well established and is documented as the paradigm of the cancer immunity cycle.[2] Extensive research over the last decade has elucidated that B cells can exert anti-tumor immunity through several mechanisms such as enhancing T cell function, complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity.[3] However, the prognostic role of intratumoral B cells remains variable and has been reported to be dependent on the organizational structure in which B cells reside.[4,5] The phenotype and thereby function of B cells is determined by the maturity – presence of germinal centers - of tertiary lymphoid structures (TLS). B cells whithin mature TLS results in plasma cell differentiation and antibody production,[5] which is correlated with favorable prognosis in NSCLC[6] and correlates with response to CI therapy in melanoma, sarcoma and NSCLC.[6-9] Specifically, memory B cells and plasma cells were found to be correlated with favorable outcome to CI therapy in melanoma and NSCLC.[7,8] Immature TLS induce regulatory B cells with immunosuppressive capacities that have been correlated with worse clinical outcome in bladder cancer.[5] In NSCLC, double negative (DN) B cells, lacking expression of CD27 and IgD, are abundant in the tumor compared to healthy lung tissue and negatively correlate with the number of memory B cells.[10] Due to the unavailability of clinical response data, no correlation between the presence of intratumoral DN B cells and clinical outcome was made in that study.

DN B cells are part of a heterogeneous group of B cells, also described as atypical B cells (ABC), tissue-like memory B cells, or age-associated B cells. These cells are diversely characterized by (a combination of) phenotypic markers such as CD11c and T-bet expression, and the lack of CD27, IgD, and CD21.[11,12] ABCs derive from naïve B cells under the influence of an inflammatory environment and exposure to specific cytokines, interleukins and toll-like receptors that subsequently determine the functionality of the ABC.[13,14] These ABCs have been described in elderly, auto-immunity and chronic infection and the functionality varies from hyperresponsive and auto-reactive to exhausted.[12-17]

Although literature on intratumoral B cells and cancer has become quite extensive, perturbations in B cell subtypes in peripheral blood of cancer patients and correlations with response to CI therapy have not been studied in detail. Only a low number of total B cells prior to therapy and a decrease in CD21- B cells after start of therapy has been correlated to worse clinical outcome.[18,19]

Therefore, we studied B cells, with a focus on memory- and DN B cells, in the peripheral blood of responding (R) and non-responding (NR) NSCLC patients to anti-PD-1 CI therapy.

Methods

Study design

The MULTOMAB study (local ethics board study number MEC16–011) was originally designed to sample peripheral blood mononuclear cells (PBMCs) and serum to analyze pharmacokinetics and immune cells subsets in patients receiving CI therapy. Patients that were asked to participate in the reported analysis are suffering from NSCLC or malignant pleural mesothelioma (MPM) and received treatment in the form of nivolumab (Opdivo®, 3mg/kg every 2 weeks) or pembrolizumab (Keytruda®, 2mg/kg every 3 weeks). Written informed consent was obtained from all participants prior to inclusion into the study.

Patient response evaluation

Radiological tumor evaluation was performed 6 weeks after start of therapy. Dependent on CT scan evaluation, a follow-up scan was performed 4-12 weeks later. For NSCLC, best overall response (BOR) was assessed according to Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 and modified RECIST was used for MPM. Progression free survival (PFS) was defined as time from start of CI therapy until radiological progression or death and OS was defined as time from start of CI therapy until death.

Patient selection and data collection

For the initial cohort, NSCLC patients were selected to create a group of responding (R) and non-responding (NR) patients to CI therapy between the 5th of May 2016 and the 2nd of December 2022. These patients were matched on age, histology, gender, PD-L1 expression and treatment. R were patients with a partial response (PR) or complete response (CR) as BOR to CI therapy and NR were patients with progressive disease as BOR. In total, 11 R and 8 NR were selected for analysis based on the availability of samples. For the second cohort, MPM patients were selected similar to NSCLC patients, resulting in 6 R and 10 NR, whereby one of the R had stable disease as BOR for more than 1 year. For both cohorts, 5 gender-matched healthy controls (HCs) were selected for analysis. Clinical data was collected retrospectively.

Peripheral blood collection and flow cytometry

Blood was drawn at baseline (prior to therapy) and PBMCs were isolated using ficoll gradient centrifugation and cryopreserved before analysis, using standard procedures. Fluorochrome conjugated antibodies are listed in Supplementary Table 1. Flow cytometry analyses were performed on cryopreserved PBMC samples and all stainings were performed at 4°C following previously described procedures (20) and adherent to general flow cytometry guidelines. (21) Cells were stained for cell surface markers for 30 min., followed by incubation with Fixable Viability Dye (eBioscience, ThermoFisher, Waltham, MA, USA) for 15 min. at 4°C. Next, cells were fixed and permeabilized using the FoxP3 Transcription Factor Staining Buffer Set (eBioscience) and stained intracellularly for 60 min. at 4°C (supplementary table 1). Acquisition was performed on a FACSymphony A5 (BD Biosciences, Franklin Lakes, NJ, USA) using BD FACSDiva software (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Statistics

Median OS and PFS were estimated using a Kaplan-Meier curve in combination with a log-rank (Mantel-cox) test. We presumed a not-normal distribution. In graphs comparing R, NR and HC, we used a Mann-Whitney U test as we primarily tested for a difference between R and NR and did additional analysis to see if R or NR more likely resemble the phenotype frequencies of HC. In graphs comparing different cell types we used a Kruskall-Wallis test with Dunn's posttest as we primarily were interested in a difference between any of the several cell subsets. A P-value < 0.05 was considered significant. All reported P-values were two-tailed. Statistical analyses were performed using R 3.6.0 (R Foundation for Statistical Computing) or Graphpad Prism 8.0.

Results

Clinical parameters

Baseline characteristics of NSCLC patients are summarized in Table 1. Median PFS for NR was 1.6 months and 56.9 months for R. PD-L1 status was available in all pembrolizumab treated NSCLC patients and there was no significant difference in PD-L1 expression between R and NR.

Table 1. NSCLC Patient baseline characteristics

	R (n=11)	NR (n=8)	Total (n=19)	HC (n=5)
Age (years)	68,7	68,4	68,6	29
Male	45% (n=5)	38% (n=3)	42% (n=8)	60% (n=3)
Histology	64% adeno (n=7) 27% squamous (n=3) 9% unknown (n=1)	50% adeno (n=4) 25% squamous (n=2) 12,5% mixed (n=1) 12,5% unknown (n=1)	61% adeno (n=11) 28% squamous (n=5) 5% mixed (n=1) 11% unknown (n=2)	NA
Therapy	45% nivolumab (n=5) 55% pembrolizumab (n=6)	38% nivolumab (n=3) 62% pembrolizumab (n=5)	42% nivolumab (n=8) 58% pembrolizumab (n=11)	NA
PD-L1%	72,5 (n=6)	78 (n=5) (p=0.91)	80,5 (n=11)	NA
PFS (months)	56,9 months	1,6 months (p<0.0001)	NA	NA
OS (months)	73,9 months	3,7 months (p<0.0001)	NA	NA

Data are presented as percentage and absolute number, unless stated otherwise. The P values for PD-L1 is for the comparison of R vs. NR with a Mann-Withney U test. P values for survival are for the comparison of the survival curves of R vs. NR. Abbreviations: R: responders, NR: non-responders, HC: healthy control, PFS: progression free survival, OS: overall survival, NA: not applicable.

NR show decreased B cell frequencies, but increased frequencies of CD27- IgD- DN B cells

We studied circulating B cell subtypes in R and NR patients with NSCLC and in HC. The gating strategy can be found in Supplementary Figure 1. Immunophenotypic analyses of PBMC samples revealed lower B cell frequencies in NR compared to R and HC (Fig. 1A). Next, the distribution of naïve B cells (CD27-IgD+), unswitched memory B cells (CD27+IgD+ or/and IgM+), switched memory (SM) B cells (CD27+IgD-IgM-), and DN B cells (CD27-IgD-) was analyzed between the different groups (Fig. 1B). NR had significantly higher frequencies of DN B cells and lower frequencies of naïve B cells compared to R, whilst R and HC showed similar frequencies in these B cell subsets. No correlation between age and the abundance of DN B cells (Suppl. Fig. 2A) was found. As the frequency of total B cells was lower in NR compared to R and HC, the frequencies of B cell subsets were also plotted as fractions of live cells (Suppl. Fig. 2B). This analysis showed that due to the lower abundance of B cells in NR patients, DN B cells as a fraction of total live cells was similar in NR, R and HC (Suppl. Fig. 2B). This implies that, regarding DN B cells, specifically the distribution within the B cell compartment associates with response to CI therapy. In addition, particularly the frequency of naive B cells as a fraction of total live cells was reduced in NR, compared with R and HC (Suppl. Fig. 1B). Finally, the proportion of proliferating Ki67+ cells within total B cells was significantly higher in NR than in R and a similar trend was seen in the other B cell subsets analysed (Fig. 1C).

A high frequency of antigen-experienced CD21- DN B cells is associated with a lack of response to CI therapy.

To phenotype the DN B cells in more detail, we analyzed CD21 expression in a subgroup of patients. For 5 individuals in R, NR and HC, PBMC samples were available for analysis of the CD21 marker. We found that the frequency of $CD21^-$ DN B cells was increased in NR, compared with R and HC, but the frequency of CD21+ DN B cells did not differ between the three groups (Fig. 1D). Finally, the frequency of CD21- DN B cells inversely correlated with the frequencies of total B cells (Suppl. Fig. 2C), indicating that the two specific characteristics for NR, i.e. reduced frequencies of total B cells and increased frequencies of CD21- DN B cells, are linked.

As ABCs are described to be antigen-experienced, we analyzed isotype distribution and observed that CD21- DN B cells are poised towards an IgG isotype (Fig. 1E). Especially CD21- DN B cells in NR are IgG+ as compared to R and HC (Fig. 1E), resulting in a substantial and significantly increased frequency of IgG+ CD21- DN B cells in NR compared to R an HC, even as a fraction of total live cells (Fig. 1F). The latter is quite impressive as the number of total B cells in NR is very low compared to R and HC (Fig. 1A).

To further elucidate on the potential function of CD21- DN B cells, we looked at the expression of Ki67, HLA-DR and CD86 in comparison with their CD27+ counterpart; SM B cells. Expression of Ki67 and CD86 was higher on CD21- DN B cells compared to SM B cells and HLA-DR expression trended to be higher (Fig. 1G). The expression of these markers on CD21- DN B cells did not correlate with response to CI therapy (data not shown).

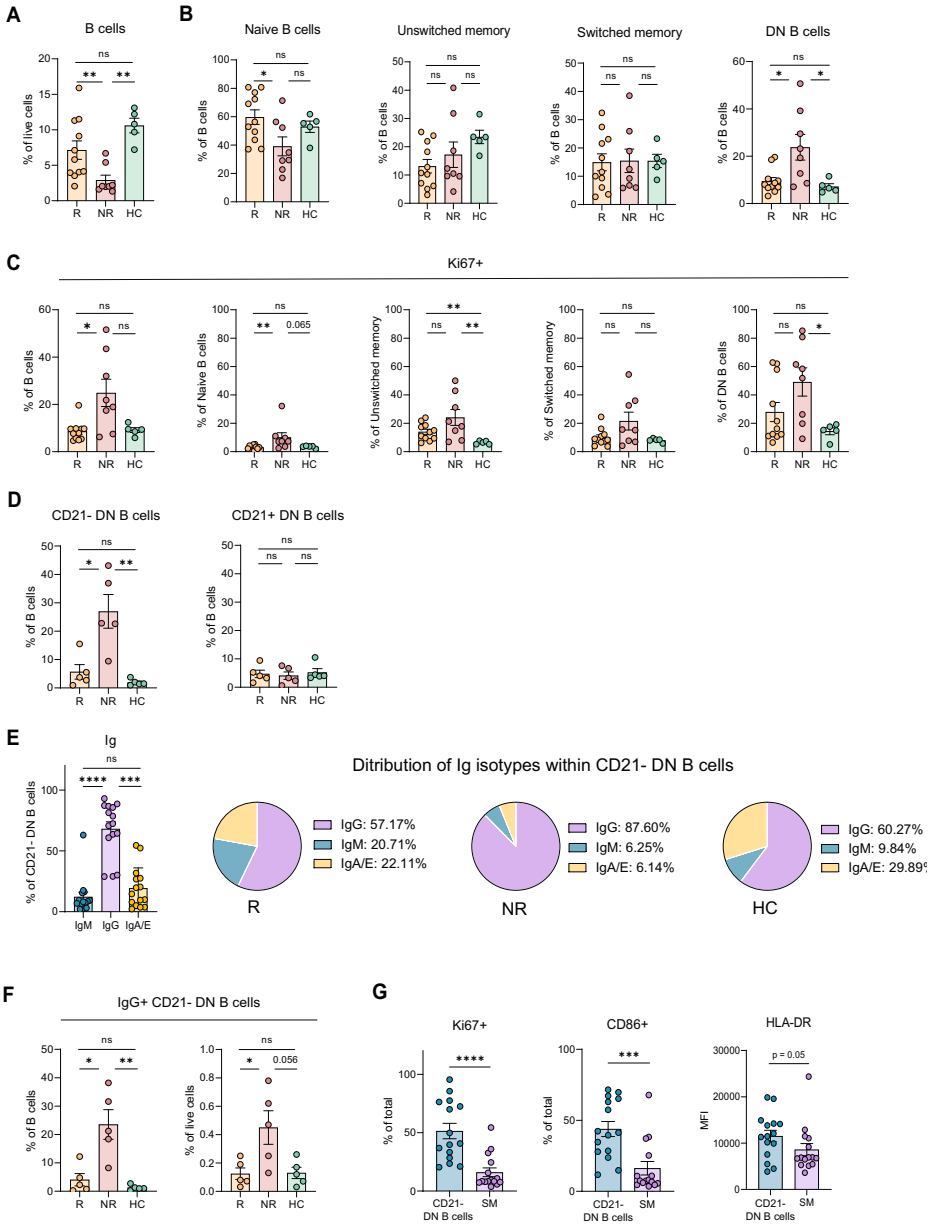

Figure 1. Perturbations of B cell subtypes in NSCLC. (A-B) Frequencies of B cells (A) and double negative (DN) B cells, naïve B cells, unswitched memory B cells and switched memory B cells (B). (C) Percentage of Ki67+ cells of B cells and B cell subsets. (D) CD21- DN B cells as fraction of total B cells (left) and as a fraction of live cells (right). (E) Immunoglobulin isotype expression on CD21- DN B cells (left) and pie charts showing isotype distribution of CD21- DN B cells per patient group (right). (F) IgG+ CD21- DN B cells as fraction of total B cells (left) and as fraction of

total live cells (right). (G) Percentage of Ki67+ (left), CD86+ cells (middle) and MFI of HLA-DR per cell subset. Means and SEMs are shown and Mann-Whitney U test or Kruskall-Wallis test withs Dunn's posttest were performed indicating statistical significance. *P < .05, **P < .01, *** P < .001, **** P < .0001. R; responders, NR; non-responders, HC; healthy controls, DN; double negative, SM; switched memory B cell, Ig; immunoglobulin.

Increased frequencies of CD21- DN B cells in NR patients is not restricted to NSCLC

To explore whether high frequencies of CD21- DN B cells are also found in NR patients with other tumors, we analyzed PBMCs from MPM patients receiving CI therapy. Median PFS for NR was 1.7 months and 24.5 months for R (Supplementary table 2). Similar to NSCLC, NR trended towards lower B cell frequencies than R and significant lower frequencies than HC (Fig. 2A). The frequency of DN B cells in NR was significantly higher compared to R and HC (Fig. 2B), which was again not age-associated (data not shown). Finally, NR had a significantly higher proportion of CD21- DN B cells than R and HC (Fig. 2C), indicating that CD21- DN B cells are more frequent in blood of patients with thoracic malignancies that do not respond to CI therapy.

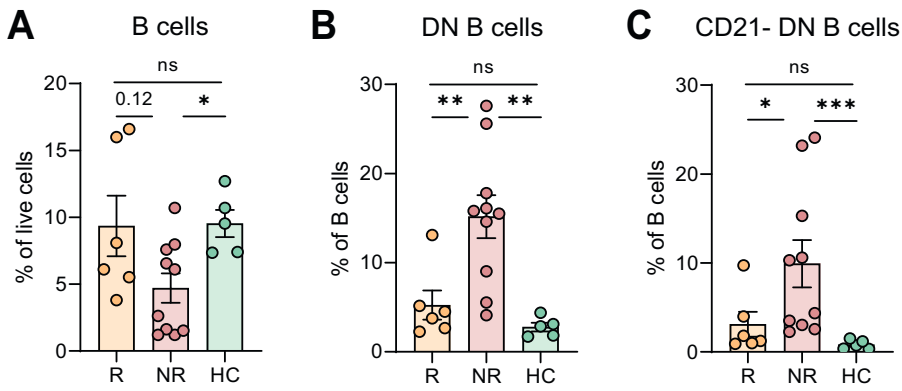

Figure 2. Perturbations of B cell subtypes in MPM. (A-C) Frequencies of B cells (A), double negative (DN) B cells (B) and CD21- DN B cells as fraction of total B cells (C). Means and SEMs are shown and Mann-Whitney U test was performed indicating statistical significance. *P < .05, **P < .01, *** P < .001, **** P < .0001. R; responders, NR; non-responders, HC; healthy controls, DN; double negative.

Discussion

Our study shows that a decreased frequency of B cells in peripheral blood is correlated with worse clinical outcome in NSCLC patients treated with CI therapy. Most importantly, a high frequency of CD21- DN B cells in peripheral blood of NSCLC and MPM patients is associated with a lack of response to CI therapy and is inversely correlated with the total

number of B cells. CD21- DN B cells are mainly class switched towards IgG, indicating these B cells are antigen-experienced.

The correlation between the scarcity of peripheral B cells and lack of response to CI therapy confirms earlier work by Xia et al.[19] The abundance of CD21- DN B cells inversely correlating with the number of B cells, complements a study in melanoma in which an increase in CD21- B cells correlated with a decrease in the number of B cells.[22] As ABCs in other diseases are prone to Fas ligand-induced cell death due to high expression of CD95,[23-28] we hypothesize that CD21- DN B cells in our study are short lived as well. NR have numerically higher frequencies of Ki67+ expression in all B cell subsets, especially in naive B cells, indicating their homeostatic proliferation.[29]

The (IgG+) CD21- DN B cells we found in NSCLC and MPM phenotypically resemble the ABCs found in the elderly, auto-immunity and chronic infection.[12-17] As our patients were age-matched and there was no correlation between age and the presence of DN B cells, it seems unlikely that the DN B cells found in cancer patients resemble classical age-associated B cells, but are rather induced by the tumor. To distinguish if ABCs in our study resemble ABCs in chronic infection or autoimmunity, one would have to look at their functionality. ABCs in autoimmunity are hyperresponsive to toll-like receptor (TLR) signaling and differentiate easily, without BCR stimulation, into auto-reactive antibody-producing plasma cells.[12-14] In chronic infections such as HIV, malaria, long COVID and HCV, the ABCs have an exhausted phenotype as a consequence of chronic antigen exposure and have high affinity thresholds for activation.[13,15,16,30] As ABCs in our study are subject to chronic tumor-antigen exposure and correlate with lack of response to CI therapy, it is most likely that they have functional similarities with ABCs seen in chronic infection, but this remains to be further investigated.

Apart from potentially being exhausted and having a high affinity threshold for activation, CD21- DN B cells in our study express relatively high levels of CD86 and HLA-DR compared to their CD27 expressing counterparts (SM B cells). CD86 has also been described to be high on CD21- B cells in HIV, but after stimulation B cells are less capable of increasing CD86 expression and are unable to activate T cells, leading to poor CD4 T cell activation and proliferation.[31] However, research has shown that in NSCLC tumor tissue CD21- CD27- B cells are capable of presenting antigen to CD4 T cells, but induce regulatory CD4 T cells (FoxP3+ IFN-g-) rather than active CD4 T cells (FoxP3- IFN-g+).[32] In a pan-cancer study, a decrease of CD21- B cells after start of CI therapy is correlated with longer survival.[18] In conclusion, lack of CD21 expression on B cells seems to be detrimental for the process of inducing an effective anti-tumor immune response.

We acknowledge that this study has some limitations. Firstly, the lack of tumor material precluded to study intra-tumoral immune cells. Establishing a correlation between our peripheral blood findings and intra-tumoral B cells is crucial to reveal if CD21-

DN B cells locally hamper a functional anti-tumor response. Secondly, due to limited sample availability, we were only able to analyze a small number of patients at one time point. Nonetheless, even with small numbers of patients we were able to identify and validate our results in NSCLC and MPM, implying that the differences in CD21- DN B cells are robust and not specific for one tumor type. In addition, the limited sample availability prevented us from conducting functional studies on CD21- DN B cells, e.g. immunoglobulin secretion capacity upon stimulation, or additional phenotyping Full validation with elaborate analyses of both the functional status and phenotype is recommended to determine if these cells can serve as a predictive biomarker. Fourth, recent literature has reported on the correlation between CD21- B cells and immune-related adverse events (irAEs) in melanoma patients treated with combination checkpoint inhibitors.[5,22,33] As patients in our study had no irAEs, we were not able to check for a correlation between the presence of CD21- DN B cells and irAEs.

In conclusion, the abundance of (IgG+) CD21- DN B cells in peripheral blood is correlated with lack of response and unfavorable clinical outcome in NSCLC and MPM patients treated with CI therapy. We speculate that naïve B cells are skewed by the tumor microenvironment – together with systemic changes that might be induced by the anti-tumor response – into CD21- DN B cells. This would parallel the proposed mechanisms in chronic infection and auto-immunity, whereby an inflammatory environment is also responsible for the induction of ABCs. The pathways by which these CD21- DN (atypical) B cells subsequently hamper CI therapy remains unknown. However, we hypothesize that CD21- DN B cells either resemble phenotypically identical exhausted B cells that are seen in chronic infection or function as antigen presenting cells that induce regulatory T cells. Future research on how CD21- DN B cells might hamper CI therapy response is needed.

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Supplementary tables and figures

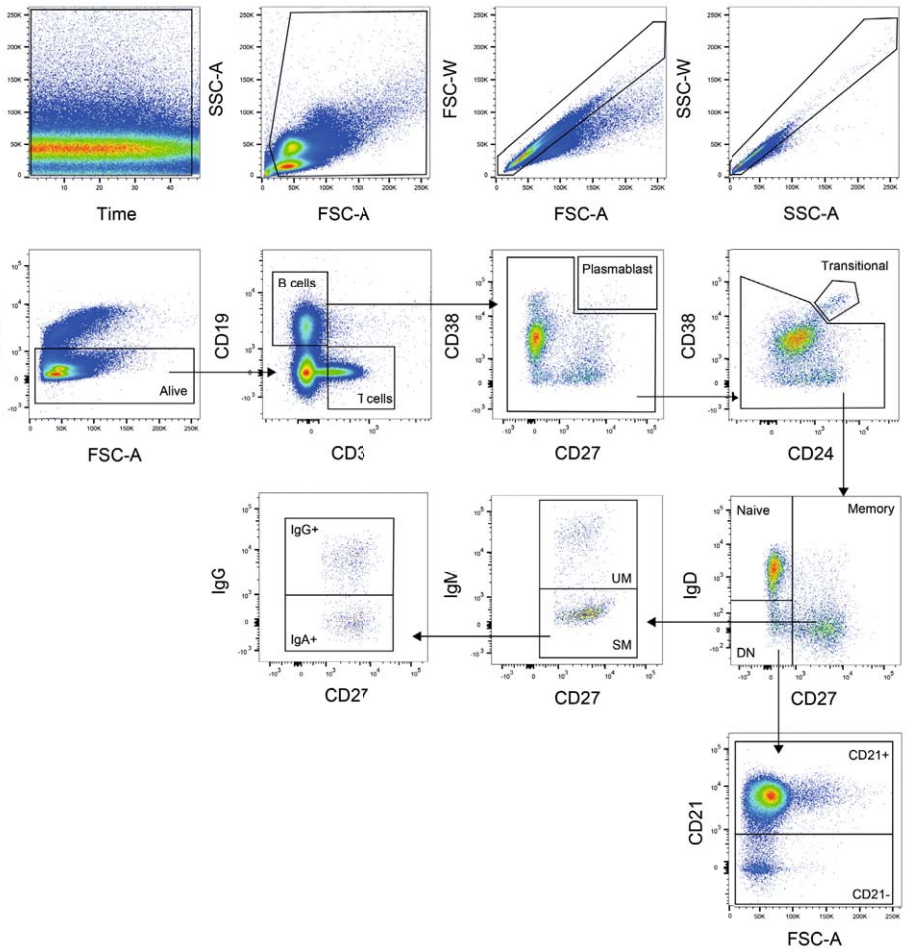
Table S1. Antibodies used for flow cytometry staining

Antibody	Fluorochrome	Manufacturer	Catalogue number
CD38	PerCP-Cy5.5	BD	566445
IgD	PE-Cy7	BD	561314
PD-1	APC	Biolegend	329908
CD24	APC-Cy7	Biolegend	311132
CD3	AF700	Introgen	56-0038-42
CD27	BV421	BD	562513
IgM	BV605	BD	562977
CD86	BV650	BD	563412
CD19	BV750	BD	747083
CD40	PE	BD	555589
HLA-DR	BV711	BD	563696
CD21	PE-CF594	BD	563474
IgA	Biotin	SouthernBiotech	2050-08
IgG	BV786	BD	564230
Streptavidin	PE-Cy5	BD	554062
Ki67	FITC	Invitrogen	11-5699-42
Aqua L/D	BV510	eBioscience	65-0866-14

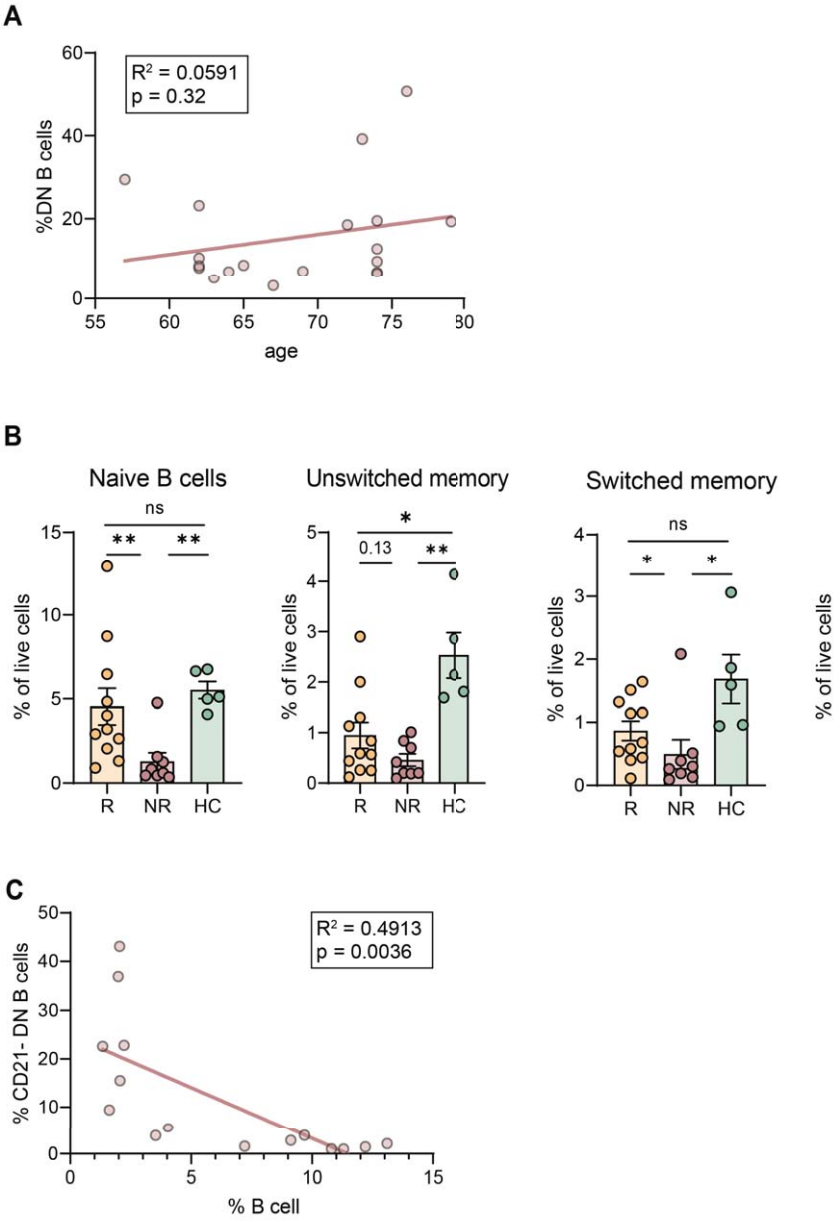
Table S2. MPM Patient baseline characteristics

	R (n=6)	NR (n=10)	Total (n=16)
Age (years)	66,3	73,1	70,6
Male	83,3% (n=5)	90% (n=9)	87,5% (n=14)
Histology	50% epithelioid (n=3) 16,7% non-epithelioid (n=1) 16,7% mixed (n=1) 16,7% unknown (n=1)	90% epithelioid (n=9) 10% non-epithelioid (n=1)	75% adeno (n=12) 12,5% squamous (n=2) 6,3% mixed (n=1) 6.3% unknown (n=1)
PFS (months)	24,5 months	1,7 months p=0.0009	-
OS (months)	Not reached	10,4 months	-

Data are presented as percentage and absolute number, unless stated otherwise. P values for survival are created by comparing survival curves of R vs. NR. Abbreviations: R: responders, NR: non-responders, PFS: progression free survival, OS: overall survival.



Supplementary figure 1. Gating strategy. Gating strategy to identify the various B cell subsets. After removing unstable measurements by applying a FCS vs. time gate, the PBMC were selected. Next, doublets were removed and dead cells were excluded from the analyses. Subsequently, B cells were selected after which the different subsets could be identified.



Supplementary figure 2. Perturbations of B cell subtypes in NSCLC. (A) Pearson correlation coefficient (R^2) between age and frequency of double negative (DN) B cells in NSCLC patients (n=19). (B) Frequencies of DN B cells, naïve B cells, unswitched memory B cells and switched memory B cells as fraction of live cells. (C) Pearson correlation coefficient (R^2) between percentage of CD21- DN B cells and total B cells in NSCLC patients. (D) Percentage Ki67+ (left), CD86+ (middle) and MFI HLA-DR (right) of IgG+ CD21- DN B cells. Means and SEMs are shown and Mann-Whitney U test or Kruskall-Wallis test withs Dunn's posttest were performed indicating statistical significance. *P < .05, **P < .01, *** P < .001, **** P < .0001. R; responders, NR; non-responders, HC; healthy controls, DN; double negative.

5

A multicentre, randomized, phase II/III study of dendritic cells loaded with allogeneic tumor cell lysate (MesoPher) in patients with pleural mesothelioma as maintenance therapy after chemotherapy: the DENdritic cell Immunotherapy for Mesothelioma (DENIM) trial

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Research in context

Evidence before this study

We searched PubMed and abstracts of major conferences for all clinical studies, published between Jan 1, 2019, and Dec 1, 2023, in English and Dutch, evaluating maintenance treatment in patients with pleural mesothelioma of both epithelioid and non-epithelioid histology using the search terms "mesothelioma" AND "maintenance" AND "randomized". We identified three studies that all found no improved overall survival outcome with maintenance treatment compared with observation or placebo. Only one study, the NVALT 19 study, showed an improved progression-free survival when comparing switch maintenance treatment with gemcitabine versus placebo. Since 2003, first-line treatment of pleural mesothelioma has consisted of four-to-six cycles of chemotherapy with platinum–pemetrexed, resulting in a median overall survival of 12.1 months. In 2014, the MAPS trial showed a significant improvement in overall survival of 2.7 months with the addition of bevacizumab, which, from then onwards, could be added to the chemotherapy. In 2021, Checkmate 743 was the first randomised trial to show increased overall survival with combination immune checkpoint inhibition versus chemotherapy. Immune checkpoint inhibitors became the recommended treatment for eligible patients. Despite the improved survival, long-term overall survival in pleural mesothelioma is still poor.

Added value of this study

To our knowledge, this study is the first randomised trial with cellular therapy in pleural mesothelioma to investigate efficacy, toxicity, and immune activation of dendritic cell therapy (MesoPher) compared with best supportive care. Although the study did not meet its primary outcome of improved overall survival, we found broad immune activation and a good safety profile. Exploratory analysis showed that patients with a non-epithelioid histology had a significantly longer progression-free survival when treated with MesoPher therapy, which was not present in the best supportive care group, although this finding should be interpreted cautiously in the light of small patient numbers in the non-epithelioid group.

Implications of all the available evidence

Immune checkpoint inhibitors are the first-line standard of care treatment in pleural mesothelioma showing the potential of activation of the immune system. However, an unmet need for improving long-term survival in most patients is present. Despite the fact that this study on cellular therapy was negative for its primary outcome, the immune activation induced by the treatment and the acceptable safety profile open the option to investigate combination treatments with immune checkpoint inhibitors and cellular therapy.

Abstract

Background

Dendritic cell immunotherapy has proven to be safe and induces an immune response in humans. We aimed to establish efficacy of dendritic cells loaded with allogeneic tumour cell lysate (MesoPher, Amphera BA, 's-Hertogenbosch, Netherlands) as maintenance therapy in patients with pleural mesothelioma.

Methods

In this open-label, randomised, phase 2/3 study, patients with histologically confirmed unresectable pleural mesothelioma, aged 18 years or older, with an Eastern Cooperative Oncology Group performance status score of 0–1, and non-progressing disease after four to six cycles of standard chemotherapy (with pemetrexed 500 mg/m² plus platinum [cisplatin 75 mg/m² or carboplatin area under the curve of 5]) were recruited from four centres in Belgium, France, and The Netherlands. Participants were randomly assigned (1:1), using block randomisation (block size of 4), stratified by centre and histology (epithelioid vs other), to MesoPher treatment plus best supportive care or best supportive care alone. Patients received up to a maximum of five MesoPher infusions, with treatment administered on days 1, 15, and 29, and weeks 18 and 30. At each timepoint, participants received an injection of 25×10^6 dendritic cells (two-thirds of the dendritic cells were administered intravenously and a third were injected intradermally). Best supportive care was per local institutional standards. The primary endpoint was overall survival, assessed in all participants randomly assigned to treatment (full analysis set) and safety assessed in all randomly assigned participants, and who underwent leukapheresis if they were in the MesoPher group. This study is registered with ClinicalTrials.gov, NCT03610360, and is closed for accrual.

Results

Between June 21, 2018, and June 10, 2021, 176 patients were screened and randomly assigned to the MesoPher group (n=88) or best supportive care alone group (n=88). One participant in the MesoPher group did not undergo leukapheresis. Mean age was 68 years (SD 8), 149 (85%) of 176 were male, 27 (15%) were female, 173 (98%) were White, two were Asian (1%), and one (1%) was other race. As of data cutoff (June 24, 2023), after a median follow up of 15.1 months (IQR 9.5–22.4), median overall survival was 16.8 months (95% CI 12.4–20.3; 61 [69%] of 88 died) in the MesoPher group and 18.3 months (14.3–21.9; 59 [67%] of 88 died) in the best supportive care group (hazard ratio 1.10 [95% CI 0.77–1.57]; log-rank p=0.62). The most common grade 3–4 treatment-emergent adverse events were chest pain (three [3%] of 87 in the MesoPher group vs two [2%] of 88 in the best supportive care group), dyspnoea (none vs two [2%]), anaemia (two [2%] vs none), nausea (none vs two [2%]), and pneumonia (none vs two [2%]). No deaths due to treatment-emergent adverse events were recorded. Treatment-related adverse events consisted of infusion-related reactions (fever, chills, and fatigue), which

occurred in 64 (74%) of 87 patients in the MesoPher group, and injection-site reactions (itch, erythema, and induration), which occurred in 73 (84%) patients, and all were grade 1–2 in severity. No deaths were determined to be treatment related.

Interpretation

MesoPher did not show improvement in overall survival in patients with pleural mesothelioma. Immune checkpoint therapy is now standard of care in pleural mesothelioma. Further randomised studies are needed of combinations of MesoPher and immune checkpoint therapy, which might increase efficacy without adding major toxicities.

Funding

Amphera BV
European Union HORIZON

Introduction

Pleural mesothelioma is a lethal disease that is causally linked to exposure to asbestos and for which available treatment options have minimal efficacy. Although immunotherapy, often administered in the form of immune checkpoint inhibitors, results in clinically significant increases in long-term overall survival and progression-free survival in other tumour types, these effects, although present, are less pronounced in patients with pleural mesothelioma than for those with other tumour types. In CheckMate 743,[1] combination immune checkpoint inhibitor therapy with anti-PD-1 and anti CTLA-4 antibodies in the first-line setting did not increase radiologically assessed response rate or progression-free survival compared with first-line chemotherapy among patients with malignant pleural mesothelioma, but a significant increase in median overall survival was found, from 14.1 months (95% CI 12.4–16.2) with chemotherapy to 18.1 months (16.8–21.4) with combination immune checkpoint inhibitor therapy. Furthermore, the 3-year overall survival was 15.4% (95% CI 11.5–19.9) in the chemotherapy group and 23.2% (18.4–28.2) in the immune checkpoint inhibitor therapy group.[2] These overall survival estimates are lower than in other cancer types treated with immune checkpoint inhibitor therapy.

Progenitor exhausted T cells are the main effector cells of tumour-infiltrating lymphocytes and are responsible for response to immune checkpoint inhibitor therapy. Continuous influx and proliferation of newly generated progenitor exhausted T cells is an essential step in cancer immunity. In a mesothelioma mouse model, we have shown that the induction of progenitor exhausted T cells that infiltrate the tumour is minimal, causing only a short-lived immune response, similar to that seen in most patients with pleural mesothelioma.[3,4] Different treatment options are under investigation to enhance this progenitor exhausted T cell induction. Among these options, adoptive cell therapies are a potentially promising tool,[5] one of which is dendritic cell therapy. Dendritic cells are the most potent antigen-presenting cells, stimulating both the adaptive and innate immune response. Dendritic cell therapy consisting of autologous monocyte-derived dendritic cells loaded with an allogenic tumour cell lysate, MesoPher (Amphera BV, 's-Hertogenbosch, Netherlands), has proven to upregulate CD4+ PD1-positive T cells and induce an anti-tumour T-cell response, both in patients with pleural and abdominal mesothelioma as well as in those with pancreatic cancer.[6,7] In a dose-escalating phase 1 study in pleural mesothelioma, we found that treatment with MesoPher was safe and feasible, induced a CD4+ T-cell response, and was associated with a promising outcome (median progression-free survival 8.8 months [95% CI 4.1–20.3], median overall survival not reached [not determined]) after a median follow up of 22.8 months (range 18.5–27.4).[8]

We aimed to assess the efficacy of MesoPher plus best supportive care as maintenance treatment after chemotherapy compared with best supportive care in patients with pleural mesothelioma.

Methods

Study design and participants

In this open-label, randomised, phase 2/3 study, we planned to enrol participants from six centres in five countries in Europe; however, due to travel restrictions during the COVID-19 pandemic, participants were recruited from four centres in three countries (Belgium, France, and The Netherlands; appendix p 6). Eligible patients had histologically confirmed unresectable pleural mesothelioma, were aged 18 years or older, with an Eastern Cooperative Oncology Group (ECOG) performance status of 0–1, adequate organ function, and had non-progressing disease after four to six cycles of chemotherapy (pemetrexed 500 mg/m² plus platinum [cisplatin 75 mg/m² or carboplatin area under the curve of 5]). Concurrent bevacizumab treatment during chemotherapy was allowed, but patients were only eligible if they stopped bevacizumab at the end of the chemotherapy period. Key exclusion criteria were an active serious infection including HIV, hepatitis B or C, or syphilis. Patients with an allergy to shellfish, a history of autoimmune disease, a previous malignancy (except adequately treated basal cell or squamous cell skin cancer, superficial or in-situ cancer of the bladder, or other cancer for which they have been disease free for at least 3 years), any other serious chronic or acute illness, an organ allograft, and pregnant women were excluded, as well as anyone who had taken more than 10 mg prednisone or equivalent per day of treatment with an immunosuppressive agent during the 6 weeks before study. Patients who were treated with any investigational product as part of another study within 4 weeks or five half-lives from screening were not eligible for inclusion. A full list of eligibility criteria is provided in the protocol (appendix). Written informed consent was obtained before study enrolment. The study was performed in accordance with the guidelines of Good Clinical Practice and the principles of the Declaration of Helsinki and the protocol and amendments were approved by the ethical committees of the countries or institutes, depending on country legislation.

Seven regular Independent Data Monitoring Committee meetings were held during the course of the study, and in light of the COVID-19 pandemic, the committee advised to continue the trial unmodified. During the COVID-19 pandemic, enrolment was halted from March 16 to May 20, 2020, but patients already enrolled in the trial were treated according to study protocol. Additionally, as a consequence of travel restrictions during the pandemic, four centres rather than six centres were involved in the study. After discussion with the European Medicines Agency in a scientific advice request procedure, a sample size reduction was agreed on, which was approved by the Independent Data

Monitoring Committee and the protocol was amended (version 9; March 15, 2021). The study is registered with ClinicalTrials.gov, NCT03610360.

Randomization and masking

Patients were randomly assigned (1:1), via block randomisation (block size of 4) and a centralised interactive web-response system with a per-centre randomisation list, to receive MesoPher plus best supportive care or best supportive care alone. Randomisation was stratified by histology (epithelioid vs other) and study centre. An investigator at each site registered patients via the web-response system and assigned them according to the randomisation sequences generated by an independent third party. All patients, investigators, study site personnel, and the sponsor were aware of the treatment assignment because treatment itself was open label, but the treating physician was masked to the randomisation procedure.

Procedures

Patients were screened and randomly assigned to treatment groups after providing written informed consent, approximately 4 weeks after the start of the last cycle of chemotherapy (appendix p 6). Sex and race were self-reported during screening. Patients assigned to the MesoPher treatment group were scheduled for a leukapheresis session 5–8 weeks after the start of the last cycle of chemotherapy at the production facility (Erasmus Medical Centre, Rotterdam, Netherlands) to collect monocytes. After 10 days of production and then another 3 weeks for batch release procedures, including sterility checks, the vials were frozen and shipped to the investigational site, which took another week. Treatment had to start 9–13 weeks after the start of the last cycle of chemotherapy and the minimum number of treatments per protocol was three, with the complete treatment schedule consisting of a maximum of five administrations (on day 1 [baseline], and thereafter on days 15, and 29, and weeks 18 and 30; appendix p 6). At each treatment visit, 25×10^6 dendritic cells were injected. Two-thirds of the dendritic cells were administered intravenously and a third were injected intradermally. Treatment was continued if no disease progression, unmanageable toxicity, or withdrawal was reported. No dose reductions were allowed. Dose interruptions were allowed in case of unforeseen circumstances per protocol definitions. Patients in the best supportive care study group were managed with best supportive care according to the discretion of institutional standards. Patients in the MesoPher group also received best supportive care. If disease progression occurred, second-line treatment (including treatment with immune checkpoint inhibitors) according to institutional policy was allowed in both groups; data were collected on treatments received. CT scans were done 3 weeks after the start of the last cycle of chemotherapy (baseline scan) and 6 weeks after the start of treatment and every 12 weeks thereafter. CT scans were assessed locally by investigators according to modified Response Evaluation Criteria in Solid Tumours (RECIST) version 1.1.

Venous blood samples were collected for safety analyses. For patients in the MesoPher group, this was done at screening, on days 1, 8, 22, and 36, on weeks 18, 20, 30, and 32, and then every 12 weeks during follow-up. For the best supportive care group, samples were collected at screening and on day 1 and weeks 6, 18, 30, and every 12 weeks during follow-up.

Adverse events were collected during each outpatient visit. During treatment with MesoPher, patients were monitored for 2 h for treatment-related adverse events. After progression, patients were contacted via telephone or outdoor patient visit once every 3 months to follow up on death and adverse events. All adverse events and serious adverse events were collected up to and including the week 36 visit. Beyond this visit, only treatment related serious adverse events were collected until study termination or death. The verbatim terms as reported in the electronic case report form by investigators to identify adverse events were coded using the Medical Dictionary for Regulatory Activities (version 19.0 or higher) and summarised by primary system organ class preferred term. The Common Terminology Criteria for Adverse Events (CTCAE; version 4.03) was used to grade toxicity throughout the study. Treatment-emergent adverse events were defined as any adverse event that occurred on or after random assignment to treatment.

Changes in quality of life were measured at screening (baseline) and 6 weeks after day 1 of treatment and every 12 weeks thereafter with the European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire (QLQ)-C30 and the lung cancer specific module EORTC QLQ-LC13.

Peripheral blood samples for exploratory immune cell analyses were taken on days 1, 8, 22, and 36 for the MesoPher group and on day 1 and week 6 for the best supportive care group. Peripheral blood was assessed with multiparameter flow cytometry analysis of co-stimulatory and co-inhibitory immune cell markers. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll gradient centrifugation (Ficoll Paque Plus van Cytiva, GE Healthcare, Chicago, IL, USA) and cryopreserved in 10% dimethyl sulphoxide (Sigma, Saint Louis, MO, USA), 40% fetal bovine serum (Capricorn, Düsseldorf, Germany), and 50% phosphate-buffered saline (Gibco, Billings, MT, USA) before analysis. Fluorochrome-conjugated antibodies that were used are listed in the appendix (p 1). Flow cytometry analyses were performed on cryopreserved PBMC samples using a FACSymphony flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and all staining was done at 4°C.

Allogeneic tumour lysate was produced under Good Manufacturing Practice-compliant conditions and consisted of five unique, clinical-grade human pleural mesothelioma cell lines, which are patented (ID P6038325PCT). For MesoPher production, monocyte derived dendritic cells were generated from monocytes in a 9-day culture protocol.

Monocytes were enriched from patient-derived apheresis products by a CD14 positive selection procedure (CliniMACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Monocyte purity after selection was greater than 97% with greater than 95% viability in all cases. Hereafter, monocytes were cultured in T225 flasks (100 million cells per flask) in X-VIVO 15 media (Lonza, Verviers, Belgium) supplemented with 2% normal human serum (NHS; Sanquin, Amsterdam, Netherlands) and adhered overnight at 37°C, in an atmosphere of 5% carbon dioxide. The following day, half of the medium was refreshed with fresh medium supplemented with 2% NHS, 1600 IU/mL recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF; Miltenyi Biotec, Bergisch Gladbach, Germany), and 1000 IU/mL recombinant human IL-4 (rhIL-4; Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were cultured for 3 additional days at 37°C, in 5% carbon dioxide. Hereafter, the immature monocyte-derived dendritic cells were transferred to six-well plates (1 million cells per well) in the presence of tumour lysate (tumour cell to dendritic cell ratio of 1:3), 800 IU/mL rhGM-CSF, 500 IU/mL rhIL4, and 10 mg/mL VACMUNE keyhole limpet haemocyanin (KLH; BioSyn, Carlsbad, CA, USA). After 2 days of culture, the cells were matured with 5 ng/mL IL-1β, 15 ng/mL IL-6, 838 IU/mL TNFα (all Miltenyi Biotec), and 10 mg/mL prostaglandin E2 (Pfizer, Brussels, Belgium). After 2 days of maturation, cells were harvested and quality controlled for sterility, to ensure cell cultures were free of endotoxins and mycoplasma, for at least 80% viability, at least 90% MHC class II CD11c cells (purity), and at least 80% CD80 cells of the MHC class II CD11c cells (activation status), and at least 30% migration in transwell assay (potency).

Outcomes

The primary endpoint was overall survival, which was measured as time from randomisation until death. For patients who were still alive at the end of the study or lost to follow-up, the overall survival time was censored on the last date they were known to be alive. Secondary endpoints were overall survival at 12 and 18 months after randomisation, progression-free survival, overall response rate, duration of response, disease control rate, and change in quality of life from baseline.

Progression-free survival was defined as the time from randomisation until radiological progression or death, whichever occurred first. Patients without progression at the end of the study or who were lost to follow-up were considered censored on the last day known to be alive. In the assessment of overall response, best disease response was defined as the best response recorded from the start of the treatment until disease progression or recurrence (including complete response, partial response, stable disease, and progressive disease). Overall response rate is defined as the proportion of participants with confirmed complete response or partial response according to the modified RECIST criteria (version 1.1; ie, complete response or partial response that was persist on repeat imaging performed more than 4 weeks after the initial documentation of response). Duration of response was defined as the time from partial response or

complete response to disease progression or death. However, due to the low number of radiological responses, duration of response and overall response rate were not analysed. Disease control rate was defined as the number of patients with a best disease response of complete response, partial response, or stable disease. Changes in quality of life were measured with the EORTC QLQ-C30 and the lung cancer-specific module EORTC QLQ-LC13.

A prespecified exploratory endpoint was systemic immune phenotyping, with evaluation of the immunogenic effect of MesoPher as determined by analysis of peripheral blood samples.

Statistical analysis

For the sample size estimation, we hypothesised a median overall survival of 21 months for patients treated with MesoPher plus best supportive care and 12 months for those treated with best supportive care alone, corresponding to a hazard ratio (HR) of 0.57 for exponential survival times. We planned the total study duration to be 36 months with an expected accrual time of 24 months. We anticipated a dropout rate of 0.5% per month. Under these assumptions, a sample size of 115 patients per treatment group for a total of 230 participants would provide 90% power with a two-sided type 1 error rate of 5%. Due to the COVID-19 pandemic, the inclusion rate dropped. The Independent Data Safety Monitoring Board met and agreed that due to the longer accrual time that would be necessitated to reach the hypothesised sample size, and hence longer follow-up time of the patients who had already been recruited, the number of events would be increased. Together with a reduction in the power of the study from 90% to 80%, while maintaining the two-sided type 1 error rate at 5%, this decision allowed the study to reduce the number of patients to be included to 82 per treatment group (for a total of 164) with an anticipated 101 events (protocol amendment version 9; March 15, 2021).

We summarised continuous variables using descriptive statistics (n, mean [SD], and median [IQR]) and categorical variables using the number and percentage of patients in each category. We did all statistical tests using a two-tailed 5% overall significance level.

We estimated the distribution of survival times and corresponding medians and 95% CIs for the overall survival (primary endpoint) time and for time to progression or death (or progression-free survival) using the non-parametric Kaplan–Meier method. We compared survival curves using the log-rank test, stratified for centre and histology (epithelioid vs other). There was no informative censoring and the number of censored patients was actually limited to seven in the MesoPher group and ten in the best supportive care alone group. We assessed the proportional hazards assumption visually using the log-log survival curve. We used a Cox proportional hazards model, including factors for centre and histology, to estimate the HR along with 95% CI. Several proposed subgroup analyses for overall survival and progression-free survival were described

in the statistical analysis plan, to potentially be completed if there were at least five participants in the level of subgroup. Hence, we did prespecified subgroup analyses for overall survival and progression-free survival using a similar Cox proportional hazards model according to age (≤65 years vs >65 years), sex (male vs female), ECOG performance status (0 vs 1), tumour stage (I or II vs III or IV), and histology (epithelioid vs other). The levels within the subgroups of age, ECOG performance status, and tumour stage were amended post-hoc due to the distribution of patients in some levels being too low or below five to be adequately powered. Additionally, the subgroup analysis of response to first-line chemotherapy (partial response vs stable disease) was added as a post hoc analysis. The null hypothesis of no difference between the overall survival times across treatment groups will be rejected should the probability of the null hypothesis being true (α) be less than 5%. We compared overall survival and progression-free between the treatment groups, by subgroup, using the log-rank test.

All patients randomly assigned to treatment were included in the full analysis set (FAS), which was used for all efficacy analyses and quality of life analysis. For response endpoints, patients who withdrew from the study were censored at that timepoint. Patients with clinical progression before CT scan was done were considered as having progressive disease. Patients who had no measurable disease at baseline could not be scored as having a partial response. The safety analysis set included all patients randomly assigned to treatment and, if they had been assigned to the MesoPher group, who underwent leukapheresis. For statistical analysis we used SAS (for Windows, version 9.4).

Immunophenotyping was an exploratory endpoint. Immunomonitoring measurements of mean fluorescence intensity (MFI) values were considered valid if the gated population consisted of at least 100 gated event counts. In case of negative phenotype MFI values, these were adjusted to zero. For all changes from baseline, we used the one-sample (paired observations) t test, and for comparison of two independent groups we used the two-sample t test. We used the Satterthwaite approximation for degrees of freedom to account for possible differences in variability between groups. Finally, we did a regression analysis to investigate the linear relationship between a change from baseline in markers that were upregulated after MesoPher treatment and progression-free survival.

Role of the funding source

The sponsor (Amphera BV) had a role in the study design, data collection, data analysis, data interpretation, and writing of the report.

Results

Between June 21, 2018, and June 10, 2021, 176 patients were screened and subsequently randomly assigned to MesoPher plus best supportive care (n=88) or best supportive care alone (n=88; figure 1; appendix p 6).

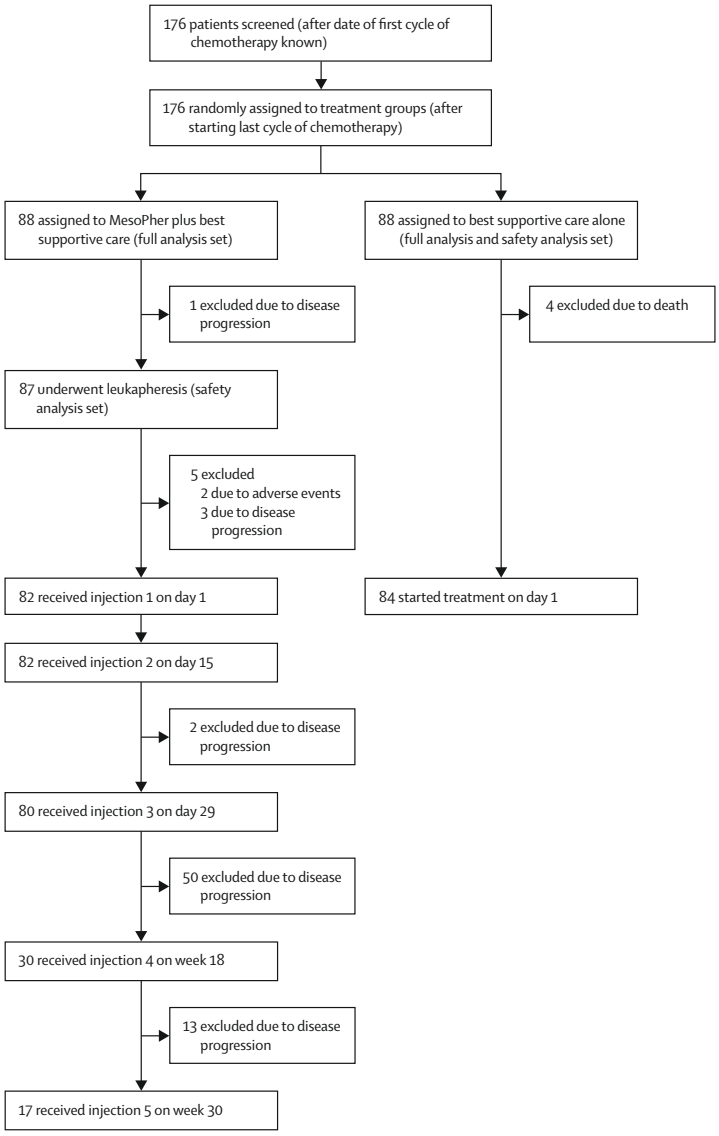

Figure 1. Schematic overview of the clinical procedure. PM, pleural mesothelioma; n, number of subjects in the analysis set; SD, stable disease; CR, complete response; PR, partial response; BSC, best supportive care; OS, overall survival; PFS, progression-free survival; DCR, disease control rate; QOL, quality of life.

Demographic and baseline characteristics were similar between the MesoPher and the best supportive care groups (table 1). Mean age was 68 years (SD 8), 149 (85%) of 176 were male, 27 (15%) were female, 173 (98%) were White, two were Asian (1%), and one (1%) was other race. By initial chemotherapy regimen, carboplatin plus pemetrexed was administered to 75 (43%) of 176 patients for four cycles, four (2%) patients for five cycles, and 16 (9%) patients for six cycles; cisplatin plus pemetrexed was administered to 38 (22%) patients for four cycles, three (2%) patients for five cycles, and seven (4%) patients for six cycles; and a combination of either cisplatin or carboplatin plus pemetrexed and bevacizumab was administered to seven (4%) patients (three had six cycles of cisplatin, three had six cycles of carboplatin, and one had four cycles of carboplatin). 25 (14%) patients started on cisplatin but switched to carboplatin (eight patients switched after one cycle of cisplatin to then receive three cycles of carboplatin, four patients received two cycles of cisplatin and then switched to receive two cycles of carboplatin, and 13 patients switched to carboplatin after three cycles of cisplatin). One patient was treated with carboplatin plus raltitrexed for four cycles. Two patients in each group withdraw consent during the study (MesoPher group n=1 on day 8 and on day 879, best supportive care alone group n=1 on day 246 and n=1 on day 393 after randomisation).

Table 1. Baseline demographic and clinical characteristics, full analysis set

	MesoPher plus best supportive care group (n=88)	Best supportive care alone group (n=88)
Age, years	68.8 (7.53)	67.2 (8.97)
18 to <65	23 (26%)	30 (34%)
65 to <75	48 (55%)	43 (49%)
75 to <85	17 (19%)	15 (17%)
≥85	0	0
Sex		
Male	78 (89%)	71 (81%)
Female	10 (11%)	17 (19%)
Race		
White	87 (99%)	86 (98%)
Asian	1 (1%)	1 (1%)
Other*	0	1 (1%)
ECOG performance status at baseline		
0	38 (43%)	27 (31%)
1	50 (57%)	60 (68%)
2	0	1 (1%)
Time since most recent chemotherapy, weeks†	11.77 (1.33)	10.87 (1.05)
BMI at screening, kg/m²	25.98 (3.02)	25.66 (3.93)

Table 1. Baseline demographic and clinical characteristics, full analysis set. (continued)

	MesoPher plus best supportive care group (n=88)	Best supportive care alone group (n=88)
Histology		
Epithelioid	73 (83%)	75 (85%)
Sarcomatoid	7 (8%)	4 (5%)
Desmoplatic	0	0
Biphasic	8 (9%)	8 (9%)
Other	0	1 (1%)
Stage at diagnosis		
IA	25 (28%)	19 (22%)
IB	33 (38%)	25 (28%)
II	8 (9%)	13 (15%)
IIIA	5 (6%)	8 (9%)
IIIB	13 (15%)	14 (16%)
IV	4 (5%)	9 (10%)
Response to first-line chemotherapy		
Partial response	27 (31%)	26 (30%)
Stable disease	60 (68%)	62 (70%)
Progressive disease	1 (1%)	0

Data are n (%) or mean (SD). Percentages may add up to more than 100% due to rounding. ECOG=Eastern Cooperative Oncology Group.*Data on other race are not collected. † Time since start of most recent cycle of chemotherapy until day 1 of the DENIM trial; n=85 for MesoPher plus best supportive care group, and n=80 for best supportive care alone group.

The median time from diagnosis to randomisation was 21.4 weeks (IQR 17.5–27.4). Randomisation took place a median of 4.3 weeks (3.7–4.7) after the start of the last cycle of chemotherapy for the MesoPher group and 4.1 weeks (3.7–4.9) after the start of the last cycle of chemotherapy for the best supportive care alone group. Of the 88 patients allocated to MesoPher treatment, six (7%) did not receive any study treatment: one (1%) of 88 patient had clinical disease progression before leukapheresis, one (1%) had a vasovagal collapse during leukapheresis and was not able to do a second leukapheresis within the time schedule, three (3%) had clinical progression of disease, and one (1%) had atrial fibrillation before start of treatment (figure 1). In the best supportive care alone group, four (5%) of 88 patients had disease progression before day 1 and eventually died.

As of data cutoff (June 24, 2023), median follow-up time for overall survival since randomisation was 15.1 months (IQR 9.5–22.4) for all patients. At the time of data cutoff, 120 deaths had occurred (61 [69%] of 88 patients in the MesoPher group and

59 [67%] of 88 in the best supportive care alone group). The cause of death was pleural mesothelioma for 119 (99%) of 120 patients and one patient (in the best supportive care alone group) died due to immune-related encephalitis caused by nivolumab (and hence not counted as a treatment emergent adverse event). Median overall survival was 16.8 months (95% CI 12.4–20.3) in the MesoPher group and 18.3 months (14.3–21.9) in the best supportive care alone group (HR 1.10 [95% CI 0.77–1.57; log-rank p=0.62; figure 2A). 12-month and 18-month overall survival estimates are shown on figure 2A.

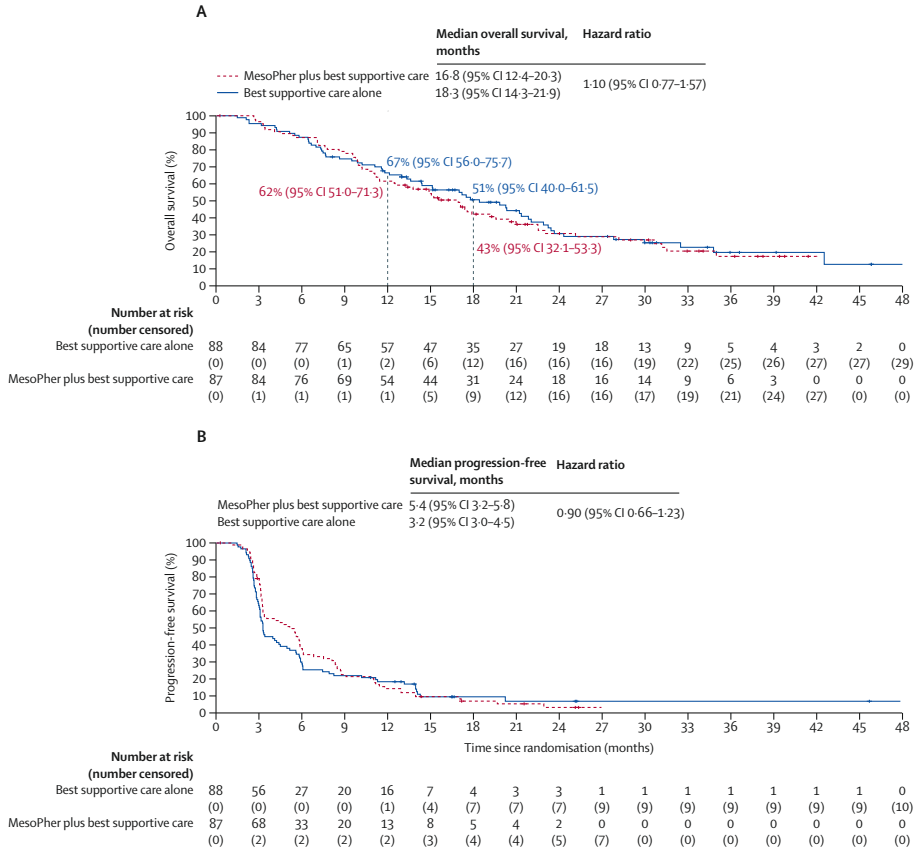

Figure 2. Overall and progression-free survival. (A-B) Kaplan Meier curves of overall (A) and progression-free survival (B) of patients treated with MesoPher + BSC (red) or BSC alone (blue). BSC, best supportive care; OS, overall survival; CI, confidence interval; PFS, progression-free survival.

As of data cutoff, 159 (90%) of 176 patients had a progression-free survival event: in the MesoPher group 81 had events [five deaths and 76 disease progressions] and in the best supportive care alone group 78 had events [seven deaths and 71 disease progressions]). Median progression-free survival was 5.4 months (95% CI 3.2–5.8) in

the MesoPher group and 3.2 months (3.0–4.5) in the best supportive care alone group (HR 0.90 [95% CI 0.66–1.23); log-rank p=0.60; figure 2B).

123 (70%) of 176 patients had measurable disease according to modified RECIST version 1.1 at baseline (57 [65%] of 88 in the MesoPher group and 66 [75%] of 88 in the best supportive care alone group). Best disease response is shown in table 2. Due to the low number of radiological responses, duration of response and overall response rate were not analysed. 14 (11%) of 176 patients had missing response evaluation data due to clinical progression or withdrawal of consent (five [6%] of 88 in the MesoPher group and nine [10%] of 88 in the best supportive care alone group). The disease control rate in the MesoPher group was significantly higher (50 [57%] of 88) than in the best supportive care alone group (35 [40%] of 88; p=0.020).

Table 2. Best disease response rate (full analysis set)

	MesoPher plus best supportive care group (n=88)	Best supportive care alone group (n=88)
Disease control rate*	50 (57%)	35 (40%)
Complete response	1 (1%)	0
Partial response	4 (5%)	4 (5%)
Stable disease	45 (51%)	31 (35%)
Progressive disease	33 (38%)	44 (50%)
Missing	5 (6%)	9 (10%)

*Defined as the sum of complete response, partial response, or stable disease.

Exploratory subgroup analyses are shown in figure 3. No differences were found between subgroups for overall survival. For progression-free survival, significant differences were found by histological subgroup.

In post-hoc exploratory analyses, patients treated with MesoPher therapy with an ECOG performance status of 0 had a significantly longer progression-free survival than those with an ECOG performance status of 1 (8.0 months [95% CI 5.4–9.0] vs 3.2 months [3.0–5.5]; log-rank p=0.0034), whereas this was not the case for the best supportive care group (4.4 months [3.0–7.4] vs 3.2 months [2.7–4.2]; log-rank p=0.28; figure 3B).

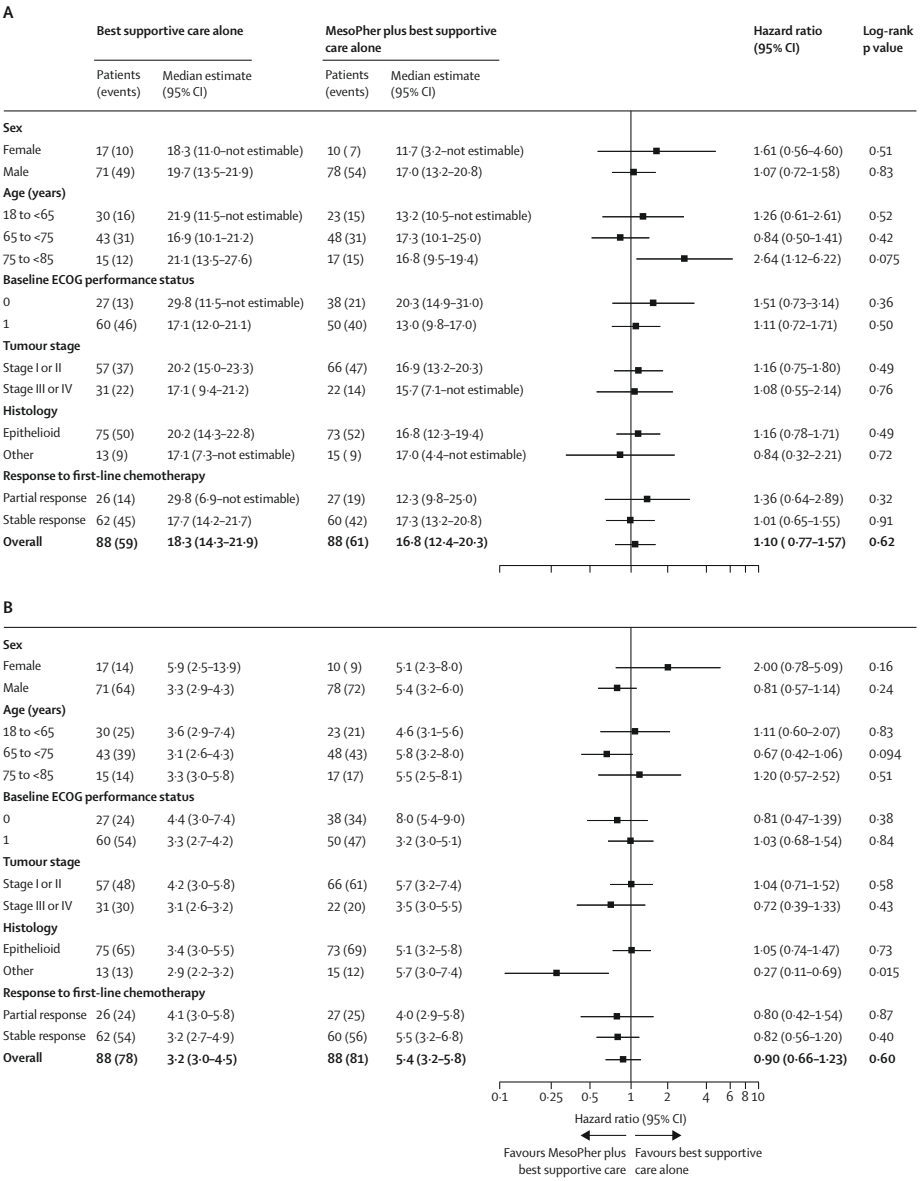

Figure 3. Subgroup analyses for overall survival (A) and progression-free survival (B). Subgroups of five or fewer participants were not included in the subgroup analysis. Response to first-line chemotherapy was a post-hoc analysis ECOG=Eastern Cooperative Oncology Group.

According to the quality of life questionnaires, global health status results between the MesoPher group and best supportive care alone group were similar over time (appendix pp 96–383). Results for each of the separate domain scores (ie, physical functioning, role functioning, cognitive functioning, social functioning, fatigue, nausea

and vomiting, pain, dyspnoea, insomnia, appetite loss, constipation, diarrhoea, and financial difficulties) were similar.

87 (99%) of 88 patients in the MesoPher group and all 88 patients in the best supportive care alone group were assessable for safety (table 3). The most common grade 3–4 treatment-emergent adverse events were chest pain (three [3%] in the MesoPher group vs two [2%] in the best supportive care alone group), dyspnoea (none vs two [2%]), anaemia (two [2%] vs none), nausea (none vs two [2%]), and pneumonia (none vs two [2%]). No deaths due to treatment-emergent adverse events were recorded. Serious treatment-emergent adverse events were reported in seven (8%) patients in the MesoPher group (including anaemia, pleural effusion, hypo magnesaemia, pericardial effusion, increased alanine aminotransferase, increase aspartate amino transferase, calculus urinary, renal impairment, atrial fibrillation, chest pain) and eight (9%) in the best supportive care alone group (including pneumonia, hip fracture, anaemia, dyspnoea, chronic obstructive pulmonary disease, and nausea; with some patients reporting more than one event). No treatment-related serious adverse events were reported. No deaths that occurred were determined to be treatment-related. The reported treatment-related adverse events were infusion-related reactions (fever, chills, and fatigue), occurring in 64 (74%) of 87 patients in the MesoPher group, and injection-site reactions (itch, erythema, and induration), occurring in 73 (84%) patients, all of which were grade 1–2 in severity. Dose reductions did not take place in the study and no patients discontinued study treatment due to treatment-emergent adverse events.

Table 3. Treatment-emergent adverse events, safety analysis set

	MesoPher plus best supportive care group (n=87)			**Best supportive care alone group (n=88)**		
	Grade 1–2	Grade 3	Grade 4	Grade 1–2	Grade 3	Grade 4
Injection-site reaction	73 (84%)	0	0	0	0	0
Infusion-related reaction	64 (74%)	0	0	0	0	0
Dyspnoea	13 (15%)	0	0	12 (14%)	2 (2%)	0
Chest pain	10 (11%)	3 (3%)	0	9 (10%)	2 (2%)	0
Arthralgia	12 (14%)	0	0	2 (2%)	0	0
Fatigue	10 (11%)	0	0	6 (7%)	0	0
Anaemia	0	2 (2%)	0	5 (6%)	0	0
Nausea	5 (6%)	0	0	0	2 (2%)	0
Renal impairment	0	1 (1%)	0	0	0	0
Pericardial effusion	0	1 (1%)	0	0	0	0
Lipase increase	0	1 (1%)	0	0	1 (1%)	0
Decreased appetite	0	1 (1%)	0	0	2 (2%)	0
Anxiety	0	1 (1%)	0	0	0	0

Table 3. Treatment-emergent adverse events, safety analysis set (continued)

	MesoPher plus best supportive care group (n=87)			Best supportive care alone group (n=88)		
	Grade 1–2	Grade 3	Grade 4	Grade 1–2	Grade 3	Grade 4
Headache	0	1 (1%)	0	0	0	0
Hypoacusis	0	1 (1%)	0	0	0	0
Bronchitis	0	1 (1%)	0	0	0	0
Exacerbation chronic obstructive pulmonary disease	0	1 (1%)	0	0	1 (1%)	0
Atrial fibrillation	0	1 (1%)	0	0	0	0
Calculus urinary	0	1 (1%)	0	0	0	0
Pneumonia	0	0	0	0	2 (2%)	0
COVID-19 pneumonia	0	0	0	0	1 (1%)	0
Depression	0	0	0	0	1 (1%)	0
Hip fracture with arthroplasty	0	0	0	0	1 (1%)	0
Alanine aminotransferanse or aspartate aminotransferase increased	0	0	1 (1%)	0	0	0
Hypomagnesemia	0	0	1 (1%)	0	0	0

Treatment-emergent adverse events of grade 1 or 2 that occurred in at least 10% of participants and all grade 3–4 events are shown; no grade 5 events occurred. Treatment emergent adverse events are defined as an adverse event that started on or after random assignment and that possibly related, probably related, or related to treatment.

Treatments received after progression are summarised in the appendix (p 4). 102 (58%) of 176 patients received at least one line of immune checkpoint inhibitor therapy as second-line treatment (53 [60%] of 88 in the MesoPher group and 49 [58%] of 88 in the best supportive care alone group), mainly anti-PD-1 antibodies.

Immune cell profiling was done on peripheral blood samples of 110 patients. Due to sample availability, flow cytometry analyses were done on 67 (76%) of 88 patients in the MesoPher group and 43 (49%) of 88 patients in the best supportive care alone group. In this exploratory analysis, MesoPher treatment led to a significant increase in the expression of the T-cell activation marker inducible costimulator (ICOS) on CD4+ T cells, and in the frequency of CD4+ T cells expressing the proliferation marker Ki67 (figure 4A, B).

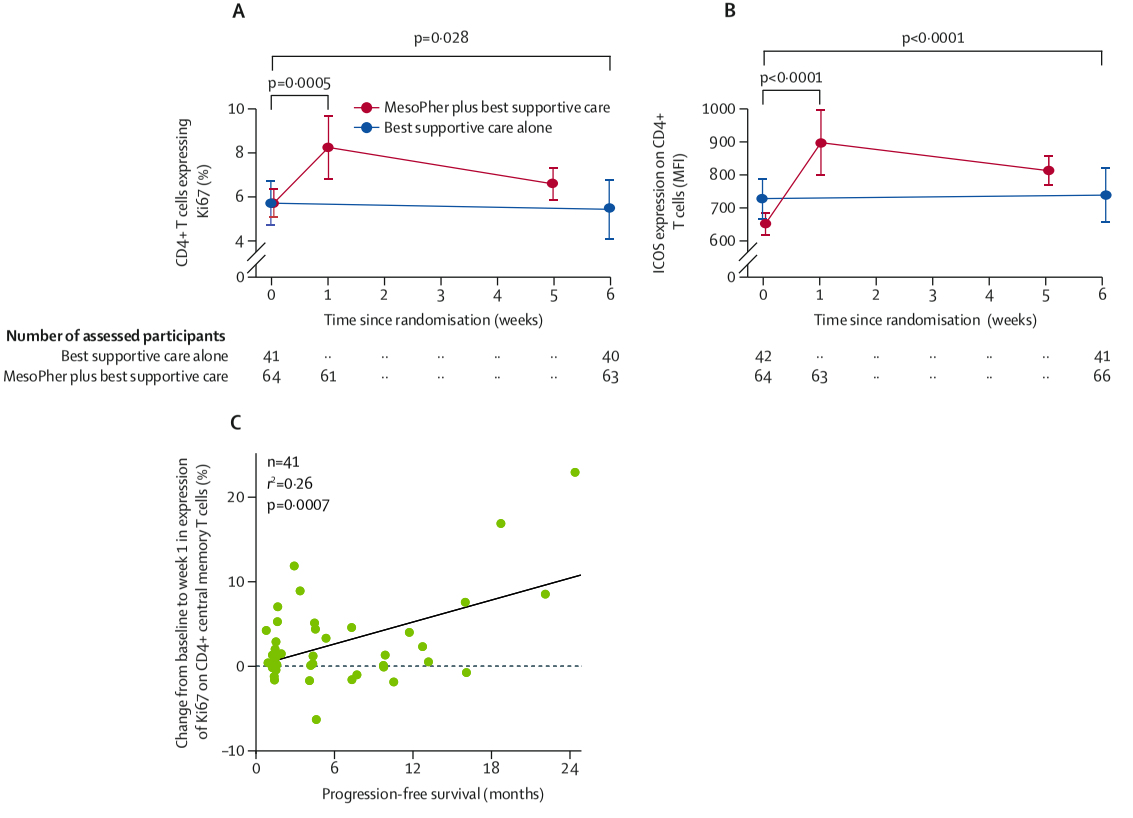

Figure 4. Peripheral blood immune phenotypic analyses. (A-B) Boxplots showing the % change in Ki67+ of CD4+ T cells (A) or change in MFI of ICOS on CD4+ T cells (B) for patients receiving MesoPher + BSC treated (red) or BSC (blue). (C) Linear regression analysis between progression-free survival for the % change in Ki67+ CD4+ central memory T (Tcm) cells from baseline to day 8. BSC, best supportive care; R^2, coefficient of determination.

These increases were most pronounced at day 8, and still present at day 36. The increase in expression of ICOS on CD4+ T cells was present in different CD4+ T-cell subsets (appendix p 5). The increase in expression of ICOS on CD4+ T cells and increased frequency of Ki67+ CD4+ T cells was present in both memory T cells and regulatory T cells. However, the fold increase of ICOS and Ki67 was more pronounced on immunostimulatory CD4+ effector memory T cells than on immunosuppressive CD4+ regulatory T cells (appendix p 5). MesoPher-induced CD4+ T-cell activation and proliferation also correlated with progression-free survival (appendix p 8). We analysed 41 (47%) of 88 patients in the MesoPher group who had a sufficient quantity of cells and sequential samples from day 1 and day 8, and found that increase in Ki67-positive central memory T cells at day 8 correlated with longer progression-free survival (r^2=0.26, p=0.0007; figure 4C).

Discussion

To our knowledge, this is the first multicentre, randomised study to investigate the value of cellular therapy in pleural mesothelioma. MesoPher therapy plus best supportive care after completion of first-line chemotherapy did not improve overall survival compared with treatment with best supportive care alone. Our study was based on phase 1 data that found promising signs of overall survival and immunological activation after MesoPher with a median overall survival that was not reached after a median follow-up of 22.8 months. Acknowledging the small sample size of this previous phase 1 trial, these data combined with our previous phase 1 trials with MesoPher,[8-10] led us to proceed with the DENIM randomised trial. In the trial, we chose to administer MesoPher as a maintenance treatment in patients with non-progressing disease after standard of care chemotherapy. This treatment plan was chosen because anticancer vaccines, and immunotherapy in general, are less effective in patients with progressing cancer,[11-13] which impairs immune activation. However, a major difference between the previous trials and our current trial is the time interval between the administration of the last cycle of chemotherapy and the first injection of MesoPher. In the DENIM trial, the mean interval between the start of the last chemotherapy cycle and first administration of MesoPher was 11.8 weeks, which is almost double the time of the earlier trials. The increased time interval was due to lengthy study procedures, such as screening and randomisation, and the extended sterility tests, which were much quicker or not done as part of the other studies.

When looking in detail at the progression-free survival curve, although no CT scans were taken during the period, we assume that a substantial proportion of patients had disease progression in the interval between completion of chemotherapy and day 1 (start of treatment in the MesoPher group). This assumption is supported by the fact that in the best supportive care group 44 (50%) of 88 patients had a progression-free

survival event at the time of first CT evaluation. Although the disease control rate was significantly higher in the MesoPher group, 33 (38%) of 88 patients still had progressive disease at the first evaluation. Additionally, six of eight patients in the MesoPher group who did not receive all of the first three injections had disease progression. To shorten the interval in future trials, a work-up similar to that in the trial of Adusumilli and colleagues[14] could be implemented, in which patient screening and leukapheresis were done during first-line treatment. Moreover, sterility testing could be shortened by use of a rapid real-time PCR compared with the now performed method (direct inoculation followed by 14 days of culture to conform to European standards). Four (5%) of 88 patients in the best supportive care alone group had a partial response, which might indicate a delayed response to the chemotherapy and the known difficulties in assessing pleural tumours.[15-17]

Assuming that patients with an ECOG performance status of 0 have a better immune system (and so are able to mount a better response to injection) than those with an ECOG performance status of 1, we found that the difference in progression-free survival between patients with each of these ECOG performance status scores was more pronounced in the MesoPher group than in the best supportive care alone group. Although these are only surrogate markers, this finding might suggest that MesoPher was given too late to induce an effective sustained immune activation in the majority of patients.

Immune profiling of peripheral blood samples from a phase I dose-escalation study demonstrated increased expression of HLA-DR, PD-1 and ICOS on CD4+ T cells and LAG-3 on CD8+ T cells.[18] Additionally, MesoPher treatment led to an increase in Ki-67 on CD4+ T cells in patients with pancreatic cancer.[19] Similar results were seen in a phase II clinical trial for peritoneal mesothelioma.[6]

Our exploratory immunomonitoring data showed that MesoPher treatment led to an increase in proliferation (as measured by Ki67) and activation (as measured by ICOS) of CD4+ T cells and that the magnitude of increase in proliferation correlated with progression-free survival in the subset of patients in whom this analysis was performed. The increase in expression of ICOS on CD4+ T cells confirmed results in the phase 1 trial with MesoPher[18] and results of the current study suggest that the magnitude of induction of memory CD4+ T-cell proliferation could be indicative of an effective anti-tumour response. CD4+ central memory T cells might not rapidly produce protective cytokines, but systemic challenge can rapidly increase the generation of new effector cells in secondary lymphoid organs.[20]

The increase in CD4+ T cells is considered to be one of the criteria for evaluation of efficacy of vaccination therapies.[21] The role of CD4+ T cells in cancer immunology has been an area of increasing interest. Although most cancer immunotherapies

focus on the CD8+ T-cell response, CD4+ T cells have a pivotal role in developing and sustaining an effective anti-tumour response. CD4+ T cells are key players in obtaining an optimal immune effect by providing help to CD8+ T cells, but also via the production of effector cytokines, such as INF-γ and TNF-α, with direct anti-tumour activity. CD4+ T-cell signalling is also essential for the formation and survival of memory CD8+ T cells, contributing to a durable immune-mediated tumour response.

In previous studies,[22,23]MesoPher was given in an adjuvant setting after surgery in patients with peritoneal mesothelioma and pancreatic cancer. In these trials, MesoPher induced a broader immune response also increasing expression of other markers such as PD-1 and CD28, indicating the effect of (tumour-derived) immune suppression that we identified herein the DENIM trial.[6,24] Currently, we are investigating the potential synergy of extended pleurectomy and decortication and MesoPher in pleural mesothelioma (NCT05304208).

In Checkmate 743, patients with a non-epithelioid histology responded better to combination immune checkpoint inhibitor therapy, consisting of anti-PD-1 and anti-CTLA-4 antibodies, than to chemotherapy. In our study, we found that patients with a non-epithelioid histology treated with MesoPher, had a significantly longer progression-free survival than those treated with best supportive care alone. However, no such association was found for overall survival. Notably, the small number of patients with non-epithelioid tumours means that these subgroup analyses should be interpreted with caution. Additionally, the effect on overall survival might be diluted due to the fact that most patients received immune checkpoint inhibitor monotherapy (anti-PD-1) after progression.

Overall survival in the best supportive care alone group was higher than expected when extrapolated from other published data in a similar patient population. In the switch maintenance study that compared gemcitabine with best supportive care, overall survival in the best supportive care group was 13.4 months (95% CI 12.4–17.8).[16] In the COMMAND study[15] that compared defactinib with placebo, overall survival was 12.7 months (95% CI 9.1–21) in the defactinib group and 13.6 months (9.6–21.2) in the placebo group. A potential reason for the longer overall survival in best supportive care alone group in the DENIM study might be a higher number of patients with stage I and II disease, whereas the other studies had a higher proportion of patients with a more advanced stage of disease. Another explanation might be that most patients received immune checkpoint inhibitor monotherapy after progression.[19] Although progression-free survival in the best supportive care group is similar to that in the placebo group in the COMMAND study (4.0 months (95% CI 2.9–4.2), median overall survival in the best supportive care alone group in the DENIM study was approximately 5 months longer.

Treatment-related adverse events in the MesoPher group were limited to infusion-related adverse reactions (ie, fever, chills, and fatigue) and injection site-related reactions (ie, itch, erythema, and induration) and were all CTCAE grade 1–2. The treatment had no negative effect on quality of life measures. This safety and tolerability profile, together with the induced immune activation, makes MesoPher a potential candidate for combination treatment with other types of immunotherapy. In fact, combining dendritic cell therapy with immune checkpoint inhibitor therapy has been shown to work synergistically in murine models and shows promising results in a clinical setting.[4]

Our results need to be interpreted in the context of several limitations. Specifically, given that mesothelioma is a rare disease and performing a randomised trial in cellular therapy is a challenge, sample size was an issue. Other limitations are the open-label nature of the study, the heterogeneity of the patients included, and the second-line treatment received by some patients after progression. For the immune profiling, a limitation is that in the best supportive care alone group blood samples were taken at fewer timepoints than in the MesoPher group due to ethical considerations, which hampers between-group comparison.

In summary, treatment with MesoPher is feasible without major side-effects or negative effects on quality of life. The safety profile and the immunological effect warrant studies with combination treatment and as first-line (combination) treatment or after surgical resection. Immune checkpoint inhibitor combination therapy is now standard of care in pleural mesothelioma. Combination therapy of MesoPher and an immune checkpoint inhibitor might increase efficacy without adding major toxicities.

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Data sharing agreements between parties are made in the EU Horizon 2020 Consortium Agreement. No separate data sharing plan has been included in the trial registration as the study started before January 1, 2019.

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Supplementary Tables

Supplementary table 1. Used reagents

Product	Fluorochrome	Supplier	Catalogue #
CD45RA	PE-Dazzle 594	Biolegend	304146
CD3	APC-Cy7	Invitrogen	47-0038-42
CD4	BV785	BD Biosciences	563877
CD8	AF700	Biolegend	344724
CCR7	BV421	Biolegend	353208
CD56	BV605	BD Biosciences	562780
FOXP3	PE	eBioscience	12-4777-42
CD28	PE-Cy7	Biolegend	302926
CD137/4-IBB	PerCP-Cy5.5	Biolegend	309814
PD-1	BV711	Biolegend	329928
HLA-DR	APC	BD Biosciences	559866
ICOS	BV650	BD Biosciences	563832
Ki67	FITC	Invitrogen	11-5699-42
LAG-3	PE-Cy7	Biolegend	369310
TIM-3	BV650	Biolegend	345028
TOX	APC	Miltenyi Biotec	130-118-335
CTLA-4	PerCP-Cy5.5	Invitrogen	46-1529-42
Human TruStain FcX™	NA	Biolegend	422302
Fixable Viability Dye eFluor™ 506	BV510	eBioscience	65-0866-14
CS&T Research beads	Various	BD Biosciences	655051
Multicolor CompBeads	NA	BD Biosciences	644204

Abbreviations: NA, not applicable

Supplementary table 2. Data output

Subset	Marker expression	% positive of	% positive of parent	MFI of parent and MFI of Ki67+ parent								
T cells	CD3+	Viable cells	KI67	-	-	-	-	-	-	-	-	-
CD4+ T cells	CD3+ CD4+	Viable cells, CD3+	KI67	CD28	CD137	PD-1	HLA-DR	ICOS	LAG-3	TIM-3	TOX	CTLA-4
CD4+ Naive T cells	CD3+ CD4+ CCR7+ CD45RA+	Viable cells, CD3+, CD4+	KI67	CD28	CD137	PD-1	HLA-DR	ICOS	LAG-3	TIM-3	TOX	CTLA-4
CD4+ Central memory T cells	CD3+ CD4+ CCR7+ CD45RA-	Viable cells, CD3+, CD4+	KI67	CD28	CD137	PD-1	HLA-DR	ICOS	LAG-3	TIM-3	TOX	CTLA-4
CD4+ Effector memory T cells	CD3+ CD4+ CCR7- CD45RA-	Viable cells, CD3+, CD4+	KI67	CD28	CD137	PD-1	HLA-DR	ICOS	LAG-3	TIM-3	TOX	CTLA-4
CD4+ Terminally differentiated effector memory T cells	CD3+ CD4+ CCR7- CD45RA+	Viable cells, CD3+, CD4+	KI67	CD28	CD137	PD-1	HLA-DR	ICOS	LAG-3	TIM-3	TOX	CTLA-4
CD4+ Regulatory T cells	CD3+ CD4+ FOXP3+	Viable cells, CD3+, CD4+	KI67	CD28	CD137	PD-1	HLA-DR	ICOS	LAG-3	TIM-3	TOX	CTLA-4
CD8+ T cells	CD3+ CD8+	Viable cells, CD3+	KI67	CD28	CD137	PD-1	HLA-DR	ICOS	LAG-3	TIM-3	TOX	CTLA-4
CD8+ Naive T cells	CD3+ CD8+ CCR7+ CD85RA+	Viable cells, CD3+, CD8+	KI67	CD28	CD137	PD-1	HLA-DR	ICOS	LAG-3	TIM-3	TOX	CTLA-4
CD8+ Central memory T cells	CD3+ CD8+ CCR7+ CD85RA-	Viable cells, CD3+, CD8+	KI67	CD28	CD137	PD-1	HLA-DR	ICOS	LAG-3	TIM-3	TOX	CTLA-4
CD8+ Effector memory T cells	CD3+ CD8+ CCR7- CD85RA-	Viable cells, CD3+, CD8+	KI67	CD28	CD137	PD-1	HLA-DR	ICOS	LAG-3	TIM-3	TOX	CTLA-4
CD8+ Terminally differentiated effector memory T cells	CD3+ CD8+ CCR7- CD85RA+	Viable cells, CD3+, CD8+	KI67	CD28	CD137	PD-1	HLA-DR	ICOS	LAG-3	TIM-3	TOX	CTLA-4
NK cells	CD3- CD56+	Viable cells	KI67	CD28	CD137	PD-1	HLA-DR	ICOS	LAG-3	TIM-3	TOX	CTLA-4
NK cells CD56 bright	CD3- CD56++	Viable cells, CD3-	KI67	CD28	CD137	PD-1	HLA-DR	ICOS	LAG-3	TIM-3	TOX	CTLA-4
NK cells CD56 dim	CD3- CD56+	Viable cells, CD3-	KI67	CD28	CD137	PD-1	HLA-DR	ICOS	LAG-3	TIM-3	TOX	CTLA-4

Abbreviations: MFI, mean fluorescence intensity

Supplementary Table 3. Treatment beyond progression

Treatment	MesoPher + BSC	BSC alone	Total
	n=88 (%)	n=88	n = 176
Carboplatin/ pembrolizumab	6 (6.8)	11 (13.0)	17 (9.7)
Cisplatin/pembrolizumab	0 (0)	1 (1.1)	1 (0.6)
Ipilimumab	3 (3.4)	1 (1.1)	4 (2.3)
Ipilimumab/nivolumab	6 (6.8)	1(1.1)	7 (4.0)
Lenvatinib/pembrolizumab	4 (4,5)	3 (3.4)	7 (4.0)
Nivolumab	39 (44.3)	43 (48.9)	82 (46.6)
Pembrolizumab	5 (5.7)	3 (3.4)	8 (4.5)
Lurbinectidin	9 (10.2)	10 (11.4)	19 (10.8)
Gemcitabine	7 (8.0)	11 (13.0)	18 (10.2)
Other	17 (19.3)	18 (14.8)	35 (19.9)

Abbreviations: BSC, best supportive care; n, number of subjects in the analysis set.

Supplementary table 4. Changes in CD4+ T cell markers

CD4	Subset	Marker	MesoPher + BSC				BSC alone	
			Change from baseline		X-times change		Change from baseline	Fold change
			Day 8	Day 36	Day 8	Day 36	Week 6	Week 6
CD4	Total	ICOS	**P<0.0001** (MFI +234.5)	**P<0.0001** (MFI +151.1)	1.35	1.24	Ns (MFI +3.4)	NA
CD4	CD4+ Effector memory T cells	ICOS	**p=0.0001** (MFI +169.5)	**P<0.0001** (MFI +206.6)	1.31	1.39	Ns (MFI -9.6)	NA
CD4	CD4+ Regulatory T cells	ICOS	**p<0.0001** (MFI +204.9)	**p<0.0001** (MFI +198.0)	1.20	1.20	Ns (MFI -44.1)	NA
CD4	Total	Ki67	**p=0.0005** (+2.3%)	**p=0.0276** (+0.7%)	1.50	1.25	Ns (-0.3%)	NA
CD4	CD4+ Effector memory T cells	Ki67	**p=0.0080** (+2.8%)	**p=0.0052** (+1.7%)	1.34	1.31	Ns (-0.2%)	NA
CD4	CD4+ Regulatory T cells	Ki67	**p=0.0244** (+1.8%)	Ns (+0.7%)	1.23	NA	Ns (-0.6%)	NA
CD4	CD4+ Central memory T cells	Ki67	**p=0.0016** (+2.8%)	**p=0.0407** (+1.1%)	1.78	1.43	Ns (+1.4%)	NA

Abbreviations: BSC, best supportive care; MFI, mean fluorescence intensity; Ns, not significant; NA, not applicable

Supplementary table 5. Timeline MesoPher production and treatment. The following table shows the timing of actions during the initial phase of the study

Start of last chemo	Week - 11 (+/- 2 weeks)
Randomization	Week -8 (+/- 2 weeks)
Leukapheresis	Week -7 (+/- 2 weeks)
Manufacturing, batch release, shipment	approx. 6 weeks
First vaccination	Day 1
Second vaccination	Day 15
Third vaccination	Day 29
CT-scan	Week 6
Fourth vaccination	Week 18
Fifth vaccination	Week 30

Supplementary table 6. List of recruitment

Centre	PI	Number of inclusions
Erasmus medical centre, Rotterdam	Robin Cornelissen	94
Netherlands cancer institute, Amsterdam	Paul Baas	33
University Hospital Antwerp	Jan van Meerbeeck	33
Centre Hospitalier Régional Universitaire de Lille	Arnaud Scherpereel	16

Supplementary Table 7. Significant correlations between selected output parameters and progression free survival

CD4/ CD8	Subset	Marker	Comparison	Arm A
				PFS
CD4	CD4+ Effector memory T cells	Ki67	Change from baseline to day 8	**p=0.0055** R^2=0.1555
CD4	CD4+ Effector memory T cells	ICOS	Change from baseline to day 8	**p=0.0302** R^2=0.0812
CD4	CD4+ Central memory T cells	Ki67	Change from baseline to day 8	**p=0.0007** R^2=0.2575

6

Enhancing Dendritic cell Therapy in Solid Tumors with Immune Modulating Conventional Treatment

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Abstract

Dendritic cells (DCs) are the most potent antigen presenting cells and are the key initiator of tumor-specific immune responses. These characteristics are exploited by DC therapy, where DCs are *ex vivo* loaded with tumor-associated antigens (TAAs) and used to induce tumor-specific immune responses. Unfortunately, clinical responses remain limited to a proportion of the patients. Tumor characteristics and the immunosuppressive tumor microenvironment (TME) of the tumor are likely hampering efficacy of DC therapy. Therefore, reducing the immunosuppressive TME by combining DC therapy with other treatments could be a promising strategy. Initially, conventional cancer therapies, such as chemotherapy and radiotherapy, were thought to specifically target cancerous cells. Recent insights indicate that these therapies additionally augment tumor immunity. by targeting immunosuppressive cell subsets in the TME, inducing immunogenic cell death (ICD) or blocking of inhibitory molecules. Therefore, combining DC therapy with registered therapies such as chemotherapy, radiotherapy or checkpoint inhibitors could be a promising treatment strategy to improve efficacy of DC therapy. In this review, we will evaluate various clinical applicable combination strategies to improve the efficacy of DC therapy.

Keywords: Dendritic cell-based therapy, chemotherapy, radiotherapy, checkpoint inhibitors, immunotherapy, tumor microenvironment, regulatory T-cells, myeloid derived suppressor cells, immunogenic cell death, macrophages

Introduction

Dendritic cells (DCs) are professional antigen presenting cells (APCs) capable of inducing a potent immune response through presentation of exogenous antigens.[1] Immature DCs, efficient at engulfing and processing antigens, reside in the periphery and will mature upon encounter with danger associated molecular patterns (DAMPs) and pattern associated molecular patterns (PAMPs).[2] Upon encounter with these danger signals, DCs will upregulate costimulatory molecules (CD80, CD86, CD40) and chemokine receptors (e.g. CCR7), produce pro-inflammatory cytokines and migrate to the lymph node to activate T-cells.[3] T-cell activation will be induced by antigen presentation (signal 1), co-stimulation (signal 2) and secretion of pro-inflammatory cytokines (signal 3).[4] In contrast, antigen presentation in the absence of signal 2 and 3 will induce tolerance.[5,6] In a tumor setting, both tumor cells and immunosuppressive cells in the tumor microenvironment (TME) can hamper anti-tumor immune responses.[7]

In DC therapy production, DCs are often matured and loaded *ex vivo*, to circumvent the initial immunosuppressive influence of the TME and tumor cells on endogenous DC maturation. In addition, administration of autologous DCs could induce and improve the *in vivo* tumor-specific immune response. It is believed that DC therapy has not yet reached its full potential.[8-10] The rather limited clinical efficacy of DC therapy can be dependent on DC therapy related aspects, such as the choice of antigen, method of loading, or type of DCs used. Next to that, active immunosuppression by the tumor and the TME, could also hamper the immune activating potential of the administrated DCs and suppress the function and infiltration of activated T-cells.[11-13]

Therefore, targeting these immunosuppressive features of the TME using FDA approved treatment modalities, such as chemotherapy, radiotherapy or more recently developed checkpoint inhibitors (CIs) in combination with DC therapy could improve DC therapy efficacy[1,7,8,12,14-17] (Fig.1). In this review, we discuss the immunological barriers DC therapy faces and potential synergistic immunomodulating treatment modalities. In addition, we review clinical trials that have combined DC therapy with additional treatments. Data regarding these conducted clinical trials were found using a search string of relevant terms, as described in the supplementary material.

Immunosuppressive mechanisms of the TME and tumor cells that hamper the efficacy of DC therapy.

Both tumor cells and immunosuppressive immune cells in the TME hamper effectivity of DC therapy through various mechanisms, such as expression of inhibitory molecules, secretion of inhibitory cytokines or enzymes, creation of a dense extracellular matrix and induction of tolerogenic cell death[18, 19] Tumor cells recruit immunosuppressive immune cells, fibroblasts[20] and endothelial cells to the TME through secretion of growth factors, chemokines and cytokines, thereby hampering infiltration of

DCs and other pro-inflammatory cells into the TME.[21,22] Moreover, fibroblasts and immunosuppressive immune cells interact synergistically with each other to maximize the immunosuppressive character of the TME.

Tolerogenic and immunogenic cell death

Cancer cell death can be tolerogenic or immunogenic depending on the stimulus of apoptosis.[23] Immunogenic cancer cell death, will lead to secretion of DAMPs, attract pro-inflammatory cells, and subsequently elicit a tumor specific immune response (Box 1). Non-immunogenic cell death of malignant cells occurs without secretion of pro-inflammatory DAMPs. Tumor cells undergo non-immunogenic cell death through chemo-attraction of immunosuppressive phagocytes and induction of immunosuppressive phagocytosis.[24] Tumor cells actively impair DC maturation through secretion of immunosuppressive cytokines, leading to the presentation of TAA by immature DCs. Presentation of antigens by immature DCs will induce T-cell anergy and activation of TAA-specific Tregs, resulting in TAA-specific tolerance.[1,25-27].

Box 1. ICD in cancer cells

Cancer cells undergoing ICD start expressing calreticulin (CRT) on the cell surface due to stress on the endoplasmatic reticulum and excrete ATP and high mobility group box1 (HMGB1). ATP is a stimulus for the migration of DCs into the tumor, CRT stimulates phagocytosis of antigens and HGMB1 upregulates antigen presentation to $CD8^+$ T-cells. Through these processes DCs are matured and activate CTLs in the lymph node to engage cytotoxic activity on the tumor cells. [23, 120, 146] ICD can be induced by several chemotherapeutics, fractioned radiotherapy, oncolytic viruses and some targeted therapies. [1]

Regulatory T-cells

Tregs are recruited to the TME through CCR4 chemokine signaling and expand in the TME upon TGF-β and IL-10 exposure.[28] Tregs enable tumor progression by suppressing tumor-specific immune responses. In general, Tregs induce immune suppression directly through cell-cell contact via inhibitory receptors, such as PD-1 or CTLA-4, or indirectly through secretion of immunosuppressive cytokines, as IL-10 and TGF-β, IL-2 consumption or pore-forming proteins, as granzyme and perforin.[29] Via these mechanisms, Tregs suppress a wide array of pro-inflammatory immune cells that can be induced upon DC therapy, such as $CD8^+$ T-cells, $CD4^+$ T-cells, NK cells, NK T-cells and B-cells. Additionally, Tregs can also suppress macrophages and DCs, thereby hampering the induction of an initial anti-tumor immune response.[8, 14] Moreover, tumor-infiltrating Tregs have a higher affinity to TAAs derived from self-antigens presented on tumor cells than $CD8^+$ T-cells, thereby affecting the activation of TAA-specific $CD8^+$ T-cells in

the TME.[22] The Treg functions create a hostile and competitive environment for DC therapy-induced tumor-specific CD8+ T-cells, hindering their cytotoxic functions.

Myeloid derived suppressor cells

MDSCs are immature myeloid cells with immunosuppressive effects. MDSCs are one of the most abundant immune cells in the TME and are attracted to the tumor site by chemokines secreted by the tumor cells.[30-32] MDSCs comprise two subsets: monocytic MDSC (mMDSC) and polymorphonuclear MDSC (pMDSC).[33,34] MMDSCs tend to be more immunosuppressive than pMDSCs as they are capable of both antigen dependent and antigen independent inhibition of T-cell responses.[18,35] The hypoxic environment of the TME induces release of *hypoxia-inducible factor 1*-alpha (HIF-1α), which causes mMDSCs to upregulate the enzymes arginase 1 (Arg1) and iNOS that break down L-arginase.[35] Two products of this enzymatic reaction, urea and nitric oxide (NO), induce T-cell depletion and inhibit T-cell function.[18,31,36,37] Moreover, mMDSCs attract Tregs through CCL4 and CCL5 production, secrete IL-10[38] and upregulate PD-L1 on their cell surface, which inhibits tumor specific T-cell cytotoxicity.[39] Furthermore, HIF-1α induces differentiation of mMDSCs into TAMs through downregulation of pSTAT3, indicating that the TME can influence both immune cell function and differentiation.[40,41]

Tumor associated macrophages

Monocytes are derived from the bone marrow and are recruited to the TME through CCL2 signaling where these cells can differentiate into macrophages. Phenotypically macrophages can broadly be divided into two subtypes: a pro-inflammatory M1 phenotype and an immunosuppressive M2 phenotype. Differentiation of M1 macrophages is induced by pro-inflammatory cytokines such as IFN-**γ** and bacterial components such as lipopolysaccharide (LPS). M1 macrophages secrete pro-inflammatory cytokines, interleukins such as IL-12 and tumor necrosis factor-α (TNF-α) leading to inflammation.[19] Macrophages are skewed into a M2 phenotype through secretion of immunosuppressive cytokines by tumor cells or immune cells in the TME and inhibit CD8+ T-cell function.[41] TAMs display a M2-like phenotype and secrete IL-10, PGE2 and chemokines to attract and induce Tregs.[41,42] Moreover, TAMs express iNOS and Arg 1, and upregulate PD-L1 on their cell surface which inhibits CD8+ T-cell function[18,43] These mechanisms hamper DC therapy induced anti-tumor immunity.

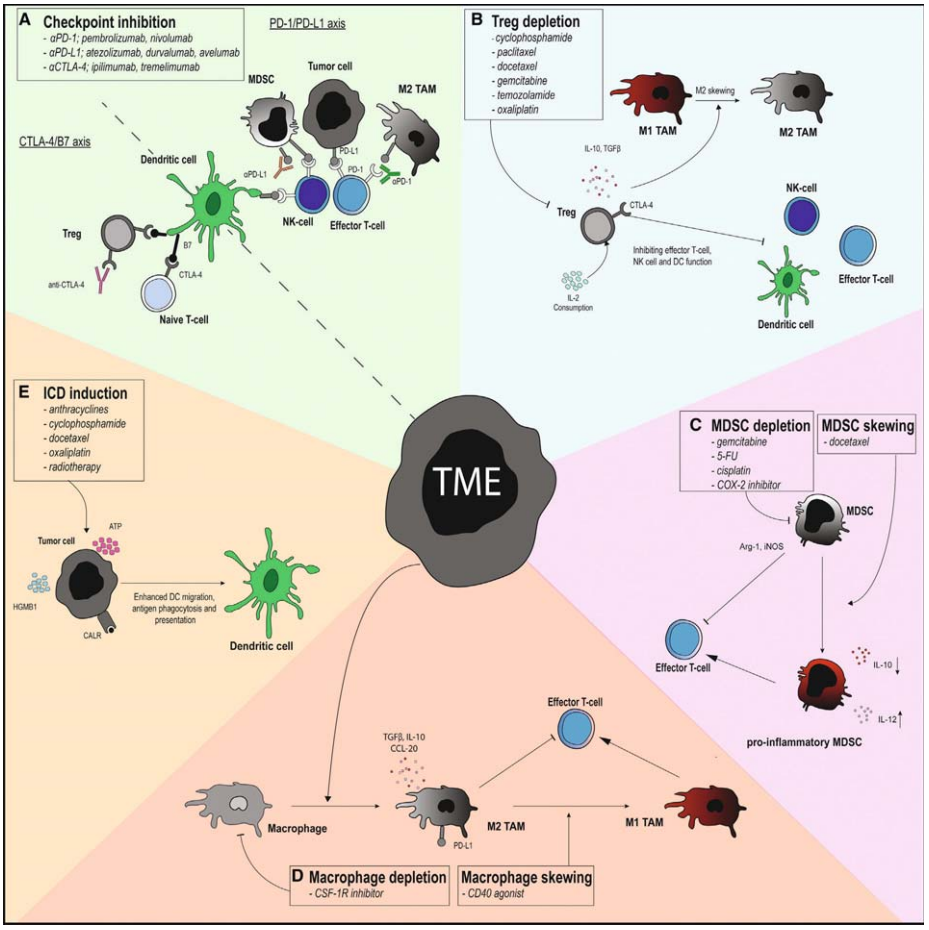

Figure 1. Targeting the TME with conventional treatment modalities. A. Inhibitory molecules (PD-(L)1, CTLA-4) inhibit T-cell effector, dendritic cell and NK-cell function and T-cell activation in the lymphnode. Checkpoint inhibitors targeting (PD-(L)1, CTLA-4) can reinvigorate the anti-tumor immune response induced by DC therapy by blocking PD-(L)1 signaling in the tumor and CTLA-4 in the lymph node. B. Tregs exert their immunosuppressive mechanisms through inhibitory molecules (CTLA-4), secretion of immunosuppressive cytokines (IL-10, TGFb) and IL-2 consumption thereby inhibiting NK- cells, T-cells and DCs and skewing TAMs in an unfavorable M2 phenotype. Tregs can be depleted with several chemotherapeutics (cyclophosphamide, paclitaxel, docetaxel, gemcitabine, temozolamide and oxaliplatin). C. MDSCs can exert their immunosuppressive function by relieving Arginase 1 (Arg1) and iNOS to deprive T-cells of metabolites. MDSCs can be depleted by chemotherapeutics gemcitabine, 5-FU, cisplatin and docetaxel and skewed into a M1 phenotype by docetaxel. D. M2 TAMs secrete IL-10 and TGFb and are involved in tissue remodeling, wound healing and tumor progression. M2 TAMs can be depleted by CSF-1R and skewed into an M1 phenotype by CD40 agonists. E. ICD is characterized by secretion of ATP and high mobility group box 1 (HGMB-1) and expression of Calreticulin (CRT) on the cell surface which stimulates DC phagocytosis, antigen presentation, and migration. ICD can be induced by chemotherapeutics, cyclophosphamide, oxaliplatin, paclitaxel, docetaxel and anthracyclines and radiotherapy.

Dendritic cell therapy

Different DC therapy strategies

DC therapy aims at eliciting a tumor-specific immune response, by loading DCs *in vitro* with tumor antigens and additional maturation stimuli. DC therapy can be historically divided into three categories: first-, second- and next- generation DC therapy.[1] In first- and second- generation DC therapy, monocyte-derived DCs (moDC) were used. MoDCs are generated from monocytes upon culture with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4. MoDCs have been shown to promote T-cell differentiation and $CD8^+$ T-cell activation.[44] In first-generation DC therapy, moDCs were loaded with a tumor lysate, TAAs or synthetic peptides without additional maturation stimuli. Not surprisingly, without a proper maturation stimulus, clinical results were disappointing with tumor regression rate of 3,3%.[45]

In second-generation DC therapy, moDCs were additionally matured after loading these immature moDCs, using 'maturation cocktails', including IL6, TNF, IL1β, PGE2 and polyinosinic:polycytidylic

acid (poly(I:C)).[46] Maturing these tumor antigen loaded moDCs significantly improved clinical results with overall response rates (ORR) of 8-15%, depending on the tumor type.[9] Median overall survival (OS) was increased with 20% in multiple clinical trials with second generation DC therapy, which is the threshold for clinical relevance.[9,46,47] Furthermore, the IMPACT trial showed an increase in median OS of 3,9 months for castration-resistant prostate cancer patients treated with sipuleucel-T (DC therapy) compared to the placebo group, leading to FDA approval in 2010.[48]

In next-generation DC therapy, naturally occurring DCs (nDCs), such as plasmacytoid DCs (pDCs) and conventional DCs (cDCs) are used for vaccination. cDCs can be divided into two main subtypes: cDC1 and cDC2.[49] cDC1s are superior in cross-presenting antigens and thereby inducing $CD8^+$ T-cell activation. Recent studies have shown that cDC1s are critically important for anti-tumor immune responses and that their presence in the TME positively correlates to OS and clinical responses upon PD-1 mAb in melanoma.[50-52] Classically, cDC2s mainly activate $CD4^+$ T-cells. The characterization of human cDC2 function remains difficult, as this subset is very heterogeneous, shares markers such as CD11b and CD172a with macrophages and moDCs and has functional overlap with cDC1s.[51] pDCs in the TME produce type 1 IFN, which attracts NK-, B-, and T-cells.[53] However, pDCs have questionable antigen presenting skills, as $CD123^+$ pDCs were found to be contaminated with pre-cDCs.[54] Naturally occurring DCs can therefore be selected based on their superior functional properties, and can be obtained without an additional culture period, leading to reduced production costs.[1]

DCs can also be targeted *in vivo* using Toll-like receptor (TLR) ligands, intra-tumoral injection of TriMix mRNA or attenuated viral agents (virotherapy).[55-57] Furthermore, FMS-like tyrosine kinase 3 Ligand (FLT3L) injection increases cDC proliferation and infiltration into the tumor site which enhanced PD-L1 mAb efficacy in a melanoma mouse model.[58,59] Clinical trials exploiting next generation DC therapy are currently performed and coming years will indicate whether the use of nDCs further improves clinical efficacy of DC therapy.[60,61] Currently, clinical studies comparing the effectivity of different DC subtypes for vaccination purposes are lacking and are urgently needed to determine which DC subset would induce the most effective anti-tumor immune response.[62]

Immune monitoring

Immunological responses induced by DC therapy are generally measured by IFN-γ ELISPOTs, tetramer analysis, co-cultures with lysate-loaded DCs and DTH skin tests. Correlating these immunological parameters to clinical response remains challenging as not all patients show increased IFN-γ production in an ELISPOT upon DC therapy, and even positive ELISPOTS can be encountered before DC therapy.[63,64] Furthermore, tumor-specific IFN-γ production by PBMCs as determined by an ELISPOT analysis often does not correlate with clinical outcome, or allergic reaction measured by the DTH skin test.[63,65,66] However in some studies, immunological parameters have been correlated with clinical parameters in both hematological and solid malignancies.[60,67,68]

DTH skin tests have been shown to correlate with clinical outcome in DC therapy trials in melanoma and colorectal cancer patients.[69-71] Furthermore, in mesothelioma patients treated with DC therapy, two patients with a negative DTH skin test had progressive disease and had the shortest OS,[72] indicating that the DTH skin test could correlate with clinical outcomes. Additionally, in pancreatic cancer patients treated with WT1 I/II peptide-loaded DC therapy, patients with a positive DTH skin test had longer PFS and OS than patients with a negative DTH skin test.[73] However, all other studies combining DC therapy and chemotherapy did not show any correlation between DTH skin testing and clinical outcome.[63,65,66,73-75] The lack of correlation between DTH skin tests and clinical outcome, could be dependent on the timing of the DTH skin test, the evaluation of response to the skin test and the lack of a negative control. The classification of a positive DTH skin test varies from 2-5 mm erythema, whilst one could argue that induration is a more important parameter.[76] Also timing of DTH skin testing varies between studies or is not mentioned.

Combination strategies to optimize DC therapy

Combining Checkpoint Inhibitors with DC therapy

Inhibitory molecules that hamper anti-tumor immune responses, such as PD-1, PD-L1 and CTLA-4 can be expressed on both tumor cells and various immune cells.[77,78] Blocking these inhibitory molecules has been shown to restore tumor-specific T-cell activity.[77] There are many other inhibitory and even co-stimulatory molecules identified that could function as potential targets for immunotherapy such as; lymphocyte activation gene-3 (LAG-3), B and T lymphocyte attenuator (BTLA), programmed death-1 homolog (PD-1H), T-cell immunoglobulin (TIM-3), T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT); Glucocorticoid induced TNF receptor (GITR) and natural killer cell inhibitory receptor NKG2A.[79-83] Efficacy of these co-inhibitory and co-stimulatory molecules is currently investigated in preclinical and/or clinical studies, and will therefore not addressed in this review.

CTLA-4 blockage inhibits T-cell activation in the lymph node, whereas blocking the PD-1/PD-L1 axis mainly inhibits the effector function of activated T-cells in the TME.[84] Anti-CTLA-4 (ipilimumab, durvalumab), anti-PD-L1 (atezolizumab, durvalumab and avelumab) and anti-PD-1 (nivolumab and pembrolizumab) are registered for treatment of solid tumors because of striking clinical effects. The efficacy of these CIs, especially PD-(L)1 mAb, often depends on and correlates with PD-L1 expression in the TME, mutational burden and the number of tumor-infiltrating lymphocytes (TILs).[13,85-88] High ORRs of 57% are reported in immunogenic cancers, such as melanoma, which is ascribed to a high mutational burden and high numbers of TILs.[89,90] In tumors with lower mutational burden (e.g. mesothelioma) ORRs remain between 9-25%[91] likely due to the relative low frequency of TILs. DC therapy induces infiltration of tumor-specific $CD8^+$ T-cells and upregulates PD-1 expression on these TILs, which could render tumors with low TIL numbers susceptible to anti-PD-(L)1 treatment.[92-95]

It is likely that the limited efficacy of DC therapy trials is in part due to inhibitory signaling in the TME and lymph node. Additional administration of CIs can block inhibitory signaling on tumor cells, and immunosuppressive cells in the TME. CIs could even inhibit the signaling of these inhibitory molecules on the DCs administered during DC therapy, as DCs express both PD-1, PD-L1 and PD-L2.[96] Expression of PD-1, and its ligands likely limits the induction of tumor-specific immune responses, as high PD-L1 expression on DCs suppresses $CD4^+$ and $CD8^+$ T-cell proliferation and promotes Treg proliferation in various diseases, including cancer.[8,97-102] This suggests that combining DC therapy with CIs can result in a two-sided synergy by targeting not only tumor cells and immunosuppressive cells in the TME, but also DCs administered during DC-therapy and even T-cells induced by DC therapy (Fig.1A). Different DC subtypes differentially express these inhibitory molecules, suggesting that specific CIs should be used in combination with certain DC subsets.[103] The rationale for using CI in combination with DC therapy

is further supported by the finding that addition of a PD-1 mAb to *ex vivo* cultured autologous T-cells and DCs of patients with myeloma improved IFNg production while limiting Treg expansion.[104] Furthermore, combining DC therapy with systemic PD-1 blockade in mice bearing intracranial glioma tumors improved survival compared to both single treatments.[92] In addition, PD-1 blockage on DCs administered in a breast tumor-bearing mice model that were subsequently systemically treated with anti-PD-1 mAb, reduced tumor growth and increased survival compared to untreated mice.[105]

Clinical Trials combining Checkpoint Inhibitors with DC therapy

Until now, three clinical studies combined DC therapy with CI treatment. In all of these studies CTLA-4 mAb has been used. Clinical responses were retrospectively observed in patients with stage III and IV melanoma that were treated with ipilimumab upon disease progression after receiving at least 3 bi-weekly vaccinations with gp100 and tyrosinase loaded DCs.[106] Especially patients with stage III melanoma responded well with an OS rate of 51% after 2 years. The presence of tumor specific T-cells obtained from DTH skin biopsies did not correlate to OS in patients with stage III and IV melanoma. Ipilimumab-related adverse events were not increased in patients pretreated with DC vaccination (58%) compared to patients treated with ipilimumab monotherapy (61-70%).[107]

In a clinical phase 1 dose escalation trial, patients with stage IIIc or IV melanoma received three bi-weekly intradermal vaccinations with MART-1 loaded DCs and concurrent systemic treatment with a dose escalation of tremelimumab (3, 6, 10 mg/kg), an CTLA-4 blocking mAb.[108] Four out of 16 patients developed a clinical response upon treatment, of which 2 patients developed a complete response (CR), and 2 patients a partial response (PR). This indicates that response rates upon combination therapy are promising compared to response rates to tremelimumab monotherapy (7-10% ORR) [108-111] and DC vaccination monotherapy (15% ORR)[9]. Remarkably, responses were also observed in patients treated with only 3 mg/kg tremelimumab, achieving plasma levels of 30 μ/ml, which is below the target level determined by prospective clinical trials.[109] This suggests that these results are not solely the effect of tremelimumab or DC therapy alone, and indicate a synergistic effect. However, immunological analysis was inconclusive with a minority of patients showing response to tetramer or ELISPOT analysis.[109]

A phase II open label single arm clinical trial combined ipilimumab treatment with DCs electroporated with Trimix-mRNA (CD40L, CD70, constitutive active TLR4) and mRNA encoding for MAGE-A3, MAGE-C2, tyrosinase3 or gp100 in patients with stage III and IV melanoma.[112] Radiological responses were assessed with immune-related response criteria (irRC) which showed an ORR of 38%. Twenty percent of the responding patients showed CR and 18% had a PR. A disease control rate of 51% at 6 months follow-up was observed and the ORR was better than ORRs observed in patients treated with ipilimumab as monotherapy.[113] Furthermore, the number of CRs was similar to clinical trials investigating combination therapy of ipilimumab and nivolumab.[107] Immunological

analysis showed an overall increase of $CD4^+$ and $CD8^+$ T-cells in the peripheral blood and a positive tumor antigen-specific ELISPOT analysis in two out of ten patients. [112]

Combining different CIs often leads to increased toxicity such as dermatologic toxicity, colitis or pneumonitis,[114, 115] whereas combining DC therapy with CIs does not increase the immune related adverse event profile of CI monotherapy. Furthermore, ORRs, PFS and OS in clinical trials investigating combination therapy consisting of DC therapy and CTLA-4 mAb treatment are promising as compared to clinical trials that investigated these therapies as monotherapy. However, phase III randomized controlled clinical trials are needed to determine efficacy improvement of combining DC therapy and CI to either treatment modality alone. Furthermore, combining PD-(L)1 targeting CIs with DC therapy still needs to be evaluated in clinical trials. Immunological analysis was inconclusive in all studies and did not correlate with clinical outcome. Concurrent intensive immunological analysis of blood and tumor material could provide proof of principle, expand current knowledge and possibly lead to objectifiable immunological parameters for immunotherapy. Currently, many phase I/II trials are being conducted that combine PD-(L)1 mAb with DC therapy.[57,116] However, to date, there are no clinical phase III trials conducted to observe synergy of CI treatment in combination with DC therapy (https://clinicaltrials.gov/).[117]

Combining Chemotherapy with DC therapy

Apart from specifically targeting cancerous cells, it is becoming apparent that chemotherapy can also actively influence the immune system by depletion of specific cell types, such as Tregs and MDSCs and by induction of immunogenic cell death (ICD). Furthermore, chemotherapy can skew immunomodulatory cells in a more pro-inflammatory subset. Depletion of Tregs[12] and MDSCs[118] in the TME after chemotherapy treatment was already observed in preclinical and clinical studies.[11,12,17,23,119,120] Such immunological changes were even associated with clinical response.[120-124] Furthermore, ICD inducing chemotherapy, anthracyclines, has a suboptimal result in immunodeficient mice.[23,125] This indicates that chemotherapy treatment can affect both the immunosuppressive TME by cell-specific depletion and improve immune responses by the induction of ICD and thereby prove beneficial when combined with DC therapy.

In most clinical trials, DC therapy was administered in combination with registered chemotherapy treatment. Therefore, studies that combined treatment of chemotherapeutics with DC therapy were not often designed to improve DC therapy efficacy. Consequently, most of the immunological parameters analyzed, such as IFN-γ ELISPOTs, tetramer analysis and DTH skin tests determined immunological response to DC therapy rather than the immunological effects induced by chemotherapy.[63,66,73,126-128] Consequently, monitoring of immunomodulatory effects and therefore objectifying the attributable effect of chemotherapy on DC therapy is difficult to achieve. Clinical trials that investigated treatment of DC therapy in combination with chemotherapy in esophageal, prostate, pancreatic cancer, mesothelioma, glioblastoma and melanoma, are summarized in Table 1.

Table 1. Overview of clinical trials combining moDC therapy with chemotherapy

Disease	Peptide(s) of DC therapy	maturation cocktail	CTX (+ other additional treatments)	Immunomodulatory effect[a]	Immunological rationale[b]
glioblastoma	autologous tumor lysate	TNF-a, IFN-a and POLI I:C	RTX + TMZ 75 mg/m2 TMZ 200 mg/m2	Treg depletion	none
pancreatic cancer	WT1 peptide + OK-432 injection	OK-432 and PGE2	S1 or S1 + gemcitabine dose not stated	Treg depletion MDSC depletion	none
pancreatic cancer	WT1-I, -II, I/II peptide	OK-432 and PGE2	gemcitabine 1000 mg/m2	Treg depletion, MDSC depletion	none
glioblastoma	autologous	monocyte-derived conditioned medium (MCM)	TMZ 150-200 mg	Treg depletion	none
esophageal cancer	WT1 peptide	OK-432 andPG-E2	DTX 50 mg/m2	Treg depletion, MDSC skewing, ICD induction	non-specific immune enhancement
melanoma	WT1, gp100 tyrosinase, MAGE-A3 or MAGE-A2	Matured with OK-432 and PG-E2	carboplatin (AUC 5) and paclitaxel (175 mg/m2)	Treg depletion	Treg depletion and decrease IL-10 and TGF-β secretion
prostate cancer	PSA, PAP mRNA	TNF-a IL-1B, IL-6, PGE2	DTX 75mg/m2	Treg depletion, MDSC skewing, ICD induction	MDSC depletion and ICD
melanoma	p53, survivin, and hTERT mRNA	TNF-a IL-1B, IL-6, PGE2	cyclophosphamide 50 mg	Treg depletion, ICD induction	Treg depletion
mesothelioma	autologous tumor lysate	PGE2 TNF-a IL-1B IL-6	cyclophosphamide 2x50 mg	Treg depletion, inducing ICD	Treg depletion
prostate cancer	killed LNCaP prostate cancer cells	poly I:C	50 mg cyclophosphamide, 75 mg/m2 DTX	Treg depletion, MDSC skewing, ICD induction	Treg depletion, enhancement of T and NK cell activation
melanoma	autologous pulsed DC	TNF-a IL-1B, IL-6 PGE2	TMZ 75mg/m2 (IL-2)3,000,000 IU/day	Treg depletion	Treg depletion
melanoma	autologous tumor lysate or survivan, hTERT, p53	TNF-a IL-1b IL-6 PGE2	IL-2, cyclophosphamide and a COX-2 inhibitor	Treg depletion, MDSC depletion	Treg depletion, MDSC depletion

a. *The hypothesized immunomodulatory effect of chemotherapeutics as described in preclinical studies and reviews.*
b. *The immunological rationale for the use of the chemotherapeutic agent described in the respective article.*
c. *The results of the immunological analysis done to evaluate immunomodulatory effects of chemotherapeutics.*
d. *The results of the immunological analysis done to evaluate immunomodulatory effects of DC therapy.*

CTX immune readout[c]	DC therapy immune readout[d]	n	CO	CO corresponding to IR	Reference
none	8/25 pos ELISPOT	31	PFS 12.7m, OS 23.4m	no	Inoges 2018
none	7/8 pos ELISPOT	8	4 PD 4 alive 2 years post-treatment	pos ELISPOT correlated to 2-year OS	Yanagi-shawa 2018
none	4/11 pos DTH	10	7 SD, 3 PD	pos DTH positively correlated with PFS	Koido 2015
none	2/9 pos ELISPOT 0 pos DTH	14	2 PR, 3 SD, 4 PD median OS 23m	no	Hunn 2015
none	5/8 pos ELISPOT 3/7 pos DTH 3/7 pos tetramer 5/8 pos HLA	10	10 PD	no	Matsu-da 2018
none	4/9 pos ELISPOT	9	1 PR, 4 SD, 5 PD OS 12m PFS 2,3m	no	Fukuda 2017
DC + DTX: decrease in MDSC	9/18 pos ELISPOT 5/18 pos DTH	19 DTX 21 DTX + DC	no difference in PFS and OS	Decreasing levels of MDSC were correlated to better PFS	Kong-sted 2017
$CD4^+$ T-cell depletion	6/17 pos ELISPOT	22	9 SD, 13 PD OS 10,4m PFS 3,1m	no	Borch 2016
Treg depletion	8/10 pos DTH	10	1 CR, 4 SD, NA 3, PD 2	no	Cornelis-sen 2016
Treg depletion	increased $CD8^+$ T-cells, increased PSA-specific IFN- γ production	24	OS 19m	no	Podrazil 2015
Treg depletion	9/17 pos DTH	17	1 PR, 6 SD, 10 PD	no	Ridolfi 2013
Treg increase, MDSC depletion	8/17 pos DTH at baseline 1/17 pos DTH after vaccination	28	16 SD, 12 PD PFS 4,5m	no	Elle-beak 2012

Abbreviations: DC: dendritic cell, CTX: chemotherapy, CO: clinical outcome, IR: immunological readout, n: number, LNCaP: androgen-sensitive human prostate adenocarcinoma cell line, WT: Wilms tumor, MAGE: melanoma-associated antigen, PSA: prostate specific antigen, PAP: prostate acidic phosphatase, p53: tumor protein p53, hTERT: Telomerase reverse transcriptase, gp100: glycoprotein 100, mRNA: messenger RNA transfected, RTX: radiotherapy, S-1: Tegafur/gimeracil/oteracil, TMZ: temozolomide, DTX: docetaxel, COX:

Cyclooxygenase, IL: interleukin, Treg: regulatory T-cell, MDSC: myeloid derived suppressor cell, ICD: immunogenic cell death, PFS: progression free survival, m: months, OS: overall survival, PR: partial response, SD: stable disease, PD: progressive disease, ELISPOT; Enzyme-Linked ImmunoSpot assay, DTH: delayed type hypersensitivity skin test, pos: positive, AUC: area under the curve, NA: not applicable because of non-measurable lesions, TGF- β: transforming growth factor β, OK-432: penicillin-killed and lyophilized preparations of a low virulence strain (Su) of Streptococcus pyogenes

Targeting of immunosuppressive environment by chemotherapy

Regulatory T cells

Various chemotherapeutics, such as cyclophosphamide, paclitaxel, docetaxel, gemcitabine, temozolamide (TMZ) and oxaliplatin are capable of Treg depletion in a clinical and preclinical setting.[12,17,119,120,129] Cyclophosphamide is the best known and studied chemotherapeutic agent with the capability of depleting Tregs. Four clinical studies evaluated the immunological and clinical effects of potentially Treg depleting chemotherapeutics in combination with DC therapy, in which 3 studies used cyclophosphamide[65,72,74] and one study used TMZ.[75] Two other studies evaluated only clinical effects of potentially Treg-depleting chemoteherapeutics, cyclophosphamide and paclitaxel, in combination with DC therapy.

In a phase I clinical trial, melanoma patients were treated with six biweekly injections of DCs electroporated with mRNA encoding p53, survivin, and hTER and concurrent low-dose cyclophosphamide (50mg/bidaily biweekly).[130] The OS was 10,4 months and 9 out of 22 patients had stable disease (SD). Tregs as well as total $CD4^+$ T-cells were depleted upon cyclophosphamide treatment, questioning whether cyclophosphamide induced specific depletion of Tregs.[130] However, another clinical trial in mesothelioma patients that also combined concurrent low dose cyclophosphamide (2x50mg/day biweekly) with three biweekly injections of DCs loaded with tumor lysate did show selective depletion of Tregs.[72] Unfortunately, depletion of Tregs was not correlated with a better clinical outcome. However, detailed analysis of naïve Tregs (nTregs, $CD45RA^+$ $FoxP3^{int}$) and activated Tregs (aTregs, $CD45RA^-$ $FoxP3^{hi}$) showed a positive correlation between the pretreatment levels of nTregs and OS.[131] In addition, results from this clinical study are quite promising, with patients still alive up to 6 years after diagnosis.

Another phase II clinical study combined DC therapy loaded with tumor lysate or peptides (surviving, telomerase and p53) with consecutive IL-2 (2 MIU/day for 5 days), with metronomic cyclophosphamide (2x50 mg/day biweekly) and a Cox-2 inhibitor (200mg daily) in melanoma patients.[74] Melanoma patients treated with this combination therapy also showed increased numbers of Tregs after four vaccinations indicating that cyclophosphamide was not able to counteract the effect of IL-2 on Tregs, as IL-2 has the potency to increase Treg numbers. [132] In contrast to Tregs, mMDSCs were significantly decreased after four vaccinations, indicating that the combination treatment depletes

mMDSCs. However, the changes observed in Treg numbers and mMDSC frequency did not correlate with clinical outcome. Clinical results were significantly improved compared to a previous trial where DC therapy was only combined with IL-2.[133]

Combining neoadjuvant TMZ (75mg/m2/day for fourteen days) treatment followed by autologous tumor lysate loaded DC therapy with consecutive IL-2 (3 MUI/day for 5 days) in 17 melanoma patients, also significantly depleted Tregs, although this did not correlate to clinical outcome. One patient showed a PR and six patients had SD. [75]

In another study, patients with metastatic castration-resistant prostate cancer were treated with neoadjuvant metronomic cyclophosphamide (50mg/day for 1 week) followed by LNCaP (androgen-sensitive human prostate adenocarcinoma cells) loaded DC therapy and docetaxel (75mg/m2 every three weeks). Predicted OS was 11,3 months, whereas in this study an OS of 19 months was observed, suggestive of a synergistic effect upon the combination of these treatments.[134,135]

In a clinical trial in patients with stage IV melanoma treated with DCs loaded with a multi-peptide (WT1, gp100, tyrosinase and MAG-E3 or MAGE-A2) combined with paclitaxel (175mg/m) and carboplatin (area under the curve 5), OS up to 24 months was observed.[126] Unfortunately, Treg numbers were not assessed in these clinical trials. Taken together, these clinical trials indicate that chemotherapy is capable of depleting Tregs and induce promising clinical responses in combination with DC therapy. Alterations in Treg numbers upon treatment could not be correlated with clinical outcome although nTreg frequencies at baseline were predictive of clinical response.[72,74] However, further research is needed to determine whether this is also observed in other malignancies and combination therapies. (Fig.1B)

Myeloid-derived suppressor cells

Numerous chemotherapeutics, such as gemcitabine, 5-FU, cisplatin and docetaxel, have been shown to specifically deplete MDSCs.[12,74,119,120] Furthermore, docetaxel possibly improves immune stimulatory effects of total MDSCs by skewing them into a more favorable pro-inflammatory/migratory phenotype (CCR2, CCR5, CX3CR1, CCR7) rather than an immunosuppressive phenotype.[136] This indicates that chemotherapeutics not only deplete immunoregulatory cells but can also change the phenotype of immunosuppressive cells. In contrast to the above described chemotherapeutics, cyclophosphamide increases the amount of specifically pMDSCs, but not mMDSCs in the peripheral blood of mice and human.[137] The increase in pMDSCs induced by cyclophosphamide can be counteracted by the addition of chemotherapeutics targeting MDSCs, as combining cyclophosphamide and gemcitabine treatment decreased both Treg and GR1^{high} MDSC numbers and reduce tumor growth.[138]

One study in patients with stage IV pancreatic ductal adenocarcinoma treated with WT1 loaded DC therapy and gemcitabine (1000mg/m2 three times every 28 days) showed that combining these treatments is safe and feasible.[73] They also found a positive correlation between DTH skin testing and PFS. Unfortunately, MDSC numbers were not assessed in this study, so it remains inconclusive whether gemcitabine administration in combination with DC therapy affected MDSC numbers in these patients.

In a clinical study in patients with metastasized adenocarcinoma of the prostate, docetaxel (75mg/m2 every 3 weeks) mono-treatment was compared to combined treatment of docetaxel with DCs transfected with mRNAs encoding PAP (prostate acidic phosphatase) and PSA (prostate-specific antigen).[65] There was no significant difference in OS and PFS between both treatment arms. Patients with decreased MDSCs frequencies in cryopreserved PBMC upon treatment had a longer PFS as compared to patients with increasing frequencies of MDSCs upon treatment. A decrease of MDSCs in cryopreserved PBMCs was only observed 6 weeks after start of combination therapy, but not upon docetaxel monotherapy, which suggests that docetaxel monotherapy is not sufficient to decrease MDSCs in peripheral blood.[65] Paradoxical, a preclinical study observed a decrease in total MDSC numbers upon treatment with docetaxel monotherapy.[136] Additionally, docetaxel treatment skewed total MDSCs towards a more pro-inflammatory phenotype in a preclinical study (Fig.1C).[136] Characterization of MDSCs remains challenging due to lack of specific markers and the need to assess these cell populations in freshly isolated blood, as especially pMDSCs are lost upon cryopreservation.[33] In addition, most clinical trials focus on evaluating MDSC numbers rather than MDSC characteristics leading to lack of evidence for the phenotypical switch of MDSCs upon docetaxel treatment in humans.

Immunogenic cell death

Various chemotherapeutics, such as cyclophosphamide, oxaliplatin, paclitaxel, docetaxel and anthracyclines are able to induce ICD (Fig.1E). The antineoplastic effects of these chemotherapeutics is also dependent on ICD.[139-142] Current monitoring of ICD occurs via vaccination assays that are not applicable in clinical trials.[142] This limits the possibility for evaluation of ICD induced synergistic effects. Consequently, ICD is not often used as a rationale for combining chemotherapy and DC therapy (Table 1). A phase III clinical study in patients with metastatic castration resistant prostate cancer comparing docetaxel treatment combined with DC therapy docetaxel monotherapy has finished accrual end results are awaited (NCT02111577). Hopefully, immunological data will lead to better understanding of the immunomodulatory effects of docetaxel. A phase III trial in glioma patients evaluates the additional effect of DC therapy to current treatment consisting of TMZ and radiotherapy (NCT03548571). Addition of a DC monotherapy arm to both these studies could have revealed the synergistic effect of docetaxel and TMZ on DC therapy.

Combining Radiotherapy with DC therapy

Radiotherapy has been used as local tumor treatment for the last century. Recently, radiotherapy was also found to affect non-radiated tumor lesions, which is called the abscopal effect. This suggests systemic effects of radiotherapy that can be explained by the upregulation of radiation-induced double-stranded DNA in the cytosol which serves as a DAMP for the instigation of ICD. Subsequently, the secretion of type I interferons by tumor cells will attract cDC1s to the tumor site, that canengulf the released tumor antigens and initiate an immune response.[16,143] Therefore, radiation induced ICD can act as *in situ* vaccination.

Apart from inducing ICD, radiation induces upregulation of adhesion molecules on the vascular endothelium of tumor cells which enables T-cell infiltration (Fig.1E).[144] Furthermore, non-lethal radiotherapy dosage increases surface expression of first apoptosis signal (Fas) ligand, carcinoembryonic antigen and MHCI on tumor cells enabling tumor-specific $CD8^+$ T-cells to recognize the tumor cells and exert their cytotoxic effects.[145] Together, these immunomodulatory effects are hypothesized to be responsible for the abscopal effect.[146] However, the theoretical immunomodulatory effect of radiotherapy lacks clinical support, as a recent review found only 46 reported cases of abscopal effect from 1969 to 2004.[147] This could be dependent on the irradiation dose used, radiation schedule and lack of additional immune stimulation. This is supported by a recent study in breast and colorectal tumor-bearing mice, where different radiation doses were compared in combination with CI. Here they found that repetitive radiation at low dose (5-8Gy) was more effective than a high (20Gy) single dose.[148,149] High dose radiation is thought to indirectly downregulate cytosolic dsDNA and thus inhibit radiation dependent ICD.[16]

Clinical trials combining DC therapy with radiotherapy

In a phase I clinical trial, 14 patients with advanced hepatoma received immature DC therapy followed by 8 Gy radiotherapy. The clinical results varied, with 2 PR, 4 minor response, 3 SD and 4 PD.[150] Seven out of ten immunologically evaluated patients developed an IFN-γ ELISPOT response upon treatment, which did not correlate with clinical response.

In an observational study, patients with esophageal cancer that either received autologous tumor lysate loaded DC therapy in combination with concurrent radiotherapy (60Gy) were compared to radiotherapy alone. Two-year survival was significantly improved upon combination therapy (67.8%) as compared to single radiotherapy treatment (33.3%).[151] Additionally, in patients with metastatic solid tumors, radiotherapy (35Gy) combined with *in situ* DC therapy using GM-CSF administration, induced an abscopal effect in 11 out of 41 patients, which was significantly higher as compared to abscopal effects induced by radiotherapy alone.[152]

These data suggest that regional radiotherapy can act synergistically when combined with DC therapy. The synergy likely depends on the release of tumor antigen that boosts the anti-tumor immune response, and should be further investigated in ongoing clinical trials. A recently registered trial for patients with metastatic melanoma will evaluate the additional effect of different immune stimulatory agents, including radiotherapy (24-32Gy), to autologous tumor lysate loaded DC therapy (NCT01973322).[153] Additionally, the effect of sipuleucel-T therapy combined with stereotactic ablative body radiation in patients with metastatic castrate resistant prostate cancer will be observed in a single arm study (NCT01818986). These studies will allow further investigation into the exact immunological mechanism of action of radiotherapy combined with DC therapy.

Other combination strategies: targeting tumor associated macrophages

Immunoinhibitory TAMs are abundant in many solid tumors.[154,155] These TAMs can be either depleted via colony stimulating factor 1 receptor (CSF-1R) blockade, or skewed into an immunostimulatory M1 phenotype by CD40 agonistic mAb.[156-162] CSF-1R blockade increased efficacy of chemotherapy in pancreatic tumor-bearing mice.[163] In glioblastoma tumor-bearing mice, TAM depletion by CSF-1R monotherapy induced tumor reduction and increased survival.[164] In contrast, in a mesothelioma mouse model, CSF-1R kinase inhibitor PLX3397 (pexidartinib) mediated TAM depletion as monotherapy did not improve survival.[156] However, when pexidartinib treatment was combined with DC therapy, improved survival was observed when compared to both monotherapies, which was accompanied by increased numbers of proliferating T-cells and effector T-cells.[156]

These studies indicate that TAM depletion can improve the immunosuppressive character of the TME and thereby act synergistically when combined with DC therapy. In addition to TAM depletion, skewing TAMs towards an immunostimulatory M1 phenotype using CD40 mAb may even hold more clinical potential, as CD40 mAb activated macrophages in pancreatic cancer infiltrate the tumor and facilitate tumor stroma depletion.[165] Especially in tumors with dense stromal, targeting the stroma indirectly through macrophage skewing could facilitate and improve tumor specific T-cell infiltration upon DC therapy. (Fig.1D)

Future Perspectives

Currently, most clinical trials that combine DC therapy with other treatments, such as chemotherapy, radiotherapy or CI often lack immunological rationale. This is likely due to already existing registrations of these treatments based on other rationales leading to mandatory use of certain therapies in specific dosage and schedules. In an experimental setting, lack of consistency in dosing and schedule of treatments complicates the

comparison of both immunological responses and clinical efficacy between different studies. For example, "metronomic" dosage of cyclophosphamide varies from 50 mg bidaily to 100 mg/day between different studies,[72, 130] whereas Treg depletion is dependent on dose and schedule of chemotherapy.[29] Separate studies should be performed to analyze the immunomodulatory effects of chemotherapy, radiotherapy, targeted therapies and immunotherapies alone. During these studies, adequate reports of the used methods and analyzation strategy are necessary to facilitate standardization of type and timing of immunomonitoring assays.[166] This will create the possibility to replicate and subsequently compare the immunomodulatory effects between studies and finally leads to a better understanding of the immunomodulatory effects of monotherapies.[116] With this knowledge, specific therapies with the right dosing can be combined with DC therapy based on the TME characteristics (Fig.1).

Variations in DC therapy, in terms of antigen loading, maturation, use of different DC subsets, dosage per injection and the interval or total amount of vaccinations makes it difficult to compare clinical studies with each other. Adequate immunomonitoring in DC therapy is mandatory to create the possibility to compare studies and evaluate the effect of different antigen loading methods, maturation cocktails and administration schedules or injection sites. Additionally, registration of DC therapies will enable the investigation of clinical efficacy of DC therapy in combination with other treatments as compared to DC monotherapy. This will inevitably lead to an increase in the number of randomized trials and rapid release and approval for registration of immunomodulatory therapies in combination with DC therapy. To date, sipuleucel-T is the only registered DC therapy.[48] Phase III trials that could eventually lead to registration of DC therapy are ongoing for melanoma (NCT02993315), glioblastoma (NCT03548571), mesothelioma (NCT03610360) and colorectal cancer (NCT02503150).

Hopefully, trials combining DC therapy with other therapies, based on a solid rationale and performed with adequate immunomonitoring and uniformity in administration schedules, will lead to registration of already existing treatment modalities for new purposes. In this way chemotherapeutics, radiotherapy, CIs or other targeted therapies can be used as an off-the-shelf, affordable immunomodulating agents to support DC therapy in a personalized manner. In order to accomplish this, intensive cooperation between clinicians and basic scientists will be needed.

Other targets for cancer therapy such as additional co-inhibitory molecules, co-stimulatory molecules or even targeted therapies such as IDO are upcoming. These targets could all hypothetically be used as immune modulators in the future. However, we have to be reluctant as some of these therapies are not yet found to be effective in phase III trials in humans.[167] In the process of proving clinical efficacy of these drugs, immunomonitoring data should already be obtained in an earlier stage, whereby the immunomodulatory effect of these therapies is known before registration.

Conclusion

Apart from improving DC therapy itself, influencing the immunosuppressive character of the TME by targeting immune cells such as Tregs, MDSCs or TAMs with already registered therapies could improve response rates upon DC therapy. To accomplish this, phase III clinical trials are urgently required that investigate clinical efficacy upon DC therapy combined with other treatments and registration of DC therapy for multiple malignancies. Additionally, elucidating the underlying immunological mechanism of these synergistic effects upon combination therapy will further boost the combination of DC therapy with other therapies. A better understanding will also lead to personalized combination therapy wherein DC therapy will be combined with other therapies based on composition of the TME, the expression of inhibitory molecules on the surface of tumor and immunosuppressive cells, and tumor mutational burden. (Fig.1)

Conflicts of interest/ Acknowledgements

J.G.J.V. Aerts reports receiving commercial research grants from Amphera and Roche, holds ownership interest (including patents) in Amphera BV, and is a consultant/ advisory board member for Amphera, Boehringer Ingelheim, Bristol-Myers Squibb, Eli-Lilly, MSD, and Roche. No potential conflicts of interest were disclosed by the other authors.

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Supplementary materials

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7

Combination of PD-1/PD-L1 checkpoint inhibition and dendritic cell therapy in mice models and in patients with mesothelioma

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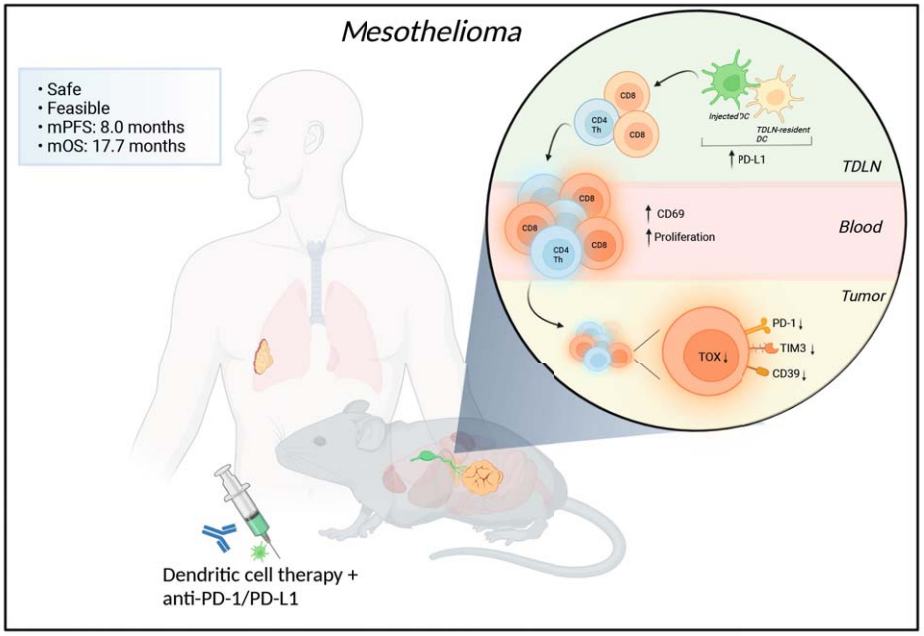

Novelty and Impact

Immunotherapy with anti-PD1/PD-L1 is effective in only a subgroup of patients with malignant pleural mesothelioma, possibly due to a lack of pre-treatment T-cell infiltration in nonresponding patients. On the other hand, dendritic cell therapy has been shown to enhance intra-tumoural T-cell infiltration. In this study, the authors investigated the efficacy of dendritic cell-based immunotherapy in combination with checkpoint blockade in malignant pleural mesothelioma patients and a murine mesothelioma model. The results suggest that the combination treatment is safe and capable of improving anti-tumor immunity, pointing to a promising new treatment option for malignant pleural mesothelioma patients.

Abstract

Immunotherapy with anti-PD1/PD-L1 is effective in only a subgroup of patients with malignant pleural mesothelioma (MPM). We investigated the efficacy of a combination of anti-PD1/PD-L1 and dendritic cell (DC) therapy to optimally induce effective anti-tumor immunity in MPM in both humans and mice. Data of nine MPM patients treated with DC therapy and sequential anti-PD1 treatment were collected and analyzed for progression-free survival (PFS) and overall survival (OS). Survival and T-cell responses were monitored in AC29 mesothelioma-bearing mice treated concurrently with the combination therapy; additionally, the role of the tumor-draining lymph node (TDLN) was investigated. The combination therapy resulted in a median OS and PFS of 17.7 and 8.0 months, respectively. Grade 3 to 4 treatment-related adverse events had not been reported. Survival of the mesothelioma-bearing mice treated with the combination therapy was longer than that of untreated mice, and coincided with improved T-cell activation in peripheral blood and less T-cell exhaustion in end stage tumors. Comparable results were obtained when solely the TDLN was targeted. We concluded that this combination therapy is safe and shows promising OS and PFS. The murine data support that PD-L1 treatment may reinvigorate the T-cell responses induced by DC therapy, which may primarily be the result of TDLN targeting.

Introduction

The median survival after diagnosis for patients with malignant pleural mesothelioma (MPM) remains between 13 and 18 months.[1,2] Therefore, novel therapeutic strategies that effectively induce anti-tumor responses are warranted. PD-1 checkpoint inhibition has shown remarkable responses in multiple cancer types. Anti-PD-1 therapy induces responses in 9-29% of MPM patients and as second line treatment it has been associated with a median progression-free survival (PFS) of 2.5 months and median overall survival (OS) of 10.7 months.[1,3-5] In combination with ipilimumab (anti-CTLA-4), response rates were even higher and more durable. Still, the majority of patients failed to respond which could be due to lack of T-cell infiltration before treatment.[2,6]

Dendritic cell (DC) therapy has been shown to be safe, feasible and able to induce radiological responses in MPM coinciding with enhanced intratumoral T-cell infiltration (7-9). As DC therapy-induced infiltrating T cells may in turn become exhausted through PD-1/PD-L1 signaling, we investigated the efficacy of adjuvant anti-PD-1 immunotherapy in DC-treated MPM patients. Additionally, as PD-L1 is expressed on DCs, the effects of concurrently combining DC- and anti-PD-L1 therapy were analyzed in a MPM murine model.

Material and methods

Patient data collection.

Data were collected of nine patients with histologically proven MPM treated in second or third line with CI therapy after progression on treatment with autologous monocyte-derived DCs (moDCs) loaded with allogeneic (n=8) or autologous (n=1) tumor lysate (NCT02395679, NCT01241682). Five patients had received first line chemotherapy prior to DC therapy.

Patient treatment.

Intravenous anti-PD-1 treatment, consisting of nivolumab (3 mg/kg every 2 weeks) or pembrolizumab (2 mg/kg every 3 weeks) was administered, irrespectively of PD-L1 expression. One patient received nivolumab and ipilimumab at dosages described in the INITIATE trial.[10]

Patient response evaluation.

Radiological tumor evaluation was done 6 weeks after start of treatment and every 4 to 12 weeks thereafter; the interval depended on the previous CT evaluation. The tumor response was assessed using the modified Response Evaluation Criteria in Solid Tumors (mRECIST) for mesothelioma (final data check November 19th, 2021).[11] OS was defined as the time from start of CI therapy until death. PFS was determined from the time

of start of CI therapy until radiological progression or death of any cause. The overall response rate was defined as the percentage of patients with a partial response (PR) or complete response. Disease control rate was defined as the percentage of patients without progressive disease as best overall response (BOR).

In vivo experiment in murine AC29 tumor model.

Female 8-12-week-old CBA/J mice and C57BL/6 mice were purchased from Envigo and housed under specific pathogen-free conditions in individually ventilated cages at the animal care facility of the Erasmus University Medical Center (Erasmus MC), Rotterdam.

For tumor inoculation, mice were intraperitoneally (i.p.) injected with 10^6 AC29 mesothelioma tumor cells (RRID:CVCL_4407) in 300 µl PBS, as described previously.[12] Mice with established i.p. tumors were killed at indicated time points for immune cell profiling or when profoundly ill according to the body condition score for therapy efficacy experiments. Mice were randomly assigned to experimental groups.

For bone marrow derived dendritic cells (BMDC)-transfer, AC29 tumor lysate was produced and DCs were cultured as previously described.[13] Briefly, tumor lysate was produced by disrupting frozen tumor cells by four cycles of freeze-thaw cycles with liquid nitrogen followed by sonication. BMDCs were generated using recombinant murine GM-CSF (provided by B. Lambrecht VIB)., Ghent) in DC-culture medium followed by loading with tumor lysate and activation with CpG (Invitrogen) on day 9 and injection at day 10. Where applicable, DCs were labeled at day 10 with carboxyfluorescein succinimidyl ester (CFSE) according to the manufacturer's instructions (Thermo Fisher). Dependent on treatment arm, mice were treated with either 200 µg isotype (clone 2A3, BioXCell) or 200 µg anti-PD-L1 antibody (clone MIH5, provided by L. Boon, Bioceros B.V., Utrecht, the Netherlands) in 300 µl PBS in the peritoneal cavity. In case of intrapleural injection, 200 µL PBS was injected in the pleural cavity of mice that were under short-term anesthesia. All experiments were performed with mycoplasma-free cells.

Preparation of single cell suspensions from mouse tissues.

Single-cell suspensions were generated from isolated inguinal lymph node (non-tumor draining lymph node (non-TDLN), mediastinal lymph node (TDLN), blood and tumor tissue of mice from each group as previously reported (13). In brief, 30 µl blood was collected in EDTA tubes (Microvette CB300, Sarstedt) and erythrocytes were lysed using osmotic lysis buffer (8.3% NH4Cl, 1% KHCO3, and 0.04% Na2EDTA in Milli-Q). Tumors were collected and dissociated using a validated tumor dissociation system (Miltenyi Biotec) according to protocol.

Statistical analysis.

Median OS and PFS were estimated using a Kaplan Meier curve in combination with a log-rank (Mantel-cox) test. Survival data were plotted as Kaplan-Meier survival curves,

using the log-rank test to determine statistical significance. A P-value of 0.05 or below was considered to indicate statistical significance. All reported p-values were two tailed. Statistical analyses were performed using R 3.6.0 (R Foundation for Statistical Computing) or Graphpad Prism 8.0.

Results

We identified strong PD-L1 upregulation on *in vitro* matured patient-derived moDCs used for vaccination (Fig 1A). Vaccination of mice with DCs induced $CD8^+$ T-cell (CTL) infiltration, which coincided with increased PD-L1 expression by tumor cells, likely due to increased IFN-γ production by CTLs (Fig. 1B-C). Due to the upregulation of PD-1 on CTLs and PD-L1 on both tumor cells and exogenous DCs, we investigated whether checkpoint blockade could re-induce T-cell mediated immunity and responses in patients. We assessed nine MPM patients receiving pembrolizumab (n=2) or nivolumab (n=7; one patient combined with ipilimumab) upon progression after DC therapy (Fig. 1C). The median PFS following start checkpoint blockade was 8.0 months and the median OS was 17.7 months (Fig. 1E). Three patients exhibited partial responses, five stable disease, and one progressive disease; thus, the objective response rate was 33% (Fig. 1D). At 6 months, five patients (55.6%) showed disease control. Application of the Common Terminology Criteria for Adverse Events (CTCAE 5.0) did not reveal any grade 3/4 adverse events.

Similar to the PD-L1 upregulation on patient-derived moDCs, we identified increased PD-L1 expression on both transferred and endogenous DCs in the TDLNs of tumor-bearing mice (Fig. 2A). Therefore, we wondered whether concurrent treatment with DC therapy and checkpoint blockade could enhance anti-tumor immunity. To investigate this, we concurrently treated mesothelioma-bearing mice with DC therapy and anti-PD-L1, enabling us to assess PD-1 expression on T cells following treatment. This combination treatment resulted in longer survival compared to untreated mice (Fig. 2B-C). This was accompanied by synergistic and rapid CD69 upregulation (early activation marker) on T cells in peripheral blood, followed by increased proliferation (assessed by Ki-67), which was most prominent for CD4-Th cells (Fig. 2D, S1A). Moreover, the expression of the exhaustion-program driver TOX on tumor-infiltrating CTLs was most profoundly decreased following combination treatment, indicating a less-exhausted T-cell phenotype. This phenotype was confirmed by a decreased percentage of cells positive for PD-1, TIM3 and CD39, and lower TOX expression within this triple-positive cell subset (Fig. 2E, S2B).

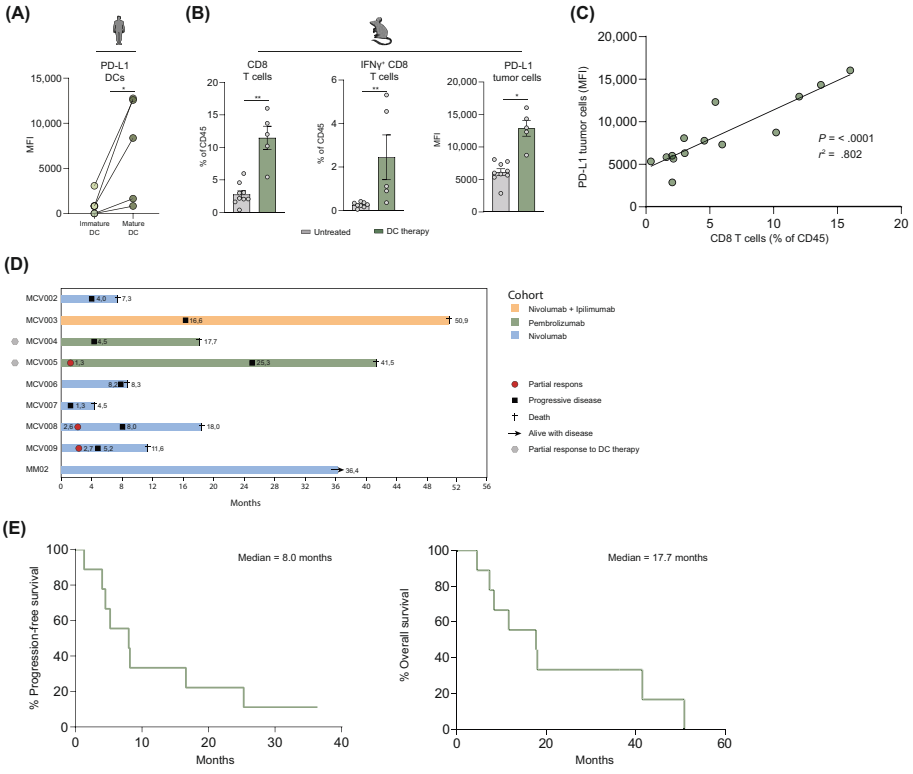

Figure 1. Rationale and clinical responses to treatment with checkpoint blockade upon progression to DC therapy. MFI of PD-L1 on immature and mature patient-derived DCs cultured in vitro for therapy (A). $CD8^+$ T-cell infiltration, IFNγ production and PD-L1 expression on tumor cells was assessed in AC29 bearing female CBA/J mice untreated (n=9) or treated with DC therapy (n=5) on day 15 (B). Correlation of PD-L1 expression on tumor cells and $CD8^+$ T-cell levels and a Pearson correlation coefficient was calculated (r^2) (n=14) (C). Swimmer plot of patients treated with checkpoint blockade upon progression to DC therapy. Overall survival of patients since date of first vaccination is represented by the filled bars. Start and end of RECIST responses are depicted by the red circles and black squares, respectively. First evaluation of response was after 6 weeks for all patients. (D) Kaplan-Meier curves showing progression-free survival (left) and overall survival (right) for all patients. (E) Means and SEMs are shown and Mann-Whitney U tests were performed indicating statistical significance. *p < 0.05, **p < 0.01.

We have previously shown a critical role for PD-L1-expressing DCs in suppressing anti-tumor T cells in TDLNs of mesothelioma-bearing mice.[12] This potentially suggests that TDLNs may be important in mediating the efficacy of PD-L1 blockade combined with DC therapy. To investigate whether the efficacy in mice resulted from PD-L1 blockade in TDLNs or in tumors, we targeted PD-L1 specifically and solely in TDLNs using an established method in which anti-PDL1 is administered at a low dose in the pleural cavity (12). Blocking PD-L1 solely in TDLNs mimicked systemic anti-PD-L1 treatment for survival (Fig. 2C) and for alterations in immune phenotype (Fig. 2D-E). This implies that

the efficacy of concurrent combination treatment may primarily depend on blocking the PD-1/PD-L1 axis in the TDLN, thereby resulting in improved T-cell priming by DCs. These findings could indicate the importance of optimizing T-cell priming in TDLNs for maximum anti-tumor T-cell capacity and provide a preclinical rationale for concurrent treatment in MPM patients.

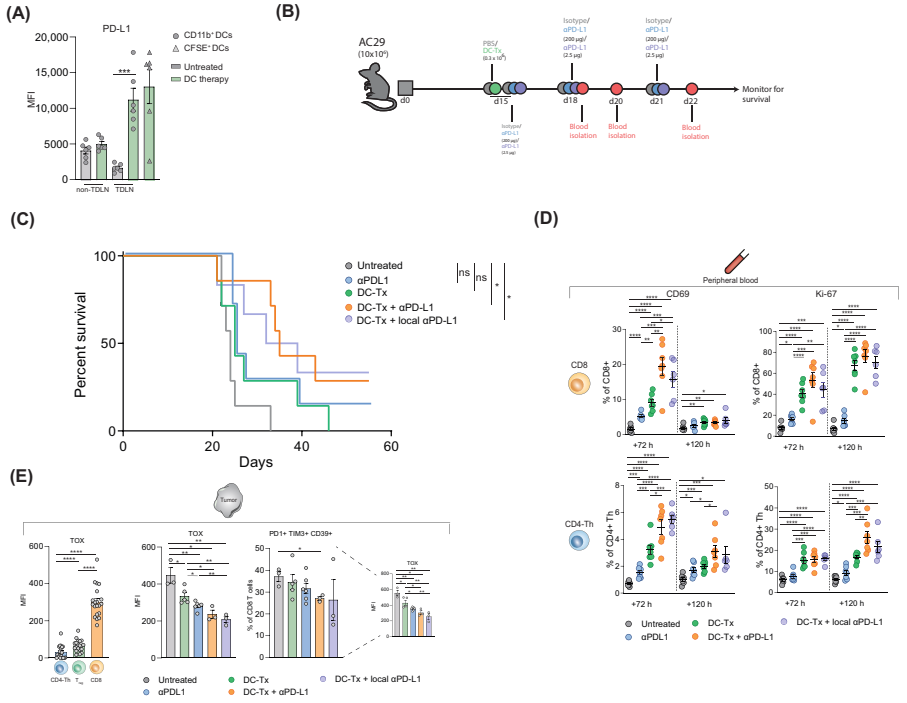

Figure 2. Concurrent treatment with anti-PD-L1 and DC therapy results in improved survival and anti-tumor immunity. MFI of PD-L1 on injected CFSE labeled DCs and endogenous DCs in non-TDLN and TDLN of AC29 mesothelioma bearing CBA/J mice (n=6/group; total n=12) 24 hours after DC therapy (A). Mice bearing AC29 tumors (n=7/group; total n=35) were treated with DC therapy or PBS at day 15. At day 15, 18 and 21, mice were also treated with either isotype, low-dose anti-PD-L1 (2.5 μg) or systemic dose (200 μg) (B). Mice were monitored for survival which is depicted in a Kaplan-Meier curve (C). From the experiment in B, blood was isolated at day 18 and 20 and expression of early-activation marker CD69 and proliferation marker Ki-67 were determined for $CD8^+$ T cells and CD4-Th cells (D). Expression of TOX on CD4-Th cells, T_{reg} cells and $CD8^+$ T cells and the percentage of triple-positive ($PD\text{-}1^+$ $TIM3^+$ $CD39^+$) and their TOX expression level was determined in end-stage tumor material from experiment in B (E). Means and SEMs are shown and paired- and unpaired t tests were performed indicating statistical significance. *p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.0001. MFI = median fluorescence intensity, non-TDLN = non tumor-draining lymph node, TDLN = tumor-draining lymph node, CD4-Th = CD4 T helper, SEM = standard error of the mean.

Discussion

In this study, we have shown that anti-PD-1 following DC therapy is safe and feasible in MPM patients. The response rate (33%), PFS (8.0 months) and OS (17.7 months) are promising when compared to anti-PD-1 monotherapy. Still, the potential bias in patient selection calls for caution in the interpretation of these findings. To support the potential synergy, combining DC therapy with concurrent blockade of the PD-1/PD-L1 axis reinvigorated T cells and prolonged survival in the mesothelioma-bearing mice. This synergistic effect of concurrent treatment may be the result of the high PD-L1 expression on DCs *in vivo* and on moDCs given as DC therapy. The data suggest that this effect could be primarily derived from the TDLN, as TDLN-specific blockade of PD-L1 resulted in comparable immune-stimulating effects as did systemic anti-PD-L1 treatment. By releasing progenitor-exhausted tumor-specific T cells, PD-L1 blockade on DCs in the TDLN has been shown to induce effective tumor immunity.[12,14] As we observed a less-exhausted tumor-infiltrating CTL phenotype in combination therapy-treated mice, these results could indicate that concurrent treatment may eventually result in more efficient T-cell priming in the TDLN by DCs. Since we treated MPM patients sequentially with PD-1 blockade, our data indicate that clinical responses might even be further improved by concurrent treatment.

Limitations of our study include the lack of pre-and post-treatment biopsies in MPM patients treated with DC therapy. This precluded investigation of the PD-L1 upregulation that we observed in mice. Furthermore, due to rapid tumor growth, we could not include a murine treatment arm of DC- and anti-PD-L1 therapy administered sequentially. Lastly, while MPM patients were treated with PD-1 blocking agents, mesothelioma-bearing mice were treated with antibodies blocking its ligand, PD-L1. Although both antibodies block the same axis, it has recently been demonstrated that anti-PD-1 and anti-PD-L1 may have different immune modulating effects due to *cis* interactions with CD80 on antigen-presenting cells which could potentially influence efficacy of the combination treatment.[15] Whether anti-PD-L1 leads to suboptimal anti-tumor immunity, compared to anti-PD-1, needs to be further investigated in our models.

In conclusion, our data from both patients and mice indicate that the combination of DC therapy and anti-PD-1/PD-L1 could be a promising treatment for MPM, as it was found feasible and safe, and did show clinical efficacy.

Acknowledgements

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Ethics statement

All patient data in our cohort were retrospectively collected (NCT02395679, NCT01241682). Therefore, according to Dutch guidelines approval from a medical research and ethics committee was not required. The murine experiments were monitored by the Erasmus MC animal welfare committee, and had been approved by the national central committee of animal experiments (CCD) under the permit number AVD101002017867, in accordance with the Dutch Acton Animal Experimentation and EU Directive 2010/63/EU.

List of abbreviations

BMDC	bone-marrow derived dendritic cells
BOR	best overall response
CFSE	carboxyfluorescein succinimidyl ester
CI	checkpoint inhibiting
CTCAE	Common Terminology Criteria for Adverse Events
CTLA-4	cytotoxic T-lymphocyte associated protein 4
DC	dendritic cell
GM-CSF	granulocyte-macrophage colony-stimulating factor
i.p	intraperitoneal
i.pl	intrapleural
moDC	monocyte-derived DC
MPM	malignant pleural mesothelioma
mRECIST	modified Response Evaluation Criteria in Solid Tumors
OS	overall survival
PBS	phosphate buffered saline
PD-1	programmed death 1
PD-L1	programmed death ligand 1
PFS	progression-free survival
PR	partial response
TDLN	tumor-draining lymph node

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Supplementary

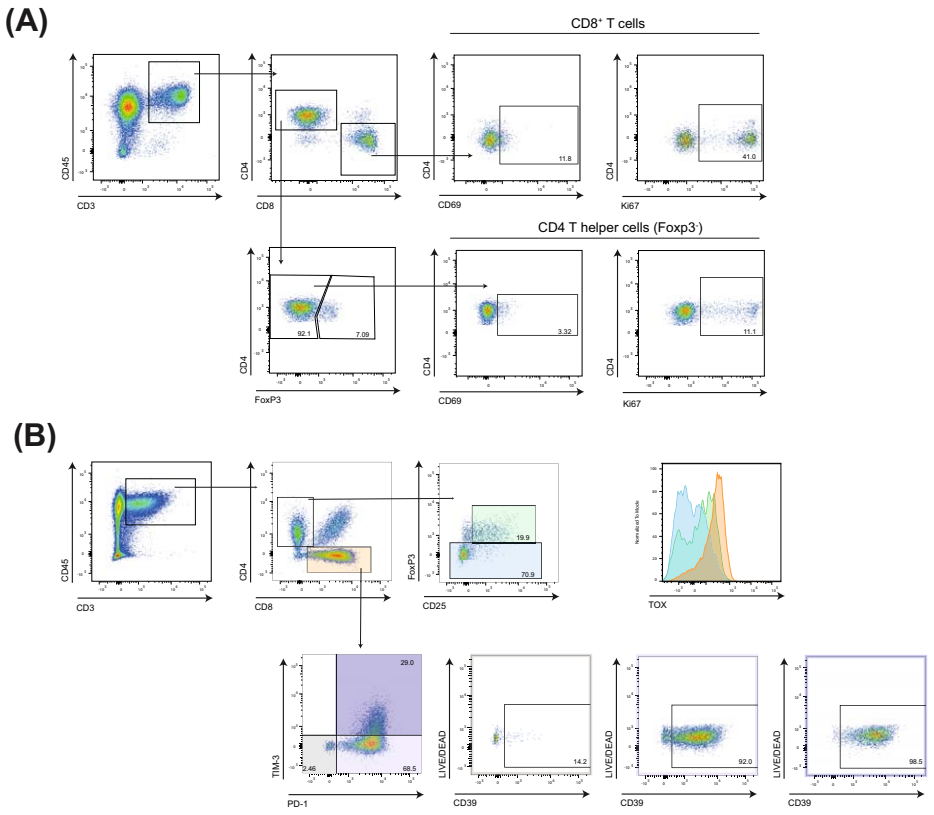

Figure S1. Characterization of T cells in peripheral blood and tumor in AC29 bearing mice treated with anti-PDL1. (A) Gating strategy of T cells (CD8 T cells and CD4 T helper cells) and related expression levels of GITR in peripheral blood isolated 72- and 120 hours following start treatment, related to figure 2D. (B) Gating strategy for the characterization of end stage tumor infiltrating T cells (CD8 T cells, CD4 T cells) and related expression levels of TOX. For CD8 T cells, gating for triple-positive cells was according to TIM3 and PD-1 (PD-1⁻ TIM3⁻ (grey) / PD-1⁺ TIM3⁻ (light purple) / PD-1⁺ TIM3⁺ (dark purple)), followed by TOX, related to figure 2E.

8

Dendritic cell therapy (MesoPher) combined with extended-Pleurectomy/Decortication in resectable mesothelioma (ENSURE trial): induction of increased T-cell infiltration and vaccine specific T-cells in the tumor

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Statement of translational relevance

In patients with early-stage pleural mesothelioma, surgical treatment via an extended pleurectomy decortication (eP/D) can be considered, but recurrence rates are high. We hypothesized that perioperative dendritic cell (DC) therapy after standard-of-care chemotherapy could reduce the risk of recurrence. The first patient included in this trial completed treatment according to protocol and has a very promising ongoing recurrence-free survival of 15 months. After DC therapy, we detected increased diffuse CD8+ T cell infiltration in the tumor and formation of tertiary lymphoid structures. We also observed vaccine-specific T cell receptor clonotypes in the tumor post-DC therapy, which were absent prior to treatment. These results are promising since they are highly suggestive for DC therapy-induced T cell infiltration in the tumor, which may potentially contribute to better survival in patients with pleural mesothelioma.

Abstract

Introduction

In patients with early-stage pleural mesothelioma (PM), surgical treatment via an extended pleurectomy decortication (eP/D) can be considered, but recurrence rates remain high. We initiated a trial to evaluate the feasibility of perioperative dendritic cell (DC) therapy (MesoPher) after standard-of-care chemotherapy. Secondly, efficacy, safety, and intratumoral immune response are assessed. This report presents the study protocol and the results of the first patient.

Methods

The ENSURE trial is an open-label, single-arm, phase II trial in patients with resectable (stage I-IIIA) epithelioid PM. Before eP/D, patients receive 2-4 cycles of standard-of-care chemotherapy followed by two biweekly MesoPher vaccinations. Four weeks after surgery, three adjuvant biweekly vaccinations are administered. Tumor material is collected before neo-adjuvant MesoPher and afterwards during eP/D. Before eP/D, a delayed-type hypersensitivity (DTH) skin test from a MesoPher injection site is obtained.

Results

The first patient was a 52-year-old female with T2N1M0 epithelioid PM. She completed treatment according to protocol and has an ongoing recurrence-free survival of 15 months. No severe postoperative or MesoPher-related complications were observed. Immunohistochemistry of the tumor showed diffuse CD8+ T cell infiltration and increased formation of tertiary lymphoid structures (TLS) post-MesoPher. In addition, there was an overlap in T cell receptor (TCR) clonotypes in the skin biopsy and tumor post-MesoPher, which were absent prior to treatment, suggestive of MesoPher-induced T cell infiltration in the tumor.

Conclusions

In this first patient, perioperative MesoPher vaccination with eP/D was feasible. Immunological analysis suggested that MesoPher induced a tumor-directed T cell response and induced TLS.

Introduction

Pleural mesothelioma (PM) is an aggressive cancer with a median overall survival (mOS) of 18 months when treated with combination checkpoint inhibition (anti-PD-1 & anti-CTLA-4) in first line.[1] For patients with early-stage PM, extended pleurectomy/decortication eP/D can be considered if complete surgical cytoreduction is deemed feasible.[2] Current guidelines recommend surgery only in the context of multimodal treatment (i.e. with systemic chemotherapy and/or radiotherapy) or a clinical trial. With the results of the MARS2 study the indication for eP/D may even be debated.[3] However, in selected patients, eP/D can result in a mOS up to 46 months, but recurrence rates are high.[4,5] New combination strategies should aim to improve recurrence-free survival (RFS) after eP/D.

Dendritic cell (DC) therapy is a form of immunotherapy that is able to induce a tumor-specific immune response.[6] Treatment with monocyte-derived DCs (moDCs) loaded with autologous tumor or allogeneic lysate (MesoPher) has shown promising results in different early phase trials in patients with PM and pancreatic cancer.[7,8] No severe DC-related toxicity was observed and patients showed radiographic responses and promising survival outcomes. In patients with a clinical response, DC therapy resulted in robust systemic immune cell activation and showed the potential to initiate tumor-lysate-specific immune responses.[8,9] DC therapy has also been shown to be feasible following surgery.[10]

In a recently completed phase III trial, the DENIM trial, treatment with MesoPher as maintenance therapy after first-line chemotherapy (without surgery) did not improve mOS when compared to patients treated with best supportive care (BSC).[11] The discrepancy between the results of this trial and the earlier phase I trials might be explained by the time of MesoPher administration. In the DENIM trial, MesoPher was administered at a later time point when the tumor is assumed to be already progressing, thus resulting in a higher tumor load and increased tumor-induced immunosuppression. This hypothesis is supported by a preclinical mesothelioma model, in which DC therapy showed better efficacy in mice treated with low tumor burden.[12] In line with this, a phase II trial in peritoneal mesothelioma studying MesoPher administration after cytoreductive surgery, demonstrated that patients with complete macroscopic resection showed longer progression-free survival (PFS) and had a more pronounced immunological response.[13] These studies indicate that DC therapy is likely to be more effective in case of low tumor burden, presenting a rationale for a combined (neo-) adjuvant approach with surgery in patients with PM.

The aim of the ENSURE trial is to evaluate the feasibility of DC therapy with MesoPher as a perioperative treatment in patients with resectable epithelioid PM after standard-of-care systemic chemotherapy. In this report, we present the study protocol and the

clinical results of the first patient together with the intratumoral immunological effect of DC therapy.

Methods

Study design

The ENSURE trial is an open-label, single-arm, phase I trial, conducted in the Erasmus MC, University Medical Center in the Netherlands (NCT05304208). This study was approved by the Medical Ethics Committee of the Erasmus Medical Center (MEC-2021-0669). A timeline of study procedures is provided in **figure 1**. The production of DC therapy (MesoPher) has been described for a previous phase I trial and a detailed description is provided in the **supplementary data**.[6] Briefly, before standard-of-care chemotherapy (pemetrexed and cisplatin/carboplatin), leukapheresis will be performed after which monocytes are isolated and used for further differentiation into moDCs. To formulate the drug product MesoPher, the moDCs will be loaded with an allogeneic PM tumor cell line lysate (Pheralys). Four weeks after completing chemotherapy, the first two bi-weekly MesoPher vaccinations will be administered. Four weeks after the first MesoPher administration, patients will undergo eP/D surgery, followed by three bi-weekly MesoPher administrations, starting four weeks after surgery. If available, a fourth and fifth adjuvant vaccination at three and six months after the last MesoPher administration can be considered by the treating physician. Tumor material will be collected before starting neo-adjuvant DC therapy and at time of surgery. Pretreatment tumor material will be obtained via a computerized tomography (CT)-guided needle or Video-Assisted Thoracoscopic Surgery (VATS) surgical biopsy. Moreover, a delayed-type hypersensitivity (DTH) skin test will be performed after the neo-adjuvant MesoPher vaccinations and prior to surgery. During a DTH skin test, a small amount of MesoPher in combination with Keyhole Limpet Hemocyanin (KLH) will be injected subcutaneously. In case of a local inflammatory response, a 3-5 mm punch biopsy of the induration will be collected.

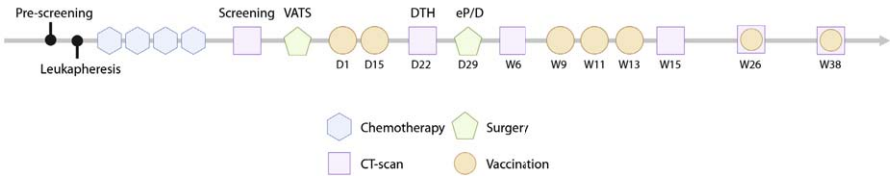

Figure 1. Timeline of study procedures. Patients with pleural mesothelioma will undergo perioperative dendritic cell (DC) therapy and eP/D after standard-of-care chemotherapy. A CT-scan (pink square) will be made at baseline, before and after surgery, after the fifth MesoPher administration, and every 12 weeks thereafter until follow-up is completed. Tumor material will be collected via a VATS biopsy and during eP/D.

Eligibility criteria

The study population consists of adult patients with ECOG performance status 0 or 1, diagnosed with resectable (cT1-3, N0-1, M0; stage I to IIIA) and histologically proven epithelioid PM, who are eligible for two to four cycles of platinum-based chemotherapy. Patients who progress after chemotherapy will not be discontinued from the trial if they are still eligible for eP/D. A detailed description of the eligibility criteria is provided in the **supplementary data**.

Primary & secondary objectives

The primary endpoint of this study is to determine the feasibility of (neo-)adjuvant MesoPher in combination with eP/D in patients with resectable epithelioid PM after standard-of-care chemotherapy. Feasibility is defined by the number of participants who complete perioperative MesoPher treatment (i.e., administration of at least five MesoPher injections or less in case of production shortage) and surgery within the predefined timeline. Completion of the study protocol can be complicated by several factors. Based on previous trials, we expect that roughly half of the patients will drop out of the study due to ineligibility for surgery after neo-adjuvant treatment or postoperative complications.[4,14,15] Other, less likely, factors are production failure of autologous treatment (MesoPher) or the absence of tumor tissue after chemotherapy due to a complete response. In total, we expect approximately 43% of patients to complete the study protocol within the predefined timeline (**supplementary data**).

A secondary endpoint of this study is to assess the safety of this treatment strategy, evaluated through the assessment of the adverse events, including injection site and infusion-related reactions, as well as surgical outcomes (**supplementary data**). The efficacy of this treatment strategy will be measured by RFS and OS. Radiological tumor evaluation will be performed six weeks after start of adjuvant DC therapy. Follow-up CT scans will be performed every 12 weeks thereafter. Tumor size will be assessed according to modified Response Evaluation Criteria in Solid Tumors (RECIST).[16] RFS is defined as time from surgery until radiological progression or death, or last date of follow-up in censored cases. OS is defined as time from start of surgery until death, or last date of follow-up in censored cases. Efficacy will be compared with a historical cohort of patients treated with eP/D in combination with chemotherapy at the Erasmus MC.

Exploratory objectives

Immunohistochemical analysis will be done on tumor tissue collected prior to and after MesoPher treatment to assess T cell infiltration in the tumor. In addition, T cell receptor (TCR) sequencing will be performed on both tumor tissues as well as tissue derived from skin biopsies after the DTH skin test to assess TCR clonotypes after MesoPher injection.

Immunohistochemistry

CD8+ T cell infiltration was determined by automated IHC using the Ventana Benchmark ULTRA (Ventana Medical Systems Inc.). Sequential 4 µm thick (FFPE) sections were stained for CD8 (clone C8/144B, Agilent) using optiview (OV) (#760-700, Ventana). In brief, following deparaffinization and heat-induced antigen retrieval with CC1 (#950-500, Ventana) for 32 minutes the tissue samples were incubated with CD8 for 32 minutes at 37˚C. Incubation was followed by optiview detection and hematoxylin II counter stain for 8 minutes, followed by a blue coloring reagent for 8 minutes according to the manufacturer's instructions (Ventana).

TCR sequencing

For T cell receptor beta chain (TRB) sequencing 500 ng of DNA was amplified through a multiplex polymerase chain reaction (PCR) of TRBV-TRBD-TRBJ gene rearrangements following the BIOMED-2 protocol.[17] Purification of PCR products and library preparation was performed as described previously.[18] Paired-end sequencing was performed using the MiSeq Reagent Kit v3 (2 × 300 bp) on the MiSeq Benchtop Sequencer (Illumina, San Diego, CA, USA). To increase library diversity, PhiX was spiked in at a 20% concentration. Raw FASTQ files were uploaded to the ARResT/Interrogate immunoprofiler for clonotype annotation and data exploration.[19] After cleanup of primer dimers, a mean of 47,729 high-quality sequences were retrieved per sample. Clonotypes were defined as unique pairings of TRBV genes, TRBJ genes and CDR3 amino acid sequences within a given sample. Additional analyses were performed in R (Version 4.2, R Foundation for Statistical Computing, Vienna, Austria). For determination of overlap of clonotypes among samples, clonotypes below 3 reads were removed in view of potential sequencing artefacts.

Results

Clinical outcomes

The first patient included in the ENSURE trial was a 52-year-old female patient diagnosed with a T2N1M0 epithelial PM. She was treated according to protocol. The patient received four cycles of neo-adjuvant chemotherapy (radiological response stable disease) and two neo-adjuvant MesoPher administrations. According to protocol the patient underwent eP/D, with a macroscopic complete resection except for one part. As the phrenic nerve was not visible due to abundant mediastinal fat, In the area where the phrenic nerve was expected, a tiny spot of mesothelioma tissue was present. This was untouched to save the phrenic nerve function. The patient was admitted to the hospital for a total of seven days. No severe postoperative complications occurred. After surgery, the patient received three bi-weekly MesoPher vaccinations followed by an additional fourth and fifth adjuvant administration at three and six months. The only DC therapy-related adverse events (AEs) consisted of mild infusion-related reactions (i.e., fever, cold chills, malaise) and injection site reactions (i.e., erythema), all limited to CTCAE grade 1 toxicity. The patient has an ongoing RFS of 15 months. **Figure 2** shows a CT scan prior to surgery (after neo-adjuvant chemotherapy and two DC vaccinations) and after the fifth adjuvant MesoPher vaccination (10 months after surgery).

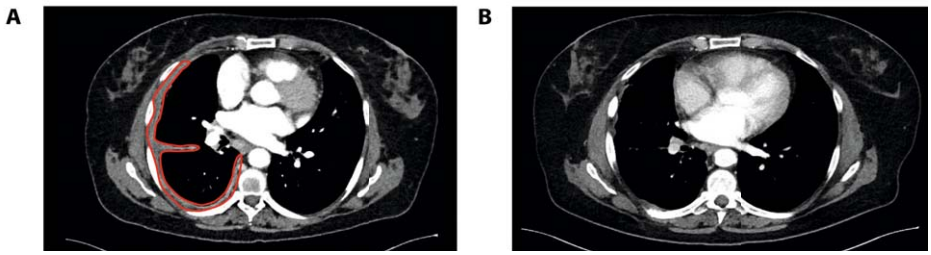

Figure 2. CT-scans of the first patient. Image of a CT scan performed as a response evaluation after neo-adjuvant chemotherapy and two DC vaccinations prior to surgery (A). The tumor location is indicated by the red outline. Image of a CT scan performed as a response evaluation after the fifth adjuvant MesoPher vaccination (10 months after surgery) (B).

Diffuse CD8+ T cell infiltration upon DC vaccination

CD8+ tumor-infiltrating lymphocytes (TIL) and tertiary lymphoid structures (TLS) appeared to be more abundant post DC therapy (**figure 3**). More specifically, whereas CD8+ TILs were restricted to TLS prior to treatment, these cells occurred more diffuse throughout the tumor upon treatment, suggesting DC therapy may improve infiltration of CD8+ T cells into the tumor microenvironment.

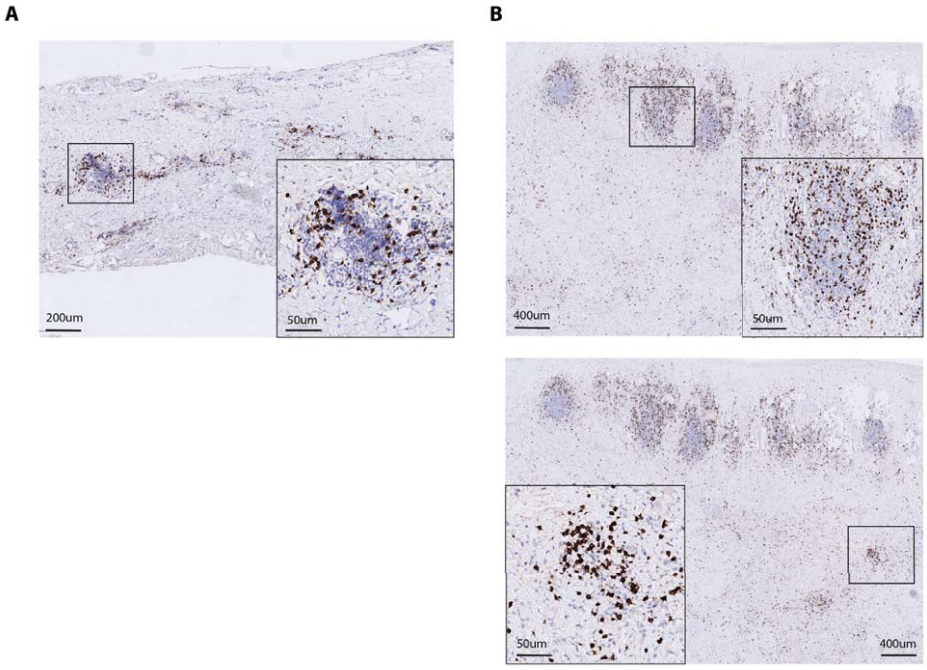

Figure 3. CD8+ T cell infiltration in pre- and post-vaccination tumor biopsies. Paired tumor material prior to DC therapy (A), obtained by a VATS biopsy, and after DC therapy (B), obtained by eP/D. DAB staining for CD8+ (brown) was performed. CD8+ T cells are present in the tumor prior to DC therapy as there are some tertiary lymphoid structures (TLS)(A). CD8+ tumor-infiltrating lymphocytes (TIL) and TLS seem more abundant post-DC vaccination, as well as diffuse CD8+ T cell infiltration (B). Enlarged images of the red square areas show detailed CD8+ infiltration (brown) in the tumor and in TLS.

TCR analysis shows overlapping TCR clonotypes

TCR sequencing identified the T cell receptor beta (TRB) gene repertoire from the skin biopsy which was compared to the TRB gene repertoire in the tumor material both prior to and after DC therapy. TCR sequencing showed four overlapping TCR clonotypes between the skin biopsy and the tumor post-DC therapy that were absent prior to DC therapy. This could suggest that there is MesoPher-induced T-cell infiltration into the tumor (**figure 4**). Clonotypes overlapping between skin and tumor after treatment were present at a low frequency, at <0.1% of total reads in the tumor and <1% of total reads in the skin. The top three expanded clonotypes in the tumor prior to treatment remained expanded after treatment, suggesting the existing anti-tumor response was maintained after treatment. Shannon diversity of the TRB gene repertoire was largely stable after treatment (data not shown), suggesting that treatment potentially did alter the composition of the anti-tumor response, but did not result in significant skewing towards a particular clonotype.

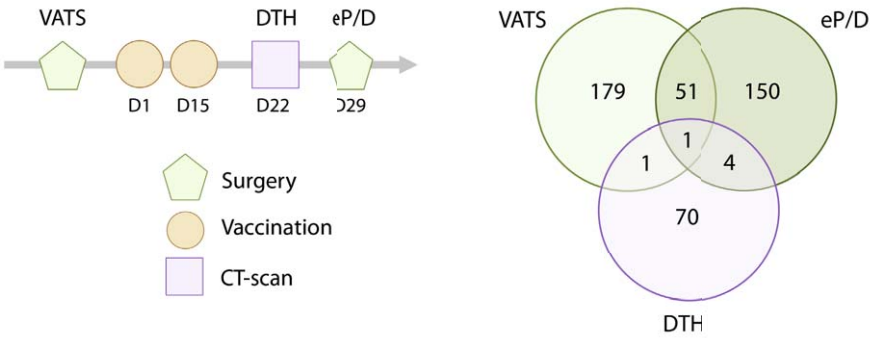

Figure 4. TCR sequencing reveals overlapping TCR clonotypes. The number of different TCR clonotypes present in the tumor prior to DC therapy (VATS, light green), in the DTH skin biopsy (purple), and in the tumor post-DC therapy (eP/D, dark green).

Discussion

The first patient that was included in the ENSURE trial has successfully completed treatment according to protocol without the occurrence of DC-related severe toxicity and has an ongoing RFS for over 15 months. Increased numbers of CD8+ TIL and TLS after DC therapy, in combination with overlapping TCR clonotypes between the skin biopsy and post-DC therapy tumor material that were absent prior to DC therapy, hint towards a MesoPher-induced anti-tumor immune response. These results are consistent with a recent study of our group that showed feasibility for MesoPher combined with surgery in patients with peritoneal mesothelioma.[13]

Since autologous DCs are loaded with allogeneic tumor lysate consisting of a wide variety of proteins, derived from five tumor cell lines, it is notoriously difficult to evaluate the immunological effect of MesoPher. In studies where DCs are loaded with one specific antigen, activated T cells can be detected through tetramer staining and flow cytometry.[20] In earlier studies, MesoPher was shown to enhance frequencies of peripheral CD4+ T cells expressing the HLA-DR, PD-1, or ICOS activation markers.[8,9] In our most recent study of peritoneal mesothelioma, we showed that MesoPher after surgery-induced CD4+ T helper cell proliferation as well as expression of co-stimulatory molecules ICOS, HLA-DR, and CD28, which was not seen for immunosuppressive CD4+ regulatory T cells.[13] In addition, an increase in the proportion of CD8+ terminally differentiated effector memory T (Temra) cells positively correlated with progression-free survival (PFS). In line with these studies, the activation of peripheral CD4+ T helper cells following DC therapy was observed in patients with pancreatic cancer.[8] In addition, MesoPher-specific CD4+ and CD8+ T cell responses have been detected *in vitro*. In the DENIM trial, we showed that MesoPher induced proliferation and activation of CD4+ memory T cells.[11] In addition, the extent of the increase in proliferation correlated with

OS, demonstrating that the magnitude of induction of memory CD4+ T cell proliferation can be indicative of an effective anti-tumor response.

A correlation between immune activation following DC therapy and PFS has been observed in earlier studies. We have, however, never investigated post DC-treatment tumor biopsies. Although not quantified and considering the heterogeneity of PM together with sampling bias, we detected increased (diffuse) CD8+ T cell infiltration in the tumor and the formation of TLS after MesoPher treatment in this first patient. The overlap of TCR clones in the DTH skin test biopsy and in the tumor post-DC therapy suggests that MesoPher-specific T cells are infiltrating the tumor. This is a promising result due to the cytotoxic effector function of CD8+ T cells, which is heavily reliant on the help of CD4+ T cells. These results support the hypothesis that MesoPher treatment induces not only systemic T cell activation and proliferation, but also migration into the tumor. Although these preliminary clinical results are promising, we have to wait for full inclusion to conclude on safety and feasibility, and to further explore the intratumoral immune response.

Conclusions

The first patient was successfully treated with DC therapy as a (neo-)adjuvant treatment to eP/D after standard-of-care systemic chemotherapy. The ongoing RFS together with the intratumoral immunological response after DC therapy in this patient point towards an immune-activating effect of DC therapy.

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Data supplement

Production of MesoPher

MesoPher is produced at the department of Pulmonary Medicine at the Erasmus MC Cancer Institute according to the protocol as published by Aerts et al.(6) Dendritic cells (DC) are derived from peripheral blood mononuclear cells, by differentiating monocytes towards immature DCs using specific culture conditions. Immature DCs are exposed to tumor specific antigens in a co-culture with allogeneic mesothelioma cell lysate (PheraLys). PheraLys is derived from five well-specified mesothelioma cell lines. After exposure to PheraLys, the immature DCs are differentiated towards mature DCs using the Jonuleit cytokine cocktail.

Eligibility criteria

Adult patients with resectable (cT1-3, N0-1, M0; stage I to IIIA) and histologically proven epithelioid PM were screened to participate in the ENSURE trial. Patients need to have an ECOG performance status of 0 or 1 and need to be eligible for two to four cycles of platinum-based chemotherapy. Patients with any previous malignancy are excluded, except for adequately treated basal cell or squamous cell skin cancer, superficial or in situ cancer of the bladder or other cancer for which the subject has been disease-free for at least three years. Patients with a history of autoimmune disease, except for diabetes mellitus type 1, are also excluded.

Sample size calculation

The primary objective of the study is to determine the feasibility of applying MesoPher as neoadjuvant and adjuvant maintenance therapy in combination with eP/D in PM patients.

A specific statistical calculation assessing the number of patients to prove feasibility is challenging. We will start enrolling 16 patients with PM. Multiple factors can complicate completion of the study protocol, and thus feasibility, such as progression during chemotherapy, post-operative complication, production failure of autologous treatment (MesoPher), and having no tumor tissue available after chemotherapy due to a complete response. Based on previous trials, we expect that roughly half of the patients will drop out of the study due to ineligibility for surgery after neoadjuvant treatment or postoperative complications.(4, 14, 15) We expect that the occurrence of batch failures and the absence of tumor tissue available after chemotherapy will be rare. Assuming one batch failure and one patient with the absence of tumor tissue, we estimate that approximately 43% of all patients will be able to complete the DC therapy schedule (i.e., at least five MesoPher injections or less in case of production shortage) within a 95% confidence interval of +/-24%. To declare that treatment is feasible, at least seven out of 16 enrolled patients should complete the DC therapy schedule.

Safety evaluation

Safety and tolerability will be evaluated through assessment of the adverse events (AEs), including local and systemic injection site reactions as well as surgical outcomes (blood loss, duration of surgery, admission time). Moreover, laboratory assessments and vital signs will be evaluated during treatment as well as performance status. All serious adverse events (SAEs) and suspected unexpected serious adverse reactions (SUSARs) related to the DC vaccination were monitored and reported.

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Ki67 (MIB-1) as a prognostic marker for clinical decision making prior to extended pleurectomy decortication in malignant pleural mesothelioma

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Abstract

Background

The role of surgery for early stage Malignant Pleural Mesothelioma (MPM) remains controversial. Current expert opinion is only to treat patients surgically as part of multimodality therapy. It is still challenging to identify patients who will not benefit from surgery. We specifically assessed tumor-related parameters in combination with clinical parameters to identify prognostic markers for survival.

Methods

Clinical data were collected and analyzed of 27 consecutive MPM patients treated with extended pleurectomy/decortication (eP/D) within a multimodality approach. Several tumor (immuno-)histopathological characteristics were determined on resected tumor material, among which MTAP and Ki67 (MIB-1) Ki67. Univariable and multivariable analysis served to correlate clinical and tumor-related parameters to overall survival (OS) and progression free survival (PFS).

Results

The median PFS (mPFS) was 15.3; the median OS (mOS) was 26.5 months. Patients with a Ki67 score >10% had a significantly shorter PFS (mPFS 8.81 vs. 25.35 months, p=0.001), and OS (mOS 19.7 vs 44.5 months, p=0.002) than patients with a Ki67 score ≤10. ROC curve analysis for Ki67 showed an area under the curve of 0.756 with a sensitivity of 90% and specificity of 71% for a cut-off of 10% for Ki67. Patients with loss of MTAP had a significantly shorter mPFS (9 vs. 21.1 months, p=0.014) and mOS (19.7 vs. 42.6 months, p=0.047) than patients without MTAP loss.

Conclusion

In our study, Ki67 was prognostic for OS and PFS in MPM patients treated with eP/D in a multimodality approach. Determination of Ki67 prior to surgery combined with specific clinical parameters could assist in clinical decision making by identifying patients with high Ki67, who are unlikely to benefit from surgery.

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive cancer of the pleural linings of the lung with a median overall survival (OS) of approximately 12 months from diagnosis.[1] Current first line treatment consisting of platinum/antifolate combination chemotherapy has shown a three month increase in OS compared to cisplatin alone.[2] Addition of bevacizumab to first-line chemotherapy increases the median OS (mOS) with 2.8 months but is currently only accepted as standard of care in France.[3] Very recently, the Checkmate 743 phase III trial, comparing first-line nivolumab combined with ipilimumab to combination chemotherapy, yielded an improvement in OS of 4 months for patients treated with combination immunotherapy leading to a mOS of 18.1 months.[4]

For early stage MPM the role of surgery remains controversial. Current expert opinion is only to treat patients surgically in a clinical trial setting as part of multimodality treatment.[5] Two different surgical techniques for complete macroscopic resection of the visceral and parietal pleura can be discerned: extrapleural pneumonectomy (EPP) and extended pleurectomy decortication (eP/D). Historically, EPP was advocated as the standard operation of choice in patients eligible for surgery, but at the cost of considerable perioperative mortality and morbidity.[6] The MARS trial has shown that EPP vs. no EPP in patients treated with chemoradiation is not beneficial.[7] Since a large multicenter study failed to demonstrate a survival difference between EPP and eP/D, most mesothelioma centers have shifted to eP/D.[8-10] For eP/D, the mOS ranges from 10.4 to 32 months.[6] Surgically treated patients seem to have a longer OS than patients who are not operated. Nevertheless, this comparison is likely to be confounded by the selection of patients for surgery who have a high performance status, low tumor stage and limited comorbidities.[11,12] Surgery might severely affect the quality of life in patients with a relatively short expected OS. If surgery is not effective, the impact on quality of life can be detrimental.[13] Therefore, there is an unmet need to identify who will or will not benefit from surgery within a multimodality approach.

Several factors that determine prognosis in MPM are: histological subtype, sex, age, TNM stage, performance score (PS), weight loss and several peripheral blood values (e.g. Albumin, CRP, lymphocytes). Prognostic score models combine some of these parameters, such as the European Organization for Research and Treatment of Cancer (EORTC) score,[14-16] the modified Glasgow Prognostic Score (mGPS) and the Neutrophil to Lymphocyte Ratio (NLR).[17] These models have proven to be prognostic for MPM and the EORTC score has been validated in surgically treated MPM patients. Unfortunately, it remains difficult to identify patients who will benefit from surgery based on these parameters.

Several tumor-related parameters have been found to determine prognosis in MPM in general, such as: nuclear atypia, mitotic rate, necrosis, percentage of solid growth, BAP1

loss and Ki67 (MIB-1Ki67).[18-26] Ki67 antigen is exclusively expressed by cells in the active phases (G1,G2, S and mitosis) of the cell cycle and is used as a proliferation marker in analogy to the mitosis score. Ki67 is used as a predictive marker in several types of cancer including peritoneal mesothelioma.[24] In MPM, however, Ki67 or other tumor markers are not used for clinical decision making although many of these parameters have a prognostic value in MPM.

In this article we report the clinical outcomes of MPM patients treated in a multimodality treatment setting including eP/D. In addition, we report the results of our evaluation of the prognostic value of both tumor-related and clinical parameters to identify patients that might benefit from surgical treatment.

Methods

We retrospectively collected data from all patients surgically treated with eP/D at the Erasmus University Medical Center (Rotterdam, NL) from May 2010 until May 2019. Our research proposal was submitted to the medical research and ethical committee and no formal approval was required according to Dutch guidelines due to the retrospective nature of the study (MEC-2020-0546). These patients had all been treated with a multimodality approach consisting of eP/D with (neo-)adjuvant chemotherapy and/or adjuvant radiotherapy. Chemotherapy consisted of platinum-based chemotherapy and pemetrexed. All patients had a histologically proven diagnosis of MPM and stage cT1-3, N0-2, M0 according to TNM classification available at time of diagnosis. If available, FDG-PET-CT scans were used to evaluate the presence of M1, supraclavicular and coeliac node involvement. All eP/D procedures had been performed according to the surgical technique reported by Maat et al.[9] Follow-up was done at Erasmus MC or in referring peripheral hospitals. For patients with a follow-up at Erasmus MC, radiological tumor assessment was done retrospectively according to modified Response Evaluation Criteria In Solid Tumors (mRECIST) version 1.1. CT-scans had been made every 3-4 months. For patients with clinical follow-up in peripheral centers, CT-scans were requested from the peripheral centers and reassessed at the Erasmus MC as stated above. If CT-scans for patients with follow-up in referring centers were not available, the response that was evaluated by the treating pulmonologist according to mRECIST and communicated with Erasmus MC. Overall survival was defined as time from surgery to death of any cause and censored at the last contact date for patients who were alive at time of data cutoff. Progression free survival (PFS) was measured from time of surgery until radiological progression or recurrence of disease or death of any cause.

Data Collection

Patient characteristics were collected from the electronic patient files. The following clinical variables were collected: age, gender, performance score, weight loss (defined as

>5% in last 3 months), number of treatment lines received (only first-line vs. more than one line of treatment), neoadjuvant chemotherapy, adjuvant chemotherapy, adjuvant radiotherapy, in-hospital mortality and 90-day post-operative mortality. The following peripheral blood parameters were collected at baseline (0-6 weeks before surgery): CRP, leucocytes, platelets, neutrophils, lymphocytes, monocytes and albumin.

In addition, the following tumor (immuno-)histopathological characteristics were determined on resected tumor material during eP/D: histological subtype, extent of solid pattern, grading of nuclear atypia, mitotic rate, mitosis score, necrosis, combined mitosis/necrosis score, BAP-1 expression, MTAP expression, percentage of Ki67 expression and nuclear grading.

Both tumor (immuno-)histopathological characteristics and patient characteristics were evaluated in the statistical analysis.

Immunohistochemistry

The tumor material was processed by the pathology laboratory at the Erasmus MC according to routine procedures. A 4-µm section of Formalin Fixed Paraffin Embedded (FFPE) tissue was mounted serially on adhesive glass slides. Deparaffinization was performed according to the Ventana BenchMark Ultra protocol. Antigen retrieval was performed by CC1 antigen retrieval solution (ref. 950-124, Ventana Medical Systems, Inc., Oro Valley, Arizona, United States). Specimens were incubated with the primary antibody, followed by detection with OptiView DAB (ref. 760-700, Ventana Medical Systems, Inc.), UltraView-DAB (ref. 760-500, Ventana Medical Systems, Inc.) or UltraView-AP (ref. 760-501, Ventana Medical Systems, Inc.) with amplification (Amplification Kit ref: 760-080 or OptiView Amplification Kit ref: 760-099, Ventana Medical Systems, Inc.). Next, the specimens were counterstained with hematoxylin II (ref: 790-2208, Ventana Medical Systems, Inc.) and coverslipped.

Each slide contained a positive control. All stains were performed on the Ventana BenchMark Ultra (Ventana Medical Systems, Inc.). Primary antibodies, detection and amplification methods used are mentioned in Table 1S.

(Immuno-)histopathological Assessment

Morphological assessment was done on hematoxylin-eosin stains, and included scoring of growth pattern (epithelioid, mixed (mesenchymal and epithelioid) and mesenchymal (which can be divided into sarcomatoid and transitional)), grading of nuclear atypia on a three-tier scale (1-3), the presence of necrosis (<50% (0) vs. >50% (1)), mitotic rate (mitoses / 10 high-power fields (HPF)), mitosis score (≤4/10 HPF (0) vs. >4/10 HPF (1)), and combined mitosis+necrosis score (sum of necrosis and mitosis score; 0-2). Scoring was done according to previously published methods.[23,26] Nuclear grading was scored in three categories as described by Kadota et al.[23]

Scoring of immunohistochemistry for Ki67, BAP-1 and MTAP was performed as follows: Ki67 was scored as percentage positive tumor cells; BAP1 and MTAP were scored as either present or absent in tumor cells (using surrounding stroma as a positive internal control).

Homogeneity of Ki67 expression within the tumor of MPM patients

Overall analysis of IHC-derived, tumor-related parameters was initially done on whole slides of selected blocks of tumor material resected during eP/D. While expression of Ki67 in breast cancer is known heterogeneous, in MPM the heterogeneity of Ki67 expression has never been analyzed.[27] Implementation of Ki67 in clinical decision making requires homogeneity to prevent unreliable outcomes of Ki67 expression determined on small tissue samples, such as CT-guided needle biopsies. To evaluate the homogeneity of expression of Ki67 within the tumor of patients with MPM we analyzed randomly selected circular areas of 2mm matching the size of needle biopsies and tissue microarray (TMA) samples. We excluded samples of patients for whom fewer than three 2mm regions were available for analysis. If possible, 10 randomly picked regions within the tumor were assessed for levels Ki67. These were determined by digitally selecting, coding and randomly presenting these areas to the scoring pathologist to avoid scoring bias. Results of scoring these regions were compared (1) with other regions selected from the same specimen and (2) with the originally determined overall Ki67 expression. Finally, we checked if patients would fall into the same category (high or low Ki67 expression) based on 2mm region Ki67 scores.

To test the reproducibility of Ki67 expression through time, sequentially collected tumor material was also stained for Ki67 and MTAP. The availability of biopsies taken prior to surgery within the Erasmus MC from surgically treated MPM patients was checked. Analysis on sequential biopsies was not done if any form of therapy (chemotherapy, radiotherapy, immunotherapy) was given between the biopsy and the operation, as (chemo)therapy can influence the expression levels of Ki67.[18]

Statistical Analysis

Patient and tumor characteristics are presented as count and percentage for categorical variables, and as median and range for continuous variables. Median follow up time was calculated by reversed Kaplan-Meier analysis for OS. Median OS and PFS were estimated with a Kaplan-Meier curve. Differences in probability of survival between the strata were evaluated by log-rank (Mantel-Cox) test. The hazard ratios (HR) of progression and death and their associated 95% confidence intervals (95% CI) for clinically important factors (e.g. adjuvant radiotherapy, adjuvant chemotherapy, gender) were calculated using univariable Cox proportional hazard model. For all continuous variables, except post-operative admission time, a cutoff was chosen to identify comparable groups. For Ki67 and age, the cutoff was set at the median; for mitosis the cutoff was based on literature describing a cutoff of 0-4 vs >4.[23] For lymphocyte count, leucocyte count, monocyte

count, platelet count and neutrophil count the cutoff was set at the median. For albumin, the cutoff was set on 35g/L, for CRP the cutoff was set to 10 mg/L according to literature. The cutoff for the EORTC score was set to 1.27 according to a validation study in MPM performed by Fennel et al.[16] Patients with an NLR above 3 were considered to have a high NLR, in accordance with recent literature.[28]

Parameters that had a p-value < 0.1 based on the univariable Cox regression model for PFS as well as OS were then evaluated in a Cox multivariable proportional hazard regression model. Due to the low number of events, only two coefficients could be estimated in the same model. The Cox multivariable proportional hazard regression model was repeated until all parameters that met the requirements had been tested with each other. Ki67 and mitosis score are both parameters by which the proliferative capacity of tumor cells is measured. Therefore, the parameter representing proliferation with the highest HR and lowest p-value was applied in multivariable analysis.

A significance level of 0.05 with a two-sided alpha was chosen to determine statistical significance. Statistical analyses were performed using R 3.6.0 (R foundation for statistical computing).

Results

From May 2010 until May 2019, twenty-seven patients had been treated surgically with eP/D in a multimodality approach. Data of all patients were available for analysis. Histological subtyping showed that epithelial MPM was found in 74% of all MPM tumors. Neither inhospital mortality nor 90-day mortality was reported. All baseline characteristics are summarized in Table 1.

Table 1. Patient baseline characteristics

Characteristics		
Total =27	**n**	**%**
Median age	60	Range: 36-73
Gender, male	22	81
Histological subtype		
Epithelioid	20	74
Non-epithelioid	7	26
Mixed	6	22
Mesenchymal: Transitional	1	4

Table 1. Patient baseline characteristics (continued)

Performance score (ECOG)		
0	15	56
1	9	33
2	3	11
Weight loss		
yes	3	11
no	23	85
Data unavailable	1	4
Perioperative treatment		
Prior first line of chemotherapy	2	7
Neo-adjuvant chemotherapy	9	33
Adjuvant chemotherapy	16	59
Neo-adjuvant or adjuvant chemotherapy + radiotherapy	8	30
Adjuvant radiotherapy	9	33
Median admission time (days)	11	Range 5-37
Tumor markers		
BAP-1 loss	12	44
MTAP	16	59
Median Ki67	10	Range 0-70
Ki67 >10	13	48
Peripheral blood parameters (median)		
Albumin g/L	42	Range 32-48
CRP mg/L	17	Range 0,5-240
Hemoglobin mmol/L	8,4	Range 5,7-10,6

Data are presented as an absolute number with according percentages, unless stated otherwise. Abbreviations: ECOG: Eastern Cooperative Oncology Group, Weight loss: >5% weight loss in the last 3 months, Prior first line of chemotherapy: chemotherapy administered more than 3 months prior to surgery.

At median follow-up time of 60.7 months, 21 patients had progressed of whom 17 had died. The median OS was 26.5 months (95%CI: 22.0-NA) and median PFS was 15.3 (95%CI: 9.6-31.5) (Figure 1).

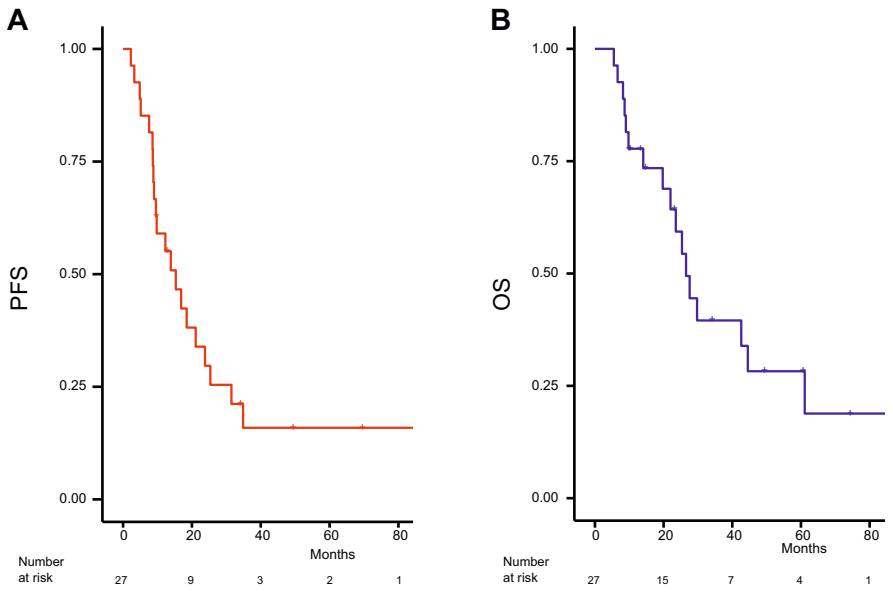

Figure 1. Kaplan Meier curves of survival in the entire cohort of MPM patients treated within a multimodality approach including eP/D. (A) Progression free survival for the entire cohort. (B) Overall survival for the entire cohort.

Association of clinically important factors with survival outcomes

Log-rank test and univariable Cox proportional hazard regression analysis of clinical parameters revealed that PFS and OS were significantly longer in female patients, for whom the median PFS and median OS was not reached during follow-up, whereas the mPFS for male patients was 11 months (log-rank $p = 0.01$, [HR 8.389, 95%CI 1.113-63.258, $p = 0.039$]) and mOS was 25.4 months (log-rank $p = 0.03$, [HR 7.105, 95%CI 0.930-55.86, $p = 0.062$]) (Figure 2A&B).

Other clinical parameters, such as weight loss before surgery, (neo-)adjuvant chemotherapy and PS or age (>60) did not significantly correlate to PFS or OS (Table 2). Hemoglobin, albumin and CRP did not correlate to PFS and OS, just as the number of leucocytes, lymphocytes, monocytes, platelets and neutrophils (data not shown). Prognostic score indexes such as the EORTC score, mGPS and NLR did not correlate to survival (Table 2).

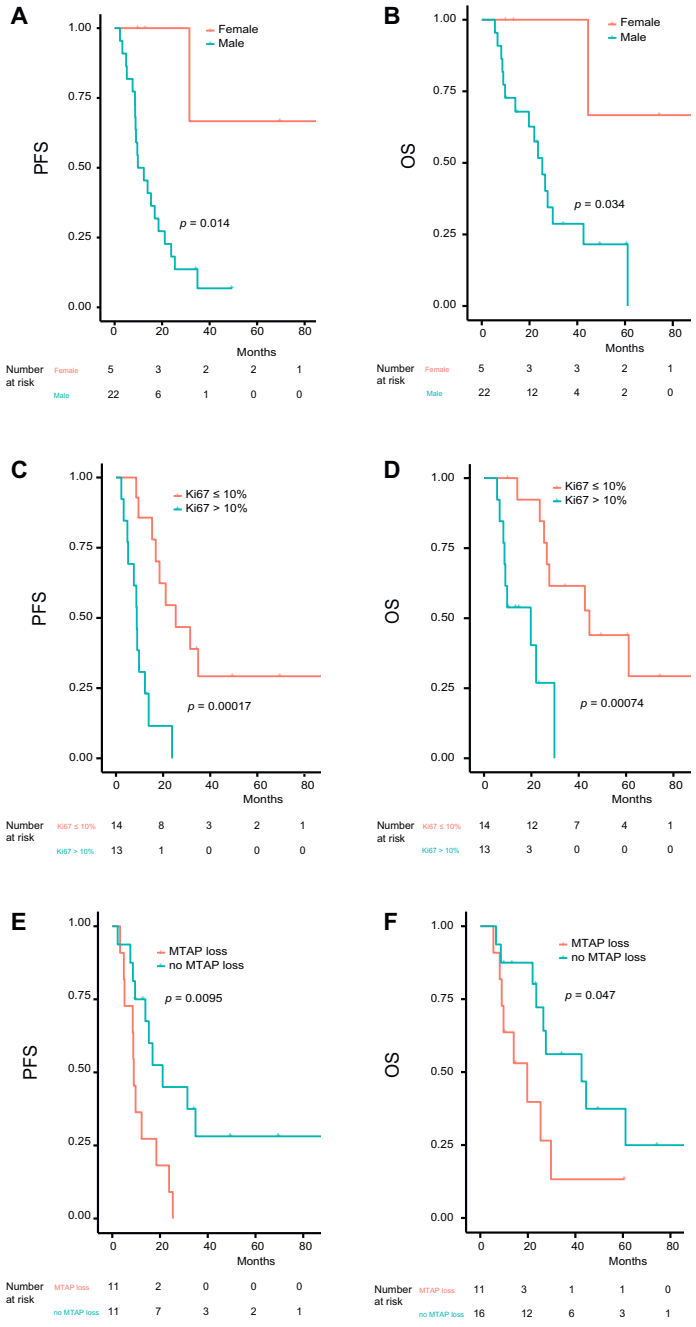

Figure 2. Kaplan-Meier curves of survival for subgroups based on KI67, MTAP and gender. (A) Progression free survival and (B) overall survival by gender. (C) Progression free survival and (D) overall survival in patients with a KI67 expression >10% versus those with a KI67 expression ≤10%. (E) Progression free survival and (F) overall survival in patients with MTAP loss versus patients without loss of MTAP expression.

Table 2. Univariable analysis of PFS and OS for clinical and tumor-related parameters

Parameter	PFS			OS		
	HR	95% CI	p-value	HR	95% CI	p-value
Age (> 60 vs <60)	0.904	0.382- 2.142	0.819	0.8799	0.3367-2.3	0.794
Gender (Male vs Female)	8.389	1.113-63.258	0.039	7.105	0.930-55.86	0.062
ECOG PS	0.6693	0.3313-1.352	0.263	0.6258	0.2777-1.411	0.258
Weight loss (Yes vs No)	1.544	0.4415-5.399	0.497	1.521	0.4252-5.441	0.519
Histology (epithelioid vs non-epithelioid)	0.338	0.132-0.868	0.024	0.7287	0.2308-2.3	0.589
Postoperative admission time (days)*	1.002	0.964-1.041	0.928	1.019	0.976-1.064	0.385
Neoadjuvant chemotherapy (Yes vs No)	1.260	0.498-3.190	0.625	1.295	0.461-3.642	0.624
Adjuvant chemotherapy**(Yes vs No)	0.509	0.213-1.220	0.130	0.467	0.178-1.230	0.123
Adjuvant radiotherapy (Yes vs No)	0.382	0.139-1.055	0.063	0.568	0.197-1.636	0.295
Solid pattern (Yes vs No)	2.061	0.855-4.967	0.107	1.977	0.685-5.702	0.207
Atypia 2vs1	2.070	0.724-5.919	0.175	2.793	0.862-9.056	0.087
Atypia 3vs1	6.800	1.434-32.257	0.016	4.954	0.768-31.941	0.092
Mitosis score	3.216	1.217-8.497	0.018	3.881	1.284-11.73	0.016
Necrosis (Yes vs No)	2.040	0.706-5.900	0.188	1.026	0.290-3.624	0.968
Mitosis necrosis score 1vs0	3.052	1.127-8.268	0.028	1.677	0.555-5.073	0.360
Mitosis necrosis score 2vs0	4.373	0.864-22.127	0.075	3.796	0.734-19.641	0.112
BAP1 (Yes vs No)	1.298	0.548-3.075	0.553	2.364	0.896-6.236	0.082
MTAP loss (No vs Yes)	0.313	0.124-0.788	0.014	0.373	0.136-1.021	0.055
Ki67 (>10 vs <10)	6.301	2.200-18.047	0.001	6.594	1.998-21.754	0.002
Nuclear grade 2vs1	1.362	0.536-3.455	0.516	1.400	0.504-3.890	0.518
Nuclear grade 3vs1	4.753	1.151-19.620	0.031	2.731	0.511-14.600	0.240
EORTC score*	0.961	0.3962-2.333	0.93	1.239	1.452-3.395	0.677
mGPS (0 vs 1 vs 2)	1.274	0.551-2.944	0.572	0.928	0.3715-2.319	0.873
NLR*	1.937	0.7681-4.884	0.161	1.512	0.5469-4.179	0.426

The univariable Cox proportional hazard model was used to calculate the HRs of tumor progression or death, and the univariable logistic regression was used to calculate the ORs of response.

*Continuous variable.

** n = 26, data of a patient who received adjuvant dendritic cell therapy removed for this analysis.

PFS, progression-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group Performance Status; Weight loss: >5% weight loss in the last 3 months; EORTC, European Organization for Research and Treatment of Cancer; mGPS, modified Glasgow prognostic score; NLR, neutrophil to lymphocyte

Association of tumor markers with survival outcomes

The median score for Ki67 was 10%, which was determined to be the cutoff value. A tumor with a score higher than 10% was considered a highly proliferating tumor. Log-rank tests and univariable cox proportional hazard analysis of tumor-associated parameters showed that patients with a high Ki67 score had a significantly shorter PFS (mPFS 8.81 vs. 25.35 months, log-rank p = 0.00017, [HR 6.301, 95% CI 2.200-18.047, *p* = 0.001]), and OS (mOS 19.7 vs 44.5 months log-rank *p* = 0.0007, [HR 6.594, 95% CI 1.998-21.754, *p* = 0.002]) than patients with a low Ki67 score (Figure 2 C&D). A high mitosis score also was significantly associated with shorter PFS and OS, but with a lower HR and higher *p* value than Ki67 (Table 2). In multivariable analysis only Ki67 retained its significant value (data not shown). As both markers are used to quantify proliferating cells, Ki67 was used in further analysis as the marker for proliferation.

MTAP loss was correlated with worse clinical outcome. The mPFS for patients with MTAP loss was 9 months vs. 21.1 months for patients with MTAP expression (log-rank *p* = 0.0095, [HR: 0.313, 95%CI 0.124-0.788; *p* = 0.014]). There was also a significant difference in mOS between patients with and without MTAP loss (mOS 19.7 vs 42.6 months log-rank *p* = 0.047, [HR 0.373, 95%CI 0.136-1.021; *p* = 0.055]) (Figure 2F). In figure 3, we report the Ki67 expression and MTAP expression of one patient with a long OS and PFS and one patient with a short OS and PFS. MTAP and Ki67 did not seem to correlate with each other, as the mean Ki67 score did not significantly differ between MTAP positive and negative tumors (data not shown).

Combining Ki67 and MTAP in a prognostic index by scoring 1 point for Ki67>10 and 1 point for a MTAP negative tumor did not improve the identification of patients who benefit from surgical treatment compared to Ki67 alone (Figure S1). Patients with an epithelioid histology had a longer PFS (mPFS 16.9 vs. 8.6 months log-rank *p*=0.02, [HR:0.338, 95%CI: 0.132-0.868; *p* = 0.024) but a non-significant difference in OS (mOS 29.7 vs 22.0 months log-rank p=0.6 [HR: 0.464, 95%CI 0.182-1.184; p = 0.108]) compared to patients with a non-epithelioid histology. Nuclear grading, nuclear atypia and the mitosis-necrosis score all showed strong relations with the survival outcome and were significantly correlated to PFS, but not to OS (Table 2).

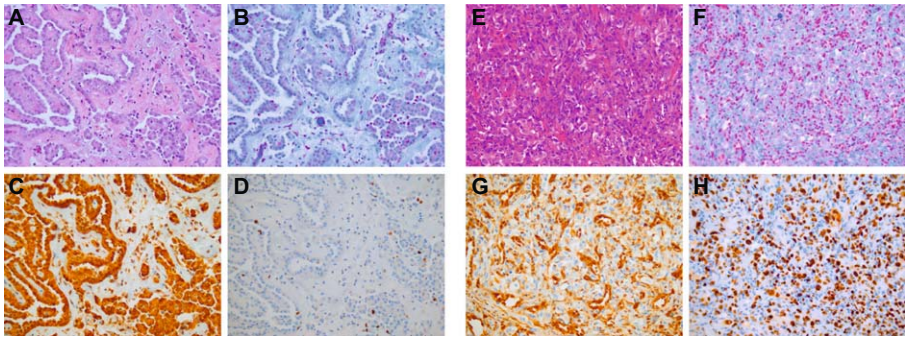

Figure 3. Illustrative stains of representative cases. A-D. Stains of a malignant pleural epithelioid mesothelioma with relatively long patient survival (PFS 69.6 months, OS 74.4, patient still has no progression of disease): A. H&E stain revealing tubulopapillary growth pattern with limited nuclear atypia (grade 1) and low mitotic rate (<1 mitoses / 10 HPF), lacking necrosis; B. Absent nuclear BAP1 staining in tumor cells; C. retained, strong cytoplasmic and nuclear MTAP staining in tumor cells; D. low proliferative activity (Ki-67, <1%). E-H. Stains of a malignant pleural mixed-type mesothelioma with relatively short patient survival (PFS 4.8 months, OS 10.3 months, patient has progressed, but is still alive with stable disease on nivolumab treatment): E. H&E stain showing predominantly solid growth pattern with evident nuclear atypia (grade 3) and high mitotic rate (26 mitoses / 10 HPF), but lacking necrosis; F. Absent nuclear BAP1 staining in tumor cells; G. absent cytoplasmic and nuclear MTAP staining in tumor cells with positive staining in stromal cells; H. high proliferative activity (ki67, 60%). H&E, hematoxylin and eosin; HPF, high-power field; OS, overall survival; PFS, progression-free survival.

Multivariable analysis and ROC curve

Gender, nuclear atypia, MTAP loss, and Ki67, as marker for proliferation, were the only markers with *p* values <0.1 for both PFS and OS. Because nuclear atypia is a categorical variable with three categories, two coefficients are needed to estimate this covariate and the lack of power prohibits adding this covariate to a multivariable model. Multivariable Cox proportional hazard regression analysis with the three remaining variables (Table 3) showed that only Ki67 retained its prognostic value, indicating that Ki67 with a cutoff of 10% remains an independent prognostic marker, conditional on gender and MTAP loss.

Table 3. Multivariable analysis of PFS and OS for gender and Ki67

Parameter	PFS			OS		
	HR	95% CI	p-value	HR	95% CI	p-value
Gender (M vs F)	6.394	0.829-49.33	0.075	5.373	0.649-44.48	0.119
Ki67 (>10 vs <10)	5.040	1.773-14.33	0.002	5.196	1.578-17.11	0.007

Parameter	PFS			OS		
	HR	95% CI	p-value	HR	95% CI	p-value
MTAP loss (no vs yes)	0.622	0.214-1.81	0.348	0.748	0.232-2.415	0.628
Ki67 (>10 vs <10)	1.578	1.469-15.96	0.009	5.581	1.436-21.68	0.013

Parameter	PFS			OS		
	HR	95% CI	p-value	HR	95% CI	p-value
Gender (M vs F)	6.246	0.786-49.62	0.083	5.726	0.689-47.585	0.106
MTAP loss (no vs yes)	0.457	0.178-1.17	0.103	0.528	0.19-1.465	0.220

Variables with p-values <0.1 for OS and PFS in univariable analysis were included in the multivariable models. The multivariable Cox proportional hazard model was used to calculate the HRs of progression or death and the univariable logistic regression was used to calculate the ORs of response. PFS, progression-free survival; OS, overall survival; HR, hazard ratio; CI, confidence interval; M: male; F; Female;

Therefore, we performed a receiver operating curve (ROC) curve to test the sensitivity and specificity of detecting death before mOS of 26.5 months, which revealed an area under the curve of 0.756 (Figure 4). With the Youden index, an optimal cutoff was determined to be 12.5% (sensitivity 90%, specificity 71%). The sensitivity and specificity with a Ki67 cutoff of 10% were the same, as none of the patients had a Ki67 expression between 10% and 12.5%. A sensitivity of 100% with a specificity of 47% was reached when the Ki67 cutoff is set to 20%, implying that patients with a Ki67 expression above 20% prior to surgery do not reach the mOS of 26.5 months.

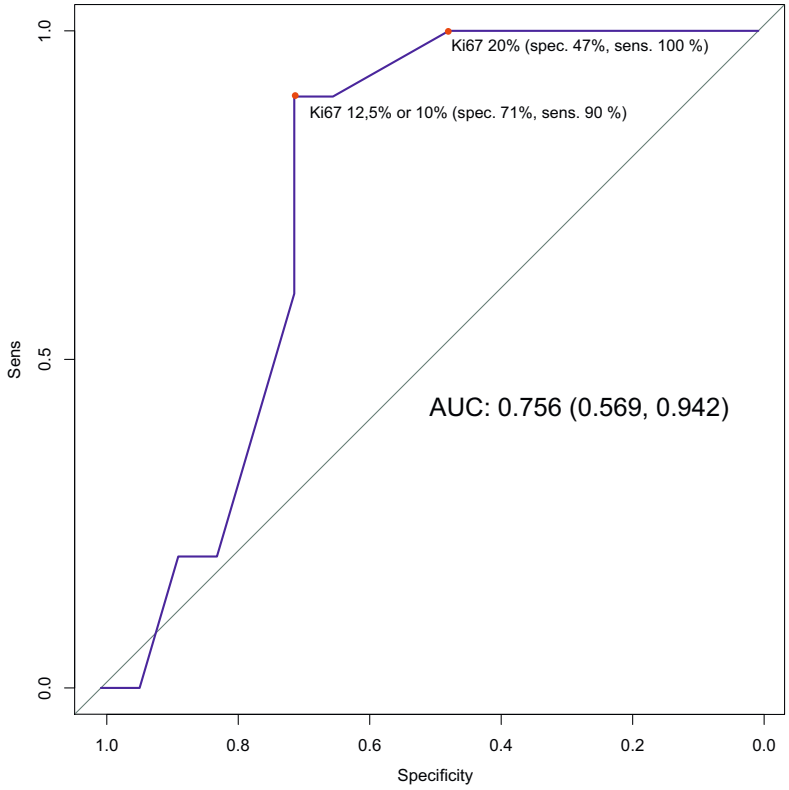

Figure 4. Receiver operating curve. A receiver operating characteristic curve for showing the sensitivity and specify of Ki67 for detecting death before median OS of 26.5 months.

Homogeneity of Ki67 expression in MPM tumors

In total, 24 biopsies were available for additional IHC analysis to check for the similarity between the originally determined Ki67 expression on the resected material and the Ki67 expression in 2mm-wide, randomly selected circular areas of the tumor (Table S2). In 22 out of 24 cases, 9 or 10 2mm spots were available for analysis, and in the 2 remaining cases, 5 and 8 2mm spots were analyzed, respectively. In 19 of 24 patients, determination of Ki67 on these 2mm spots resulted in a congruent categorization of high (>10%) or low (£10%) Ki67 expression compared with overall scoring of the specimen block (see Figure 5). In 5 patients, however, results of the pseudo-TMA analysis were not congruent with the originally determined Ki67 expression on the resection material. For patients 3, 18 and 24 a subselection of the additional Ki67 analyses resulted in a different risk profile for the relevant patient. In patient 18, 10% of the additional stainings resulted in a different risk profile. For patients 3 and 24 this was 30% and 70%, respectively. The originally determined Ki67 expression for patient 7 was 15%, but all additional focal analyses were below 10%. For patient 21, with an original Ki67 expression of 25%, all additional focal analyses showed a Ki67 expression

below 10%. Importantly, patient 21 and patient 7, who both had a high Ki67 expression after the original Ki67 analysis on the resected tumor material, had a respectable mPFS of 13.9 and 12.3 months. To conclude, 19 out of 24 patients would have been scored in the correct overall Ki67 expression category based on determination of Ki67 on randomly selected 2mm wide spots in the tumor. Two of 24 patients would have been scored in a different risk category based on these analysis, and 3 out of 24 patients would have potentially been scored in a different risk category. In total, 229 additional IHC subanalyses were done, and 199 had a Ki67 expression that was congruent with the overall Ki67 expression determined originally on the resected tumor material, leading to a true positive score of 87%.

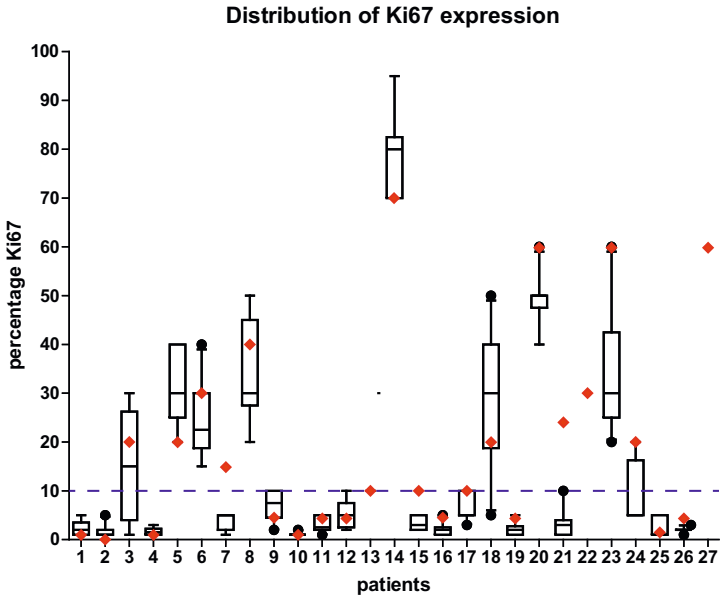

Figure 5. Distribution of Ki67 expression per patients. Boxplots of additional analysis of Ki67 expression on randomly selected 2mm wide parts of tumor tissue per patient. The red diamonds show the expression of Ki67 determined originally on the resected tumor material.

For the analysis of sequentially collected tumor samples, 11 patients were excluded because they had received chemotherapy between their biopsy and eP/D. Unfortunately, from the remaining patients, only 2 had sequentially collected tumor material that was available at the Erasmus MC. One patient (Patient 21) was also included in the overall analysis. The other patient (Patient A) was excluded from the overall analysis as this patient underwent a pleuropneumonectomy. Analysis of the biopsies and surgically resected material from eP/D showed a variability of maximally 5% (Table 4). More importantly, patients with a low Ki67 expression (<10%) did not have high expression of

Ki67 (>10%) in the tumor material resected during eP/D and vice versa. MTAP expression remained the same in these patients.

Table 4. Expression of Ki67 and MTAP in sequentially collected tumor material

Patient	Material	Date of material collection	MTAP	Ki67 (%)
21	VATS biopsies	19-12-2017	pos	20
21	eP/D	06-03-2018	pos	25
A	Thorascopical biopsies	23-07-2001	pos	5
A	Pleurapneumonectomy	06-02-2002	pos	1

Patient 21 is a patient that was in our original analysis cohort. Patient A was excluded from the original cohort because this patient got a pleurapneumonectomy. In both patients a similar Ki67 expression in sequential biopsies is observed and MTAP expression is constant.

Discussion

In this retrospective study of 27 MPM patients treated with eP/D in a multimodality approach, we found a mPFS of 15.3 months and a mOS of 26.5 months, in line with currently reported PFS and OS in surgically treated patients. Whilst Ki67 expression has been correlated to clinical outcome in epithelioid MPM and MPM patients treated with chemotherapy and EPP, we hereby report for the first time a correlation between high Ki67 expression and poor survival in MPM patients treated with eP/D in a multimodality approach irrespective of histology. All patients with a high expression of Ki67 (>10%) had died within 30 months, whereas the mOS for the patient group with a low expression of Ki67 was 44.5 months. Pre-operative tumor sampling and immunohistochemical staining in MPM could therefore be of great value for clinical decision making in a multidisciplinary setting. In this retrospective analysis, we were able to identify Ki67 as a prognostic marker. The predictive value of Ki67 is unfortunately not assassable, as a control group was lacking. A validation study of patients treated with either eP/D in a multimodality approach or chemotherapy alone could identify the potential predictive value of Ki67 for eP/D.

In several types of cancer, proliferation and thus Ki67 expression, is related to tumor growth and progression.[29,30] In MPM, chemotherapy is known to reduce Ki67 expression and thus proliferation of cancer cells. In a study where patients were treated with neo-adjuvant chemotherapy in combination with EPP, the median Ki67 expression prior to chemotherapy was 20%, and 11.25% after chemotherapy.[18] The survival analysis showed a significant relation between shorter mOS and high Ki67 expression (>20%) in tumor samples collected prior to chemotherapy as well as for high Ki67 expression (>11.25%) in tumor samples collected after chemotherapy. In our study, some patients had received neoadjuvant chemotherapy. As all samples were collected during surgery, tumor samples of these patients were collected after chemotherapy, whereas for patients receiving adjuvant chemotherapy tumor samples were collected prior to chemotherapy.

In patients treated with neoadjuvant chemotherapy, Ki67 expression might have been higher prior to chemotherapy. Although we did not correct for this, there is still a significant relation between Ki67 expression and clinical outcome. This implies that irrespective of prior treatment, a cutoff of 10% for Ki67 prior to eP/D is prognostic and clinically relevant for patients treated within a multimodality approach. In a study by Verma et al., the survival time for neoadjuvant chemotherapy was similar to that for adjuvant chemotherapy in combination with surgery, but neoadjuvant chemotherapy was related to longer hospitalization and higher 30-day mortality. This finding may be a reason to limit the clinical implementation of neoadjuvant chemotherapy. Nevertheless, considering that chemotherapy decreases Ki67 expression, neoadjuvant chemotherapy might still be of benefit to patients with high Ki67 prior to surgery. Analysis of biopsies prior to, and after chemotherapy in the EORTC1205 trial might elucidate whether neoadjuvant chemotherapy enhances the prognosis of patients whose Ki67 expression has been decreased by chemotherapy prior to surgery.

Several studies have shown the prognostic value of Ki67 in peritoneal and pleural mesothelioma, but with different cutoff values, ranging from 10-25%.[18,22,24] In individual studies, the cutoff value is determined on the basis of the available data and results. For example, low Ki67 expression (<15%) has already been reported to have a prognostic value in epithelioid MPM irrespective of treatment, except for patients treated with surgery alone.[22] In peritoneal mesothelioma, pre-operative determination of Ki67 has already been implemented in clinical decision making.[31] In our study, ROC curve analysis indicated an optimal cutoff value of 10% with a sensitivity of 90% and a specificity of 71%. Additionally, our data showed that patients with a Ki67 expression above 20% will definitely not benefit from a multimodality approach including eP/D. For clinical implementation, a universally accepted cutoff value for Ki67 should be determined in future research on a large dataset derived from multiple centers. Determination of this cutoff value is essential for the use of Ki67 as a selection criterion for surgery in MPM. From our analysis it seems that a high Ki67 can be best used as a negative selection criterion. Given that the survival in these patients is short, the adverse effects associated with surgery are not justified in patients with high Ki67.

If Ki67 is to be used as a negative selection marker in the future, determination of Ki67 in needle biopsy material should represent the Ki67 expression in the tumor. The expression throughout the tumor should thus be homogeneous. To check if Ki67 expression in needle biopsies was congruent with the Ki67 expression throughout the tumor, we determined Ki67 expression in randomly selected 2mm wide circular areas of the tumor. Here we found that in 80% of the patients, all of the additional focal analyses classified patients in their originally determined Ki67 expression category. Furthermore, in 87% of all 229 additional Ki67 analyses, the score was congruent with the originally determined Ki67 expression. In all cases where the score of the TMA did not match the originally determined score, the pseudo-TMA score was lower than

suspected. From these results we can conclude two things: (1) If a patient has a high (>10%) Ki67 expression in their needle biopsy, the patient is probably not going to have benefit from eP/D; 2. If a patient has a low Ki67 score in their needle biopsy material and is a potential candidate for surgery, additional tumor material could be collected during video-assisted thoracic surgery (VATS) pleurodesis prior to eP/D to confirm the Ki67 expression levels.

In our analysis, Ki67 has a stronger relation to PFS and OS than mitosis score. Mitoses are determined by counting of the number of mitoses per 10 high power fields by the pathologist who selects the regions of interest. By contrast, Ki67 staining is less dependent on morphological interpretation, and is relatively easy to recognize in a whole slide, which lowers the probability of a sampling error due to analysis of different regions of the biopsy. A meta-analysis has shown that assessment of proliferation by Ki67 leads to a higher interobserver agreement than for mitotic count.[32] Ki67 seems to be a better prognostic factor in our study and has been proven to be more reliable for multicenter use in clinical practice as well.

MTAP is located adjacent to CDKN2A and is co-deleted in 90% of mesothelioma tumors with a CDKN2A homozygous deletion. CDKN2A is a gene located on chromosome 9 which encodes for the p16 protein. Homozygous deletion of CDKN2A detected by fluorescence in situ hybridization (FISH) is a diagnostic marker for malignancy in mesothelioma. Immunohistochemical staining with an MTAP antibody is less labor-intensive and has lower costs than CDKN2A FISH. The interobserver agreement and interlaboratory reproducibility for MTAP was excellent and MTAP loss was 78% sensitive and 96% specific for CDKN2A homozygous deletion.[19] CDKN2A homozygous deletion has been correlated to poor survival in MPM, but MTAP has not yet been evaluated as a prognostic marker in MPM. For malignant peritoneal mesothelioma, MTAP has been proven to be significantly associated with OS and disease-specific survival.[33] Importantly, we hereby describe for the first time a significant positive relation between loss of MTAP expression and shorter PFS and OS in MPM. In the Cox regression models no significant association was found with OS, although the effect size was substantial (HR 0.373), which can be caused by a power issue with our small data set. In the multivariable model combined with Ki67, the effect of MTAP seemed attenuated (HR 0.748) and also not significant. Larger cohorts are needed to further investigate the usefulness of this marker. In our study, MTAP and Ki67 did not correlate with each other and therefore the hypothesis was that combining both parameters might have an additive prognostic effect. Unfortunately, this was not found in our data.

An obvious limitation of our study is the low number of patients, which can be explained by the fact that eP/D in MPM is not standard of care in the Netherlands. Extended P/D is rarely carried out, only in selected hospitals and after discussion in a multidisciplinary team or within a clinical trial. An extra parameter that can support the clinical decision

for or against eP/D would be helpful. Due to the low number of events, a multivariable analysis for all collected parameters was not possible. Additionally, the low number of events resulted in low power for the analyses, resulting in covariates with high hazard ratios and corresponding statistically non-significant *p* values. We were, however, able to identify Ki67 as an independent prognostic factor for OS and PFS in patients treated with eP/D within a multimodality approach. Histological subtype is the only tumor-related parameter that is currently taken into account in clinical decision making. In our analysis, however, histology was related only to PFS, not to OS. Furthermore, clinical parameters that are prognostic for mesothelioma such as gender, significantly correlated with survival in univariable analysis. But, in a multivariable analysis with Ki67, gender no longer significantly correlated with survival. This possibly indicates that Ki67 is a more accurate prognostic factor than the currently used and accepted prognostic parameters.

Clinical Implication and Surgical Decision Making

Patients eligible for surgery need to be strictly selected as only few may benefit from surgical treatment.[34] Patients with stage I-II according to the latest TNM 8 version[35] and non-sarcomatoid tumor histology are potential candidates for surgery.[34,36,37] However, their physical condition and frailty needs to be evaluated prior to major thoracic surgery.[34,36,37] Several laboratory prognostic factors such as serum hemoglobin (Hb) level,[38] serum albumin level,[38] white blood cells count[39] and platelet count[39,40] have been described which can have significant implication on surgical outcome.

Screening of the patient by a dedicated surgeon and anesthesiologist is an indispensable part of the pre-operative evaluation. Based on the radiological findings, which are often combined with a diagnostic video-assisted thoracic surgery (VATS) procedure, the surgeon should determine the likelihood of macroscopic complete resection (MCR) which should be the goal of the operation.[34,36,37] The use of validated comorbidity scores, as described in the literature, is recommended for surgical risk stratification.[41,42]

Surgery is not recommended in the presence of severe comorbidities such as: congestive cardiac failure, severe (peripheral) vascular disease, cerebrovascular disease (hemiplegia or paraplegia, dementia), chronic pulmonary disease, severe rheumatological disease, liver disease, diabetes mellitus with end organ damage, renal disease, any other malignancy, obesity BMI>35, previous heart/lung surgery, immobility, or severe mental disorders. In this study we, therefore, present our patient selection flowchart for surgical decision making combining the already available prognostic factors with Ki67 to define the best surgical candidates (Figure 6). Especially, when it proves difficult to reach a consensus within a multidisciplinary meeting, Ki67 might provide the decisive factor. Clinical implementation of this flow chart can only be justified after validation of Ki67 expression and its correlation to prognosis after surgery in larger cohorts.

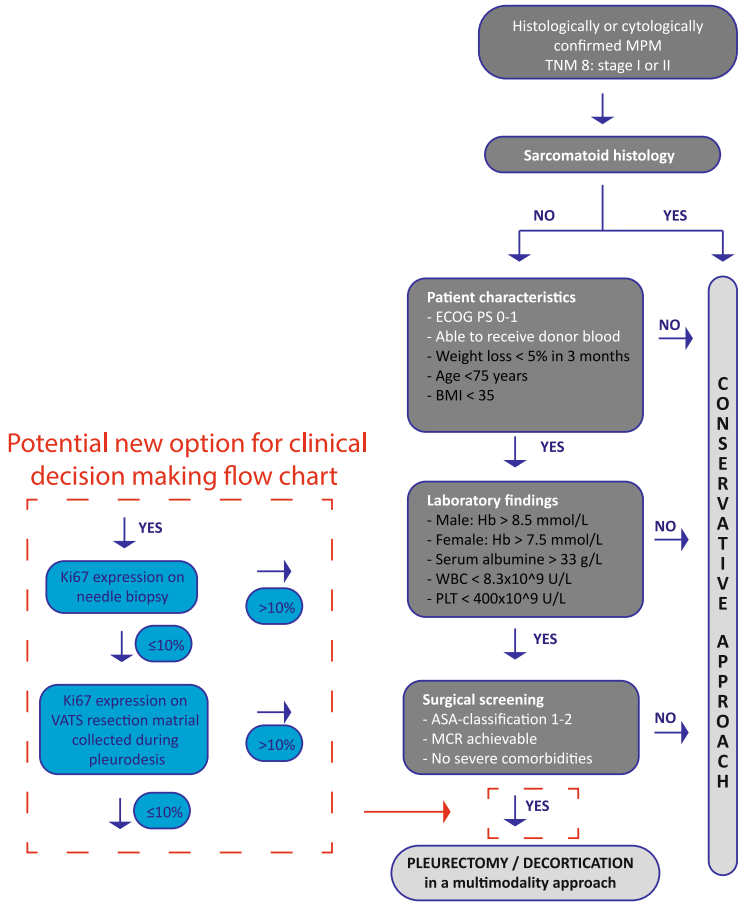

Figure 6. Flow diagram showing different steps in clinical decision making with inclusion of Ki67. The parameters written in white are mandatory. The proposed parameters in black should provide guidance, but are not absolute cutoff values, e.g. a patient aged 76 that meets all other criteria should still be eligible for surgery. Abbreviations; MPM: malignant pleural mesothelioma, TNM: Tumor Nodes Metastasis Classification for malignant tumors, ECOG PS: Eastern Cooperative Oncology Group – Performance score , BMI: body mass index, Hb: hemoglobin, WBC; white blood cell count, PLT:platelets , MCR; macroscopical complete resection, ASA; American Society of Anesthesiologists Severe comorbidities: Previous heart / lung surgery, heart failure, COPD GOLD 3-4, Hemiplegia or paraplegia, Dementia, Moderate / severe liver disease, Moderate / severe renal disease, Diabetes with severe organ damage, Severe mental disorder

Conclusion

Ki67 is prognostic for PFS and OS in MPM patients treated with eP/D in a multimodality approach and is likely to be reliably assessable from needle biopsy-collected tumor material. Determination of Ki67 prior to surgery combined with the already available clinical prognostic factors could be helpful in clinical decision making by identifying

patients with high Ki67 (>10%) who are unlikely to benefit from surgery. Due to the low number of patients in our study and no consensus for the cutoff of Ki67 in literature, a definitive cutoff for Ki67 in clinical decision making has to be validated. Large retrospective studies on tumor material from surgically treated patients might lead to consensus for a cutoff of Ki67. Furthermore, future cohort studies can evaluate the effect of combining currently existing prognostic scores, such as the EORTC score, mGPS and NLR, with tumor related parameters such as Ki67.

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Supplementary

Table S1. Primary antibodies, detection and amplification methods used

Antigen	Clone	Supplier	Ref. no.	Detection method	Antigen retrieval: duration/ temperature	Primary antibody incubation: duration/ temperature	Amplification step
MTAP	EPR6893	Abcam	ab-126770	OptiView-DAB	CC1 32 min/ 97°C	56 min/ 36°C	Yes
BAP1	C4	Santa Cruz	sc-28383	UltraView-AP	CC1 32 min/ 97°C	32 min/ 37°C	Yes
Ki-67	30-9	Ventana	790-4286	UltraView-DAB	CC1 36 min/ 97°C	28 min/ 37°C	No

Table S2. Ki67 expression per patient

Patient nr	Ki67 Surgery	TMA1	TMA2	TMA3	TMA4	TMA5	TMA6	TMA7	TMA8	TMA9	TMA10
1	1	5	3	2	5	2	1	2	2	1	1
2	0	1	1	1	2	2	5	1	1	1	1
3	20	25	30	30	15	25	10	5	1	1	15
4	1	3	1	2	3	2	1	2	1	1	1
5	20	30	40	30	25	20	25	40	40		
6	30	40	20	25	25	20	15	15	30	30	20
7	15	5	5	2	5	2	1	5	5	5	5
8	40	50	40	30	50	40	25	30	20	30	
9	5	5	5	10	10	10	10	10	5	2	3
10	1	2	1	1	1	1	1	1	1	1	1
11	5	3	5	2	5	2	5	2	5	2	1
12	5	10	10	2	5	3	5	5	2	5	
13	10										
14	70	95	80	90	80	70	70	80	70	80	70
15	10	5	3	3	5	5	5	2	2	2	3
16	5	3	2	1	2	5	2	1	1	2	
17	10	10	5	10	5	10	5	3	5	5	5
18	20	20	30	15	25	40	5	50	30	40	30
19	5	5	2	2	2	5	2	1	1	1	2
20	60	50	50	50	40	50	50	50	50	40	60
21	25	3	10	5	3	1	2	1	3	1	
22	30										
23	60	30	25	60	40	30	50	25	20	30	25
24	20	5	5	20	5	20	15	5	5	5	10
25	2	5	5	1	1	1					
26	5	3	2	2	1	2	2	2	2	2	2
27	60										

Ki67 Surgery: determination of Ki67 expression originally done on the surgically resected tumor material.
TMA: Ki67 expression on randomly selected spots of 2mm within the surgically resected tumor material.

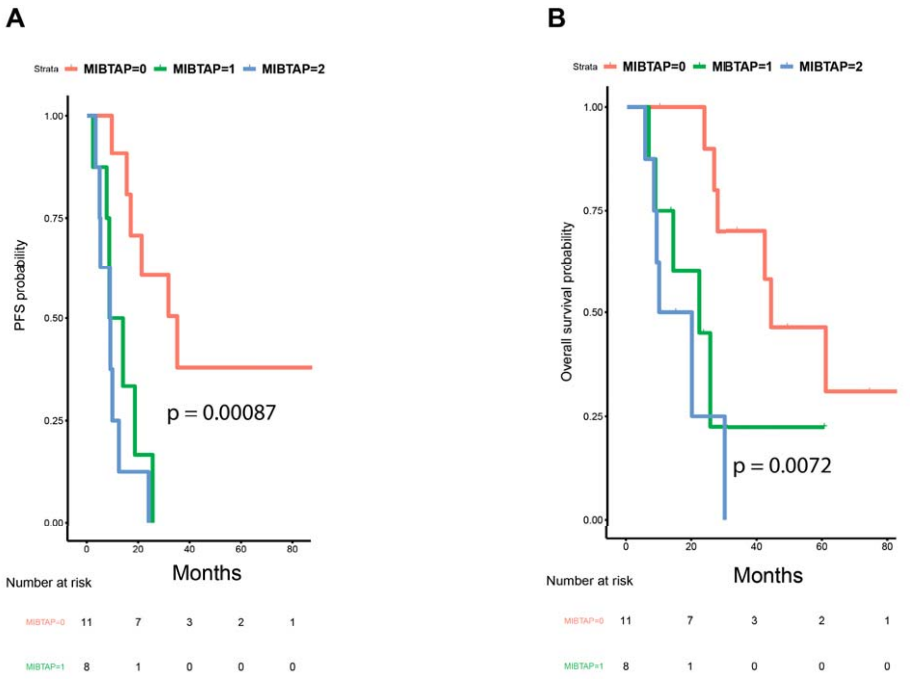

Figure S1. Kaplan Meier curves of survival in patients groups defined by a combination score of Ki67 and MTAP.

10

Summary and Discussion

Summary

Pleural Mesothelioma (PM) represents a great clinical challenge, characterized by its poor prognosis and limited response to conventional therapies, such as chemotherapy, radiotherapy and surgery.[1-3] PM is related to asbestos exposure,[4] but due to a lengthy latency time (± 40 years),[5] its incidence in the Netherlands was not yet reported to be declining, despite the ban on the use of asbestos in 1993. In this thesis we have shown that the incidence in the Netherlands is finally decreasing, showing the effect of asbestos regulations **(chapter 2)**. However, the decrease in incidence is minor and environmental asbestos exposure will be a continuous risk factor in the future. Therefore, improvement of treatment outcomes for patients with PM is essential. The main focus of this thesis has been on improving immunotherapy in PM, with a focus on dendritic cell (DC) therapy. In **chapter 3**, we showed that checkpoint inhibitor (CI) therapy (anti-PD-1) as second- or third-line therapy has limited impact on overall survival (OS). However, the 10% of patients that did have a radiological response also benefited in terms of OS, indicating heterogeneity of PM. Looking for a mechanism of response to CI therapy in PM, we identified patients with high levels of atypical B cells in the peripheral blood have no benefit of CI therapy **(chapter 4)**.[6] Dendritic cells (DCs), initiators of anti-tumor immune responses, are low in number and impaired in function in PM.[7] This might lead to impaired tumor-specific cytotoxic T cell responses and can be a potential explanation for the limited effect of CI therapy in PM. We therefore assessed the effect of DC therapy (MesoPher) as maintenance therapy in PM, which had no impact on OS, but did show MesoPher-induced CD4+ T cell activation that correlated with progression-free survival (PFS) **(chapter 6)**. The complexity and variability of DC therapy itself and the immune system leads to a boundless range of potential barriers and appropriate solutions. These could include; refining the MesoPher product itself, but also intervening in the (tumor microenvironment) TME through surgery, radiotherapy or systemic therapies **(chapter 7)**. In **chapter 8,** we showed that sequential MesoPher and CI therapy is safe and feasible, leading to promising survival outcomes in patients, with proven synergy when given as concurrent treatment in mice. Tumor load is associated with tumor-induced immunosuppression and tumor load reduction might enhance the effect of MesoPher. A combination of extended Pleurectomy/Decortication (eP/D) and (neo) adjuvant DC therapy has resulted in a promising ongoing recurrence-free survival of 15 months in a 52-year old female patient with PM **(chapter 9)**. As the role of surgery in PM remains controversial, in **chapter 10** we identified PM patients with a low expression of the proliferation marker Ki-67 that seem to derive clinical benefit from surgery.

Discussion

Incidence and treatment patterns in PM

The main risk factor for PM is asbestos exposure. Notably, the Netherlands has historically experienced a relatively high incidence of PM, prompting legislative action in 1977 culminating in the ban of asbestos usage in 1993. In **chapter 2**, we showed a decline in PM incidence since 2010, despite an anticipated peak around 2020 due to a 30-40 year latency time and a ban since 1993. The regulatory interventions implemented in 1977, following the seminal thesis (1969) by J. Stumphius[8] correlating asbestos exposure with PM, appear to have played a pivotal role in curtailing asbestos usage prior to its eventual ban. These measures likely have had a major effect on the observed decline in PM incidence, as elucidated by our investigation. Hopefully, the shown decrease in incidence as a consequence of regulations serves as motivation for other nations still mass-producing asbestos to reduce exposure.

We also looked at the incidence of peritoneal mesothelioma (PeM), which remained relatively stable, suggesting that asbestos might not be the primary instigator of PeM. Germline mutations, notably BRCA1 associated protein 1 (BAP1), and aberrations in other genes such as BRCA2 and cyclin-dependent kinase 2 A (CDKN2A), are likely contributors to the incidence of PeM.[9]

We analyzed treatment data spanning from 1993 to 2018 and our findings revealed a rise in the utilization of chemotherapy following the seminal study by Vogelzang et al. (2003), correlating with improved survival rates extending to 12 months. [1] Intriguingly, treatment was administered to only 40% of patients, with 42% actively opting against it. This observation suggests the presence of robust advanced care planning, with patients consciously weighing the trade-off between proper survival gains and compromised quality of life. Notably, Damhuis et al.[10] showed that chemotherapy was given to 60% of patients in Belgium, without a significant increase in OS compared to the Netherlands. This shows limited effect of chemotherapy and well implemented advanced care planning in the Netherlands. Furthermore, our analysis revealed that surgical intervention was administered to only 3% of patients, whereas 27.6% of PM patients receive surgery in the USA,[11] reflecting the controversial role of surgery and different views on its efficacy seen in the American and British guidelines.[12-14]

Improving CI therapy in pleural mesothelioma

As previously reported, first-line chemotherapy improved median OS (mOS) to approximately 12 months for selected patients with PM.[1] CI therapy, targeting immune checkpoints such as PD-1 and CTLA-4, has demonstrated significant success in improving clinical outcomes in cancers like non-small cell lung cancer (NSCLC)[15] and

melanoma[16] by reinvigorating the anti-tumor immune response and is therefore been tested in other tumors as well.[17] Although CI monotherapy in PM has shown promise in phase I/II trials,[18-22] it has not demonstrated a significant survival benefit in phase III trials.[23,24] In **chapter 3**, we have showed that treatment with PD-1 inhibitor nivolumab in pretreated PM patients resulted in an overall response rate (ORR) of 10%, mOS of 6.7 months, and mPFS of 2.3 months. These results are not encouraging for the group as a whole and are inferior to those observed in clinical trials. The latter is probably due to patient selection. However, we noted that mOS was not reached with a follow up time of 14.1 months in patients who achieved a partial response (PR) to CI therapy, implicating clinical benefit in this subgroup of patients. The phase III trial conducted by Popat et al. (2019) comparing pembrolizumab to single-agent chemotherapy in relapsed PM did not demonstrate an improvement in mPFS and mOS, despite observing a higher overall response rate (ORR) in the pembrolizumab arm (22% vs. 6%).[24] This absence of a significant benefit in mPFS and mOS, despite a better ORR in the pembrolizumab arm of the PROMISE-meso trial might be due to the relative low ORR and the quick disease progression in non-responding patients. For example, if only a small fraction (10–20%) of patients benefit from the therapy, mPFS and mOS metrics are unlikely to change significantly because more than half of the patients will experience early progression or mortality, reflecting the natural progression of the disease. Thus, six-month PFS and one-year OS might be more appropriate endpoints for evaluating (immune) therapies with low response rates. Another hypothesis for the lack of increased mPFS despite a relative high ORR might be a lack of sustained CI therapy induced anti-tumor immune response. Further analysis of patients who achieved a partial response (PR) to pembrolizumab in the PROMISE-meso study has not yet been released and could offer additional insights.

The Checkmate 743 trial showed that combination CI therapy (anti-PD-1 and anti-CTLA-4) compared to combination chemotherapy (antifolate and platinum) improved mOS from 14.1 months to 18.1 months.[25] Unfortunately, long-lasting responses as seen in melanoma and lung cancer are less frequently seen. Why response to CIs is less pronounced in PM is still unclear. Several hypotheses are postulated, such as: low tumor mutational burden (TMB), relatively low T cell infiltration, loss of HLA heterozygosity. Yet, none of these parameters are exclusively explaining the lack of response to therapy. Moreover, toxicity to combination immunotherapy in PM is quite severe, leading to discontinuation of treatment in 25% of patients.[25] Therefore, there is an unmet need to improve clinical outcome through patient selection or by enhancing CI therapy through combination strategies. To understand what therapies could be effective, the biological mechanisms behind therapy resistance should be elucidated.

Shifting from response prediction to mechanistic understanding

The presence of an anti-tumor immune response is essential for the effectivity of CI therapy. Extensive research has been done to identify predictive biomarkers for response to CI therapy, acknowledging that durable responses are limited to a subset of patients. Some biomarkers are a measure of the antigeneic potential of the tumor (TMB), whilst others directly reflect the potential binding capacity of CI therapy (PD-L1). Various invasive biomarkers, such as PD-L1 expression, histology, TMB, neoantigen load, germline mutations, and immune cell infiltration within the tumor have been investigated. Among these, PD-L1 expression and TMB are used in other tumors but not in PM.[15,26] We will discuss the following parameters in more detail: serum albumin, TMB, PD-L1 expression and histology.

Albumin

In **chapter 3**, we have analyzed peripheral blood markers and our findings demonstrated that low albumin levels are correlated with poorer overall survival (OS), a relationship corroborated by a meta-analysis that associates albumin levels with progression-free survival (PFS), OS, and response rate in cancer patients treated with CI therapy.[27] Although the underlying mechanisms remain to be elucidated, a hypothesis suggests that CIs, which are based on IgG antibodies, share the same homeostatic pathway as albumin. Consequently, serum albumin levels may reflect the pharmacokinetic rate at which IgG antibodies are cleared from the system. In addition, low albumin levels have been correlated with cachexia and chronic inflammation and might also reflect a general dysfunction of the immune system.[28] This is why we initiated a new study (NL76015.078.21, NCT05902260) looking at the effect of nutritional support on the variability of clearance of pembrolizumab during a 12-week period in patients with non-small cell lung cancer and checking for immune activation status.

TMB

In solid tumors with a TMB exceeding 10 mutations per megabase (mut/Mb), the FDA has approved the use of pembrolizumab.[26] The CheckMate 743 study, revealed that the median TMB in PM was 1.75 mutations per megabase and as expected the highest tertile >2.05 mut/Mb did not correlate to survival.[29] As only 1,2% of PM tumors reaches the cutoff level of 10 mut/Mb,[30] there have to be other factors explaining response rates around 10% CI therapy in PM. Although all studies show relatively low TMB for PM, different sequencing assays and depths employed in various studies have led to incomparable results and disparate potential cutoffs, without significant clinical impact. Given that PM is specifically induced by mutagens, a low TMB is unexpected. Recent studies have demonstrated that despite the low TMB, chromosomal rearrangements (chromoplexy and chromothripsis) are present in 86% of PM samples and are potentially linked to neoantigen formation.[31] Unfortunately, these rearrangements did not correlate

with improved outcomes following CI therapy. However, these rearrangements were correlated with better outcomes when they were associated with gene signatures related to "antigen presentation and antigen processing." This suggests that the neoantigenic potential of chromosomal rearrangements is dependent on the tumor cells' ability to present neoantigens.[32] In summary, it is essential to precisely define the metrics being assessed and to thoroughly understand their biological implications for the potential therapy being administered.

PD-L1 expression and histology

PD-L1 expression and histology should be discussed together as these parameters have been correlated with each other.[33] PD-L1 expression is used in NSCLC to guide treatment decisions between CI monotherapy and combination therapy with chemotherapy. However, in PM, the predictive value of PD-L1 expression for CI monotherapy remains unclear. Several trials (Nivomes,[20] CONFIRM[23] and Promise-meso[24]) failed to show a positive correlation between PD-L1 expression and response to CI therapy, while the MERIT trial reported a positive correlation.[21] In **chapter 3**, we showed that the overall response rate (ORR) was 36% in the PD-L1 positive group compared to 9% in the PD-L1 negative group. The heterogeneity of intratumoral expression of PD-L1 in pleural mesothelioma (PM) may account for these inconsistent findings. Furthermore, the role of histology in predicting response to CI therapy remains ambiguous.[23] Results from trials with CI combination therapy were more consistent as the Initiate,[34] MAPS-2[35] and Checkmate 743 trial[29] reported on an advantage of PD-L1 positive tumors receiving immunotherapy. Notably, the Checkmate 743 trial reported that non-epithelioid tumors did not show superior responses to immunotherapy compared to epithelioid tumors (18.1 months vs. 18.2 months), yet non-epithelioid tumors have a negligible response to chemotherapy compared to immunotherapy (8.8 months vs. 16.7 months). Collectively, these findings underscore that investigating the TME could refine our ability to identify (non-)responders. In this context, Alcala et al. (2019) introduced a novel and imperative approach to prognostication, based on a continuum of molecular profiles resulting in a five-protein based prognostic score incorporating CD8, PD-L1, VEGFR3, VEGFR2, and VISTA.[36] Finally three molecular profiles were determined and two of these profiles encompassed mainly non-epithelioid tumors, categorized as either hot (PD-L1+ & CD8+) or cold (PD-L1- & CD8-). These profiles were prognostically similar but were measured independent of treatment. This molecular profiling could potentially explain why only a subgroup of patients respond to CI therapy, even in the non-epithelioid group. This hypothesis is supported by the exploratory analysis of the Checkmate 743 trial, which revealed that patients with a high four-gene inflammatory score (CD8A, STAT1, LAG3, and PD-L1) in baseline tumor biopsies who received combination immunotherapy had significantly longer mOS (21.8 months) compared to those with a low inflammatory score (16.8 months).[29] Furthermore, analysis of PM patients treated with nivolumab of nivolumab/ipilimumab showed that a higher density of CD4, CD8 FoxP3 and PD-1 at baseline was significantly correlated with partial responses.[37] Therefore, expanding

our focus beyond histology and PD-L1 and integrating histological information into a comprehensive predictive score may enhance clinical outcomes through personalized therapy with optimized benefit while minimizing unnecessary side effects.

DN B cells in peripheral blood

Non-invasive biomarkers, which are not influenced by spatial heterogeneity issues, have also been studied extensively. Given that anti-PD-1 antibodies primarily act by reinvigorating CD8+ T cells within tumors or tumor-draining lymph nodes, immunomonitoring has concentrated on these cells. Despite correlations between various CD8+ T cell subsets and clinical responses,[38] none have proven robust enough for clinical application. Given the complexity of the immune system and the dynamic interactions among various immune cell subsets, it is essential to investigate these and other immune cells in greater detail. This is not solely to identify a biomarker for response, which remains challenging, but also to gain a deeper understanding of the underlying biological mechanisms. Recent studies have highlighted the correlation between intratumoral memory B cells within tertiary lymphoid structures and response to CI therapy.[39-42] Additionally, double negative (DN) (CD27- IgD-) B cells have been found to be abundant in non-small cell lung cancer (NSCLC) compared to healthy lung tissue,[43] and these cells inversely correlate with the presence of intratumoral memory B cells.[43] In **chapter 4**, we showed that a high frequency of B cells in the peripheral blood is correlated with response to CI therapy, but that a relative abundance of (CD21-) DN B cells is associated with a lack of response to CI therapy. Based on literature there are two potential hypotheses to explain this finding. As CD21- DN B cells are phenotypically similar to atypical B cells observed in chronic infections, they might share functional traits as well. At the time of publication of our manuscript, atypical B cells in chronic infection were often, but not always, described as being exhausted and anarchistic.[6,44,45] A more recent study has evaluated B cells in chronic infection with single cell sequencing and found 3 subtypes of atypical B cells, with all different attributes ranging from quite functional to anarchistic. We don't know if any of these atypical B cell subtypes were overrepresented in our study, only that the clinical consequence is detrimental. Another hypothesis is based on a study that found that DN B cells function as antigen presenting cells that rather activate regulatory T cells than cytotoxic T cells. In conclusion, atypical B cells in peripheral blood correlate to bad prognosis or impaired response to CI therapy in NSCLC and PM, but should be defined in more detail phenotypically and functionally.

Despite the probability that the aforementioned parameters may not serve as definitive biomarkers for response with a stringent cutoff for clinical implementation, they significantly enhance our understanding of the underlying reasons for treatment response. Serum albumin and CD21-DN B cells seem to be surrogate markers for patients with a malnourished clinical status and exhausted immune system, respectively. This translates into a bad prognosis and dismal responses to CI therapy. It is perhaps the treating physicians' responsibility to consult patients on the likelihood

of a good clinical response based on available clinical data, tumor tissue and peripheral blood analysis, enabling the patient to make a well-informed decision which can reduce unnecessary side effects and improve clinical outcomes. Although for these patients, cancer-induced deterioration is causing therapies to fail, an abundance of patients with PM in relatively good condition is also failing to respond to CI therapy. As doctors, we always strive to improve outcomes for a broader patient population and CI therapy can potentially be enhanced by inducing an anti-tumor immune response in patients with an immunosuppressive tumor like PM. This can be achieved through dendritic cell (DC) therapy, which can induce an anti-tumor immune response and has demonstrated efficacy in mesothelioma mice models and is feasible and safe in patients with PM.[46-49]

DC therapy in pleural mesothelioma

In **chapter 5**, we reported the outcomes of a phase III clinical trial assessing the efficacy of DC therapy (MesoPher) compared with BSC as maintenance therapy in PM following first-line chemotherapy. Our analysis revealed that MesoPher, when administered after chemotherapy, did not lead to a survival benefit over best supportive care (BSC) in terms of OS.

Comparing immune cell activation in pleural mesothelioma, peritoneal mesothelioma and pancreatic cancer

On a positive note, we did see a correlation between the magnitude of MesoPher-induced proliferation of CD4+ central memory T cells (Tcm) and PFS. Parallel to the DENIM study, two other studies were done with MesoPher in pancreatic cancer and PeM.[50,51] These studies were both phase II single arm studies where MesoPher was given as adjuvant treatment after surgery.

In PeM, surgery in combination with MesoPher led to a mPFS of 12 months and mOS was not reached after a 26-month median follow-up. Furthermore, following MesoPher treatment, there was a notable increase in the proliferation of CD4+ T cells, accompanied by an upregulation of co-stimulatory molecules, such as ICOS, HLA-DR, and CD28, specifically on memory CD4+ T cells. Additionally, a modest rise in the production of TNF-α and IFN-γ by memory CD8+ T cells was observed.

In pancreatic cancer, clinical outcomes were promising with a recurrence free survival (RFS) of 64% compared to 40% in a comparable cohort from the PREOPANC trial treated with adjuvant chemotherapy. In this study, no correlation was seen between MesoPher-induced immune cell activation and RFS, but there was a broader activation of CD4+ T cells (HLA-DR, ICOS, Ki-67, PD-1, CD39). More importantly, *in vitro* a strong MesoPher-specific CD4+ and CD8+ T cell response was shown and T cell receptor (TCR) sequencing demonstrated overlapping clonotypes between tissue from a metastasis and the vaccine, that were not present in the primary tumor. These results suggest DC therapy-induced T cell activation and tumor infiltration.

To conclude, in pancreatic cancer and peritoneal mesothelioma MesoPher led to comparable, but potentially more extensive immune cell activation. Clinical results were promising and adjuvant MesoPher was safe and feasible. Of course, clinical results should be interpreted with caution as these are phase II trials. A potential reason for the more extensive immune activation could be due to the fact that these patients had to be fit enough for surgery as well as the fact that tumor load and thus tumor -induced immunosuppression was reduced. This hypothesis is confirmed by the fact that in earlier mice studies we already showed that high tumor load impairs efficacy of DC therapy.[46] Moreover, in the DENIM trial we have shown that performance status (ECOG 0 vs ECOG 1) did result in longer PFS in patients that received MesoPher treatment (8.1 months vs. 3.2 months, $p < 0.01$), whereas this was not seen in patients receiving BSC (4.4 months vs. 3.2 months, $p = 0.28$). To see if reduction of tumor load could enhance the effect of DC therapy, we started the ENSURE trial to combine (eP/D) with neoadjuvant DC therapy, which will be discussed later **(chapter 8)**.

Improving DC therapy in pleural mesothelioma

Negative clinical results urge us to reassess our assumptions, revisit our methods and refine our approach. A potential flaw in the design of the DENIM trial that could have negatively impacted the clinical outcome of DC therapy was a long interval between MesoPher treatment and chemotherapy. Similar to CI therapy, patients with a non-epithelioid histology seem to benefit more from DC therapy than chemotherapy, leaving room for patient selection. Whereas there was a correlation with MesoPher-induced CD4+ T cell proliferation in peripheral blood and PFS, we did not see CD8+ T cell proliferation or activation in peripheral blood, whilst CD8+ T cells are main contributors to tumor cell killing as well. It is important to note that the lack of CD8+ T cells in the peripheral blood does not necessarily indicate a lack of CD8+ T cell infiltration in the tumor. In **chapter 7**, we discussed several options of optimizing DC therapy through selection of naturally occurring DCs, usage of different maturation cocktails or loading peptides, intervening in the TME (depletion of Tregs or tumor-associated macrophages (TAMs)) or combining treatment with CI therapy or surgery. All these potential flaws and improvements will be discussed below.

Timing of DC therapy

The decision to employ DC therapy as a maintenance treatment was based on the hypothesis that a non-progressive tumor would be associated with reduced immunosuppression, resulting in optimal efficacy of MesoPher. However, the mean interval between the final chemotherapy cycle and the initiation of DC therapy was 11.7 weeks, which is nearly double the time observed in our phase I trial, where a mOS of 26.7 months was accomplished (updated, yet unpublished data). Given the natural history of PM, it is anticipated that a large proportion of patients would experience

disease progression within the 11.7-week interval. This is corroborated by the fact that 44 patients (50%) in the BSC arm and 33 patients (37.5%) in the MesoPher arm exhibited progressive disease (PD) at the time of the first CT evaluation. In addition, 6 out of 8 patients that did not receive the first three vaccinations, had PD prior to the first vaccination. One factor contributing to the extended interval was the sterility testing of MesoPher, which could be expedited by utilizing rapid real-time polymerase chain reaction (PCR) methods. Additionally, monocyte collection could be performed before the initiation of chemo, [52] or, alternatively, naturally occurring dendritic cells (nDCs) could be harvested to reduce the conventional culture time. However, these strategies offer only marginal improvements compared to a potentially more effective approach: administering DC therapy prior to first-line treatment, particularly given its very mild side effect profile. This strategy is especially pertinent in clinical practice, where therapy initiation is sometimes delayed in patients with good performance status, due to the limited number of available therapeutic options. Furthermore, as mentioned before, preclinical studies suggest enhanced efficacy when tumor burden is low, supporting the rationale for early intervention with DC therapy.[46]

Non-epithelioid histology outperforming epithelioid histology

In the DENIM trial we found that there was a prolonged PFS in patients with a non-epithelioid histology that were treated with MesoPher. This is in line with the results shown in the checkmate 743 study and **chapter 3**. These results are just scratching the surface as histology might be just a small part of the TME, which is already discussed previously in "*CI therapy in PM".*

Lack of objectified peripheral CD8+ T cell activation despite extensive CD4+ T cell activation and proliferation

We showed that MesoPher treatment led to an increase of proliferation and activation of CD4+ T cells. Furthermore, we showed that the magnitude of increase of proliferation (marked by Ki67) on CD4+ Tcm correlated to PFS. Moreover, the magnitude of increased proliferation marker expression on CD4+ effector memory T cells (Tem) also correlated to PFS and OS. In addition, increased activation, measured by ICOS expression on CD4+ Tem cells also correlated to PFS (see table 1).

Table 1. Significant correlations between selected output parameters and clinical responses

CD4	Subset	Marker	Comparison	Arm A	
				OS	PFS
CD4	CD4+ Effector memory T cells	Ki67	Change at day 8	**p=0.0281** R^2=0.1005	**p=0.0055** R^2=0.1555
CD4	CD4+ Effector memory T cells	ICOS	Change at day 8	Ns	**p=0.0302** R^2=0.0812
CD4	CD4+ Central memory T cells	Ki67	Change at day 8	Ns	**p=0.0007** R^2=0.2575

OS: overall survival, PFS: progression-free survival, Ki67: proliferation marker, ICOS: activation marker.

Whilst CD8+ T cells have long been regarded as the central mediators of anti-tumor immunity, the role of CD4+ T cells has been historically underestimated, despite evidence as early as 1998 demonstrating their crucial role in a melanoma mouse model (B16).[53] Furthermore, in 2005 a study by Antony et al. demonstrated that CD8+ cytotoxic T lymphocyte (CTL) responses require CD4+ T cell assistance to sustain their anti-tumor activity.[54-57] These studies highlight the critical role of CD4+ T cells in anti-tumor immunity. The question remains how CD4+ T cells mediate anti-tumor immunity. Apart from CD8+ T cell activation,[58] CD4+ T cells also play a pivotal role in antibody responses within tertiary lymphoid structures by activating B cells through CD40/CD40 ligand interactions and can exert direct cytotoxicity similar to CD8+ T cells through MHC class II antigen recognition on tumor cells.[59,60] To conclude, CD4+ T cell activation seems to play a bigger role than we assumed in anti-tumor immunity. However, we can't overlook the importance of CD8+ T cell activation, which should be a logical consequence of CD4+ T cell activation. There are three potential explanations for the observed lack in CD8 T-cell activation. First, a specific CD8+ subset may be proliferating and activating, but the appropriate phenotypic markers for this subset have not yet been identified or the CD8+ T cells have migrated into the tumor. Alternatively, CD8+ T cells may not be adequately activated by the DC therapy, possibly due to insufficient MHC class I expression on the DCs. At last, the immune system has to be capable of mounting an immune response, which is perhaps impaired in patients with a low performance status or high tumor load as discussed earlier.

Choosing the right dendritic cells, optimal maturation cocktail and best pulse material

In our studies, monocytes are collected from the peripheral blood and then cultured *ex vivo* by using IL-4 and GM-CSF to **differentiate** them into monocyte-derived DCs (moDCs). Subsequently, moDCs are **loaded** with allogeneic tumor lysate and **matured** using interleukins and cytokines (e.g. IL-6, TNF-a). The DCs are **injected** intravenously and intradermally *(see introduction for a detailed description of our production process)*.

The differentiation cocktail, maturation cocktail, loading antigens and way of injection is always under debate. Some reports have suggested that culturing monocytes into moDCs with IL-4 and GM-CSF results in less potent tumor-specific CD8+ T cell activation compared to monocytes cultured with IL-3 and IFN-B and that migration of these moDCs is impaired due to overstimulation with IL-4.[61,62] Other studies have stated that different maturation cocktails (IL-15 and TLR7/8 ligand) could result in more optimal maturation, activation, and migratory potential.[63,64] Furthermore, several studies have suggested that naturally circulating DCs (nDCs) could outperform moDCs in terms of migration and immune activating potential.[65] These claims often come from preclinical studies or *in vitro* culturing assays and not from clinical comparative studies. Moreover, nDCs have to be isolated through positive selection using magnetic antibody-coupled beads, optionally preceded by depletion of monocytes and B cells and need to be expanded to reach a sufficient amount, which directly questions the feasibility of this approach.[65,66] Lastly, DCs loaded with tumor cell lysate seem to outperform DCs loaded with neoantigen peptides selected with in silico predictors of peptide-MHC affinity.[67] A meta-analysis showed that clinical responses in patients treated with DCs loaded with tumor cell lysate were significantly higher compared to DCs presenting specific antigens (8.3% vs 2.6%).[68] A potential reason for this is that a lot of tumor associated antigens are unknown and not all tumor cells express the same antigen. Therefore, continuing to use tumor cell lysate seems the best option. Injection of DCs directly into the lymph node could circumvent migration issues to the lymph node, but this approach has a feasibility issue if hilar lymph nodes need to be injected through EUS or EBUS for example. Taken together, there are a lot of unanswered questions related to the production process as well as the injection method of DC therapy. Although the antigeneic potential of DC therapy has improved over the last decade, clinical results did not improve as expected. Perhaps the focus of future studies should be on clinical hurdles such as collection of naturally occurring DCs and injection methods.

Targeting the TME

Another option of enhancing DC therapy efficacy is by skewing the immunosuppressive milieu of the TME by reducing the abundance of Tregs (e.g. with low-dose cyclophosphamide) or tumor associated macrophages (CSF-1R inhibitor).[48,69,70] In PM we showed that we could deplete Tregs using low-dose cyclophosphamide and that this was feasible and safe in combination with autologous mo-DC therapy even leading to an impressive 26 months mOS.[48] The number of naïve Tregs before treatment negatively correlated to clinical outcome.[69] Unfortunately, no correlation was made between the decrease of Tregs and clinical outcome. Our group also showed that depletion of TAMs using CSF-1R inhibitor together with DC therapy synergistically enhanced survival in a mesothelioma mouse model.[70] Vascular endothelial growth factor (VEGF) is released by hypoxic cancer cells and promotes tumor growth by increasing neovascularization, but also enhances the proliferation of Tregs, mobilizes TAM and skews them into an immunosuppressive M2 phenotype.[71] Moreover, MDSC become activated and DC

maturation and antigen presentation is impaired. Anti-VEGF treatment could inhibit all these immunosuppressive effects of VEGF and potentially enhance DC therapy. Lenvatinib (anti-VEGF antibody) combined with CI monotherapy resulted in a 45% response rate but a rather disappointing mOS of 12.8 months treated after progression on first line chemotherapy.[72] Moreover, toxicity was immense with a 71% grade III or higher prevalence and serious adverse events in 26% of patients. Anti-VEGF might enhance the potential of DC therapy, but toxicity should be closely observed.

Combining DC therapy with CI therapy

Recognizing that T cells infiltrating tumors after dendritic cell (DC) therapy may undergo exhaustion mediated by PD-1/PD-L1 signaling, we conducted a study investigation **(chapter 7)** into the potential benefits of combining DC therapy with adjuvant anti-PD-1 immunotherapy in patients with PM. The rationale behind this approach was to counteract the immune exhaustion that could diminish the effectiveness of the T cell response, thereby enhancing the therapeutic outcome. We showed that this treatment regime was safe without increased toxicity compared to monotherapy CI therapy. Furthermore, an impressive disease control rate of 55.6% at 6 months and a mOS of 17.7 months was observed. Additionally, we showed PD-L1 upregulation on DCs upon *in vitro* culturing. Therefore, we extended our study with a murine model of PM and showed that concurrently administering DC therapy alongside anti-PD-L1 therapy enhanced survival. This combined approach not only aimed at preventing T cell exhaustion, but also at potentially enhancing the overall immune response by targeting multiple points of immune regulation, thereby offering a more robust and sustained anti-tumor effect. This concomitant strategy could provide an enhanced effect to current first-line combination CI therapy. In this way, DC therapy is given as early as possible when tumor load is low and the condition of the patient is relatively good. The current consensus on vaccine therapies is to start as early as possible which fits this approach. Toxicity is low in DC therapy and not expected to increase toxicity of CI therapy.

Another way to circumvent immunosuppression through PD-L1 on DCs is through PD-L1 silencing, but this will not affect tumor cell/T cell interaction.[64] CI therapy has already been combined with CAR T-cell therapy in PM with promising outcomes[73] and a new study in PM has commenced in which DC therapy will be combined with anti-PD-L1 antibodies and chemotherapy.[74] Although chemotherapy could potentially harm DC therapy-induced T cell activity, the approach of starting DC therapy in an early stage is probably wise.

Surgery in combination with DC therapy

As discussed already, tumor load reduction might enhance the efficacy of MesoPher treatment. We therefore started the ENSURE trial to combine eP/D with neoadjuvant DC therapy **(chapter 8)**. in the first included patient we were able to evaluate not only

safety and feasibility, but also look at primary clinical results and intratumoral effect of DC therapy as it was given before and after surgery. The first patient included in this trial completed treatment according to protocol and has a very promising ongoing RFS of 15 months. After DC therapy, we detected increased diffuse CD8+ T-cell infiltration in the tumor and formation of tertiary lymphoid structures, which could imply that there is CD8+ T-cell activation and that these cells have migrated to the tumor and are therefore not detectible in the peripheral blood after DC therapy. We also observed vaccine-specific T cell receptor clonotypes in the tumor post-DC therapy, which were absent prior to treatment and is highly suggestive for DC therapy-induced T-cell infiltration in the tumor. Although these results seem to prove the principle of DC therapy- induced T-cell infiltration, we need to interpret them with caution as these are only the results derived from one patient.

Improving pre-operative decision making with Ki67

The role of surgery in pleural mesothelioma remains highly controversial resulting in conflicting guidelines, as previously discussed in this thesis.[13,14] This is exemplified by the great contrast in surgical practice between the Netherlands, where only 3% of patients undergo surgery, and the USA, where approximately 25% of patients receive surgical intervention.[11] Given the high morbidity associated with surgery and the limited number of patients who derive significant benefit, there is a pressing need to identify those who are most likely to benefit from surgical treatment.[3]

In this context, Ki67, a well-established prognostic marker in pleural mesothelioma, is used as a preoperative marker in PeM to guide surgical decision-making.[75] **Chapter 10** investigates the potential utility of Ki67 expression in PM. Our analysis revealed that high Ki67 expression, using a 10% cutoff, was predictive of shorter OS, indicating limited clinical benefit from surgery. We also showed that a cutoff of 20% will lead to a sensitivity of 100% for not reaching the mOS of 26.5 months. Notably, we demonstrated that the heterogeneity of Ki67 expression does not undermine the validity of this cutoff, addressing concerns previously raised in the literature.

Following our publication, two responses affirmed the potential of Ki67 as a marker for guiding surgical decisions but underscored the need to establish a definitive cutoff value in a larger cohort of patients undergoing eP/D. Additionally, artificial intelligence -guided Ki67 quantification was suggested as a means to enhance the objectivity and accuracy of Ki67 assessment.[76]

A large study involving 940 patients demonstrated increased accuracy over Ki67 alone in a pathological grading system incorporating histology, necrosis, mitotic count, and Ki67 scoring.[77] In contrast, recent analyses using digital imaging of the Ki67 index have correlated it with tumor grade and mitotic count, showing predictive value for OS comparable to the mitotic score, and suggesting its potential as a surrogate for tumor

grade.[78] Given that nuclear grading and mitotic counting are labor-intensive, expensive, and subjective, Ki67 could serve as a viable alternative.

Further evidence of Ki67's clinical relevance comes from a recent study indicating that Ki67 expression (with a 15% cutoff) can predict treatment benefit in patients undergoing lung-sparing surgery as part of multimodality therapy. Patients with low Ki67 expression demonstrated a median OS of 48.1 months, compared to 24.3 months in those with high Ki67 expression.[79] However, these results should be interpreted with caution, as the surgical treatment also included tumor debulking, and the distribution of debulking versus P/D in the high and low Ki67 groups was not clearly defined. This suggests that while a small subset of patients may benefit from surgery, definitive conclusions would require validation in a randomized controlled trial (RCT).

In this context, the results of the MARS II trial are noteworthy, showing worse mOS in patients who underwent surgery (19.3 months) compared to those who received chemotherapy alone (24.8 months).[3] Additionally, surgery was associated with a 3.6-fold increased risk of serious adverse events, higher costs, and fewer quality-adjusted life years. These findings from an RCT suggest that earlier positive surgical outcomes may have reflected patient selection bias, and that surgery in PM could be inconsistent with the Hippocratic oath.

Before initiating an RCT in epithelioid PM patients with a low Ki67 index, it is important to reconsider whether Ki67 is merely a prognostic marker. If possible, a thorough review of historical data is needed, focusing on patients eligible for surgery with low Ki67 scores who were not operated on, and comparing their survival outcomes with those of patients in our study. Perhaps there is only a role for surgery in PM in the context of tumor load reduction and thus impairment of immunosuppression, enhancing the effect of immunotherapies. More specifically, tumor load reduction could enhance the effect of DC therapy as discussed above.

Aggressive tumor growth and large tumor load are taken into consideration when thoracic oncologists choose for the addition of chemotherapy to CI therapy in first-line treatment in stage IV NSCLC. In a neo-adjuvant setting CI therapy is always given in combination with chemotherapy in NSCLC. In PM, we have shown that relative slow proliferation as marked by low Ki67 expression results in better effect of eP/D. Perhaps this specific group might benefit from immunotherapy in a (neo-)adjuvant setting, such as DC therapy, considering this might be a tumor with a less aggressive tumor growth. Perhaps a study like the ENSURE study should be done specifically in patients with a low ki67 expression. On the other hand, patients with a high ki67 and thus quickly proliferating tumor cells, might derive benefit from upfront chemotherapy instead of DC therapy and surgery. Whether this should be combined with CI therapy, DC therapy is up for debate. In addition, as chemotherapy is capable of reducing Ki67 expression,

one could argue if a chemotherapy-reduced Ki67 expression makes patients eligible for eP/D.

Future perspectives

This thesis sought to elucidate the biological mechanisms that hamper responses to (immuno)therapy in PM and tried enhancing therapeutic efficacy of DC and CI therapy by addressing potential efficacy barriers. Our findings underscore that PM is a heterogeneous disease, with a subset of patients demonstrating potential benefits from CI therapy or DC therapy. Identifying patients unlikely to benefit from specific therapies is critical, given the generally low mOS in PM and the importance of maintaining quality of life for patients in their final stages.

Given the multitude of biomarker studies and the limited number of biomarkers that reach clinical application, future research might benefit from emphasizing the underlying biological mechanisms that explain why patients with specific biomarker values exhibit differential responses. Understanding why a particular parameter is changing and how this change leads to an improved or worse clinical response has greater potential to influence treatment and the development of new therapies than merely identifying biomarkers. There is a crucial role for comprehensive immunomonitoring with a holistic approach as the TME, peripheral blood assessments, as well as the patient's clinical performance predict outcome. In addition, we need to be willing to adjust to paradigm shifts, for example new histological subtyping of a tumor.

DC therapy has led to promising clinical results in a phase I trial in PM and in phase II trials in pancreatic cancer and PeM, DC therapy has induced radiological responses in PM and a promising 64% 2-year recurrence-free survival in pancreatic cancer when administered as adjuvant treatment after surgery. However, DC therapy has not yet reached its full potential in the phase III DENIM trial. As discussed, the immunosuppressive TME in PM can be targeted with systemic therapies and the DC product itself can be enhanced to improve outcomes to DC therapy. The most important improvement in outcome might result from better timing of administration. Evolving insights on vaccination highlight the importance of initiating treatment at the earliest possible stage. However, the current research landscape primarily restricts the development of novel drugs when standard -of-care treatments have been administered. This requirement is sensible for systemic agents that should be given for months or until disease progression and have potential treatment-related adverse events that cause missing out on standard-of-care treatments. For vaccination therapies that can be administered in weeks with minimal treatment-related adverse events, testing in earlier disease stages in combination with standard-of-care treatment might yield better results. The most logical and immediate step in the current treatment landscape would be to integrate DC therapy with CI

combination therapy as a first-line treatment approach. In this way DC therapy is given at the earliest stage possible and CI therapy might enhance induction of the anti-tumor immune response by blocking inhibitory signaling in the lymph node. The subsequent increase of tumor-specific cytotoxic T cells might increase the effect of CI therapy.

I would like to conclude with a minor remark on our role in medicine and medical research as doctors. One excerpt from the Hippocratic oath is as follows: "I will apply, for the benefit of the sick, all measures that are required, avoiding those twin traps of overtreatment and therapeutic nihilism." I cannot ignore the fact that we occasionally fail to adhere to this principle. I am not only referring to the issue of over-treatment of individual patients, but also to the repetitive studies using the same pharmaceutical agents. When we engage in mechanistic thinking and attempt to build upon previously conducted studies, there is no need to subject a large number of patients to investigate outcomes we already anticipate.

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A

Appendices

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Nederlandse samenvatting

Inleiding

Pleuraal mesothelioom (PM) is kanker van het borstvlies. Bij 90% van de patiënten is er sprake van asbest expositie in het verleden, daarom wordt het ook wel asbestkanker genoemd. PM is een lastig te behandelen maligniteit met een zeer slechte prognose. De mediane overleving voor alle PM patiënten tezamen is ongeveer 9 maanden vanaf het moment dat de diagnose gesteld wordt. Wanneer we kijken naar de patiënten die nog in aanmerking komen voor behandeling is dit iets langer. Ten tijde van de start van dit proefschrift bedroeg de mediane overleving voor patiënten die chemotherapie kregen tussen de 12-14 maanden. Immunotherapie, met name de "checkpoint inhibitors" (CI), liet al prachtige resultaten zien bij de behandeling van niet-kleincellig longkanker (NSCLC) en melanoom. Helaas bleef het indrukwekkende klinische effect uit bij de behandeling van PM. Fase 3 studies waarbij CI, zoals anti-CTLA-4 of anti-PD-(L)1, werden gebruikt in PM lieten geen verbetering zien van de mediane overleving. Tijdens het schrijven van dit proefschrift is er een studie uitgekomen waarbij de CI, anti-CTLA-4 en anti-PD-1, gecombineerd werden bij patiënten met PM. Dit heeft wel geleid tot een verbetering van de mediane overleving tot 18 maanden ten opzichte van 14 maanden bij de klassieke chemotherapie behandeling. Echter zien we in mindere mate de langdurig aanhoudende respons op therapie zoals bij NSCLC.

Het mechanisme van CI berust voornamelijk op ontketenen van een reeds bestaande immuun respons tegen de tumor. Hier is dus een actieve anti-tumor respons voor nodig met cytotoxische T cellen die de tumor herkennen en attaqueren. Hoe meer mutaties een tumor cel bevat, hoe meer potentiële antigenen de tumor cel tot expressie kan brengen, welke herkend kunnen worden door cytotoxische T celen. Deze cytotoxische T cellen moeten wel eerst in de lymfeklier geactiveerd worden door dendritische cellen. Deze dendritische cellen fagocyteren de necrotische tumor cellen en brengen vervolgens de tumor antigenen op hun eigen cel oppervlak tot expressie. Hierna migreren de dendritische cellen van de tumor naar de lymfeklier om de tumor antigenen te presenteren aan cytotoxische T cellen. Wanneer de cytotoxische T cel het tumor antigeen herkent, zal deze ook geactiveerd worden, zich prolifereren en de lymfklier verlaten om op zoek te gaan naar tumor cellen. In PM ligt het aantal mutaties vrij laag en is er met name een verminderd aantal dendritische cellen, welke ook minder goed functioneren. Wellicht dat we voor immunotherapie in PM dus niet een bestaande anti-tumor respons moeten ontketenen middels CI, maar de anti-tumor respons moeten initiëren met dendritische cel therapie.

In dit proefschrift ligt de nadruk op het onderzoeken en begrijpen van het onderliggende mechanisme van respons op immunotherapie in PM, waarbij we ons in het bijzonder

focussen op dendritische cel therapie. Daarnaast bekijken we retrospectief het verloop van de incidentie en behandeling van PM in de afgelopen decennia in **Hoofdstuk 2.** Naast systemische therapie voor PM richten we onze blik ook nog op de rol van chirurgie in PM in **Hoofdstuk 9**.

Historisch besef en trends in incidentie en behandeling van PM

Zoals reeds genoemd is PM gerelateerd aan asbest expositie, waarbij de latentie periode 30-50 jaar is. Gezien het feit dat er in Nederland sinds 1993 een verbod is op het gebruik van asbest, werd aangenomen dat de piekincidentie rond 2020 zou liggen. In **Hoofdstuk 2** onderzochten we de trends in incidentie en behandeling van 1993 tot en met 2018. Hierin beschrijven we dat incidentie van PM niet meer stijgt sinds 2010 en er een niet-significante dalende trend waargenomen is. De dalende trend is nog niet significant, omdat er in de leeftijdsgroep van 80+, waar veel mensen onbeschermd zijn blootgesteld aan asbest, nog een stijging wordt gezien. In alle andere leeftijdsgroepen (0-54, 55-64, 65-79) zien we wel een significante daling van incidentie en kunnen we dus verwachten dat dit ook zo zal zijn in de toekomst voor de 80+ groep. Er is wel een goede verklaring waarom de incidentie van PM eerder lijkt te dalen dan we zouden verwachten op basis van de latentie tijd en het asbest verbod in 1993. Het asbest gebruik is namelijk streng gereguleerd sinds 1978 waarbij de import en het verwerken van asbest sterk verminderde. Deze maatregelen werden genomen als gevolg van het proefschrift van J. Stumphius (1969) waarin onomstotelijk de correlatie tussen asbest expositie en PM werd aangetoond.

Daarnaast hebben we in **Hoofdstuk 2** gekeken hoe PM in de afgelopen decennia in Nederland werd behandeld. Nadat er in een fase III studie in 2003 (Vogelzang et al.) werd bewezen dat combinatie chemotherapie de mediane overleving met 3 maanden verlengd tot een totaal van 12 maanden, is er een significante stijging in het gebruik van chemotherapie en overleving te zien. Desalniettemin wordt er nog steeds 'maar' bij 40% van de PM patiënten chemotherapie voorgeschreven, wat het gevolg zou kunnen zijn van late diagnostiek ofwel goede "advanced care planning". De rol van chirurgie is in Nederland minimaal, waarbij maar 3% van de patiënten wordt geopereerd. In Amerika is dit ongeveer 25%. Onderzoeksresultaten waren tot voor kort ook contrasterend over het klinische voordeel van chirurgie, wat heeft geleid tot positievere adviezen in de Amerikaanse richtlijn dan in de Britse (Europese) richtlijn. Een recent gepubliceerde fase III gerandomiseerde studie (MARS 2) heeft aangetoond dat chirurgie leidt tot een kortere overleving en meer morbiditeit. Hierdoor lijkt de rol van chirurgie wellicht niet meer houdbaar in PM.

Immunotherapie in PM

Checkpoint remming

Na de studie van Vogelzang et al. in 2003 is er 18 jaar geen fase 3 studie geweest welke verbetering van de mediane overleving liet zien. In deze periode is er in andere solide tumoren zoals melanoom en NSCLC enorme vooruitgang geboekt met CI. Derhalve zijn CI ook intensief onderzocht in PM. In de periode dat deze studies liepen, was er ook de mogelijkheid om deze middelen in een "patient-acces program" aan patiënten met PM te geven. Binnen het Erasmus MC (Rotterdam) en het Nederlands Kanker Instituut (Amsterdam) zijn PM patiënten behandeld met nivolumab (anti-PD-1) in de tweede of derde lijn na progressie op chemotherapie. In **hoofdstuk 3** laten we zien dat de mediane progressie-vrije overleving (2,3 maanden) en mediane overleving (6,7 maanden) teleurstellend zijn. Ondanks de teleurstellende resultaten voor de gehele groep, zagen we dat de patiënten met een radiologische respons (10%) na behandeling met CIs een significant langere mediane overleving hebben. Hoewel dit logisch lijkt, is het een bevestiging dat er een kleine groep patiënten is die daadwerkelijk klinisch voordeel heeft van CI behandeling. Wanneer we begrijpen waarom deze patiënten responderen op CI behandeling, kan dit zorgen voor verbetering van klinische uitkomsten en het voorkomen van onnodige bijwerkingen bij patiënten die geen baat hebben bij CI behandeling. In **hoofdstuk 3** zien we ook dat patiënten met een laag albumine gehalte in het bloed een kortere mediane overleving hebben dan patiënten met hoog albumine. Het lage albumine gehalte zien we vaak bij patiënten met kanker als gevolg van een verhoogd metabolisme. Dit verhoogde metabolisme kan mogelijk ook de oorzaak zijn van een snellere afbraak van CIs, waardoor deze minder hun werk kunnen doen.

We weten dat de rol van cytotoxische T cellen cruciaal is voor een anti-tumor respons. Daarnaast is inmiddels ook duidelijk dat de aanwezigheid van B cellen in en rond de tumor ook cruciaal is voor anti-tumor immuniteit, maar het exacte mechanisme waarmee de B cellen bijdragen aan de anti-tumor immuniteit is nog niet duidelijk. In NSCLC is er een positieve correlatie tussen geheugen B cellen in tertiaire lymfoïde structuren in nabijheid van de tumor en respons op CIs. Echter zien we in NSCLC relatief meer dubbel negatieve (CD27-IgD-) (DN) B cellen en minder geheugen B cellen dan in gezond longweefsel. Deze DN B cellen afkomstig uit NSCLC tumoren activeren ook meer regulatoire T cellen dan cytotoxische T cellen, wat theoretisch zorgt voor meer immunosuppressie dan anti-tumor activiteit. In perifeer bloed zijn DN B cellen beschreven in patiënten met chronische infecties, waar ze in overmaat aanwezig zijn en worden beschreven als "uitgeput" en mogelijk verminderd functioneren. De rol van de DN B cel in perifeer bloed in PM en NSCLC was in de literatuur nog niet beschreven. In **hoofdstuk 4** laten we zien dat, zoals verwacht, het totaal aantal B cellen in perifeer bloed positief correleert met respons op CI in NSCLC en PM. We zien dat patiënten die geen baat hebben bij CI behandeling, juist relatief hoge aantallen

CD21- DN B cellen hebben in perifeer bloed. Het exacte mechanisme achter deze correlatie is nog onduidelijk en zou te maken kunnen hebben met de eerder verschenen literatuur over DN B cellen in NSCLC tumoren die regulatiore T cellen activeren. De rol van DN B cellen in chronische infecties zou ook nog een mogelijke verklaring kunnen geven voor de gevonden correlatie, waarbij de B cellen "uitgeput" zijn. Hierbij is de chronische blootstelling aan antigenen de overeenkomst tussen patiënten met kanker en chronische infecties, die als potentiële oorzaak dient voor de "uitputting" van de B cellen.

Dendritische cel therapie

Het combineren van CI (anti-CTLA-4 & anti-PD-1) blijkt in 2021 wel te leiden tot langere mediane overleving dan chemotherapie in PM. De verbetering in OS van 14 naar 18 maanden lijkt voornamelijk terug te leiden op het feit dat patiënten met niet-epitheliale tumoren beter reageren op de immunotherapie dan op de chemotherapie. Daarnaast blijkt uit exploratieve analyses dat patiënten met een hoge inflammatie score, meer infiltratie van cytotoxische T cellen, beter reageren op CI combinatie therapie. De werking van checkpointremming is dus afhankelijk van de aanwezigheid van tumor-specifieke T cellen. In PM zijn er relatief weinig dendritische cellen (DCs) en functioneren deze minder goed. Gezien DCs verantwoordelijk zijn voor de activatie van tumor-specifieke cytotoxische T cellen, zou het goed kunnen dat de anti-tumor immuunrespons in PM onvolledig is. Middels DC therapie, waarbij we de DCs *in vitro* activeren, matureren en vervolgens injecteren bij de patiënt, kunnen we een anti-tumor respons induceren. In fase 1 studies is reeds getoond dat dit veilig is en dat er bij een deel van de patiënten ook een radiologische respons is op de DC therapie, wat uiteindelijk heeft geleid tot de start van een fase 3 studie (DENIM studie).

In **hoofdstuk 5** wordt de DENIM studie beschreven en laten we zien dat DC therapie als onderhoudstherapie na standaard eerstelijns chemotherapie niet leidt tot een verlenging van de mediane overleving. Een positief resultaat van deze studie is dat we voor het eerst een correlatie aantonen tussen DC therapie-geïnduceerde immuun cel activatie, CD4+ geheugen T cellen, en mediane (progressie-vrije) overleving. Mogelijk is er dus bij een groot aantal patiënten onvoldoende immuun cel activatie, waardoor er geen klinisch voordeel is in overleving. Er zijn meerdere verklaringen mogelijk voor de verminderde DC therapie-geïnduceerde immuun cel activatie; te lang interval tussen de chemotherapie en DC therapie (onderhoudstherapie), gebrek aan CD8+ T cel activatie en/of onvoldoende migratie van de DCs naar de lymfeklier. Daarnaast kan een klinische response uitblijven door remming van de tumor-specifieke T cellen door immunosuppressieve cellen in de "tumor micro environment" (TME) of door checkpoint remming van de tumor cellen middels PD-L1.

Combineren van dendritische cel therapie met andere behandelingen

Er zijn tal van barrières die moeten worden geslecht voor een optimale werking van DC therapie. Hierbij kan je denken aan verbetering van de activiteit van de DCs zelf of verbeterde migratie van de DCs naar de lymfeklier, maar ook aan de immunosuppressieve werking van de TME en de checkpoint remming van de tumor cellen. In **hoofdstuk 6** bespreken we hoe we deze hordes kunnen nemen door gebruik te maken van reeds bestaande therapieën, zoals chemotherapie, radiotherapie en immunotherapie. Met name CI en DC therapie zouden theoretisch synergistisch kunnen werken. DC therapie activeert namelijk tumor-specifieke cytotoxische T cellen in de lymfklier welke daarna migreren naar de tumor en in principe de tumor cellen zullen attaqueren. Wanneer de tumor cel de cytotoxische T cel probeert te deactiveren door middel van checkpoint remming (PD-L1), kunnen CIs deze remming stoppen waardoor de cytotoxische T cellen actief blijven.

Specifiek de potentiële synergie van CI en DC therapie onderzochten we zelf verder in **hoofdstuk 7**, waarin we laten zien dat CI behandeling na DC therapie veilig en uitvoerbaar is en leidt tot klinisch veelbelovende resultaten bij patiënten met PM. Daarnaast tonen we in muizen aan dat gelijktijdige behandeling middels DC therapie en CIs resulteert in een langere overleving dan wanneer we beide therapieën alleen geven. Gezien de huidige eerstelijns behandeling voor PM patiënten bestaat uit dubbele CI behandeling, zou gelijktijdige DC therapie hier een aanvulling op kunnen zijn. Uiteraard is hier aanvullend onderzoek voor nodig om de veiligheid en effectiviteit uit te zoeken.

De “tumor load”, en dus het aantal tumor cellen en potentieel immunosuppressieve cellen in de TME, correleert met de mate van tumor-geïnduceerde immunosuppressie wat resulteert in een remming van de anti-tumor respons. In muisstudies hebben we laten zien dat het vroegtijdig vaccineren, wanneer de “tumor load” lager is, resulteert in een langere overleving. Derhalve zijn we een studie (ENSURE studie) gestart waarbij we chirurgie combineren met DC therapie. In deze studie wordt DC therapie voor en na chirurgie gegeven, waardoor we ook kunnen kijken naar het effect van DC therapie op tumor niveau. Inmiddels is er 1 patiënt geïncludeerd waarvan de resultaten in **Hoofdstuk 8** laten zien dat er 15 maanden na start van de behandeling nog geen terugkeer van ziekte is. Daarnaast zien we op het oog een toegenomen infiltratie van cytotoxische T cellen in de tumor evenals een toename van tertiaire lymfoïde structuren na DC therapie. Wanneer we specifiek kijken naar de T cellen in de tumor ná DC therapie, zien we specifieke T cel klonen die er vóór start van de DC therapie niet zaten. Deze klonen zien we wel terug in een huidtest waarin alleen DC therapie-specifieke klonen zitten. Dit is zeer suggestief voor het feit dat er na DC therapie infiltratie is van DC therapie-specifieke T cellen in de tumor. Gezien deze resultaten van maar 1 patiënt zijn, moeten we deze resultaten voorzichtig interpreteren en meer patiënten includeren voor een bevestiging van bovenstaande.

Chirurgie in PM

De rol van chirurgie in PM is lang onduidelijk geweest, waarbij er gebrek was aan een gerandomiseerde studie die het voor- of na- deel van chirurgie zou laten zien. In Nederland werd er bij geselecteerde patiënten nog wel chirurgie toegepast in de vorm van een uitgebreide pleurectomie en decorticatie (eP/D) in combinatie met systemische therapie. De resultaten na chirurgie zijn zeer wisselend en wij wilden uitzoeken in **Hoofdstuk 9** of we konden voorspellen welke patiënten baat hebben bij eP/D. We weten dat patiënten met PM die een snel prolifererende tumor hebben, lees een hoge expressie van proliferatie marker Ki67, het prognostisch slechter doen dan patiënten met een lage Ki67 expressie. Na retrospectieve analyse van de data van de patiënten die eP/D hebben ondergaan in het Erasmus Medisch Centrum, blijkt dat de groep patiënten met een lage expressie van Ki67 door de tumor cellen, een significant langere mediane overleving heeft dan de groep met een hoge Ki67 expressie. Het is tot op heden nog onduidelijk of Ki67 alleen een prognostische marker is of dat het ook daadwerkelijk een predictieve waarde heeft bij PM patiënten die eP/D ondergaan.

Na deze publicatie, zijn de resultaten van de MARS 2 studie gepubliceerd. Dit was een fase 3 gerandomiseerde studie, waarbij chemotherapie werd vergeleken met chemotherapie in combinatie met chirurgie. Hieruit blijkt dat chirurgie in combinatie met chemotherapie in PM leidt tot een hogere mortaliteit en morbiditeit dan chemotherapie alleen. In het licht van deze resultaten is de rol van chirurgie voor PM onzeker en is er wellicht nog meer behoefte aan patiënten selectie.

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PhD portfolio

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Description		**EC**
		07-01-2025
Erasmus MC - BROK® (Basic course Rules and Organisation for Clinical researchers) (2018)		1.50
Erasmus MC - Follow-up Photoshop and Illustrator CC (2018)		0.30
Erasmus MC - Microsoft Excel 2010: Basic (2018)		0.30
Scientific Integrity (2018)	Erasmus MC Graduate School	0.30
Course Basic and Translational Oncology (2018)		1.80
Erasmus MC - Biomedical English Writing (2019)		2.00
Course on R (2019)		1.80
Erasmus MC - Advanced course on Applications in flow cytometry (2019)		0.50
WCLC 2019 (2019)		2.00
Cicon 2019 (2019)		0.60
Oral presentations for patient federation (2020)		1.00
Supervision of medical student (2020)		4.00
NRS educational program (2020)		5.00
Coaching bachelor students (2021)		3.00
WCLC 2020 (2021)		3.00
Weekly research meeting (2021)		2.00
Weekly meeting (2021)		2.00
ELCC 2023 (2023)		2.00
IMIG 2023 (2023)		1.20
Total EC		------ + **34.30**

Dankwoord

Een proefschrift zonder dankwoord is als een frikandel speciaal zonder uitjes, net niet af. Ik ben me bewust van het feit dat deze pagina's moeten compenseren voor de vele ongelezen pagina's elders in dit proefschrift en ik zal mijn uiterste best doen om het leeswaardig te maken.

Gezien ik tijdens mijn 3-jarige "carrière" als wedstrijdroeier kennis heb mogen maken met een vorm van fysieke zelfkastijding, wilde ik de mentale variant daarvan ook graag ervaren voor eenzelfde termijn. Wegens de onverwachte en bijzonder goede match tussen de wetenschap, basaal onderzoek en mijzelf, maar vooral vanwege lichte vertraging bij de inclusie van de DENIM studie, heb ik er 7 jaar van mogen genieten.

Een promotietraject dwingt je tot het ontwikkelen van zelfstandigheid, assertiviteit, geduld, incasseringsvermogen en doorzettingsvermogen. Het gaat niet altijd zoals gepland en 80% van je ideeën zien het daglicht niet. Precies om die reden is het voor mij essentieel geweest een fijne sfeer te hebben op werk en met name veel plezier, humor en een gezonde dosis relativeringsvermogen daarbuiten. Naast het feit dat ik ervan overtuigd ben dat interdisciplinaire samenwerking leidt tot grotere impact en verbetering van kwaliteit, had ik het überhaupt niet gekund zonder veel steun en hulp in het lab, de kliniek en buiten het ziekenhuis. Kortom, er zijn veel mensen die een directe of indirecte bijdrage hebben geleverd aan dit proefschrift en die ik daar graag voor wil bedanken.

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Verder heb ik geleerd dat ik ook moet reageren op mails met de aanhef Bib of Bop en heb ik een bijzonder talent ontwikkeld voor het lezen en ontcijferen van cryptische teksten zonder interpunctie. Hierbij verdenk ik je zelfs van opzet bij de multi-interpretabiliteit van de mails of opmerkingen in manuscripten om ons zelf te laten nadenken en de beslissing te laten maken.

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Curriculum vitae

Bob Robert Anton Belderbos was born on May 19th 1992 in Breda, the Netherlands. After graduating from secondary school (Stedelijk Gymnasium, Breda) in 2010, he studied medicine at Erasmus University Rotterdam. After he obtained his medical degree in 2017, the opportunity arose to do a PhD at the pulmonary department in the Erasmus Medical Center in Rotterdam, where he worked as a fulltime PhD student from March 2018 until March 2020 under the guidance of Prof dr. J.G.J.V. Aerts. With his special interest in thoracic oncology, the PhD focused on immunotherapy, more specifically dendritic cell therapy, in pleural mesothelioma. As a consequence of COVID-19, from March 2020 he started working in the clinic in the Erasmus Medical center and started his residency in September 2020 for pulmonary medicine in the Erasmus Medical center and continued his PhD during his residency. He currently has 2 years left in his residency.

Between 2011 and 2014 Bob has rowed at a top-level at A.R.S.R. Skadi and was selected for the R.V. Minerva crew to participate in the Head of the Charles Regatta, Boston U.S.A. After stopping with rowing, Bob initiated a biking trip from Rotterdam to Barcelona to collect funds for the “Fonds Gehandicaptensport”.