EMERGING THERAPIES FOR MALIGNANT MESOTHELIOMA

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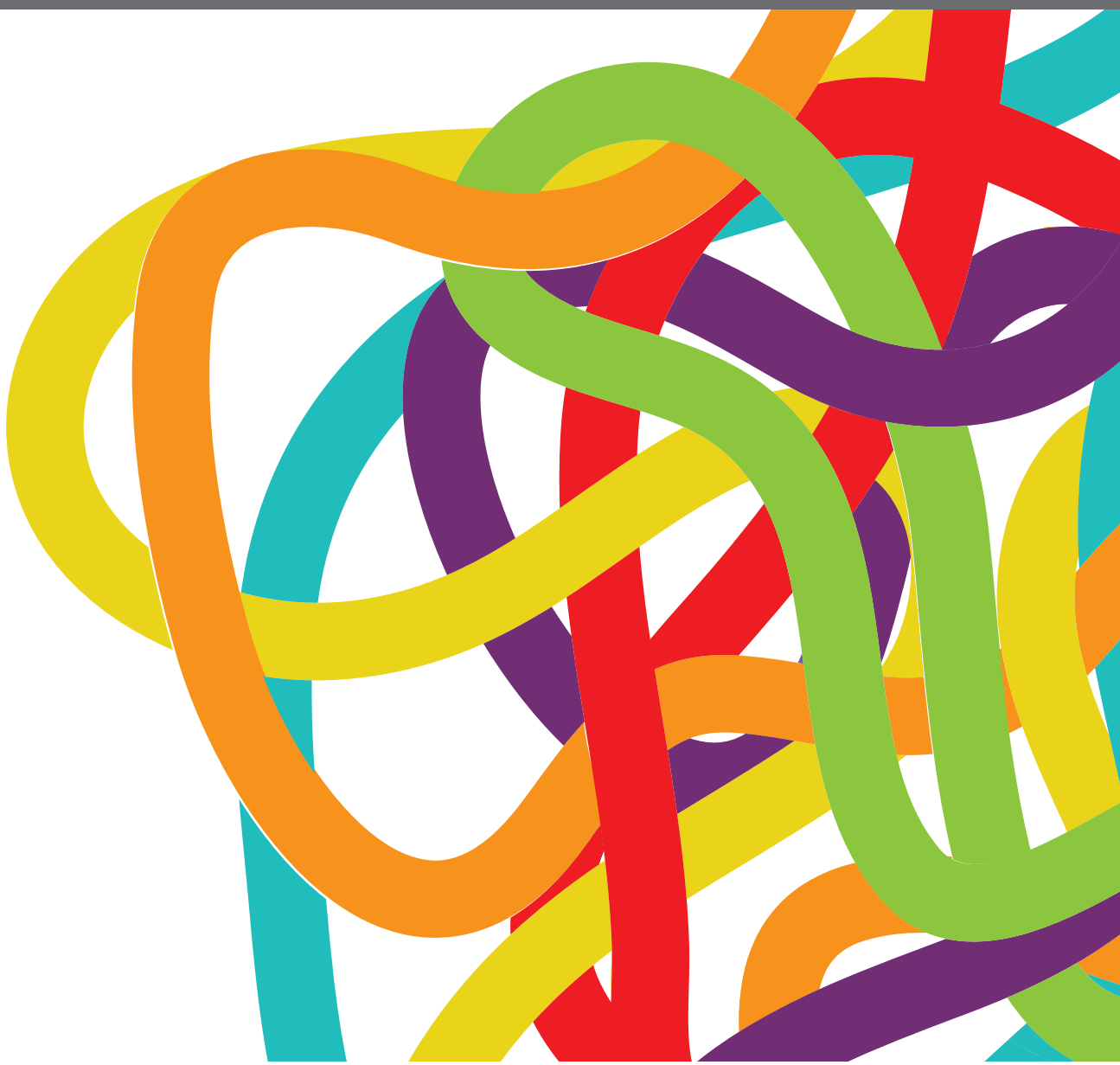

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EMERGING THERAPIES FOR MALIGNANT MESOTHELIOMA

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EDITORIAL
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Editorial: Emerging Therapies for Malignant Mesothelioma

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Keywords: malignant mesothelioma, tumor biology, preclinical models, immune environment, angiogenesis inhibitors, chemotherapy, immunotherapy, microRNA

Editorial on the Research Topic

Emerging Therapies for Malignant Mesothelioma

Malignant mesothelioma, resistant to most currently available therapies, is associated with the lowest survival rates of any major cancer type. Despite intensive efforts, significant improvements in patient outcomes have remained out of reach. Although some activity of experimental approaches such as intrapleural pro-inflammatory cytokines was noted in the 1990s (1–4), chemotherapy-based therapies, surgery and radiotherapy dominated clinical research into mesothelioma treatment through the 1990s and 2000s. This was particularly the case after the pemetrexed/cisplatin combination was established as the backbone of systemic therapy for malignant pleural mesothelioma (MPM) in 2003 (5). Although radical surgery continues to be associated with superior survival figures, it is unable to shift survival beyond the 2-year mark (6) and the reality is that <10% of patients will be judged eligible for radical multimodality therapy. Moreover, the peri-operative mortality of extra-pleural pneumonectomy turned out to be considerable, eliciting discussions about acceptable levels of surgical morbidity/mortality and the feasibility of aggressive multimodality approaches (7–9).

It has taken many years for mesothelioma research to take a different direction, and this has largely followed advances in the treatment of other cancer types. However, despite the promise of these new approaches, failures have outnumbered successes. In stark contrast to the beneficial effects of targeted therapy in non-small cell lung cancer and other cancers driven by mutated oncogenes, targeted therapy approaches were largely unsuccessful in MPM. Despite frequent overexpression of EGFR in MPM, TKIs, and antibodies blocking the receptor lacked sufficient clinical activity. The addition of bevacizumab to pemetrexed/cisplatin led to a significant survival advantage, this gain was only a modest 3 months (10). In retrospect, these observations should not have surprised us, considering the relatively low mutational burden in mesothelioma and relative lack of oncogenic drivers (11, 12).

After little improvement in patient outcomes despite the intensive efforts of the past two decades, the recent advances using novel clinical and experimental approaches for MPM provide new hope. The rapid changes in prognosis of melanoma and non-small cell lung cancer as a consequence of treatment with immune-checkpoint inhibitors have now found their way into the mesothelioma field (13). As a consequence of some positive studies in the second-line setting, the National Comprehensive Cancer Network (NCCN) guidelines have recently accepted pembrolizumab and nivolumab with or without ipilimumab as salvage therapy (NCCN guidelines Version 2.2019-April 1, 2019). At the same time the mesothelioma community is also paying attention to other immunotherapy approaches, such as tumor vaccines, immunotoxins, and targeted T-cells.

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Additional experimental approaches including microRNA replacement therapy, have also shown signs of clinical efficacy (14).

Therefore, it is appropriate to review recent translational research studies and the early clinical experience with novel treatment approaches for mesothelioma. Thirty-five mesothelioma researchers from around the world have made a contribution, and it is a great privilege for the editors to introduce this series of 10 articles which summarize our increasing insight into mesothelioma biology and the gradual change in treatment approaches for MPM.

Our article collection begins with pre-clinical lab studies before discussing new clinic approaches. Testa and Berns are the first to review rodent models that have greatly assisted in increasing our understanding of the pathophysiology of mesothelioma. Blanquart et al. have a similar goal and discuss the pros and cons of the different preclinical mesothelioma models used, including organoids. In an opinion paper, Felley-Bosco and Gray concentrate on tumor suppressor genes, ferroptosis, and resistance of mesothelial cells against apoptosis. Chu et al. seek explanations for the mixed results of immunotherapy trials by reviewing the tumor micro-environment of mesothelioma, and Reid et al. highlight the potential of restoring levels of tumor-suppressive microRNAs in MPM in the lab and clinic.

The strong rationale behind the inhibition of angiogenesis in a highly inflammatory tumor such as mesothelioma is detailed by Nowak et al. while de Gooijer et al. provide an overview of the rapidly expanding clinical experience with immune checkpoints inhibitors in MPM. The promise of cellular immunotherapy in MPM is given by Belderbos et al.. Finally, the last two decades of clinical trials in MPM are comprehensively reviewed by two separate groups (Cantini et al.; Nicolini et al.). Both reviews underline the importance of well-designed clinical trials to improve treatment outcomes in MPM and to incorporate biomarkers validated in the translational setting. Considering past experience, it is very unlikely that we will discover a one-size-fits-all therapy for MPM patients. However, with the spectacular increase in translational mesothelioma data witnessed in the last decade, there is hope that this will eventually translate into better treatment outcomes for patients affected by one of the most recalcitrant solid tumors.

AUTHOR CONTRIBUTIONS

NZ, GR, and PB contributed to the writing and reviewing of this editorial.

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OPINION
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Mesothelioma Driver Genes, Ferroptosis, and Therapy

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If a given cell has a propensity to die in a certain manner, the logical step for this cell to become a cancer cell is to insure its survival by installing mechanisms circumventing the predestined regulated cell death. A clear example of this occurs in follicular lymphoma where chromosomal re-arrangements result in Bcl2 overexpression, allowing escape from apoptosis and tolerance to undesired generation of otherwise physiological mutations and double strand breaks necessary to produce the variability necessary for antigen recognition site by immunoglobulin (1).

The predestined regulated cell death mechanism for mesothelial cells is not known, but recent data have linked two frequent drivers of mesothelioma, *NF2* and *BAP1* (2, 3), to ferroptosis (4, 5). The latter is a more recently described type of iron-dependent regulated cell death (6).

An additional driver of mesothelioma, which is however less specific to this cancer type, is loss of *CDKN2A* (7–9). One of the products encoded by *CDKN2A* gene is p16, which is one of the effectors of senescence (10). The latter is a state of stable cell cycle arrest with active metabolism where resistance to ferroptosis induction has been observed due to decreased iron bioavailability, linked to increased ferritin (*FTH1*) levels, and accompanied by increased levels of iron regulatory protein 2 (*IREB2*) and decreased levels of iron-cluster assembly enzyme (*ISCU*) (11).

The aim of this Opinion paper is to complement the editorial by Fennell (12) with some additional considerations, which include potential ideas regarding treatment, based on data from our own model of mesothelioma development (13) and the mesothelioma TCGA database (3).

In ferroptosis (**Figure 1A**), cell death is executed by reactive oxygen species (ROS)-mediated peroxidation of polyunsaturated fatty acids (PUFAs). The origin of ROS includes incomplete reduction of oxygen during electron transport to form superoxide, and a direct generation of superoxide by the membrane bound NADPH oxidases (NOX) (14). Lipid peroxidation is prevented by glutathione peroxidase 4 (GPX4), which uses glutathione (GSH) as reducing agent [reviewed in (15)]. GSH is synthesized from cysteine, which is either derived from methionine through methionine-R-sulfide reductase B2 (MSRB2), or it is imported. Interestingly, *MRSB2* expression is significantly higher in epithelioid compared to tissues with a sarcomatoid molecular profile (2). Import of cysteine is mediated by SLC7A10 transporter, but cysteine can also be derived from the reduction of cystine (product of the oxidation of two cysteine molecules, which are then linked via a disulfide bond). Cystine is transported into the cell through the system Xc^- transporter, which includes SLC7A11 subunit. It is worth noting that only cystine is present in cell culture medium, and, as for cells like lymphocytes [reviewed in (16)], mesothelial and mesothelioma primary cells grow better in the presence of beta-mercaptoethanol (17, 18). This effect is likely due to formation of beta-mercaptoethanol dimers with cystine facilitating its uptake by other transporters (19).

BAP1 decreases the expression of SLC7A11 (5), leading to increased sensitivity to ROS and erastin in mesothelioma cells.

PUFA abundance, and hence predisposition to ferroptosis, is dependent on the expression of acyl-CoA synthetase long-chain family member 4 (ACSL4). In the absence of a negative control downstream NF2/Hippo pathway, the transcriptional co-activator YAP increases *ACSL4* expression (4). Resistance to ferroptosis is associated with high expression levels of *aldo-keto reductase*

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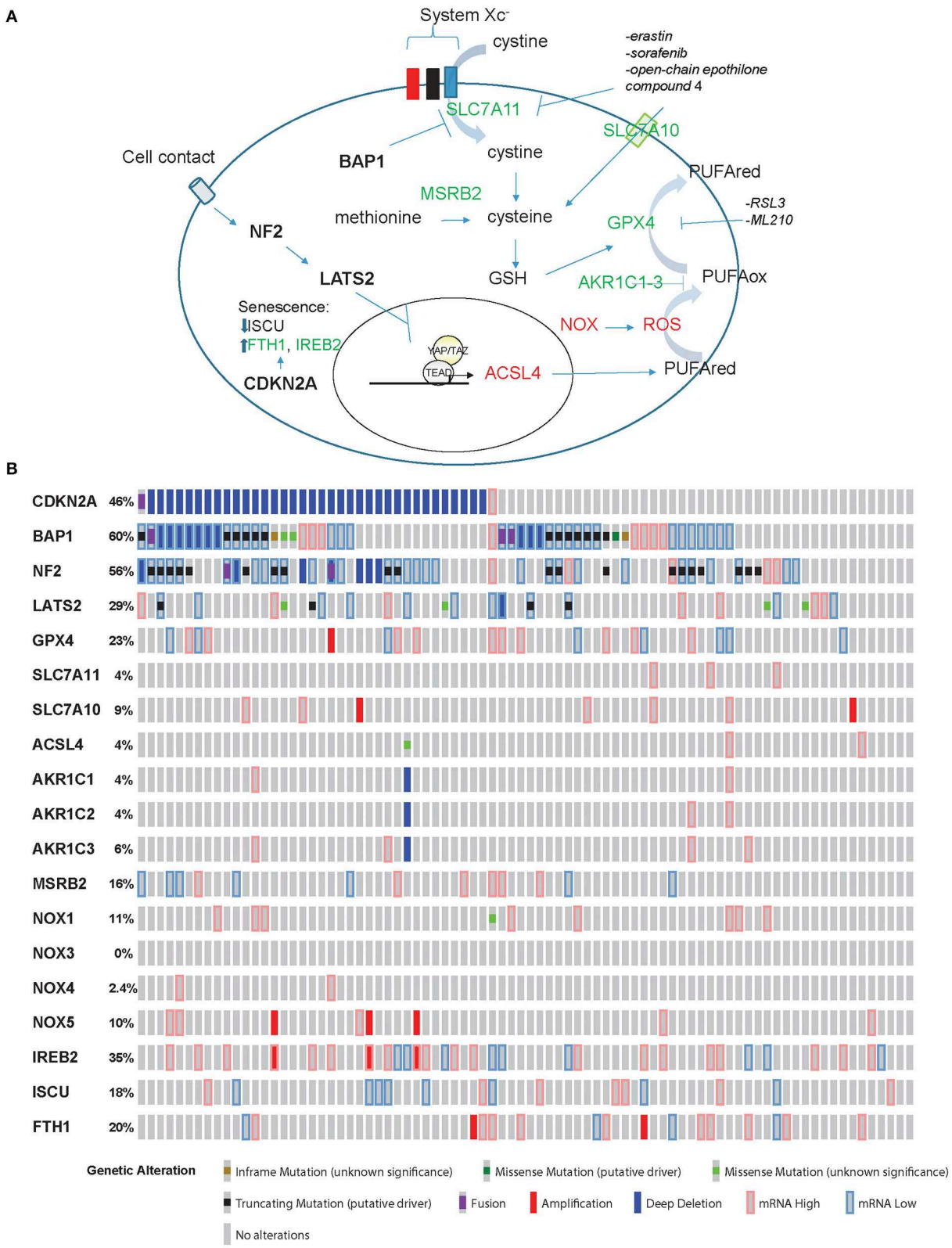

FIGURE 1 | Ferroptosis effectors in mesothelioma. **(A)** Model for ferroptosis pathway. Promoters of ferroptosis (red) include ACSL4, NOX, and ROS, while SLC7A11, SLC7A10, MSRB2, GPX4, and AKR1C1-3 (green) are ferroptosis scavengers. *ACSL4* expression is activated by YAP/TAZ while BAP1 inhibits the expression of *SLC7A11*. CDKN2A-encoded p16 is one of the effectors of senescence where ferroptosis is prevented by increased expression of FTH1 and IREB2 accompanied by decreased levels of ISCU. **(B)** "Oncoprint" analysis of ferroptosis effectors in TCGA data performed using cBioportal (www.cBioportal.org).

1-3(AKR1C1-3) (20). These enzymes have been shown to participate in the detoxification of reactive aldehyde generated downstream of the oxidation of various PUFA.

Taking into account all this information, a mesothelial cell losing BAP1 function becomes resistant to ROS and ferroptosis, while mesothelial cells losing NF2 function become "primed" for ferroptosis, while loss of p16 expression will be associated with impaired senescence-driven ferroptosis resistance.

Loss of BAP1 is mostly associated with epithelioid histotype (21), while loss of NF2 function is mostly associated with high S-score, which identifies tumor samples with a high sarcomatoid phenotype component (22). This is consistent with the observation that cells in a mesenchymal state, which are less sensitive to chemotherapeutics, have been shown to rely on GPX4 function to avoid ferroptosis (23–25). Intriguingly, Nagai et al. observed that iron chelation did not prevent mesothelioma development in rats upon exposure to asbestos fibers, but tumor histotype shifted toward increased incidence of epithelioid compared to the sarcomatoid histotype observed in the control group (26). In the absence of accompanying genomic alteration analysis of those tumors it is not possible to know whether the two groups had a different genetic alteration profile or whether there was a plasticity response of cancer cells to the environment.

Recently, in our own model of mesothelioma development (13) we observed a significant ($p = 0.008971$, FDR = 0.0145) 1.4-fold increase of *Acsl4* and a significant 74 and 91% decrease of *Gpx4* (p = 6.28E-22, FDR = 8.19E-21) and *Msrb2* (p = 1.38E-88, FDR = 4.95E-86) expression, respectively, when comparing tumors to inflamed precancerous lesions. Hence, these tumors should be predisposed to ferroptosis death, as expected from their spindeloid phenotype and YAP activation. However, *Slc7a11* undergoes a significant ($p = 0.004227$, FDR = 0.007263) 4.7-fold upregulation as well, consistent with the loss of one BAP1 allele. Collectively, these observations suggest that tumors with alterations in both pathways, NF2 and BAP1, which occur in a significant fraction of MPM patients according to TCGA data (3) (**Figure 1B**), might be more resistant to ferroptosis. However, functional studies are necessary to verify this hypothesis.

Drugs modulating ferroptosis have been recently reviewed (27). Inhibitors of GPX4, such as Ras-selective-lethal 3 (RSL3) or ML210, trigger ferroptosis, while SLC7A11 inhibiting agents, such as erastine or sorafenib, lead to glutathione depletion and endoplasmic reticulum stress. The mechanism behind sorafetinib inhibition of cysteine Xc^- transporter is not clear and is possibly indirect (20). Dr. Fennell pointed to two clinical trials in mesothelioma (28, 29), where sorafenib was used and in which objective responses were observed in only in a small proportion of unselected patients. Therefore, it will be necessary to have a translational study accompanying these trials to determine if those patients that responded had a disrupted NF2/Hippo pathway.

Relevant for the current first-line therapy of mesothelioma patients, which includes cisplatin, erastin has been shown to have a synergistic cancer cell killing effect with cisplatin in *in vitro* models (30).

Remarkably, in a recent study ferroptosis was observed in cells treated with some open-chain epothilones small molecules in a manner similar to that of erastin (25). Additionally, mesothelioma cell killing is iron-dependent in a novel therapeutic approach using atmospheric plasma therapy (31). Plasma is the fourth condition of physical state, in addition to solid/liquid/gas [reviewed in (32)].

Given the propensity of mesenchymal cells to be sensitive to ferroptosis induction, it is tempting to suggest that mesothelioma patients with high S-score might benefit from this novel therapy. However, a plethora of novel therapies for mesothelioma have emerged (33–35) and it might be worth assessing whether mesothelioma cells can undergo ferroptosis *in vivo*. Indeed, it must be noted that Carbonic anhydrase 9 (CAIX) has recently been shown to confer resistance to ferroptosis/apoptosis in malignant mesothelioma under hypoxia (36). Given that CAIX is ubiquitously highly expressed in mesothelioma (37, 38), this may have to be taken into account moving forwards. Because of the known effect of cisplatin on ROS generation [reviewed in (39)], it may also be of use to analyze the expression of *PTSG2*, encoding for COX-2, a marker of ferroptosis (40), in samples from these cisplatin-treated patients.

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The Immune Microenvironment in Mesothelioma: Mechanisms of Resistance to Immunotherapy

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Although mesothelioma is the consequence of a protracted immune response to asbestos fibers and characterized by a clear immune infiltrate, novel immunotherapy approaches show less convincing results as compared to those seen in melanoma and non-small cell lung cancer. The immune suppressive microenvironment in mesothelioma is likely contributing to this therapy resistance. Therefore, it is important to explore the characteristics of the tumor microenvironment for explanations for this recalcitrant behavior. This review describes the stromal, cytokine, metabolic, and cellular milieu of mesothelioma, and attempts to make connection with the outcome of immunotherapy trials.

Keywords: mesothelioma, microenvironment, immunotherapy, tumor-associated macrophages, myeloid-derived suppressor cells, T-cells

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INTRODUCTION

Malignant pleural mesothelioma (MPM) has a justified reputation for being resistant to therapy. Large case series of patients with mesothelioma indicate a median overall survival of only 9.5 months (1). The epithelioid histological subtype is the most common variant; it has polygonal, oval or cuboidal cells and is associated with a better median overall survival of 13.1 months (1, 2). However, the sarcomatoid variant with spindle-shaped cells has a median survival of only 4 months (1). Both surgery and radiotherapy have limited roles in the management of the disease (3). VEGF inhibition in combination with chemotherapy results in a modest increase in survival for patients with malignant pleural mesothelioma (4). However, the first randomized trial of immune checkpoint inhibition using tremulimumab, an anti-CTLA-4 antibody, failed to improve median overall survival (5). In addition, nintedanib, a multi-tyrosine kinase small molecule inhibitor targeting VEGFR1-3, PDGFRα/β and FGFR1-3 receptor signaling, did not prolong progression-free survival when added to chemotherapy (6). Various Phase 2 trials, such as the MAPS2 trial of nivolumab and ipilimumab, show promising activity and require confirmation in larger Phase 3 trials (7), While Phase 1 and Phase 2 trials of immunotherapies have produced modest signals to date, checkpoint inhibition in real-life clinical settings have reported limited effects. For example, in Phase 1b and 2 trials of pembrolizumab, the median survival is between 11.5 and 18 (8, 9), but median survival is only 7.2 months when prescribed off-label in palliative settings (10). Furthermore, the results from the randomized Phase 3 PROMISE-meso trial indicated that pembrolizumab was not superior to single-agent chemotherapy in pre-treated MPM

(11). While several trials using immunotherapy monotherapy, combination immunotherapy or immunotherapy in combination with chemotherapy are underway in mesothelioma, it is pertinent to examine the tumor immune microenvironment for explanations as to why mesothelioma is so resistant to therapy.

THE INFLAMMATORY RESPONSE AND CARCINOGENESIS

The inflammatory response to asbestos is a cardinal feature of mesothelioma's pathogenesis and microenvironment. The inflammatory response to asbestos fibers that reach the outer pulmonary parenchyma is one hypothesis for how amphibole fibers and fluid enter the pleural space in the first place (12). In addition, mesothelial cells in contact with asbestos fibers generate CCL2 (13), attracting macrophages which become embroiled in "frustrated phagocytosis" due to the size and biopersistence of amphibole fibers (12). Macrophage production of Reactive Oxygen Species (ROS) and nitrogen species augments the reactive oxygen/nitrogen species already catalyzed by the iron in asbestos fibers (14–18). The quantity of hydroxyl free radicals and nitric oxide free radicals have been associated with the extent of DNA strand breaks and gene deletions in cultured cell lines and are considered responsible for key mutagenic events (14, 15, 19).

Furthermore, cells which have sustained genotoxic damage would ordinarily undergo poly(ADP)ribose polymerase-induced programmed cell death (20) but are "rescued" by aspects of the inflammatory response. For example, macrophages are key producers of TNF-α (17), not only as a consequence of frustrated phagocytosis (21), but also in response to the release of High Mobility Group Box 1 from mesothelial cells undergoing programmed cell death (20). TNF-α acting on upregulated TNF-α receptors and the NF-κB pathway can protect human mesothelial cells from cell death *in vitro* (22). This effect can be abrogated by antibodies to TNF-α or inhibitors of NF-κB (22). While TNF-α receptor knockout mice have not yet been studied in mesothelioma models, these mice are protected from fibroproliferative lesions when exposed to asbestos (23). In summary, the innate immune system, particularly macrophages, contribute to a milieu that promotes mutagenesis as well as the survival of mutated mesothelial cells.

Abbreviations: CTLA-4, Cytotoxic T lymphocyte Associated Protein; ECM, Extracellular Matrix; FGF, Fibroblast Growth Factor; G-CSF, Granulocyte Colony Stimulating Factor; GM-CSF, Granulocyte and Macrophage Colony Stimulating Factor; HGF, Hepatocyte Growth Factor; iNOS, Inducible Nitric Oxide Synthase; M-CSF, Macrophage Colony Stimulating Factor; MMP, Matrix Metalloproteases; MPM, Malignant Pleural Mesothelioma; NF-κB, Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells; PD-1, Programmed Cell Death Protein 1; PD-L1, Programmed Death Ligand 1; PDGF, Platelet Derived Growth Factor; PMN-MDSC, Polymorphonuclear Myeloid Derived Suppressor Cells; ROS, Reactive Oxygen species; SMA, Smooth Muscle Actin; TAM, Tumor Associated Macrophages; TIM3, T-cell Immunoglobulin and Mucin-Domain Containing-3; TGFβ, Transforming Growth Factor β; VEGF, Vascular Endothelial Growth Factor.

EXTRACELLULAR MATRIX AND STROMA—MORE THAN A SCAFFOLD

In mesothelioma, the surrounding stroma is not merely a scaffold but promotes tumor growth, invasion and protection from an anti-tumor immune response. Many genes related to the synthesis of, and interaction with, extracellular matrix (ECM) are upregulated in RNA expression analyses of mesothelioma specimens (24–27). These ECM-related genes are more associated with biphasic (25), desmoplastic (27) and sarcomatoid variants (27)—the histological subtypes with poorer prognoses. Mesothelioma cell lines can also produce various ECM components such as type IV collagen, laminin and fibronectin, as well as integrins which bind to these proteins (28, 29). ECM components have autocrine and paracrine effects that stimulate mesothelioma cell chemotaxis and haptotaxis (28, 29). Under the influence of various growth factors mesothelioma cell lines can also produce matrix metalloproteases (MMP) to remodel the ECM and permit invasion (30). Some of these MMPs such as MMP2 and MMP14 are also associated with a poorer prognosis in mesothelioma (31, 32). Furthermore, there is an association with these stroma-related genes and so-called "immune deserts," tumor regions with little lymphocytic infiltrate, suggesting that the stroma and ECM are acting as a barrier to the immune response (26).

When comparing mesothelioma tissue and cell lines, we can conclude that stromal cells and cancer-associated fibroblasts or fibrocytes contribute some of the signals seen in these RNA analyses (25). Activated fibroblasts are present in most mesothelioma tissues (33) and are identified by alpha smooth muscle actin (SMA). Although not studied in mesothelioma, two separate origins of cancer-associated fibroblasts and fibrocytes have been described: α-SMA expressing fibroblasts are tissue-derived, but fibrocytes with spindle-shaped nuclei are derived from macrophages or dendritic cells (α-SMA-, HLA-DR+ with moderate expression of CD68) (**Figure 1**) (34). Mouse models suggest that fibrocytes migrate to areas of hypoxia under the influence of CXCL12 and CXCR4 (35). Cancer-associated fibroblasts and fibrocytes can synthesize ECM components such as collagens, hyaluronan, laminin, and fibronectin and remodel ECM with MMP (36). Furthermore, these spindle-shaped stromal cells develop a positive-feedback relationship with tumor cells by secreting growth factors. For example, TGF-β and IL-6 are consistent features of the mesothelioma secretome (37) and are cardinal activating molecules for fibroblasts. In addition, Fibroblast Growth Factor 2 (FGF2) is seen in most mesothelioma tissue specimens by immunohistochemistry (IHC) (33, 38, 39) and leads to proliferation of fibroblast cell lines *in vitro* and migration to the malignancy in xenograft models in SCID mice (33). Furthermore, FGF2 leads to fibroblast production of hepatocyte growth factor (HGF) and platelet-derived growth factor A (PDGF-A) which can in turn stimulate the growth and migration of mesothelioma cell lines (33, 40). The HGF-receptor (c-MET) and the PDGF receptors α and β, are detected in the majority of mesothelioma specimens by IHC (41, 42). Unexpectedly, Phase 2 and Phase 3 clinical trials of PDGFR inhibition by the small molecular tyrosine kinase

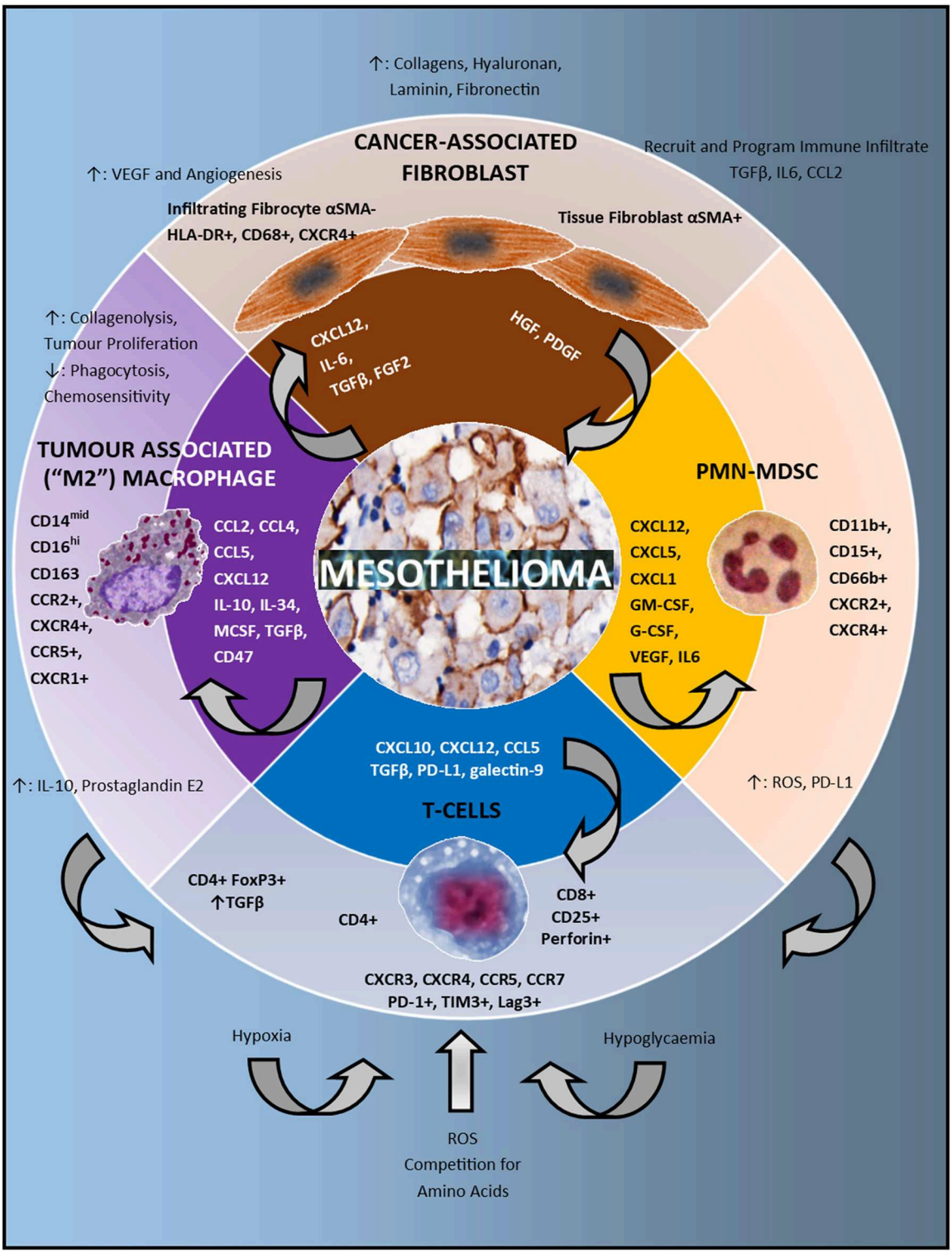

FIGURE 1 | The immune microenvironment in mesothelioma. In the center of the schematic are mesothelioma cells. The second circle lists the chemokines, growth factors and checkpoints present in the microenvironment which attract and program the immune cell infiltrate. These cells include: cancer associated fibroblasts, Polymorphonuclear (PMN) Myeloid Derived Suppressor Cells (MDSC), T-cells and Tumor Associated Macrophages (TAMs). The direction of the arrowhead depicts which cells are influenced by these signals. The outermost circle describes both the phenotype and function of the immune infiltrate. Tumor associated macrophages have immunosuppressive effects on T-cells via increased IL-10 and prostaglandin E2 production. PMN-MDSC have immunosuppressive effects on T-cells via production of Reactive Oxygen Species (ROS) and upregulation of PD-L1. At the bottom of the schematic in blue, various metabolic factors also influence the activity of T-cells including hypoxia, hypoglycaemia, reactive oxygen species, and competition for amino acids.

inhibitors vatalanib or nintedanib did not show major activity (6, 43). However, targeting FGFR using small molecules (44) or FGF-ligand "traps" (45), c-MET by tyrosine kinase inhibitors (46), or fibrosis with pirfenidone (47) continues to elicit considerable research interest.

Finally, in addition to molecules actively secreted by mesothelioma cells, cancer-associated fibroblasts have been noted to produce TGFβ, IL-6 and CCL2 (36). These molecules are detected in pleural effusions of patients with mesothelioma (37) and as such cancer-associated fibroblasts may contribute to the recruitment and differentiation of immunosuppressive cells. They can also contribute to VEGF production and subsequent angiogenesis (36, 37). In summary, the stroma and stromal cells provide a scaffold for invasion, a barrier to the immune response and stimulate tumor growth and the differentiation of immunosuppressive cells.

THE MESOTHELIOMA SECRETOME AND METABOLOME

Before describing the cellular components of the tumor immune microenvironment, it is important to recognize that the chemotaxis and differentiation of these cells is influenced by chemokines, growth factors and metabolites. Examination of pleural fluid, patient-derived tumor cells and tumor cell lines are invaluable in evaluating the "secretome." The mesothelioma secretome includes the chemokines CCL2, CCL4, CXCL10, CXCL5, CXCL1, and CXCL12, the cytokines IL-10 and IL-6, and the growth factors TGFβ, VEGF, MCSF, GM-CSF, G-CSF, FGF, and PDGF (33, 37, 48–53). These molecules can have autocrine effects and are responsible for the chemotaxis and differentiation of immune cells.

Hypoxia is one of the cardinal features of the mesothelioma metabolome. It is likely that tumor cells are exposed to fluctuating oxygen levels due to rapid tumor proliferation, stromal reactions, and angiogenesis (54). In patients with mesothelioma, this hypoxia is noted on F-fluoromisonidazole (FMISO) Positron Emission Tomography (PET) scans, and is associated with increased metabolic activity on Fluorodeoxyglucose (FDG)-PET (55). Evidence of hypoxia has also been demonstrated using immunohistochemical detection of Hypoxia Induced Factor 1α (HIF1α) (56). Hypoxia is capable of profoundly enhancing the growth of mesothelioma cell lines: including clonogenicity, stemness, resistance to chemotherapy, epithelial to mesenchymal transition, migration, morphological changes with pseudopodia, and various phenotypic changes (increased expression of HIF1α/2α, CD44 and Oct4, Bcl2, E-cadherin, vimentin and Glut1) (57). In addition, hypoxia results in the influx of additional immune cells via increased expression of CXCL12 (35) and stimulates angiogenesis by the upregulation of VEGF expression (54, 58). Furthermore, hypoxia, acting via increased HIF1α-expression, increases PD-L1 expression in tumor cell lines as well as in murine macrophage and dendritic cells (58). In myeloid derived suppressor cells (MDSCs), HIF1α expression is associated with increased *arg1* and *inos* and the suppression of T-cell proliferation in mice (59). Knockout of HIF1α was able to abrogate all these effects (59). Hypoxia also induces MDSC production of IL-6, IL-10, and TGFβ1 (58). Apart from MDSCs, murine macrophages exposed to hypoxia increase HIF1α expression and have enhanced suppression of T-cell proliferation (60). HIF1α knockout also abrogated this effect (60).

Apart from oxygen, infiltrating immune cells compete with mesothelioma cells for key nutrients. Mesothelioma cells can upregulate Glucose Transporter 1 (Glut1) in order to more efficiently access glucose and this is evident on IHC (61). Elevated Glut1 levels has been recognized as a poor prognostic factor (62). Mesothelioma is typically a low glucose environment and glucose is reduced in mesothelioma-associated pleural effusions (63). In such an environment, competition for glucose can substantially affect T-cell function (64). Similar competition occurs for essential amino acids. For example, mesothelioma can increase L-type Amino acid Transporter 1 (LAT1)-expression and this has also been associated with poor prognosis in univariate analyses (65). LAT1 transports both arginine and tryptophan and therefore the tumor can deprive T-cells of amino acids essential for T-cell proliferation and function (64). Mesothelioma cells may also express increased levels of Indoleamine-pyrrole 2,3-dioxygenase (IDO) (66) which metabolizes tryptophan into kynurenine, inhibiting T-cell glycolysis and function (64). To conclude, the mesothelioma secretome and metabolome both attract and program infiltrating immune cells.

IMMUNE CELL INFILTRATE

Tumor-Associated Macrophages

Tumor associated macrophages (TAMs) are prominent in the tumor microenvironment; they are associated with a poor prognosis and mouse models suggest that they could be a potential target for treatment. TAMs are generally the most prominent cells in the immune infiltrate when analyzed by flow cytometry of pleural effusions and constitute on average 26–42% of the cellular immune infiltrate in mesothelioma by IHC (51, 67–69). While not the subject of specific analysis in mesothelioma, most of the CD163+ TAMs in other malignancies are monocyte-derived from the peripheral blood rather that tissue-resident macrophages (34). Chemokine signals that attract monocytes in mesothelioma include CCL2, CCL4, CCL5, and CXCL12 and these appear to be of mesothelioma cell origin (**Figure 1**) (37, 52, 53). Murine experiments of asbestos-induced mesothelioma also implicate CCL7, CCL8, CCL3, and CX3CL1 but these have not been detected or investigated in humans to date (70). In relation to macrophages, CCL2 has been studied in most detail in mesothelioma with CCL2 concentrations in malignant pleural effusions being substantially higher compared to benign pleural effusions and pleural effusions from patients with lung adenocarcinoma (24, 71). CCL2 acting via CCR2 appears to be the key chemokine in monocyte trafficking in MPM. Monocytes migrate toward malignant pleural fluid or mesothelioma cell line supernatant and neutralizing antibodies to CCL2 or CCR2 substantially reduce this migration in Transwell experiments (48). However, CD14+ monocytes found in pleural and peritoneal effusions of patients with malignant mesothelioma

are also noted to express CXCR4, CCR5, and CXCR1 with varying degrees of positivity in flow cytometry (72). Other chemokine receptors that can be found on monocytes, such as CX3CR1 and CCR1, are also upregulated in RNA-seq analyses of asbestos-induced mesothelioma in mice (70).

Monocytes and macrophages are programmed into suppressor cells by various components of the mesothelioma secretome (**Figure 1**). For example, primary cells from patients with MPM that are capable of producing M-CSF and IL-34, and MCSF can be detected in pleural effusions (48, 73). These growth factors are implicated in monocyte and macrophage development but may also have autocrine functions as well (73). Other key cytokines for macrophage activation such as TGF-β and IL-10 have been identified in pleural fluid and supernatant from mesothelioma cultures, also suggesting a tumor origin (51, 74). IHC of MPM samples have confirmed the presence of TGFβ (38) and this feature appears to distinguish MPM from primary lung cancers (74, 75). An autocrine feedback loop has also been proposed for TGF-β (76). Apart from the immunosuppressive and polarising cytokines described above, the macrophage checkpoint and "don't eat me signal," CD47, was found to be expressed in high levels in the majority of patients with epithelioid mesothelioma (77).

TAMs develop an immunosuppressive phenotype in mesothelioma; human monocytes cultured with malignant pleural effusions developed a $CD14^{mid}CD16^{hi}$ immunosuppressive phenotype, resembling cells cultured with M-CSF (48). Furthermore, Izzi et al. performed a comprehensive array of macrophage function tests to show that co-culture of THP-1-derived macrophages with a single mesothelioma cell line resulted in reduced phagocytic activity, increased IL-10 production, increased collagenolytic activity for tissue remodeling, and increased arachidonic acid and prostaglandin E2 production (78). Curiously, contrasting effects were noted on monocytes (78). When co-cultured with immunosuppressive macrophages, mesothelioma cells proliferate more and have reduced sensitivity to chemotherapy with cisplatin or pemetrexed (48). The functional importance of macrophages in promoting mesothelioma is attested in a syngeneic, immunocompetent, orthotopic mouse model of mesothelioma (79). When the local macrophage population was selectively removed using liposome-encapsulated clodronate, reduced tumor number, invasiveness, and metastases were observed (79).

There have been conflicting reports on the prognostic effect of macrophages in epithelioid and non-epithelioid mesothelioma (68, 80). However, more precise biomarkers using an immunosuppressive to pan-macrophage ratio with CD163 to CD68 correlated with poor overall survival in a cohort of patients with epithelioid mesothelioma (81). Greater quantities of circulating monocytes are also associated with worse outcomes from cytoreductive surgery (68). The effect is associated with tumor bulk but is still seen when controlling for disease stage (68), suggesting that both tumor size and its distinct secretome could be influencing peripheral blood monocyte counts. A low peripheral blood lymphocyte-to-monocyte ratio has also been identified as a marker of poor prognosis (82). In summary, TAMs are numerous, programmed by the mesothelioma secretome, have an immunosuppressive phenotype and function, and are associated with poor prognosis.

T-Lymphocytes

The CD3+ T-lymphocyte is the second most common immune cell present in the mesothelioma microenvironment and constitute on average 20–42% of the immune cell infiltrate (69, 80, 83). CD8+ T-cells are almost universally present and CD4+ and CD4+ FoxP3+ T-cells are also present in the majority of patients (67, 83). Of interest, the number of T-regulatory cells in pleural effusions of MPM patients is lower than in other solid tumors (74). With regards to T-cell trafficking, apart from CXCL12 discussed previously, the mesothelioma secretome also includes CXCL10 (37). CXCL10 is produced in greater concentrations in pleural fluid compared to the supernatant of primary cells, suggesting additional origins of the chemokine rather than solely from tumor cells (37). The CXCR3 chemokine receptor for CXCL10 is upregulated in murine models of asbestos-induced mesothelioma (70). CCL5 is also substantially elevated in the peripheral blood of patients with mesothelioma compared to asbestos workers and healthy individuals (84) and the CCR5 receptor is present on T-cells in pleural effusions (72). Other chemokine receptors on T-cells in pleural effusions include CXCR4 and CCR7 (72).

The mesothelioma microenvironment includes both neoantigenic stimuli as well as checkpoint molecules which can affect T-cell programming. Although next generation sequencing of mesothelioma originally identified few neoepitope generating mutations (85), more recently mate-pair seq based analysis has identified higher numbers of neoepitope generating mutations which were probably from chromosomal rearrangements missed by NGS (86). When analyzing predicted neoantigen load and TCRβ diversity in MPM, it is noted that in general the most diverse polyclonal TCRβ repertoire is associated with fewer predicted neoantigens. In contrast oligoclonal expansion is associated with high neoantigen loads presumably due to clonal expansion (87). While neoantigens may prompt T-cell activation and proliferation, various checkpoint molecules are also evident in the mesothelioma microenvironment and are discussed in more detail elsewhere in this issue. PD-L1 is detected by flow cytometry of pleural effusions as well as IHC (88–91) and has been associated with poor prognosis (88, 89). Of interest, PD-L1 expression is associated with a higher objective response rate to nivolumab but is not entirely predictive of response (7). This finding is reflected in other malignancies treated with PD-1 or PD-L1 inhibition, indicating that other parameters including tumor mutational burden or tumor-infiltrating lymphocytes also influence response to PD-1 or PD-L1 blockade (92, 93). Galectin 9, a ligand for TIM-3 has also been detected by IHC and by flow cytometry on human macrophages (94). T-regulatory cells are consistently detected in MPM IHC and flow cytometry of associated pleural effusions (37, 67, 74, 80). The T-regulatory compartment develops in the context of abundant TGF-β and presumed inadequate stimulation by dendritic cells (37, 74). It has also been shown that PD-L1 signaling via PD-1 is responsible for the plasticity of some TH1 cells, converting them to inducible T-regulatory cells (95).

As a result of the above influences, the phenotype of infiltrating T-cells is varied. The CD8+ T-cells that are present in pleural effusions show higher levels of CD25+ compared to other malignancies, generally indicative of activation (74). In addition, there is an increase in perforin expression in CD8+ T-cells which correlated with the number of neoepitopes that are present in the tissue (87). Despite these signs of activation, CD8+ cytotoxic T-cells also display phenotypic markers of exhaustion including PD-1+, TIM3+, and LAG3+ (88). CD4+ T-helper subsets and function in mesotheliomas have not been extensively investigated but again clear signs of exhaustion are evident with significant levels of PD-1+, TIM3+, and LAG3+ detected by flow cytometry (88). Of the T-cells present in mesothelioma, the majority have an effector memory phenotype (69).

Although one cannot draw conclusions regarding causation, T-cell numbers are associated with patient prognosis. Two studies have shown that epithelioid mesotheliomas infiltrated by more CD4+ T-cells were associated with a better prognosis (67, 80). A third study showed an association with prognosis that was only statistically significant in univariate analysis (53). This association has not been confirmed in sarcomatoid tumors (80). Only one comparatively small study demonstrated a poorer prognosis in multivariate analyses of low CD8+ T-cell counts (83). Interestingly, low CD8+ T-cell count was also a poor prognostic factor in patients undergoing extrapleural pneumonectomy (96). High proportions of FoxP3 positive T-cells have been associated with a poor prognosis in analyses of epithelioid and sarcomatoid tumors (80).

Although it is presumed that this T-cells infiltrate has some functional significance, the clinical experience with intrapleural IL-2 has been disappointing. While there is yet to be any randomized trial of IL-2, in one study the overall survival did not differ substantially from historical controls who underwent the same intensive therapy with pleural decortication, intrapleural postoperative epidoxorubicin, adjuvant radiotherapy followed by chemotherapy and did not receive any IL-2 (97). Immunological effects seen in response to IL-2 include an increase in both CD8+ T-cells as well as FoxP3+ T-cells (97). This suggests that the T-regulatory cells are acting as a "sump" for IL-2 in this context. There is also conflicting evidence regarding the effects of anti-CD25 therapy in murine experiments (98, 99). In summary, T-lymphocytes are programmed by the mesothelioma secretome, neoantigens and checkpoint molecules and are associated with altered prognosis. The remaining challenge is to determine whether they can be successfully redirected into a robust anti-tumor response.

Chimeric Antigen Receptor (CAR) T-cell therapy is one such method of enhancing patient T-cell responses against mesothelioma and is discussed in more detail elsewhere in this issue. The requirement for neoantigens is bypassed by directing the CAR T-cell receptor to a tumor-associated antigen, such as mesothelin. The fibrous stroma can be circumvented by locoregional administration (100, 101), or designing CAR T-cells to target antigens that are expressed by both the tumor and cancer-associated stroma such as Fibroblast Activation Protein (102), or by adding chemokine receptors such as CCR2 to enhance trafficking to tumor (103). T-cell metabolism can be manipulated by the choice of costimulatory molecules, such as 4-1BB (104, 105). Exhaustion can also be ameliorated by the concomitant use of PD-1 inhibitors (100, 101), or designing CAR T-cells with dominant negative PD-1 receptors to prevent signaling via native PD-1 (100). Switch receptors have also been designed for mesothelin CAR T-cells with extracellular PD-1 linked to intracellular CD28 (106). Other modifications such as mutating the CAR CD3ζ Immunoreceptor Tyrosine-Based Activation Motifs have also been shown to prevent exhaustion in other disease models (107), and these principles are likely to be applicable to mesothelioma. These developments address some challenges posed by the tumor microenvironment and results of early clinical trials are eagerly anticipated.

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSC) can be polymorphonuclear (PMN-MDSC) or monocytic (M-MDSC). However, the distinction between MDSC and other immune cells such as TAMS is still unclear despite proposed standardized nomenclature and markers for identification (108). The granulocytic infiltrate is less prominent and on average is 6–9% of the cellular infiltrate (49, 69) but still has prognostic implications and functional importance. Neutrophilic infiltrate can be detected by IHC, perhaps with greater sensitivity using CD66b (which also detects eosinophils) and CD15 compared to neutrophil elastase (49, 69, 80). Apart from CXCL12 and CXCR4 previously mentioned, other neutrophil chemoattractants include CXCL5 and CXCL1 which are detected in patient-derived mesothelial cell supernatants, and CXCL5 also reaches detectable levels in pleural effusion (37). Murine mesothelioma models show upregulation of the granulocyte chemokine receptor CXCR2 for these ligands (70).

Granulocytic growth factors are produced in the mesothelioma secretome including GM-CSF, G-CSF, VEGF, and IL-6 (37, 49). Furthermore, in the mesothelioma microenvironment granulocytes develop a phenotype consistent with PMN-MDSC and express CD15+, CD11b+, CD66b+, and are CD14/CD33 double-negative (49, 108). These polarizing growth factors likely have systemic effects as increased populations CD11b+CD15+HLADR- granulocytes are also noted in the peripheral blood of patients with mesothelioma compared to healthy controls (49). These cells function as MDSCs and inhibit the proliferation of T-cells compared to CD15+ cells from normal pleura or from the peripheral blood of healthy donors (49). The inhibitory effect of these MDSC is predominantly through the generation of ROS; peripheral blood granulocytes from patients with MPM show increased ROS expression and the proliferation of T-cells can be restored with inhibitors of ROS such as N-Actyl Cysteine (49). Free radical species can also affect T-cell function by nitration of the T-cell receptor (109), downregulation of CD3ζ, and H_2O_2-mediated reduction in cytokine production (110). PD-L1 expression on granulocytes has also been associated with fewer T-cells in the tumor (49). While various alternative mechanisms of immunosuppression have been attributed to MDSCs, *in vitro* assays with peripheral blood granulocytes indicate that immunosuppressive cytokines, arginase expression

or iNOS expression were the same in patients and healthy controls (49). Moreover, arginase or iNOS inhibitors did not restore T-cell function (49). However, it is important to note is that these experiments assessed peripheral blood granulocytes in patients rather than tumor-associated MDSCs. The presence of greater neutrophilic infiltrate in tumor and an increased peripheral blood neutrophil to lymphocyte ratio is associated with a poorer prognosis in epithelioid mesothelioma (80, 111).

Chemotherapies that are recognized to reduce MDSCs have been used to treat MPM. 5-Fluorouracil or paclitaxel did not show positive effects whereas mixed results were seen with gemcitabine (112). In summary, PMN-MDSC are relatively abundant and are also associated with prognosis. However, it is remains to be seen if eliminating these cells with targeted therapy will be successful.

Other Cells

B-cells have been detected in both tumor and stroma in MPM to varying degrees (26, 53, 69, 80). Higher B-cell counts have been associated with a better prognosis in multivariate analyses of patients with epithelioid mesothelioma (53, 80). However, it is yet to be determined whether this is an epiphenomenon or whether the B-cells themselves have a functional role. Autoantibodies have been detected in the sera of a fraction of patients with mesothelioma (113). Some of these antibodies appear to be tumor-specific and target the nuclear fraction (113). However, in a more comprehensive analysis of sera from patients with MPM against a limited panel of autoantigens, the percentage of patients with autoantibodies was not markedly elevated compared to other patients with asbestos-related diseases or asbestos-exposed healthy controls (114). The antibody subclasses from B-cells taken from mesothelioma tissues appear to be predominantly IgG1 and IgG3 which are known to activate complement (115). The analysis of B-cell cytokines or B-regulatory cells is currently limited in mesothelioma (116).

CD3-CD56+ Natural Killer (NK) and CD3+CD56+ Natural Killer T (NKT) cells are found in the majority of mesothelioma tissues but only in very small numbers (69, 80, 96, 117, 118). In pleural effusions they are found to have typical inhibitory receptors (NKG2A) and activation receptors (NKG2D) but are also $CD56^{bright}$, a subset associated with poorer cytotoxicity but enhanced cytokine production (117). A greater proportion of peripheral blood NK cells also express the exhaustion marker TIM3+ (119). While pleural effusion NK cell function is reduced in degranulation assays compared to the peripheral NK cells from healthy donors, similar changes were noted in NK cells from non-malignant pleural effusions (117). The interpretation of these data is problematic given that there is no healthy control or reference range for pleural NK cell cytotoxicity (117). However, it is noteworthy that after treatment with IL-2 *in vitro*, the cytotoxicity of NK cells from various malignant effusions can be restored, suggesting some reversibility in impaired function (120). In murine mesothelioma tumor models, removing NK cells by anti-asialo GM1 antibodies did not alter tumor growth, nor was tumor growth accelerated in beige mice with impaired NK cell function (121). The presence of NK cells as detected by IHC has also not been associated with altered prognosis in either epithelioid or sarcomatoid mesothelioma (80). In conclusion, current evidence does not indicate that NK cells are key players in the mesothelioma tumor microenvironment.

Mast cells have been detected in mesothelioma tumors treated with IL-2 and high counts of tryptase-positive mast cells has been associated with a better prognosis but this is awaiting further confirmation (122). Dendritic cells do not constitute a large population in the mesothelioma tumor microenvironment when assessed with antibodies to CD123 in IHC (69).

While this review focuses on the immune aspects of tumor microenvironment, it is prudent to acknowledge that angiogenesis is a simultaneous and interlinked process that also requires therapeutic intervention. In fact, immunosuppression and angiogenesis are intrinsically interconnected repair mechanisms co-opted by malignancy (123). Both have linked physiological roles, but both occur in an unchecked and disorganized manner in the context of the tumor microenvironment (123). As we have discussed, both share metabolic and growth factor stimuli, such as hypoxia, VEGF, HGF, TGF-β, angiopoietin, and prostaglandin E2 (37, 123–125). Studies in mesothelioma and other malignancies indicate that both processes are driven by tumor cells, cancer associated fibroblasts, MDSCs, TAMS, and T-regulatory cells (33, 36, 126, 127). In addition, angiogenesis measured by microvessel density is an independent marker of poor prognosis in mesothelioma (128) and anti-angiogenic therapy with Bevacizumab improves median overall survival (4). While anti-angiogenic therapies in mesothelioma require further refinement and are discussed elsewhere in this edition, it is likely that successful immune-based treatments would also benefit from incorporating ancillary anti-angiogenic treatments.

CONCLUSIONS

While checkpoint inhibition represents an exciting development in the treatment of several solid tumors, the outcomes in mesothelioma have been less positive and may well be affected by the complex structure of the tumor microenvironment in mesothelioma. While more comprehensive descriptions of the tumor microenvironment and suppressor cells have been presented elsewhere, we have chosen to focus on research that relates specifically to mesothelioma, given the evidence that MPM poses unique challenges when compared to other malignancies. We recognize that this review may not adequately emphasize the significant heterogeneity between patients and within the tumor microenvironment itself. However, we hope that providing a better understanding of the stromal tissue, the secretome, metabolome and relevant immunosuppressive cells will assist in finding the rationale for more effective therapy combinations in the future.

AUTHOR CONTRIBUTIONS

GC wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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MINI REVIEW
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Malignant Pleural Mesothelioma: State-of-the-Art on Current Therapies and Promises for the Future

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Malignant pleural mesothelioma (MPM) is a rare, aggressive cancer of the pleural surface associated with asbestos exposure. The median survival of MPM patients is a mere 8–14 months, and there are few biomarkers and no cure available. It is hoped that, eventually, the incidence of MPM will drop and remain low and constant, given that most nations have banned the use of asbestos, but in the meantime, the incidence in Europe is still growing. The exact molecular mechanisms that explain the carcinogenicity of asbestos are not known. Standard therapeutic strategies for MPM include surgery, often coupled with chemotherapy and/or radiotherapy, in a small percentage of eligible patients and chemotherapy in tumors considered unresectable with or without adjuvant radiotherapy. In recent years, several new therapeutic avenues are being explored. These include angiogenesis inhibitors, synthetic lethal treatment, miRNA replacement, oncoviral therapies, and the fast-growing field of immunotherapy alone or in combination with chemotherapy. Of particular promise are the multiple options offered by immunotherapy: immune checkpoint inhibitors, tumor vaccines, and therapies taking advantage of tumor-specific antigens, such as specific therapeutic antibodies or advanced cell-based therapies exemplified by the CAR-T cells. This review comprehensively presents both old and new therapeutic options in MPM, focusing on the results of the numerous recent and on-going clinical trials in the field, including the latest data presented at international meetings (AACR, ASCO, and ESMO) this year, and concludes that more work has to be done in the framework of tailored therapies to identify reliable targets and novel biomarkers to impact MPM management.

Keywords: malignant pleural mesothelioma (MPM), immunotherapy, mesothelin, CAR (chimeric antigen receptor) T cells, miRNA replacement

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INTRODUCTION

Malignant pleural mesothelioma (MPM) is a rare, incurable, aggressive cancer of the pleural surface associated with asbestos exposure with a median survival of 8–14 months (1, 2). Although the incidence in some countries, e.g., the USA (3,200 cases/year) (3), is fairly constant, in Europe, it is growing and is expected to peak between 2020 and 2025 (1). Moreover, the migratory

phenomena toward western countries from nations lacking legislation on asbestos use will render MPM even more frequent. At present, no actionable driver mutations have been identified in MPM. However, MPM carcinogenesis and outcome are influenced by many factors: BRCA-associated protein 1 (BAP1) expression status, CDKN2A and neurofibromatosis type 2 (NF2) tumor suppressor inactivation, overexpression of growth factors such as vascular endothelial growth factor (VEGF), mesothelin (MSLN) promoter methylation, and Ras/mitogen-activated protein kinase and phosphatidylinositol 3-kinase/mTOR pathway activation (4, 5).

MESOTHELIOMA THERAPIES

Standard

The standard therapeutic strategies for MPM are (i) surgery for resectable tumors, often combined with radiotherapy (RT) and/or chemotherapy (CT) (trimodality treatment), and (ii) CT or RT in unresectable tumor cases. To date, the only FDA- and EMA-approved frontline therapy is the cisplatin-pemetrexed combination (6–10). Only selected patients can benefit from a complete resection, either lung-sacrificing surgery (extrapleural pneumonectomy, EPP) or lung-sparing (pleurectomy/decortication, P/D) (11–13). Surgery can be coupled with intraoperative treatments (14–17), but a general consensus on the proper multimodality approach is lacking.

Radiotherapy

RT is used as an adjuvant or neoadjuvant treatment in MPM, mainly in a palliative setting (8–10). As standard practice, patients undergoing an EPP receive adjuvant conventionally fractionated RT (50–60 Gy) in the ipsilateral hemithorax area (18, 19). In node-negative MPM patients, neoadjuvant therapy, based on intensity-modulated RT (IMRT) consisting of a fractionated irradiation of 5–6 Gy, is delivered before EPP (20–22). In contrast, prophylactic radiotherapy of chest wall tracts after surgery to prevent parietal tumor seeding is not recommended anymore by the ASCO guidelines following the results of the SMART and PIT trials (23–25). Recently, adjuvant hemithoracic pleural RT has been shown to be effective and safe (26–28). Advanced RT treatments, e.g., proton therapy (29) or Arc therapy (a novel and accurate IMRT modality) (30), alone or combined with immunotherapies, are being tested to improve RT impact in MPM management.

Angiogenesis Inhibitors

The angiogenic process plays an important role in MPM maintenance. VEGF receptor tyrosine kinase inhibitor (TKI) monotherapy yielded modest results (31–37). The addition of bevacizumab, a humanized monoclonal antibody against VEGF, to cisplatin-pemetrexed CT increases the median overall survival (OS) from 16.1 to 18.8 months and progression-free survival (PFS) from 7.3 to 9.2 months, as shown in the phase III MAPS study (NCT00651456) (38). Since this therapeutic regimen showed manageable toxicities, it has been included in the National Comprehensive Cancer Network guidelines (category 2A) (39), although it is not yet approved by the FDA or EMA. The SWOG S0905 phase I study evaluated the combination of cisplatin-pemetrexed CT with cediranib, a VEGF/PDGF receptor inhibitor, demonstrating a preliminary promising efficacy and reasonable toxicity profile (40) but, when compared to placebo, this combination failed to significantly increase OS and PFS in the following randomized phase II trial (41). Nintedanib is an inhibitor of three (triplet regimen) different growth factor receptors (VEGFR, PDFGR, and FGFR) and its administration in combination with CT improved the objective response rate (ORR) from 44 to 57% and the median PFS (9.7 vs. 5.7 months) compared to placebo in the LUME-Meso trial (42). Data from the phase III LUME-Meso trial (NCT01907100) have recently been published, and the primary PFS endpoint failed, not confirming the previous phase II trial results (43). Other TKIs, such as the anti-VEGFR axitinib (44) or the multi-target inhibitor of VEGFR1/2/3, FGFR-1, PDGFR-β, and RAF/cKit pathway sorafenib failed to improve median OS and PFS in chemonaive or CT-pretreated MPM patients (45, 46). The limited success of anti-angiogenic drugs is due to the lack of good predictive biomarkers to guide the selection of suitable patients for this therapy. Recently, blocking of FGF signaling has been pursued through the sequestration of FGFs with the GSK3052230 ligand trap molecule to avoid toxicities associated with FGFR inhibitors. A phase Ib study indicates that a combination of GSK3052230 plus cisplatin-pemetrexed-CT leads to an ORR of 44% and to a median PFS of 7.4 months with limited adverse events (47).

Synthetic Lethal Therapies

Some MPM tumors cannot synthesize arginine due to the loss of argininosuccinate synthetase 1 (ASS1) gene expression. ASS1 deficiency is twice more frequent in the biphasic/sarcomatoid histotypes than in the epithelioid subtype. *In vitro* experiments suggest that depletion of arginine through exposure to a specific deaminase leads to synthetic lethality (48). The TRAP phase I trial (NCT02029690) demonstrated a positive effect of treatment with pegylated arginine deaminase (ADI-PEG 20) combined with CT in ASS1-deficient MPM patients (49). The ATOMIC-Meso phase III trial (NCT02709512) is recruiting patients with ASS1 gene loss. Genomic studies on MPM cells reported a reduced or absent expression of an enzyme involved in DNA repair and Ca^{2+}-dependent apoptosis BAP1 in ∼50% of sporadic MPMs. *In vitro* studies demonstrated that BAP1-mutated cells are less sensitive to ionizing radiation causing DNA double-strand breaks (50, 51) or to the DNA synthesis inhibitor gemcitabine (52), highlighting the contribution of BAP1 in DNA damage signaling and repair and a possible role as a predictive biomarker (53). Inherited loss-of-function mutations in BAP1 predispose to multiple carcinomas, including mesothelioma (54–56). Interestingly, MPM patients with germline mutated BAP1 or with genetic alterations in other DNA repair genes and treated with platinum CT showed a significantly longer median OS than patients devoid of the same mutations (57). Hence the BAP1 mutational status at diagnosis could be an important factor in

predicting MPM patients' response to CT and may sensitize patients to synthetic lethality therapies that hit other components of the DNA repair machinery. Accordingly, as already suggested by Srinivasan et al. (58), the homologous repair (HR) component PARP-1 would be an excellent target for a synthetic lethality approach, given that MPM cells are frequently characterized by HR deficiency and unrepaired DNA damage accumulation due to the aforementioned BAP1 mutations. PARP-1 inhibitors, such as niraparib and olaparib, clearly decreased MPM cell survival, albeit regardless of BAP1 status. BAP1 loss also up-regulates the expression of EZH2, a Polycomb Repressive Complex-2 (PRC2) component involved in epigenetic silencing (59) and oncogenic pathways (60), suggesting sensitivity of BAP1-deficient MPM tumors to EZH2 inhibition. A phase II clinical trial (NCT02860286) is ongoing to evaluate the efficacy of the EZH2 inhibitor tazemetostat in MPM patients (61).

Finally, the synthetic lethality of inhibition of the Focal Adhesion Kinase (FAK) tyrosine kinase with loss of Merlin protein, the first involved in the survival, proliferation, and migration of tumor cells (62) and the second, a tumor suppressor encoded by the NF2 gene frequently mutated in MPM (5), has been proposed. Despite an encouraging positive trend observed in phase I trial in which FAK inhibitor GSK2256098 was tested in MERLIN-negative patients (63), a second large phase II trial (COMMAND, NCT01870609) demonstrated that neither PFS nor OS was improved by the FAK TKI defactinib as compared to placebo when administered as a maintenance treatment after frontline CT (64).

Immunotherapies

Multiple lines of evidence point to the involvement of the immune system in the pathogenesis and sensitivity to therapy of MPM (65, 66). Spontaneous regressions in some patients are attributable to an activation of the immune system (67, 68). Moreover, B cells are essential for a good prognosis (69) in murine preclinical models of mesothelioma treated with immunotherapy, indicating that antibodies are generated and contribute to the therapeutic effect. Also, the presence of cytotoxic $CD8^+$ tumor-infiltrating lymphocytes (TILs) is a good prognostic marker in MPM (70, 71).

MPM can be immunogenic but develops mechanisms to evade immune eradication. PD-L1 is the ligand for PD-1, a receptor expressed by activated T and B cells. Binding of PD-L1 to PD-1 affects effector T-cell and B-cell function and ultimately leads to exhaustion and apoptosis (72). Recently PD-L1 was shown to be expressed in 40% of MPMs, almost all of the sarcomatoid subtype, and was associated with a significantly poorer outcome, with a median survival of 5 months for PD-$L1^+$ MPM patients vs. 14.5 months for PD-$L1^-$ tumors ($p < 0.0001$) (73). However, PD-L1 expression is heterogeneous among MPM cells and could vary during treatment, limiting the efficacy of anti-PD-(L)1 therapy (74, 75).

Immune Checkpoint Inhibitors

Immune checkpoint inhibitors (ICIs) are immune-modulating agents that boost the latent immune-response kept in check by the tumor. PD-1/PD-L1 and CTLA-4 inhibitory functions are targeted by immunomodulatory therapies, allowing T- and B-cell (re-)activation (76). Recently, many ICIs, including anti-CTLA-4, a glycoprotein expressed on regulatory and on activated $CD4^+$ and $CD8^+$ T cells, or anti-programmed death 1 (PD-1)/PD-L1 antibodies, have been approved for the treatment of solid and hematological malignancies (76–78).

Despite early enthusiasm for the results of tremelimumab, an anti-CTLA-4 ICI, as first-line therapy (79), its use as a second- or third-line treatment demonstrated no benefit of CTLA-4 inhibition over placebo (DETERMINE, NCT01843374) (80). Nivolumab efficacy was tested as a second- or third-line treatment alone vs. placebo in MPM patients in two recently completed phase II studies (NivoMes, NCT02497508, and MERIT, JapicCTI-163247) with ORRs of 24.0 and 29.4% and disease control rates (DCRs) of 50.0 and 67.6%, respectively (81, 82). A clear correlation between response and PD-L1 expression was reported (81). An ongoing randomized, placebo-controlled phase III trial is testing the efficacy of nivolumab in relapsed mesothelioma (CONFIRM, NCT03063450) (83).

The anti-PD-1 ICI pembrolizumab has been evaluated in different phase I (KEYNOTE-028, NCT02054806) and II (NCT02399371) studies as a second- or third-line treatment, showing promising DCR and prolonged disease stability (84–86). The results from the randomized phase III trial PROMISE-meso (NCT02991482) were instead disappointing, with relapsed MPM patients receiving pembrolizumab or single-agent CT failing to show an improved median OS and PFS despite a superior ORR for pembrolizumab compared to a CT regimen (22 vs. 6%) (87). Popat and colleagues suggest that ICI treatment should be tested at earlier stages and on patients that are better stratified to benefit from longer periods of immunotherapy.

Other ICIs, like the Inducible T-cell COStimulator (ICOS) agonist GSK3359609, alone or in combination with pembrolizumab, are being evaluated in advanced solid tumors including MPM (INDUCE-I, NCT02723955) (88).

Combination Strategy

Two ICIs against different targets can be combined. An ipilimumab (anti-CTLA-4) and nivolumab (anti-PD-1) combination was tested in the phase-II MAPS2 trial (89) in relapsed MPM patients. The results indicated that the primary endpoint, DCR after 12 weeks, was reached by combined therapy (50%) and not by nivolumab alone (44%). An ORR of 25.9 vs. 18.5% and a modest increase of median response duration (7.4 vs. 7.9 months) were achieved in the combination and nivolumab groups, respectively. Severe treatment-related side effects were registered in 17% of patients. The same combination is being investigated in a randomized phase III trial (Checkmate 743, NCT02899299) in the front-line setting (90). Similarly, the combined therapy of tremelimumab plus durvalumab, an anti-PD-L1 antibody, tested in the phase II NIBIT-MESO-1 trial (NCT02588131), resulted in grade 3–4 treatment-related side effects in 17.5% of patients (91). A phase III study is evaluating the combination of pembrolizumab with pemetrexed and platinum-based CT vs. pembrolizumab or CT alone as first-line treatment for MPM patients (NCT02784171).

INNOVATIVE THERAPEUTIC APPROACHES FOR MALIGNANT MESOTHELIOMA

miRNA Replacement

miRNA replacement is an innovative anti-cancer approach that restores miRNA expression by delivering miRNAs or miRNA mimics. Restored miRNAs can interfere with the expression of proteins endowed with oncogenic activity (92–94) thereby inhibiting proliferation or inducing apoptosis of tumor cells (95).

miR-16 is often downregulated in MPM, while its expression in *in vitro* and in murine xenografts results in decreased cell proliferation, decreased glucose uptake, and increased mortality (95). The feasibility of miR-16 exploitation by delivering its mimic encapsulated into an anti-EGFR-coated bacterially-derived shell termed EnGeneIC Dream Vector (TargomiR) (96) was shown in the NCT02369198 trial, which reported efficacy and good tolerability in patients with relapsed MPM (97). TargomiR therapy was associated with a drop in glucose uptake in 60% of patients as measured by PET-CT, while 73% of patients achieved disease control.

Tumor Treating Fields

Recently, the FDA approved an innovative first-line treatment for MPM patients as a humanitarian use device, called NovoTTF-100L, that is based on the delivery of specific electric frequencies (Tumor Treating Fields, TTF) in combination with CT, to interfere with cancer cell proliferation. *In vitro* and *in vivo* data (98) are consistent with recent STELLAR phase II registration trial (NCT02397928) results, where a median OS of 18.2 months and low systemic toxicity have been experienced by the patients treated with TTF plus CT (99).

Oncoviral Therapies

In the wake of successful phase I and II studies (100, 101), a phase III clinical trial (INFINITE, NCT03710876) is evaluating the efficacy of an Adenovirus-Delivered Interferon Alpha-2b (rAd-IFN) in combination with celecoxib and gemcitabine in MPM patients who failed previous regimens. A phase II study (NCT04013334) is testing the efficacy of Ad-SGE-REIC/MTG201, an adenoviral vector for the expression of Reduced Expression in Immortalized Cell (REIC)/Dickkopf-3 (Dkk-3) gene in combination with nivolumab. The Dkk-3 protein is a Wnt signaling pathway antagonist that induces cancer cell death and antitumor immune response. A previous phase I/II study showed that intrapleural virus administration was safe and well-tolerated and that Dkk-3 gene expression allowed durable disease control (102). Preclinical studies evaluated the replication-competent neuroattenuated Herpex Simplex Virus (HSV-1716) as oncolytic virotherapy for mesothelioma, showing cytotoxicity in combination with CT or RT *in vitro* and reduced tumor growth also at low doses *in vivo* in MPM murine models (103). The results of a phase I/IIa trial (NCT01721018) testing the intrapleural administration of HSV-1716 demonstrated virus replication, pleural Th1 cytokine response, and anti-tumor immunoglobulin production (104). The use of other viral vectors [reviewed in (105)], such as attenuated versions of vaccinia or measles virus genetically engineered to produce human thyroidal sodium iodine symporter (NIS), is being investigated in different phase I clinical trials (NCT02714374, NCT01503177).

Dendritic Cell Vaccination

Cancer vaccines aim at inducing tumor-specific effector T cells that reduce tumor mass and induce tumor-specific memory T cells to curtail tumor relapse (106). Autologous dendritic cell vaccination (DCV) has shown efficacy in MPM treatment. The PMR-MM-002 clinical trial (NCT01241682) demonstrated the safety and feasibility of tumor lysate-pulsed dendritic cells as therapeutic adjuvants in MPM patients (107). The DENIM phase II/III randomized clinical trial (NCT03610360) will treat MPM patients with dendritic cell immunotherapy plus best supportive care (BSC) and compare the results with BSC alone (108). Other vaccination-based therapies currently under investigation are autologous DC loaded with Wilms' Tumor Antigen (WT1) (109) combined with CT (MESODEC, NCT02649829) and autologous TILs plus IL-2 (110). Based on the results obtained by PMR-MM-002 and by ICIs, a phase Ib MESOVAX clinical trial (NCT03546426) is recruiting MPM patients to test the efficacy of a tandem combination of autologous DCV and pembrolizumab at our institute.

Mesothelioma Targeting Antigens

MSLN is a glycoprotein expressed more on the cell surface of several tumors, including MPM cells, than in normal tissues (111). A phase II clinical trial (NCT00738582) testing amatuximab, a chimeric anti-MSLN mAb, plus standard CT compared to CT alone showed a promising OS of 14.8 months (112) that was not confirmed in the ARTEMIS trial (NCT02357147). Anetumab ravtansine (AR), an anti-MSLN antibody conjugated with the cytotoxic anti-tubulin drug ravtansine, showed a 50% ORR and 90% DCR in pretreated patients (113). A second phase II randomized clinical trial showed that AR did not improve survival compared to the anti-mitotic chemotherapeutic, vinorelbine, as a single agent (114). The combined regimen of pembrolizumab plus AR will be evaluated in a phase 1/2 trial (NCT03126630) that is recruiting only MSLN-positive patients. A phase I study (NCT02798536) is currently active to assess a novel low-immunogenic anti-MSLN recombinant immunotoxin, RG778/LMB-100 (115), composed of a human single-chain variable fragment (scFv)-targeting moiety directed against MSLN linked to Pseudomonas exotoxin A (PE). The phase I trial NCT01675765 evaluated the sequential administration of the cancer vaccine CRS-207, an attenuated form of *Listeria monocytogenes* expressing MSLN, with or without cyclophosphamide followed by consolidation CT, to stimulate an innate and adaptive immunity against MSLN-expressing cells. The cyclophosphamide arm showed acceptable toxicity and a DCR of 89%, a PR of 54%, an SD of 29%, and a median PFS and OS of 7.5 and 14.7 months, respectively (116).

The success of advanced cell-based therapies, e.g., Chimeric Antigen Receptor-transduced T cells (CAR-T) in hematological tumors, awoke interest as well for MPM (117). CAR-T-cell receptors directed against MSLN are being investigated in several phase I clinical trials. The critical issues in Adoptive Cell Therapy (ACT) and CAR-T treatment are the safety profile and the degree of off-tumor toxicity. Intravenous or intra-tumor administration of MSLN-CAR-T cells (NCT01355965) (118) obtained by T-cell electroporation with encoding mRNA to achieve transient expression resulted in moderate responses and low toxicity (119). A phase I study (NCT02414269) drawing on preclinical results in orthotopic mouse MPM models (120, 121) is ongoing to test the MSLN-CAR-T cells in multi-treated MPM patients. Preliminary results presented at the AACR [Abstract CT036, (122)] and ASCO [Abstract 2511, (123)] meetings this year have shown an ORR and DCR of 36.8 and 57.8%, respectively, in a cohort treated off protocol in combination with pembrolizumab.

Fibroblast activation protein (FAP) is another interesting target expressed by all MPM subtypes and by cancer-associated fibroblasts (CAFs) and exploited by FAP-targeted CAR-T cells in an ongoing phase I trial (NCT01722149) (124) Preliminary results presented at the ESMO congress this year showed

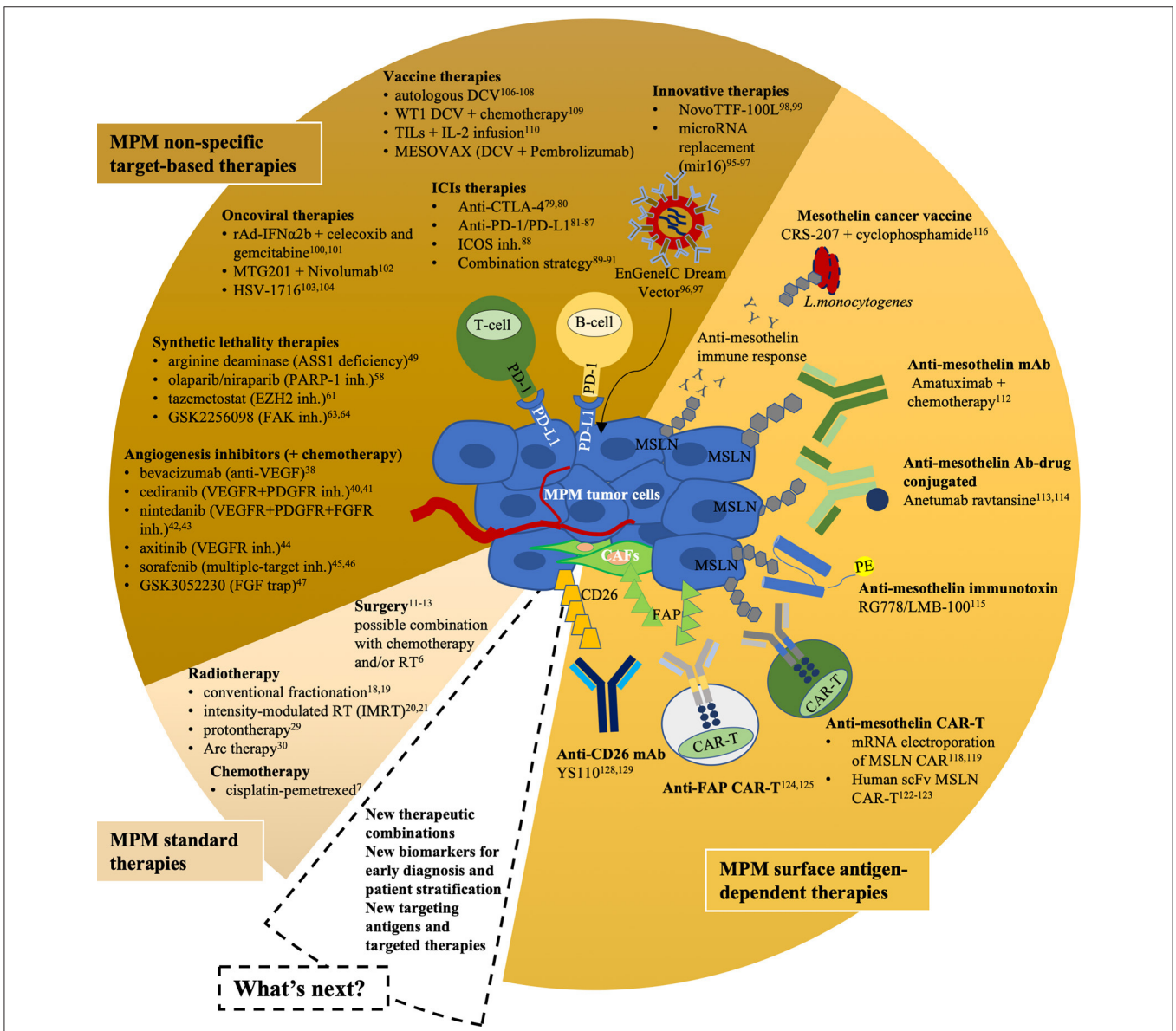

FIGURE 1 | Current and innovative clinical approaches for MPM. Different segments represent MPM standard therapies (light brown), MPM non-specific target-based therapies (brown), and MPM surface antigen-dependent therapies (orange). inh., inhibitor; DCV, dendritic cell vaccination; WT1, Wilms' Tumor Antigen; CAR, chimeric antigen receptor; IL-2, interleukin-2; TILs, tumor-infiltrating lymphocytes; MSLN, mesothelin; FAP, fibroblast activation protein; CAFs, cancer-associated fibroblasts; PE, *Pseudomonas exotoxin* A.

TABLE 1 | Overview of MPM clinical trials.

References	Clinical trial code	Acronymous	Type of study	Treatment	OS (months)	PFS (months)	ORR (%)	DCR (%)	Result or status
ANTI-ANGIOGENIC THERAPIES									
Zalcman et al. (38)	NCT00651456	MAPS	III	CT -/+ bevacizumab	**18.8**	9.2	NE	NE	Pos
Tsao et al. (41)	NCT01064648	SWOG S0905	II	CT + ceradinib or pl.	10.0	**7.2**	50.0	NE	Neg
Scagliotti et al. (43)	NCT01907100	LUME-Meso	III	CT + nintedinab or pl.	14.4	**6.8**	45.0	91.0	Neg
Buikhuisen et al. (44)	NCT01211275	–	II	CT -/+ axitinib	18.9	5.8	36.0	79.0	Neg
Dubey et al. (45)	NCT00107432	–	II	Sorafenib	9.7	3.6	**6.0**	60.0	Neg
Papa et al. (46)	NCT00794859	SMS	II	Sorafenib	9.0	**5.1**	6.0	62.0	Neg
van Brummelen et al. (47)	NCT01868022	–	Ib	GSK3052230 + CT	NE	7.4	**39.0**	86.0	Pos
SYNTHETIC LETHALITY THERAPIES									
Beddowes et al. (49)	NCT02029690	TRAP	I	ADI-PEG 20 + CT	6.3	5.2	0	80.0	Pos (primary endpoints: recommended dose, safety, and tolerability)
	NCT02709512	ATOMIC-Meso	III	ADI-PEG 20	–	–	–	–	Ongoing
Zauderer et al. (61)	NCT02860286	–	II	Tazemetostat	NE	NE	NE	**51.0**	Pos
Fennell et al. (64)	NCT01870609	COMMAND	II	Defactinib or pl.	**12.7**	**4.1**	18.0	64.0	Neg
IMMUNOTHERAPIES									
Calabrò et al. (79)	NCT01649024	MESOT-TREM-2008	II	Tremelimumab	10.7	6.2	**7.0**	31.0	Neg
Maio et al. (80)	NCT01843374	DETERMINE	IIb	Tremelimumab or pl.	**7.7**	2.8	5.0	28.0	Neg
Quispel-Janssen et al. (81)	NCT02497508	NivoMes	II	Nivolumab	11.8	2.6	24.0	**47.0**	Pos
Okada et al. (82)	JapicCTI-163247	MERIT	II	Nivolumab	17.3	6.1	**29.4**	NE	Pos
Fennell et al. (83)	NCT03063450	CONFIRM	III	Nivolumab or pl.	–	–	–	–	Ongoing
Alley et al. (84)	NCT02054806	KEYNOTE-028	I	Pembrolizumab	18.0	5.4	**20.0**	72.0	Pos
Desai et al. (85)	NCT02399371	–	II	Pembrolizumab	11.5	4.5	**19.0**	66.0	Pos
Popat et al. (87)	NCT02991482	PROMISE-meso	III	Pembrolizumab vs. CT	10.7	2.5	22.0		Neg
Angevin et al. (88)	NCT02723955	INDUCE-I	I	GSK3359609	–	–	–	–	Ongoing
Scherpereel et al. (89)	NCT02716272	MAPS2	II	Nivolumab vs. nivolumab + ipilumab	11.9–15.9	4.0–5.6	19.0–28.0	**44.0–50.0**	Pos
Zalcman et al. (90)	NCT02899299	Checkmate 743	III	Nivolumab + ipilumab vs. CT	–	–	–	–	Ongoing
Calabrò et al. (91)	NCT02588131	NIBIT-MESO-1	II	Tremelimumab + durvalumab	16.6	5.7	**28.0**	63.0	Pos
–	NCT02784171	CCTG	III	CT vs. CT + pembrolizumab vs. pembrolizumab	–	–	–	–	Ongoing
INNOVATIVE THERAPIES									
van Zandwijk et al. (97)	NCT02369198	MesomiR 1	I	TargomiRs	6.7	NE	5.0	73.0	Pos (primary endpoints: MTD and DLT)
Ceresoli et al. (99)	NCT02397928	STELLAR	II	TTFields + CT	**18.2**	7.6	40.0	97.0	Pos
ONCOVIRAL THERAPIES									
Sterman et al. (101)	NCT01119664		I/II	–/+ CT + rAd-IFNa2b + CT	21.5	–	25.0	88.0	Pos (primary endpoint: safety)

(Continued)

TABLE 1 | Continued

References	Clinical trial code	Acronymous	Type of study	Treatment	OS (months)	PFS (months)	ORR (%)	DCR (%)	Result or status
	NCT03710876	INFINITE	III	rAd-IFNa2b + celecoxib + gemcitabine	–	–	–	–	Ongoing
Goto et al. (102)	UMIN000013568	–	I/II	Ad-SGE-REIC		3.4		62.0	Pos (primary endpoints: safety and tolerability)
–	NCT04013334	MTG201-MPM-001	II	Ad-SGE-REIC + nivolumab	–	–	–	–	Ongoing
Danson et al. (104)	NCT01721018	–	I/IIa	HSV-1716	15.0	NE	NE	50.0	Pos (primary endpoints: safety and tolerability)
–	NCT01503177	–	I	Measles virus encoding NIS	15.0	2.1	0	67.0	Pos (primary endpoint: AE profile)
DENDRITIC CELL VACCINATION									
Cornelissen et al. (107)	NCT01241682	PMR-MM-002	I	Tumor lysate-pulsed DCV	NE	NE	NE	80.0	Pos (primary endpoint: number of cytotoxic T cells and regulatory T cells in the blood of patients)
Belderbos et al. (108)	NCT03610360	DENIM	II/III	Tumor lysate-pulsed DCV + BSC vs. BSC	–	–	–	–	Ongoing
Berneman et al. (109)	NCT01291420	–	I/II	WT1 DCV	32.0	5.0	NE	NE	Pos (primary endpoint: immunogenicity of intradermal DCV)
–	NCT02649829	MESODEC	I/II	WT1 DCV + CT	–	–	–	–	Ongoing
Doherty et al. (110)	NCT02414945	TILs-003-Meso	I/II	TILs + IL-2	–	–	–	–	Ongoing
–	NCT03546426	MESOVAX	Ib	DCV + pembrolizumab	–	–	–	–	Ongoing
ANTI-MSLN (IMMUNO)THERAPY									
Hassan et al. (112)	NCT00738582	–	II	Amatuximab + CT	14.8	**6.1**	40.0	91.0	Neg
–	NCT02357147	ARTEMIS	II	Amatuximab + CT	–	–	–	–	Terminated for business reasons
Blumenschein et al. (113)	NCT01439152	–	I	AR	NE	NE	31.0	75.0	Pos (primary endpoint: MTD and pharmacokinetic profile)
Kindler et al. (114)	NCT02610140	–	II	AR or vinorelbine	10.1	**4.3**	8.0	NE	Neg
–	NCT03126630	MC1721	I/II	AR + pembrolizumab	–	–	–	–	Ongoing
–	NCT02798536	–	I	RG778/LMB-100 –/+ nab-paclitaxel	–	–	–	–	Ongoing
Hassan et al. (116)	NCT01675765	ADU-CL-02	I	CRS-207 –/+ cyclophosphamide + CT	14.7	7.5	54.0	89.0	Pos (primary endpoints: AE profile and induction of an immune response to MSLN)
Zhao et al. (118) and Beatty et al. (119)	NCT01355965	UPCC 17510	I	MSLN-CAR-T (mouse scFv)	NE	NE	NE	NE	Pos (primary endpoint: AE profile)
Adusumilli et al. (122, 123)	NCT02414269	–	I	MSLN-CAR-T (human scFv) + pembrolizumab	–	–	–	–	Ongoing
IMMUNOTHERAPIES AGAINST NON-MSLN TARGETS									
Curioni et al. (125)	NCT01722149	FAPME-1	I	FAP-targeted CAR-T	NE	NE	NE	NE	Pos (primary endpoint: safety)
Angevin et al. (128)	NCT03177668	YS1101	I	YS110 (anti-CD26)	**9.5**	**3.0**	**14.0**	**71.0**	Pos

OS, overall survival; PFS, progression-free survival; ORR, objective response rate; DCR, disease control rate; CT, chemotherapy; pl., placebo; NIS, sodium/iodide symporter; DCV, dendritic cell vaccination; BSC, best supportive care; WT1, Wilms' Tumor Antigen; TILs, tumor-infiltrating lymphocytes; IL-2, interleukin-2; AR, Anetumab ravtansine; MSLN, mesothelin; CAR, chimeric antigen receptor; neg, negative; pos, positive; NE, not evaluated; MTD, maximum tolerated dose; DLT, dose-limiting toxicities; AE, adverse event; scFv, single chain fragment variable. Primary endpoints are in bold or indicated in the last column.

a good tolerance of treatment and persistence of CAR-T cells (125).

CD26 is a receptor overexpressed by all MPM histotypes and involved in immune regulation, T-cell activation, and the malignant potential of several cancers (126, 127). YS110 is a humanized mAb targeting CD26 that is currently under investigation in a phase I clinical trial (NCT03177668) in MPM patients. Preliminary results show that 50% (13/26) of patients achieved SD, with a median PFS of 43 days (128, 129).

DISCUSSION

Despite amazing efforts devoted to understanding and treating MPM better (**Figure 1** and **Table 1**), clinical practice has not changed over the past decades, and CT remains the only standard option. Anti-angiogenic therapies and also ICIs that showed impressive clinical responses in other solid malignancies have little impact on survival in MPM as single agents, while ICI combination efficiency comes at the cost of relevant toxicities. The hopes for patients with MPM are, therefore, innovative therapies such as oncoviral, TTFields, TargomiRs, and CAR therapies in combination with anti-PD-1 ICIs that have shown good preliminary efficacy, although the results need confirmation in larger trials.

AUTHOR CONTRIBUTIONS

FN prepared the manuscript and figure. MM prepared part of the manuscript, provided guidance to FN in preparing the manuscript, and proofread and edited the manuscript. MB, GB, AD, MG, and LC helped with the review and made vital modifications along with suggestions to improve the content. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Manipulating microRNAs for the Treatment of Malignant Pleural Mesothelioma: Past, Present and Future

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microRNAs (miRNAs) are an important class of non-coding RNA that post-transcriptionally regulate the expression of most protein-coding genes. Their aberrant expression in tumors contributes to each of the hallmarks of cancer. In malignant pleural mesothelioma (MPM), in common with other tumor types, changes in miRNA expression are characterized by a global downregulation, although elevated levels of some miRNAs are also found. While an increasing number of miRNAs exhibit altered expression in MPM, relatively few have been functionally characterized. Of a growing number with tumor suppressor activity *in vitro*, miR-16, miR-193a, and miR-215 were also shown to have tumor suppressor activity *in vivo*. In the case of miR-16, the significant inhibitory effects on tumor growth following targeted delivery of miR-16-based mimics in a xenograft model was the basis for a successful phase I clinical trial. More recently overexpressed miRNAs with oncogenic activity have been described. Many of these changes in miRNA expression are related to the characteristic loss of tumor suppressor pathways in MPM tumors. In this review we will highlight the studies providing evidence for therapeutic effects of modulating microRNA levels in MPM, and discuss these results in the context of emerging approaches to miRNA-based therapy.

Keywords: microRNA, malignant pleural mesothelioma, tumor suppressor miRNA, oncomiR, extracellular vesicles, drug delivery, drug formulation

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INTRODUCTION

Numerous studies in the last decade have shed light on the characteristic changes in microRNA (miRNA) expression in malignant pleural mesothelioma (MPM). MiRNAs are an important class of non-coding RNA that post-transcriptionally regulate the expression of most protein-coding genes (1). In addition to central roles in normal biology, their aberrant expression in tumors contributes to all of the hallmarks of cancer (2). In common with other tumor types, changes in miRNA expression in MPM are characterized by a global downregulation, although elevated levels of some miRNAs are also found (3). These changes have been explored in order to identify new biomarkers, as well as to better understand the role of miRNAs in MPM biology and to evaluate their potential as therapeutic targets for MPM (3, 4). In this review, we focus on miRNAs for which biological activity in MPM has been demonstrated, in particular highlighting *in vivo* findings and clinical studies. These will be discussed in relation to

the development of miRNAs (and siRNAs) as therapies for cancer and other diseases.

MODULATING microRNA LEVELS IN MPM

Tumor Suppressor miRNAs—Early Studies

Multiple miRNAs are downregulated in MPM samples when compared with non-neoplastic control tissue (see reviews) but relatively few have been characterized functionally (**Table 1**). Initial studies reported modest *in vitro* tumor suppressor activity of miR-29c-5p, miR-31-5p, and miR-145-5p, among others. In a series of surgical samples, lower levels of miR-29c-5p (the rarer passenger strand of miR-29c) were associated with poor prognosis (16). Using a mimic to restore expression levels revealed miR-29c-5p to have modest tumor suppressor activity in two MPM cell lines *in vitro*, by inhibition of proliferation and migration/invasion. The same mimic led to downregulation of the DNA methyltransferases DNMT3A and DNMT3B, as well as increasing expression of upstream signaling molecules including adiponectin. In a subsequent study, the same group demonstrated frequent loss of miR-31 expression in MPM cell lines due to co-deletion of *MIR31HG* with the *CDKN2A* locus (17). Re-expressing miR-31 with a mimic again led to modest inhibition of proliferation, clonogenic growth and migration/invasion in the same two MPM cell lines. Loss of miR-31 further correlated with the elevated expression of cell cycle and replication-associated genes.

Following these initial studies, *in vitro* tumor suppressor activity in MPM has been ascribed to a growing number of

TABLE 1 | Dysregulated miRNAs with biological activity in MPM.

	Expression change in MPM			Activity				
microRNA	**Cells**	**Tumors**	**Prognostic value?**	***In vitro***	***In vivo***	**Experimentally validated function(s)**	**TS or oncomiR?**	**References**
Let-7a	N.D.	N.D.	N.D.	√	–[¶]	Induced by EphrinA1; inhibits RAS	TS	(5)
miR-1-3p	N.D.	↓	N.D.	√	–	Inhibits proliferation and migration/invasion; targets PIM1	TS?	(6, 7)
miR-15a-5p	↓	↓	None	√	–	Inhibits growth of MPM cells	TS	(8)
miR-15b-5p	↓	↓	None	√	–	Inhibits growth of MPM cells	TS	(8, 9)
miR-16-5p	↓	↓	None	√	√[*]	Tumor suppressor functions; downregulates CCND1, BCL2, and PD-L1	TS	(8, 9)
miR-17-5p	↓	↓	High Exp = SS	√	–	Inhibits migration; targets KCNMA1	TS	(10)
miR-18a-5p	↑	N.D.	High Exp = SS	√	–	Antimir causes modest growth inhibition; targets PIAS3	OncomiR	(11)
miR-21-5p	N.D.	↑	High Exp = SS	√	–	Mimic causes modest growth inhibition; targets mesothelin	TS?	(12–14)
miR-24-3p	↑	↑	N.D.	√	(√)	Promotes migration and tumor growth in mice; targets CGN	Oncomir	(15)
miR-29c-5p	↓	N.D.	High Exp = LS	√	–	Mimic inhibits growth and migration; targets DNMT1/3A	TS	(16)
miR-31-5p	↓	↓	High Exp = SS	√	–	Mimic inhibits growth and migration; targets PPP6C; role in drug resistance	TS?	(17–19)
miR-34a-5p	↓	↓	N.D.	√	√[i]	Lost in genetically modified mouse model; targets c-Met	TS	(20–23)
miR-34b-3p	↓	↓	N.D.	√	√	Inhibits MPM growth, enhance radiosensitivity; inhibitors promote mesothelial proliferation	TS	(21, 22, 24–26)
miR-34c-5p	↓	↓	N.D.	√	√	Inhibits MPM growth, enhance radiosensitivity; inhibitors promote mesothelial proliferation	TS	(21, 22, 24–26)
miR-126-3p	↓	↓	N.D.	√	(√)	Induced by oxidative stress; alters metabolism, inhibits respiration, angiogenesis; targets IRS1	TS?	(27, 28)
miR-137-3p	↑/↓	↑/↓	High Exp = SS	√	–	Inhibits growth and migration/invasion; targets YB-1	TS	(29)
miR-145-5p	↓	↓	N.D.	√	(√)	Inhibits clonogenicity and migration, sensitizes to pemetrexed; regulates OCT4	TS	(30)
miR-182-5p	↑	N.D.	N.D.	√	–	Overexpressed, antimir inhibits growth; targets FOXO1	OncomiR	(6, 31)
miR-183-5p	↑	N.D.	N.D.	√	–	Overexpressed, antimir inhibits growth; targets FOXO1	OncomiR	(6, 31)
miR-193a-3p	↓	↓	High Exp = LS	√	√[*]	Tumor suppressor; targets MCL-1 and PD-L1	TS	(9, 32)
miR-193a-5p	↓	↓	High Exp = LS	√	–	Tumor suppressor function	TS	(32)
miR-205-5p	↓	E>non-E	N.D.	√	–	Involved in EMT, affects migration; targets ZEB1 and ZEB2	TS	(33)
miR-206-3p	N.D.	↓	High Exp = LS	√	√	Inhibits growth and migration/invasion; targets KRAS/CDK4/CCND1	TS	(7, 34)
miR-223-3p	↓	↓	N.D.	√	–	Inhibits migration; targets STMN1	TS	(35)
miR-215-5p	↓	↓	High Exp = LS	√	√[*]	P53 regulated, mimic inhibits growth; targets MDM2	TS	(36)
miR-302b-3p	N.D.	N.D.	N.D.	√	–	Induced by EphrinA1, inhibits proliferation; targets MCL1	TS	(37)

√, activity shown experimentally; –[¶], no experimental evidence of activity; √[*], in vivo activity following systemic administration; (√), in vivo activity consists of tumour cells transfected pre-implantation; √[i], in vivo activity inferred by loss of function; ↑, increased expression; ↓, decreased expression; ↑/↓, expression either up- or downregulated; SS, short survival; LS, long survival; E, epithelioid MPM; non-E, non-epithelioid MPM.

miRNAs (**Table 1**). A well-characterized example is miR-145. Restoring expression of miR-145, one of a number of miRNAs found to be down-regulated in a small series of MPM tumor samples, inhibited proliferation and migration, and induced senescence (30). MPM cells transfected with a miR-145 mimic before implantation into SCID mice formed fewer and smaller tumors compared with control mimic-transfected cells. At least part of the activity of miR-145 was linked to its targeting of OCT4, a gene involved in the hypermigratory phenotype of aggressive tumors via control of the epithelial-to-mesenchymal transition (EMT). Another miRNA influencing EMT in MPM is miR-205. In a comparison of epithelioid and non-epithelioid tumors, EMT regulators ZEB1 and ZEB2 were expressed at lower levels in biphasic and sarcomatoid tumors, along with a decrease in epithelial markers (33). These changes corresponded with a decrease in miR-205 in MPM tumor samples and cells lines. Transfecting MSTO-211H cells with a miR-205 mimic reduced ZEB1/2 expression and inhibited migration and invasion.

Tumor Suppressor miRNAs—*in vivo* Activity

Despite the increasing number of miRNAs exhibiting tumor suppressor function in MPM, only a handful have been demonstrated to have *in vivo* activity in clinically relevant models. In the case of miR-16-5p and miR-193a-3p, the growth inhibitory activity of both *in vitro* was confirmed in xenograft tumor models in two independent studies (8, 32). In these studies, mimics were loaded into bacterial minicells and targeted to MSTO-211H-derived xenografts via an EGFR-specific antibody. The minicells (known as EDVs) are formed through the asymmetric cell division of bacterial, and were previously used to deliver drugs and siRNAs to tumor xenografts (38, 39). Minicell delivery is achieved through a combination of passive accumulation via the leaky vasculature of the tumor and specific targeting using antibodies to a cell-surface antigen (EGFR) in the tumor. In both studies, systemic administration of mimic-loaded minicells led to significant inhibitory effects on tumor growth (8, 32). This was likely to be at least in part due to the inhibition of anti-apoptotic and cell cycle genes demonstrated *in vitro* in these studies.

Results from these studies laid the foundation for the phase I MesomiR-1 trial, investigating the safety and optimal dose of a miR-16-based mimic delivered in anti-EGFR antibody-targeted bacterial minicells, dubbed TargomiRs. The mimic was a novel sequence based on the consensus sequence of the miR-15 family (all of which are downregulated in MPM), which was shown to inhibit tumor xenograft growth at a similar level to native miR-16-5p (40). This trial of 27 patients demonstrated safety of the treatment as well as initial signs of activity, with one objective response (41) and stable disease in a further 15 patients (42). With miR-16-5p also impacting response to chemotherapy (8) and contributing to PD-L1 regulation (9) *in vitro*, restoration of miR-16-5p levels in combination with chemo or immunotherapy are potential future applications of this approach. In addition, recent demonstration of effective delivery of doxorubicin to MPM xenograft tumors using a mesothelin-specific antibody (43) further expands the scope of possible future trials.

Other miRNAs shown to exhibit pronounced tumor suppressor activity, including miR-137-3p and miR-193a-3p, are further candidates for clinical development using minicells. In the case of miR-193a-3p, minicell-mediated delivery inhibited tumor growth to a similar extent as miR-16-5p (32). In addition, both the 5p and 3p arms of miR-193a have growth inhibitory effects in MPM (32) and other cancers (44, 45), meaning that delivery of a mimic with two active arms would potentially increase the activity. The lower levels of both arms of miR-193a recently found to be associated with shorter overall survival in the TCGA study (46) (see below) lend support to this notion. A miR-137-3p mimic also led to pronounced inhibition of proliferation and migration in the majority of MPM cell lines tested (29). These phenotypes appeared to be predominantly due to miR-137-3p-mediated suppression of *YBX1*, previously identified as an oncogene in a range of cancer types, as there was no evidence of additivity when miR-137-3p was used in combination with a YBX1-specific siRNA (29).

While minicells remain the most clinically advanced approach to mediate systemic delivery of miRNA mimics, other vehicles have been regularly employed in preclinical cancer studies to deliver miRNAs and siRNAs (47). At this stage, however, we are not aware of any that have been tested in MPM. An early publication demonstrating the tumor suppressor activity of a miR-34b/c construct (24) was followed up by a short report describing *in vivo* delivery of an adenoviral vector expressing miR-34b/c (25). In this study, intratumoral injection of the adenoviral construct led to increased miR-34b/c expression in xenograft tumors and significant growth inhibition. More recently, atelocollagen was used to successfully deliver a miR-215-5p mimic in xenograft models of MPM (36). This study, based on the hypothesis that the well-known retention of functional p53 in MPM tumors that was recently confirmed by NGS studies (46, 48), could represent a molecular vulnerability. The expression of the p53-regulated miRNAs of the miR-192/194/215 family were assessed in MPM samples and high levels of miR-215-5p were found to be associated with increased overall survival. Mimics of all three family members were associated with growth inhibition, with miR-215-5p more effective than miR-194 or miR-192, the latter consistent with previous observations (32). The inhibitory effects of miR-215-5p were associated with decreased MDM2 protein levels and consequently an increase in p53 and its downstream effectors including p21, Bax and Puma (36). Moreover, the miR-215-5p mimic-mediated activation of p53 also caused an increase in miR-145-5p, the tumor suppressor miRNA discussed in the previous section (30). These *in vitro* studies were expanded to test miR-215-5p *in vivo* using a mimic complexed with atelocollagen to mediate local delivery. Peritumoral injection of this complex in a subcutaneous xenograft model reduced tumor volume, induced apoptosis and—importantly—increased levels of miR-215-5p in the tumor. Intrapleural administration reduced growth of orthotopic xenografts and improved the survival of tumor-bearing mice. This latter result is very relevant to MPM, where intrapleural drug delivery has been used in experimental treatment.

Oncogenic miRNAs

A number of miRNAs are consistently found to be upregulated in certain cancer types, where they have cancer promoting function and have been termed oncomiRs. In contrast to the use of mimics to restore levels of tumor suppressor miRNAs downregulated in MPM, inhibition of overexpressed oncogenic miRNAs with antisense oligonucleotides is an alternative strategy for modulating miRNA levels. This approach is attractive as it may be amenable to local delivery, avoiding the problems associated with tumor targeting via systemic administration. While the number of miRNAs found to be consistently overexpressed in MPM is relatively small, recent studies suggest that their inhibition can have profound effects on MPM growth. One such example was the report of the effects of inhibiting the overexpressed miR-182-5p and miR-183-5p (31). They are upregulated in MPM cell lines where they promote proliferation and invasion, at least in part due to suppression of FOXO1. Reducing their levels with miRNA inhibitors reversed these effects, with dual inhibition showing additive effects. An oncogenic role for miR-182-5p was first demonstrated in melanoma, in which this miRNA enhances migration, invasion and metastasis via inhibition of FOXO3 and MITF (49). Upregulation of miR-182 in melanoma is due to amplification (at 7q31) of a miRNA cluster which also contains the related miR-183 and miR-96. As this region appears to be more frequently lost in MPM, the mechanism for overexpression remains to be determined.

Another miRNA with oncogenic activity in MPM is miR-24-3p, which was identified via a screen of polysome-associated miRNAs and is upregulated in cell lines and tumor samples (15). This miRNA regulates a range of genes involved in cell adhesion and communication, many of which are associated with good prognosis, and miR-24-3p knockdown reduced migration and invasion *in vitro* and *in vivo*. Although the targets of miR-24-3p identified in this study had no obvious link to MPM biology, it is intriguing that in other cancers miR-24-3p regulates both transcripts produced by the *CDKN2A* locus. Moreover, miR-24 is part of the miR-23a/24-2/27a cluster that is regulated by c-Myc and contributes to metastasis in breast cancer (50), and miR-23a and miR-27a upregulation was previously linked to loss of expression of the tumor suppressor ZIC1 (51). Whether other well-known oncogenic miRNAs such as miR-155, and miR-10b promote MPM tumor progression remains to be seen, but the initial results with miR-182-5p, miR-183-5p and miR-24-3p warrant further pre-clinical development.

miRNAs With Unexpected Activity in MPM

Recent studies suggest that miRNAs with oncogenic function in other tumor types may have variable function in MPM. In addition, several miRNAs that are reported to be downregulated in MPM compared with control tissue are nonetheless associated with poor prognosis in tumors with higher than median expression (**Figure 1**). The case of miR-21 is a prominent example of an oncogenic miRNA with unexpected function in MPM. This miRNA is upregulated in numerous tumor types where it is associated with multiple oncogenic functions (52). In MPM, high expression of miR-21-5p in tumor samples was associated with poor prognosis in a series of surgical samples (12). MiR-21-5p was also detected in MPM but not normal tissue by *in situ* hybridization, and was inversely correlated with expression of its target gene PDCD4 (13). In light of these observations, it is surprising that the only study to date to assess miR-21-5p activity in MPM suggests that it has modest tumor suppressor function. In a study designed to identify regulators of the MPM marker mesothelin (MSLN), both miR-21-5p and miR-100-5p were found to interact with the *MSLN* 3'UTR (14). Further experimentation revealed that a miR-21-5p mimic led to modest but significant inhibition of proliferation in two MPM cell lines, with a more pronounced reduction in colony forming ability. The authors ascribed this observation to a tumor suppressor effect that was previously observed following MSLN silencing (53).

Studies from two independent laboratories also suggest that members of the miR-17∼92 polycistron, generally considered to be oncogenic (54), appear to have inconsistent functions in MPM. The first used bioinformatics to look for enriched

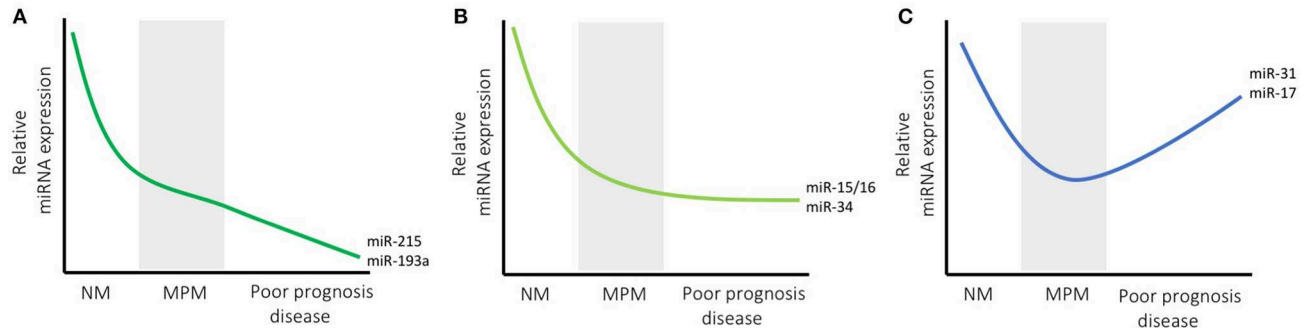

FIGURE 1 | microRNA expression changes with disease course in MPM. The expression of most miRNAs is lower in MPM than normal mesothelium (NM) levels, and is shown schematically for three representative groups (levels are shown relative to NM, and are in arbitrary units for illustrative purposes). Some miRNAs are found at lower levels in tumors with poor prognosis (e.g., miR-215 and miR-193a) which may indicate a continuing gradual decrease in expression with tumor progression (indicated by decreasing levels in **A**). Others, such as miR-15/16 and the miR-34 family are consistently decreased in MPM samples but do not appear to have prognostic value, suggesting they do not change with advanced stage **(B)**. Another group, exemplified by miR-31 and miR-17, exhibit lower levels in MPM compared with NM, but are also higher in patients with shorter survival, possibly indicating an increase in expression with tumor progression **(C)**.

microRNA binding sites in genes exhibiting upregulated mRNA expression, and found that miR-17 and miR-30 were both overrepresented (10). The upregulated mRNA expression was correlated with downregulation of members of the miR-17 and miR-30 seed families in both MPM cell lines and tumor samples compared with controls, and a miR-17-5p mimic reduced MPM cell migration corresponding to the downregulation of the KCa1.1 potassium channel. This result was somewhat unexpected as high levels of miR-17-5p and miR-19b-3p were associated with shorter survival in MPM patients undergoing surgery (12). In contrast, a third miRNA from the miR-17∼92 cluster, miR-18a, was found to have modest oncogenic activity in MPM (11). In this study, analysis of RNA-seq data from the TCGA study revealed that high expression of miR-18a, but not others from this cluster, was associated with shorter survival. Antisense inhibition of miR-18a led to a small but significant decrease in the viability of MPM cells. The apparent discrepancy of these results may be due to the complex processing of the complex sequential processing of the 6 mature miRNAs in the polycistron, which are known to be expressed at variable levels in cells (55). Together, these results provide evidence for MPM-specific activity of mature miRNAs from the miR-17∼92 cluster and warrant further investigation.

A further example of apparent inconsistencies between miRNA expression levels and activity in MPM was found in the case of miR-137-3p. Expression of this miRNA was found to be highly variable in normal mesothelium and to a greater extent in tumor samples, where evidence for both very high and very low expression was observed (29). This contrasts with most studies of miR-137-3p in cancer, where it is almost always downregulated (56). A similar range of expression was found in MPM in cell lines compared with the mesothelial control MeT-5A, but whether this correlates with the expansion of a variable nucleotide tandem repeat (VNTR) upstream of miR-137 implicated in altered processing (57) was not tested. In the series of 115 patients analyzed, high expression (defined as >2-fold increase compared with median) of miR-137-3p was associated with shorter survival. Surprisingly, an antisense inhibitor of miR-137-3p had no effect on growth whereas a mimic significantly inhibited proliferation and migration/invasion in most MPM cell lines, including those with high endogenous expression.

Another miRNA with apparent discrepancies between expression levels and functional activity is miR-31-5p. Previous studies have revealed tumor-specific functions of this miRNA, with both tumor-suppressor and oncogenic properties being observed (58). As described in the previous section, loss of miR-31-5p expression was originally linked to its tumor-suppressor activity in MPM (17). Intriguingly, this miRNA was more highly expressed in sarcomatoid tumors, albeit in a small sample set (18), and also contributed to cisplatin resistance in MPM cell lines *in vitro* (19). Whether this miRNA is solely tumor suppressive in MPM, or its activity changes over the course of the disease or in different histological subtypes, is still an open question. In contrast to downregulation in MPM, miR-31-5p was shown to be overexpressed in both mouse and human lung cancers (59), and to exhibit oncogenic activity in lung cancer cell lines (59) and in xenografts (60), with the latter observation linked to its control of BAP1 expression (60). Furthermore, a miR-31-5p antimiR repressed esophageal tumor growth *in vivo* (61), whereas in breast cancer, miR-31-5p contributed to the maintenance of the stem cell compartment and miR-31 KO compromised breast cancer tumorigenesis (62).

Pathways Commonly Dysregulated in MPM Alter miRNA Levels

As the number of miRNAs known to be altered in MPM continues to grow, it is interesting to note that critically dysregulated pathways in this disease converge on miRNA biology (**Figure 2**). Recent reports in MPM combined with earlier studies investigating the mechanistic basis of global downregulation of miRNA expression implicate the p53 tumor suppressor response as a key effector influencing miRNA levels. While MPM is unusual among solid tumors in that it generally retains wild-type p53, the p53 response is compromised by frequent loss of the upstream regulator p14ARF (via *CDKN2A* deletion) leading to upregulated MDM2 levels and increased p53 degradation. This was exploited in the study of local delivery of miR-215-5p mimic discussed earlier (36), as miR-215 is both a direct transcriptional target of p53 and a regulator of MDM2, thus forming a positive feedback loop. The study further showed a miR-215-5p-mediated upregulation of miR-145, another target of p53. Although not evaluated, it is likely that this treatment would also result in increased expression of other miRNA targets transcriptionally regulated by p53 such as miR-34a. This would be consistent with results from a mouse model of a partial *Cdkn2a* knockout, in which miR-34a suppression contributed to elevated c-Met (20). As well as direct targets, p53 is also implicated in the global downregulation of miRNAs in two important ways. First, p53 interacts with the Drosha processing complex to stimulate conversion of pri-miRNA transcripts into pre-miRs in colorectal cancer cells, thereby enhancing the maturation (without affecting transcription) of multiple tumor suppressor miRNAs, including miR-16, miR-15a and miR-145 (63). Second, because miR-145 targets c-Myc, loss of p53-regulated miR-145 expression has the added effect of relaxing post-transcriptional control of c-Myc (64). This in turn results in the transcriptional suppression of multiple miRNAs by c-Myc, including miR-15a, miR-16, miR-34a and the miR-29 family (65). This relationship was recently demonstrated directly for miR-16 in MPM (21).

Added to the central role played by dysregulated p53 activity, Hippo signaling is also implicated in altering miRNA levels in MPM. This pathway is frequently compromised in MPM through a combination of NF2 and LATS2 mutation and YAP activation (66). Like p53, this pathway stimulates maturation of primary miRNA transcripts via interaction with the Drosha processing complex (67). At low cell density, suppressed Hippo signaling culminates in nuclear localization of YAP and cellular proliferation, which is in part due to an interaction with the microprocessor protein p72 which reduces pri-miRNA processing (67). At high cell density, YAP is sequestered in the cytoplasm, the microprocessor is active and miRNA levels increase markedly. RNAi-mediated silencing of NF2 or LATS2 led to similar decreases in

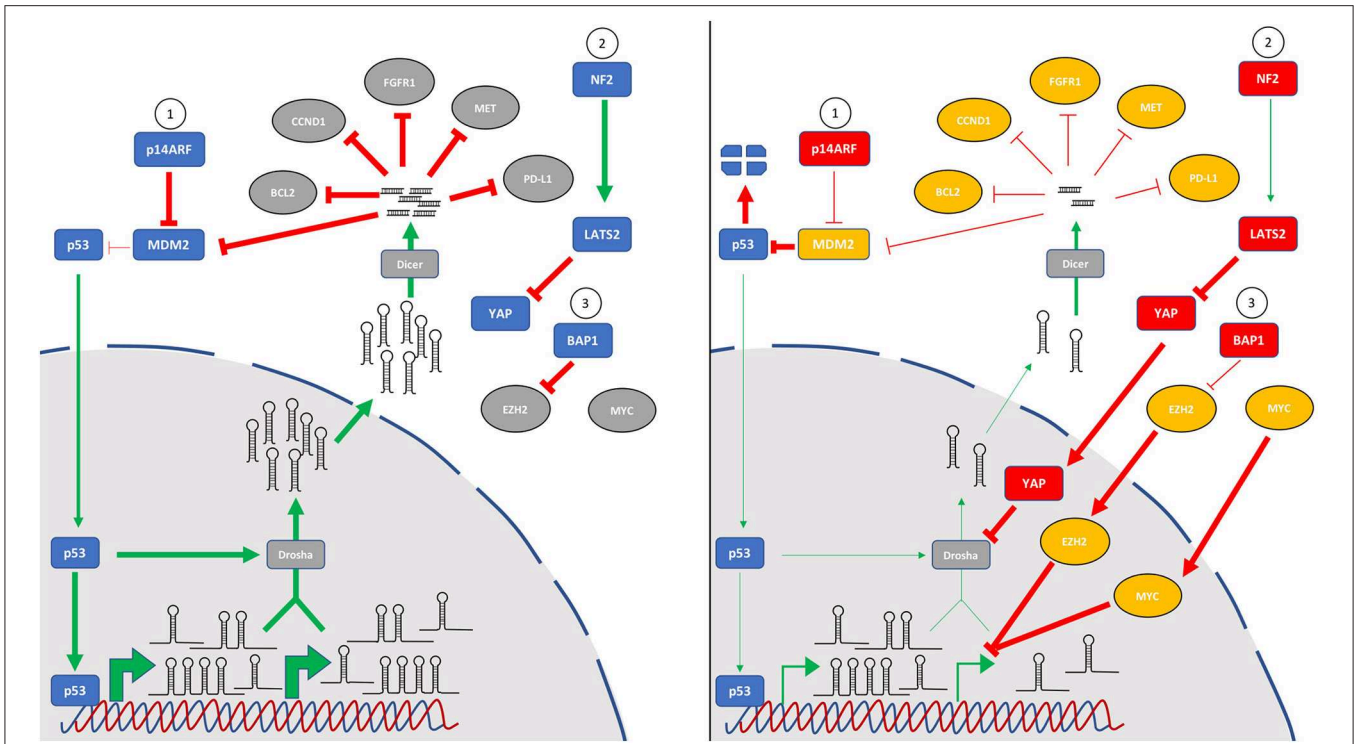

FIGURE 2 | The key pathways most frequently mutated in MPM all contribute to global downregulation of microRNA levels. Tumor suppressor miRNAs are often downregulated in MPM, and several mechanisms appear to play a role in this observation. In addition to specific control of microRNA transcription, other pathways are involved in indirect control of microRNA levels via effects on processing. In mesothelial cells (left panel), the p53 pathway (1) is intact, and cell stress induces microRNA levels via direct transcription as well as by p53-induced pri-miR processing. Similarly, normal signaling of the Hippo pathway (2) through NF2 and LATS2 phosphorylates YAP1 which is retained in the cytoplasm. BAP1 deubiquitinase activity (3) destabilizes EZH2, a key component of the polycomb repressor complex 2. In mesothelioma (right panel), frequent mutation or deletion of the *CDKN2A* locus leads to loss of p14ARF and increased MDM2-mediated p53 degradation. Loss of function mutations of NF2/Merlin or LATS2 (or gain-of-function mutations of YAP) dysregulate Hippo signaling leading to accumulation of YAP in the nucleus where it can inhibit the microprocessor complex via interaction with Drosha. Inactivating BAP1 mutations prevent control of EZH2 which can alter miRNA levels. Additionally, MYC amplification or mutation can lead to transcriptional suppression of multiple miRNA genes. Whether these changes are also involved in the upregulation of oncogenic miRNAs in MPM remains to be determined.

miRNA expression as knockdown of Drosha or p72, and affected miRNAs included let-7, miR-34a and miR-15a (67)—all found at reduced levels in MPM. Moreover, let-7 and miR-34a also target c-Myc, further exacerbating miRNA disequilibrium (68, 69). The more recently identified BAP1 mutations common in MPM (70, 71) also have a component of miRNA dysregulation. Loss of BAP1 function in MPM leads to increased expression of the polycomb repressor complex component EZH2 (72). This is consistent with the previous observations that frequent overexpression of EZH2 in MPM correlates with a decrease in levels of miR-26a and miR-101 (73), and miR-26a directly targets EZH2 in a range of cancer types (74). In turn, miR-26a is a direct target of c-Myc (75) and is downregulated in multiple cancer types. Moreover, miR-31 loss – as discussed earlier, a common event in MPM—leads to EZH2 upregulation in melanoma (76). Taken together, mutations in these three signature pathways are likely to be significant contributors to the global miRNA downregulation found in MPM.

FUTURE PROSPECTS

After a decade of research into the role of miRNAs in the biology of MPM, their potential value as biomarkers and therapeutic targets is no longer in question. Initial clinical experience from the MesomiR-1 trial suggests that miRNA modulation is safe and has the potential to alter the course of disease. With the FDA approval in August 2018 of patisiran, the first ever siRNA-based drug, gene silencing has finally reached the clinic. At the time of writing at least 20 siRNAs are being evaluated in clinical trials (47, 77). However, a number of questions remain to be answered. For instance, while many miRNAs show biological activity in MPM models, other (better) targets with more pronounced tumor suppressor function in MPM may exist. More importantly, the effective delivery of nucleic acid-based drugs in general, and miRNA mimics in particular, is a problem that is far from solved. Below we discuss these two outstanding questions and how their answers may contribute to the development of new therapies for MPM.

Additional Targets From Genomic Studies

The recent analysis of the 74 MPM samples completed by the TCGA was the first to comprehensively analyse miRNA expression in a large series of tumor samples using RNA-seq (46). Unsupervised clustering of these data revealed 5 subtypes that were associated with 5-year survival. The subgroup with the longest survival had significantly higher expression of a number of miRNAs previously identified to have tumor suppressor activity in MPM, including both miR-193a-5p and miR-193a-3p arms of miR-193a (discussed above), as well as several miR-29 family members. The prognostic value of the miR-29 family is consistent with the earlier study by Pass et al. who linked miR-29c-5p to longer survival. Their miR-29c-5p mimic inhibited growth and downregulated the DNA methyltransferases DNMT3A and DNMT3B. Overexpression of these genes in lung cancer was previously linked to reduced expression of the miR-29 family in lung cancer (78), however this was due to highly conserved targeting by the 3p arms rather than the rarer 5p arm. Similarly, the apoptosis-related gene *MCL1* and collagen genes involved in metastasis are also targeted by this family in cancer (79). As both studies used early versions of microRNA mimics it is possible that one or both were based on a pre-miR mimic containing both 5p and 3p arms. The miR-29 family also indirectly increases p53 activity by suppressing p85 and CDC42, negative regulators of p53 (80). These observations suggest that revisiting the role of the miR-29 family in MPM could reveal broader activities. Moreover, the TCGA analysis revealed a number of miRNAs with prognostic value but no known functional role in MPM such as miR-100-5p and miR-148b-3p. As these have well-characterized tumor suppressor activity in other tumor types (81, 82), they represent additional candidates for follow-up studies. In addition, histological subtype is an important determinant of MPM biology and the different subtypes are likely to be characterized by differences in miRNA expression, as mentioned in previous sections. Confirmation of the role played by miRNAs in the aggressive nature of sarcomatoid tumors awaits comparative analysis of a larger number of samples of this type.

Mesothelioma-Specific miRNA Expression

For a miRNA to make an effective biomarker or therapeutic target in MPM, it would ideally be expressed selectively (or even better specifically) in the cell or tissue of interest. Of the miRNAs investigated to date as potential biomarkers and therapeutic targets, however, almost all are evolutionarily conserved and expressed widely in most tissues and cell types. This observation is not peculiar to MPM, however, and can be seen by the predominance of relatively few miRNAs in functional preclinical studies of miRNA targeting approaches across cancer in general. Following the discovery of miRNAs in mammalian cells, the majority of human miRNAs identified were highly conserved, and this is reflected by most entries in the mirbase database. However, in a series of recent papers, a large number of cell- and tissue specific miRNAs were identified from RNA-seq data via computational approaches (83–85). These new miRNAs exhibit similar GC content and genome distribution to conserved miRNAs, and the expression of a number has been validated via RT-qPCR. While few have been characterized on a functional level, they nonetheless represent a rich source of potential biomarkers and therapeutic targets. Most recently, a study compared specific miRNA expression in lung cancer and MPM, demonstrating highly specific expression of a number of miRNAs that may prove able to assist with differential diagnosis (86). As a number are either highly expressed in MPM or present at lower levels, they represent candidate tumor suppressors and oncomiRs. Ongoing research will be required to determine whether they are altered in MPM carcinogenesis and to elucidate their functions.

Alternative Delivery Approaches

Increasing evidence supports the concept that miRNA mimics represent a valid approach to therapy in MPM, but to date the only clinical experience remains the MesomiR-1 trial of TargomiRs (42). While the FDA approval of patisiran, and ongoing development of other siRNA- and miRNA-based drugs using liposomal or direct conjugation to targeting moieties underlines the potential for miRNAs to serve as cancer drugs (47), delivery to tumor cells *in vivo* remains a major hurdle (87). As the lipid-based delivery vehicles commonly used for double-stranded RNA drugs frequently accumulate in the liver, most siRNA- or miRNA-based drugs in development target hepatocytes. However, even with this selective delivery advantage, the miR-34a-based drug MRX34 targeting hepatocellular carcinoma or liver metastases was terminated due to unexpected immune-related adverse events (88). It is notable that seed sequence-mediated hepatotoxicity has been used to screen siRNA drug candidates prior to clinical development (89), but as the miR-34a mimic used did not cause immune events or hepatocyte damage in mouse models of liver cancer (90) and there are no published results describing these adverse events in more detail, the underlying cause remains unknown. Nevertheless, reaction to the liposomal vehicle, immune stimulation by double-stranded RNA or necrotic cell death may have played a role (88). The latter may be related to the toxicity of the GC-rich miR-34a seed, shown to preferentially downregulate survival genes and cause cell death in cancer cells (91).

In terms of vehicles for systemic delivery of miRNA mimics to organs other than the liver, few have reached an advanced stage of development. Most lipid- or nanoparticle-based systems are hampered by the inefficient escape from the endosomal system following endocytosis, meaning only a small fraction of the mimic molecules entering the cell are active in the cytoplasm (87). This is illustrated by studies with patisiran which suggest that of the 60% of the total dose that is delivered to the hepatocytes, only 3% is associated with the RISC machinery (92). An alternative approach gaining traction involves the use of extracellular vesicles (EVs) such as exosomes or microvesicles for miRNA delivery. Cells release a variety of EVs that contain a range of cellular molecules including miRNAs (93). Their ability to transfer miRNAs and mRNAs to recipient cells and influence gene expression was demonstrated in early studies (94–96), and their role in intercellular communication in cancer is now widely accepted (97). The ability of EVs to deliver miRNAs has subsequently been exploited as a potential vehicle method for miRNA mimics and siRNAs. An early study purified

exosomes from dendritic cells engineered to express a neuron-specific targeting moiety, which were then electroporated with GAPDH siRNA (98). These exosomes were able to cross the blood-brain barrier and reduce GAPDH expression in neurons and other brain cells. In a similar approach, exosomes from HEK293 cells engineered to produce an EGFR-specific peptide ligand, and transfected with let-7a mimic, delivered let-7a mimic to EGFR-expressing breast cancer xenografts and inhibited their growth (99).

A clinically advanced example of this approach is represented by the delivery of engineered exosomes loaded with mutant KRAS-targeting siRNAs to inhibit pancreatic cancer (100). Following intraperitoneal injection, exosomes loaded with siRNA accumulated to a greater extent in the pancreas, and were also more growth inhibitory in a KRAS mutant orthotopic tumor xenografts model compared with liposomes carrying the same siRNA. This was demonstrated to be a result of increased retention in the circulation and cellular uptake via micropinocytosis, due in part to endogenous transmembrane proteins (100). Although many questions remain surrounding the large-scale production and purification of EVs (92), a phase I trial of KRAS siRNA-loaded exosomes, dubbed iExosomes, is scheduled to start in early 2020 [NCT03608631].

In the context of the potential use of EVs to deliver miRNAs to MPM, three recent studies are of particular relevance. The first described showed that a miR-15a mimic loaded into isolated exosomes via electroporation was able to decrease its target BCL2 when delivered to human monocytes *in vitro*, and increased miR-15a in mouse alveolar macrophages *in vivo* (101). The second study investigated the distribution of exosome-delivered miR-126 in a co-culture system combining mesothelial or MPM cells with endothelial cells and fibroblasts. MiR-126 accumulated in endothelial cells co-cultured with mesothelial or miR-126 sensitive MPM cells, but it accumulated in fibroblasts when the system contained miR-126 insensitive MPM cells (102), suggesting a role for miR-126 in controlling angiogenesis. Finally, the third study described methotrexate (MTX) delivery using autologous tumor cell-derived membrane microparticles (TMPs) in a malignant pleural effusion (MPE) model, and was based on the homotypic adhesion properties of cancer cell TMPs that increase their uptake by cancer cells (103). Immunocompetent mice with MPE resulting from intrapleural inoculation of tumor cells, were treated with MTX-TMPs via intrapleural administration. These mice developed fewer foci, had reduced MPE volume and survived longer than those treated with empty TMPs or MTX alone (103). These results were extended in a pilot clinical trial of 11 lung cancer patients with malignant pleural effusions. Treatment with MTX-TMPs proved safe, most patients had reduced MPE volume and symptomatic improvements, and assessment of fluid revealed fewer tumor cells. The continuing phase I trial aims to recruit 90 patients and has an expected completion date of December 2019 [NCT02657460]. As exosomes (and presumably other EVs) are numerous in MPE from MPM patients (104) and in cell-conditioned medium secreted from MPM cells (105, 106), and these are preferentially taken up by the cell of origin, they represent a potential vehicle for therapeutic miRNA delivery.

CONCLUSIONS

Multiple lines of evidence support the continued development of miRNA-based therapies for MPM. Numerous miRNAs have been demonstrated to contribute to cancer hallmarks in MPM cells *in vitro*, and manipulating their expression using miRNA mimics or inhibitors can inhibit the proliferation and invasion of MPM cells and their interaction with stromal and immune cells. In addition to targeted systemic delivery with minicells, local delivery via intrapleural administration of miRNA mimics complexed with atelocollagen or encapsulated in (patient-derived) EVs have enormous potential for the treatment of MPM. Continued clinical investigation and optimization of methods for EV preparation, purification and miRNA loading will be needed to realize the potential of these novel treatment approaches.

AUTHOR CONTRIBUTIONS

GR wrote the first draft of the manuscript. GR and TJ generated the figures. GR, TJ, and NZ revised the manuscript and approved the submitted version.

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REVIEW
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Preclinical Models of Malignant Mesothelioma

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Rodent models of malignant mesothelioma help facilitate the understanding of the biology of this highly lethal cancer and to develop and test new interventions. Introducing the same genetic lesions as found in human mesothelioma in mice results in tumors that show close resemblance with the human disease counterpart. This includes the extensive inflammatory responses that characterize human malignant mesothelioma. The relatively fast development of mesothelioma in mice when the appropriate combination of lesions is introduced, with or without exposure to asbestos, make the autochthonous models particularly useful for testing new treatment strategies in an immunocompetent setting, whereas Patient-Derived Xenograft models are particularly useful to assess effects of inter- and intra-tumor heterogeneity and human-specific features of mesothelioma. It is to be expected that new insights obtained by studying these experimental systems will lead to new more effective treatments for this devastating disease.

Keywords: malignant mesothelioma, preclinical rodent models, *in vivo* asbestos carcinogenesis, genetic driver lesions, mesothelioma inflammatory phenotype, conditional tumor suppressor gene knockout/oncogene mouse models, patient-derived xenograft models of mesothelioma

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INTRODUCTION

Malignant mesothelioma (MM) is a treatment-resistant malignancy causally linked to asbestos exposure. Despite recent advances in therapeutic modalities, MM patients usually die within 1 year following diagnosis. MM is particularly lethal in patients with pleural disease, particularly those whose tumors have sarcomatoid features (1). Consequently, *in vivo* models of MM are needed to investigate MM disease pathogenesis and to provide accurate preclinical models for identifying new therapies that might move forward in clinical trials.

We here summarize where we stand with regard to existing models of MM and how they might be further improved. All the desirable features will be unlikely found in a single model, but the disease evolving in the model should mimic at least several of the salient features of human MM, such as its pathology, its gene expression patterns, the genetic driver lesions, and the inflammatory phenotype that is characteristic for MM. In view of the inflammatory phenotype of MM and the prominent role the immune system fulfills in either promoting or impairing tumor development, models exhibiting this specific feature should also be part of the armamentarium. Preferentially, the model should also exhibit a reproducible and short latency period as to permit intervention studies. The models—mostly encompassing small rodents—range from graft models in which human MM cell lines or patient-derived tumor fragments are implanted to complex conditional tumor suppressor gene knockout/oncogene mouse models.

SOMATIC GENETIC AND SIGNALING ALTERATIONS IN HUMAN MESOTHELIOMA

There is abundant evidence that inactivating somatic mutations and deletions of the tumor suppressor genes (TSGs) *BAP1*, *CDKN2A*, and *NF2* represent the most frequent genetic lesions in human malignant pleural mesothelioma (MPM) (2–13). Moreover, losses of these three TSGs are frequently seen in various combinations in a given MPM (7, 14). The notion that loss of these particular TSGs is so predominant implies that MPM development critically depends on the cellular signaling pathways that are guarded by these genes.

CDKN2A encodes p16INK4A and p14ARF, two tumor suppressors that, respectively, regulate the Rb and p53 cell cycle pathways. p14ARF is a component of the p53 pathway, and *TP53* alterations have also been observed in some MPMs (6, 15). In fact, a recent report that compared next-generation sequencing of two series of MPMs—one from The Cancer Genome Atlas (TCGA) (13) and the second from a Harvard series (12)—revealed only four "significantly mutated genes at a false discovery rate of <0.05" common to the two studies: *BAP1*, *NF2*, *TP53*, and *SETD2*, each of which showed prominent levels of inactivating nonsense, frameshift, and splice-site mutations, consistent with their putative roles as driver loss-of-function lesions in this malignancy (13). In the TCGA data set, focal deletions were found to affect several TSGs, especially *CDKN2A*, with deep, apparently homozygous deletions occurring in 36/73 (49%) tumors and single-copy losses in 5 others (7%) (13). In the Harvard series, Bueno et al. found copy number losses of *CDKN2A* in 48/95 (51%) MPMs (12). In a deletion mapping analysis, homozygous *CDKN2A* deletions were identified in 36 of 40 (90%) human MPM cell lines tested, while homozygous deletions of the adjacent locus *CDKN2B* occurred in most—i.e., 32/36—of these same cell lines (6). Experiments in mice have shown that the *Cdkn2b* also exhibits a tumor suppressor role in MPM, as its deletion concomitant with *Cdkn2a* further accelerates MPM development (our unpublished results) offering a rationale for the predominant deletion of all three tumor suppressors in this locus in MPM.

Unlike these specific TSGs, mutations of protooncogenes are seldom identified in MPM. Moreover, in the TCGA cohort, no activating mutations were observed in genes encoding components of the MAPK or PI3K/AKT pathways (13). However, both PI3K/AKT/mTOR and RAS/MAPK pathways were upregulated in this series, and they were each associated with a poor-prognosis. Moreover, despite a rarity of mutations of *PTEN* in MPM, earlier immunohistochemical (IHC) studies revealed diminished PTEN protein expression in 16 to 62% of MMs in several studies (16–18). Additionally, various receptor tyrosine kinases (RTKs) were shown to be frequently overexpressed and/or activated in MPM, resulting in activation of proliferation and pro-survival signals through the PI3K/AKT/mTOR signaling pathway (7, 19–21). Thus, it is not surprising that phospho-AKT immunostaining is observed in a high percentage (65–84%) of human MPMs (6, 16, 22, 23).

In view of the prominence of TSG inactivation and the relatively rare oncogenic gain-of-function mutations in MM, high-throughput chemical inhibitor screens and gene expression analyses have been performed in MM cell lines to identify unique vulnerabilities. Chemical screens pointed to increased sensitivity to FGFR inhibitors in a subset of the MPM cell lines. This corresponded with higher FGFR3 expression specifically in cell lines not expressing BAP1 (24). BAP1-deficient MM also showed augmented sensitivity to TRAIL (25). Furthermore, loss of BAP1 function was found associated with increased expression of EZH2, with concomitant widespread epigenetic gene silencing sensitizing the cells to EZH2 inhibitors (26), whereas the impaired argininosuccinate synthase 1 (ASS1) expression likely as a result of enhanced EZH2 levels sensitized cells to arginine deprivation (27, 28). In addition, BAP1-depleted cells showed increased sensitivity to PARP inhibition (29). Another vulnerability relates to the co-deletion of *CDKN2A* and the nearby methylthioadenosine phosphorylase (*MTAP*) gene (30), the latter rendering cells dependent on protein arginine methyltransferase (PRMT5) (31, 32). NF2 depletion leads to dysregulation of the Hippo pathway by activating the transcriptional co-activator YAP1 and its association with the TEAD family of transcription factors, resulting in up-regulation of genes that promote cell proliferation and inhibit cell death. Inhibitors that disrupt the YAP/TAZ-TEAD complex are not yet available but could serve as promising drugs in view of the strong dependence of MM on activation of the Hippo pathway (33). MM also shows overexpression of RTKs such as MET and downstream PI3K, making inhibitors targeting components of this pathway other promising therapies for this disease (21). Therefore, there are a number of potential vulnerabilities that are worth exploring both as single agents and as combinations in the various preclinical models of MM.

RODENTS AS MODELS OF ASBESTOS CARCINOGENICITY AND MESOTHELIOMA PATHOGENESIS

Numerous investigators have induced MM in rats and mice via injection or inhalation of asbestos fibers (34) or in hamsters through exposure to SV40 (35). Notably, several studies have shown that the MMs induced in rats via asbestos inhalation do not exhibit cytogenetic or gene expression patterns similar to those seen in their human tumor counterparts nor do they show inactivation of genes implicated as drivers in human MM (36–39). Studies in the laboratory rat, beginning in the 1960's, documented that various forms of asbestos and other mineral fibers inoculated intrapleurally/intrathoracically (IT) developed MPM (40). Erionite, the zeolite mineral fiber that is linked to the MM epidemic in near Cappadocia, Turkey (41, 42) was shown to be more carcinogenic than asbestos in IT injection or inhalation studies (43).

While the rat was favored over the mouse as a model for mineral fiber studies due to its larger pleural space for inoculation and "… its more suitable nasal passage architecture

for inhalation studies, some of the early investigations did use mice for IT inoculation of amphiboles and serpentine mineral fibers," however, fibrosis and granulomas were mainly observed (44), with occasional papillary carcinomas seen in inhalation experiments (45). Subsequent carcinogenicity studies using intraperitoneally (i.p.)-inoculated asbestos or zeolite fibers resulted in MMs in more than 20% of wild type mice (46). Over the last two decades, various laboratories have reported variable MM incidences and survival rates in wild type mice that have been injected i.p. with asbestos fibers (6, 38, 47–56), due at least in part to the use of differing types, dimensions and amounts of fibers used, whether the injections were given chronically or as a bolus injection, the length of time the animals were followed, and variations in the genetic background of the mice.

Genetically engineered mouse (GEM) models, typically harboring heterozygous whole-body germline mutations, have been used to assess whether loss of TSGs implicated in human MPM accelerate tumor formation. Different groups have performed such experiments with GEM models carrying mutations of MM-related genes. An early investigation used *Tp53*- deficient mice (38, 47), with mice injected i.p. with crocidolite weekly for 22 weeks. $Tp53^{+/-}$ mice developed a high incidence (76%) of MMs (median latency, 44 weeks) vs. a32% of wild type mice (median latency, 67 weeks). Only 1/8 (12.5%) $Tp53^{-/-}$ mice had a MM, with others succumbing quickly due to thymic lymphomas or hemangiosarcomas, previously reported to arise spontaneously in $Tp53^{-/-}$ mice (57).

Two research groups tested whether heterozygous *Nf2* mice have increased susceptibility to the carcinogenic effects of asbestos (6, 48). Both groups independently demonstrated that $Nf2^{+/-}$ mice injected i.p. with asbestos develop a high incidence and rapid onset of MMs compared wild type littermates. Notably, the normal *Nf2* allele was deleted in most MMs from the $Nf2^{+/-}$ mice, consistent with biallelic inactivation, which similarly occurs in many human MPMs (6). Moreover, most MM cell lines from the *Nf2*-deficient mice showed homologous deletions of *Cdkn2a*/*Cdkn2b* and activation of Akt, recapitulating events that often occur in human MPM. Collectively, these findings are consistent with *Nf2* being a TSG that, when inactivated, acts as a primary driver in the formation of MM.

As noted previously, *CDKN2A* encodes p16INK4A and p14ARF (19Arf in the mouse). To test the relative contributions of these genes to MM formation, one study used mice with heterozygous deletions of *Cdkn2a* exon 1α (resulting in loss of p16Ink4a) or exon 1β (p19Arf), or with a deletion of exon 2 (deleting both p16Ink4a and p19Arf) (51). Both $p16Ink4a^{+/-}$ mice and $p19Arf^{+/-}$ mice injected i.p. with asbestos exhibited higher incidence and more rapid onset of MM than wild type control mice. Mice heterozygous for *Cdkn2a* exon 2 showed a more accelerated rate of asbestos-induced MMs vs. mice deficient for either *p16Ink4a* or *p19Arf* separately. Together, these data indicate that each of the *Cdkn2a* gene products suppresses asbestos-induced MM, and that the combined inactivation of both gene products results in further cooperation to accelerate asbestos-induced MM development and progression.

Early Sanger sequencing studies had revealed point mutations in *BAP1* in 20–25% of sporadic human MMs (7, 8), but subsequent studies of sporadic MMs using various combinations of assays, such as quantitative real-time PCR, targeted comparative genomic hybridization, next generation sequencing, and/or multiplex ligation-dependent probe amplification platforms demonstrated *BAP1* alterations in up to 60–65% of MMs (9–11). Most of the alterations not detected by Sanger sequencing were large deletions.

In addition to somatic changes, it is now well-established that *BAP1* mutation carriers are predisposed to MM and a variety of other tumors (8, 58). The use of *Bap1* knockout models has shown that heterozygosity in the germline predisposes to asbestos-induced MM (53, 59), and similar results were obtained with two knock-in models (54) that harbored different germline mutations that were identical to the ones found in two BAP1 tumor predisposition syndrome (BAP1-TPDS) families that exhibited a very high incidence of MM (8). MM cells from $Bap1^{+/-}$ mice showed biallelic inactivation of *Bap1* (53). Collectively, these data indicate that human *BAP1* mutation carriers have are more prone to the carcinogenic effects of asbestos, even when exposed to small amounts of these fibers (59), when compared to the general population.

Other work has recently demonstrated cooperation between *Nf2* and *Cdkn2a* in MM development in asbestos-exposed $Nf2^{+/-}$;$Cdkn2a^{+/-}$ mice, which exhibited significantly hastened tumor onset and disease progression vs. similarly exposed $Nf2^{+/-}$ and wild-type cohorts (56). These studies also showed that tumors from $Nf2^{+/-}$;$Cdkn2a^{+/-}$ mice had enhanced metastatic potential and an increased cancer stem cell population, in connection with p53/miR-34a-dependent activation of c-Met.

Since chronic inflammation may contribute to the formation of many types of malignancy, including MM, some investigators have employed mouse models for studies of asbestos-mediated inflammation. In one such study, $Nf2^{+/-}$;$Cdkn2a^{+/-}$ mice were used to test if inflammation-related IL-1β release promotes MM formation (55). Exposure of $Nf2^{+/-}$;$Cdkn2a^{+/-}$ mice to asbestos in the presence of an IL-1 receptor (IL-1R) antagonist known as anakinra resulted in a significant delay MM development compared to that of asbestos-exposed mice given a vehicle control, i.e., 33 vs. ∼22.5 weeks, respectively (55). Overall, this work suggested that inflammation-related IL-1β/IL-1R signaling is linked to the formation of asbestos-induced MM. Moreover, the data demonstrate the usefulness of this model for gene-environment and/or "chemoprevention" studies.

Another mouse model, MexTAg, has been used to demonstrate co-carcinogenicity between asbestos and SV40. The investigators used the mesothelin gene promoter to express SV40 large T antigen specifically in the mesothelial lining (49, 60). Several MexTAg mouse lines were created with varying copies (1–100) of the oncogenic transgene. The animals generally do not develop spontaneous MM. However, after i.p. injection of asbestos, 100% of the MexTAg mice developed MM, with disease onset occurring after 20–40 weeks vs. after 50–100 weeks in the ∼25% of wild type mice that developed MM. The investigators concluded that MexTAg mice are well-suited not only basic research, but also for testing the potential of dietary or pharmacological chemoprevention studies of MM (49). To illustrate the utility of MexTAg mice for preclinical studies,

Robinson et al. tested the effects of gemcitabine, a cytotoxic drug that has been shown to have some efficacy in the human disease (60). MexTAg mice treated with vehicle had a median survival of 33 vs. 48 weeks in the gemcitabine-treated cohort. In another investigation with MexTAg mice, treatment with celecoxib, a COX-2 inhibitor, did not diminish the rate of asbestos-induced MM, despite the fact that COX-2 is frequently overexpressed in human MM and correlates with poor prognosis (60). While the MexTAg model has several advantages (100% MM penetrance, short median survival), it does not have any of the genetic hallmarks attributed to the human disease, and a causative association between SV40 and human MM is now disproven (61, 62). However, in one study, gene expression profiling of MMs from MexTAg mice "…had a concordant set of deregulated genes compared to normal mesothelial cells that overlapped with the deregulated genes between human MMs and mesothelial cells" (63).

CONDITIONAL MOUSE MODELS OF MESOTHELIOMA AS PRECLINICAL TOOLS

Since specific genetic driver lesions had been repeatedly found to be associated with human MM by the year 2008, particularly alterations of the *CDKN2A*, *NF2,* and *TP53*, Jongsma et al. decided to establish whether various genetic alterations affecting the same signaling pathways that are dysregulated in the human disease counterpart might similarly induce MM in rodents in the absence of carcinogenic exposure to asbestos (64). Thus, they generated a variety of mutant mice carrying deletions in the Nf2/merlin, p53, and/or Ink4a pathways, hypothesizing that mice with one or more of these combinations might represent an appropriate model of human MM. To avoid possible issues such as embryonic lethality due to germline homozygous deletion of one or more targeted genes, mice with conditional knockout (CKO) of various TSGs were used in combination with the Cre-LoxP system (65). Locotemporal inactivation of the TSG(s) was carried out by injecting adenoviruses expressing the Cre recombinase (65). Upon injecting adeno-Cre into the pleural space of Rosa26 LacZ reporter mice, the investigators demonstrated expression of β-galactosidase specifically in the mesothelium (64). Moreover, MPMs arose in both *Nf2;Tp53* and *Nf2;p16Ink4a/p19Arf* CKO mice at a high frequency and short latency (20 and 30 weeks, respectively) following IT inoculation of adeno-Cre, and the tumors closely mimicked the phenotype of human MPM. Thus, these mice hold promise as a rapid, non-carcinogenic model system for preclinical selection of new combination therapies and for testing novel targeted agents.

BAP1-TPDS patients with MM have a significantly better long-term survival compared to sporadic MM patients, i.e., those without a heritable variant (11, 66). However, it remained unclear whether somatic mutations/deletions of *BAP1* have a similarly favorable prognosis in sporadic MM, or if somatic *BAP1* alterations are a poor prognostic marker, as is the case for uveal melanoma and clear cell renal cell carcinoma (67, 68). Furthermore, although most human MMs exhibit somatic alterations of *BAP1*, *NF2,* and/or *CDKN2A*—with 25/74 cases of MPM in the TCGA series having alterations of all three TSGs in combination (13)—it was not known if loss of *BAP1* could cooperate with the inactivation of *NF2* and/or *CDKN2A* to initiate a more aggressive form of MM. To address this possibility experimentally, Kukuyan et al. used CKO models, including a $Bap1^{f/f}$ mouse they generated (69). Various combinations of deletions of *Bap1*, *Cdkn2a*, and *Nf2* were introduced in the pleural cavity of the mice, focusing on the contribution of Bap1 loss. While homozygous CKO of any one of these TSGs alone gave rise to few or no MMs—similar to the results of Jongsma et al. (64)—deletion of *Bap1* cooperated with deletion of either *Nf2* or *Cdkn2a* to promote MM formation in about 20% of double-CKO mice. In contrast, a much higher incidence (22/26, 85%) of MMs was observed in $Bap1^{f/f}$;$Nf2^{f/f}$;$Cdkn2a^{f/f}$ mice injected IT with adeno-Cre (triple-CKO mice). Onset of MM was rapid in the triple-CKO mice (median survival, 12 weeks), and tumors from these mice were consistently high-grade and invasive. With regard to histological subtype, notably no epithelioid MMs were observed with any of the mouse genotypes. Sarcomatoid MMs predominated, with the only exception being the *Bap1;Nf2* double-CKO cohort, in which 6 of 7 MMs showed mixed (biphasic) histology. The MMs observed in triple-CKO mice showed enrichment for genes that are transcriptionally controlled by the polycomb repressive complex 2 (PRC2) (69). The findings suggested that loss of *Bap1* contributes to MM progression, at least partially, via loss of PRC2-mediated repression of oncogenic target genes that were identified, suggesting a novel avenue for therapeutic intervention (69).

To explore the role of individual components of the *Cdkn2a* locus by comparing models in which *Cdkn2a* (including *p19Arf*) were disrupted with or without concomitant loss of *Cdkn2b* Badhai et al. showed that the additional disruption of *Cdkn2b* further added to the aggressiveness of the resulting MMs, providing also an explanation for the predominance of deletion of the complete *CDKN2A-CDKN2B* locus in human MM over point mutations in *CDKN2A* (Badhai et al., submitted).

Because *CDKN2A* deletions encompassing the sequence encoding p14ARF, a component of the p53 pathway, have been documented in 90% of human MM cell lines (6) and *TP53* is altered in about 15% of primary MMs, and because the PI3K/PTEN/AKT pathway is activated in most human MPMs, Sementino et al. decided to determine if alterations affecting the same pathways would also induce MM in mice (70). This was thought worthwhile, given that p53 helps mediate the DNA damage response and that AKT regulates neoplastic cell survival and therapeutic resistance. The investigators demonstrated that while neither adeno-Cre-mediated homozygous deletion of *Tp53* or *Pten* alone in the mesothelium was sufficient to induce MM formation, compound deletion of these two TSGs resulted in rapid, aggressive peritoneal and pleural MMs (median latency: 9 and 19 weeks, respectively). A longer term follow-up study of the $Tp53^{f/f}$ cohort revealed MMs in 0/12 mice injected with adeno-Cre i.p. and 0/10 mice injected IT; among the $Pten^{f/f}$ cohort, MMs were observed in 0/12 mice injected i.p. and 1/10 injected IT (Sementino et al., unpublished data). In the $Pten^{f/f}$;$Tp53^{f/f}$ cohort, 23/25 (92%) mice injected i.p. developed MM, whereas

19/34 (56%) mice injected IT showed MM, with 14 histiocytic sarcomas also seen in this group.

Given the high penetrance and rapid development of MMs in *Pten*$^{f/f}$;*Tp53*$^{f/f}$ mice inoculated i.p., and the frequent involvement of p14ARF/p53 and PI3K/PTEN/AKT pathways in human MM, this GEM model holds promise for preclinical work. However, this model does have certain limitations, such as for testing agents designed to reactivate the normal cellular functions of Pten and Tp53. For instance, given that this model has homozygous loss of *Tp53*, this precludes studies of a drug such as RITA, which reactivates p53's pro-apoptotic function in tumor cells that preserve expression of mutant or wild-type p53 (71). To elude this issue using an agent such as RITA, this mouse model might be modified such that only a single *Tp53* allele were deleted, i.e., by using *Pten*$^{f/f}$;*Tp53*$^{+/f}$ mice. A second shortcoming with regard to the translational relevance of the *Pten*$^{f/f}$;*Tp53*$^{f/f}$ model is that somatic mutations of other TSGs considered to be hallmarks in human MM progression usually do not occur in tumors from these animals. However, the fact that the MMs in this model repeatedly show sarcomatoid or biphasic histology with very short latency, especially in mice injected i.p., provides advantages for certain preclinical applications.

GRAFT MODELS OF MESOTHELIOMA

Many human MM cell lines have been established over the years and used in numerous *in vitro* studies. They are also exploited for *in vivo* experiments, usually for testing their tumorigenicity and the efficacy of small molecule inhibitors as a prelude for evaluating these compounds in clinical trials. Due to often long-term *in vitro* propagation, these cell lines have invariably acquired (epi)genetic alterations that facilitate their propagation in cell culture, resulting in new vulnerabilities and resistance features. This is one of the reasons why treatments that are effective in these graft models often do not well-translate to human. Furthermore, the requirement to use immunodeficient mice as a host for these graft experiments complicates assessment of immunomodulating effects. Patient-derived xenograft (PDX) models, in which tumor fragments are grafted directly into immunodeficient recipient hosts, more closely resemble the human condition and usually retain their human stromal components for a number of passages. The capacity to establish PDX lines also correlates with the aggressiveness of the tumor in man (72). Studies in PDX models permit addressing specific questions that are difficult to assess in solely mouse based models such as inter- and intra-tumor heterogeneity as well as features imposed by the distinct genetic backgrounds (73). As potential drawbacks, we note that propagation has to be performed in immunodeficient backgrounds and retrofitting these models with a functional human immune system from the patient from which the tumor was obtained (humanized models) is still in an early stage of development (74) and also practically very demanding.

Experienced investigators seeking a mouse model that may faithfully reflect human MPM pathobiology may also find an orthotopic, intrapleural model such as the one described by Servais et al. (75) useful for preclinical therapeutic studies. This tumor model recapitulates human pleural anatomy/microenvironment and can be used in combination with quantitative, non-invasive imaging for bioluminescent monitoring of tumor burden. The parietal pleural surface contains lymphatics that offer escape of MM cells into the systemic circulation, and this immunocompetent orthotopic model of pleural cancer permit studies of inflammation on tumor progression as well (75). However, as noted by the authors, for studies of therapies targeting human antigens, immunodeficient models are required in order to perform studies on xenografted human cancer cell lines.

ARE WE MISSING ANYTHING?

First of all, it is worth emphasizing that the choice of the model depends on the question asked. Furthermore, a mouse is not a "small human" and we need to accept that we cannot simply extrapolate findings from such a model to the human condition. However, models can teach us important biological principles and can provide us with therapeutic concepts worth testing in clinical settings notwithstanding the evolutionary distance between man and mouse. Where possible, we should try to align the model on the basis of molecular aberrations found in humans, e.g., by introducing similar driver lesions in the right target cell and using comparable external carcinogens if applicable, e.g., asbestos. Evidently, PDX models might be very valuable to assess intrinsic tumor heterogeneity and to evaluate their response to drug combinations. For immunotherapy studies, it will be important to use a model with a functional immune system. To permit effective immunotherapy studies in MM mouse models, it will be important to establish these in a defined genetic background (e.g., BL6, the "work horse" of immunologists) in order to permit isogenic graft studies. Fortunately, current Crispr/Cas9 engineering has made the generation of complex conditional MM models relatively easy. This should facilitate the testing of new promising intervention strategies for this highly lethal cancer.

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JT and AB wrote the paper and are accountable for the content of the work.

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Conflict of Interest: JT has a patent on *BAP1* mutation testing and has provided legal consultation regarding the role of germline mutations of *BAP1* in mesothelioma.

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REVIEW
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Antiangiogeneic Strategies in Mesothelioma

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There is a strong rationale for inhibiting angiogenesis in mesothelioma. Vascular endothelial growth factor (VEGF) is an autocrine growth factor in mesothelioma and a potent mitogen for mesothelial cells. Further, the abnormal tumor vasculature promotes raised interstitial pressure and hypoxia, which may be detrimental to both penetration and efficacy of anticancer agents. Antiangiogenic agents have been trialed in mesothelioma for close to two decades, with early phase clinical trials testing vascular targeting agents, the VEGF-A targeting monoclonal antibody bevacizumab, and numerous tyrosine kinase inhibitors, many with multiple targets. None of these have shown efficacy which has warranted further development as single agents in any line of therapy. Whilst a randomized phase II trial combining the multitargeted tyrosine kinase inhibitor nintedanib with platinum/pemetrexed chemotherapy was positive, these results were not confirmed in a subsequent phase III study. The combination of cisplatin and pemetrexed with bevacizumab, in appropriately selected patients, remains the only anti-angiogenic combination showing efficacy in mesothelioma. Extensive efforts to identify biomarkers of response have not yet been successful.

Keywords: mesothelioma, angiogenesis, hypoxia, bevacizumab, clinical trials

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INTRODUCTION

Malignant mesothelioma is an almost uniformly fatal malignancy aetiologically linked to asbestos fiber inhalation, mainly through occupational exposure. Whilst mesothelioma can develop in the peritoneum, tunica vaginalis, and pericardium, the pleura is the primary site in around 90% of cases (1). Most systemic therapy research has been conducted in malignant pleural mesothelioma (MPM), which will be the focus of this review. Whilst some patients presenting with early disease will undergo aggressive surgery and multimodality therapy, most patients present with advanced disease and palliative systemic therapy will be their mainstay of treatment (2).

Systemic therapy for mesothelioma has not yet benefited from the paradigm shift of personalized medicine. The first demonstration of benefit from systemic therapy of mesothelioma was in 2003, with the EMPHACIS study showing a modest improvement in overall survival (OS) for patients receiving cisplatin/pemetrexed, over cisplatin alone (3). The combination of cisplatin with the antifolate raltitrexed showed similar survival benefits but reported later, and is not widely used (4). The first challenge to this standard of care came in 2016, when the MAPS trial reported a further survival benefit for the addition of bevacizumab to cisplatin/pemetrexed (5). As supported by the

NCCN and ASCO guidelines, this has changed the standard of care in some, but not all, parts of the world, due to the lack of FDA registration and universal reimbursement. Here, we discuss the history and role of anti-angiogenic strategies in mesothelioma, with an emphasis on clinical trial data and their clinical application.

ANGIOGENESIS IN MESOTHELIOMA

Tumor vasculature is highly abnormal, with tortuous vessels which can be either distended or pruned, and deviate from the orderly morphology in normal tissues (6). This results in heterogeneity of tumor blood flow, with resulting hypoxia. Excessive vascular leakiness and raised interstitial pressure can further compress the abnormal vasculature, and contribute to poor penetration of anticancer agents into tumor. These characteristics have important consequences for tumor biology and treatment.

Hypoxia is a tumor-promoting state, leading to changes in gene expression that reduce apoptosis (7), enhance receptor tyrosine kinase signaling (8), and promote metastasis (9) and invasion (10), amongst other actions. Hypoxia also has profound immunosuppressive effects and contributes to treatment resistance, most notably to radiotherapy (11). Additionally, hypoxia participates in a feedback cycle which compounds the generation of abnormal tumor vasculature, by upregulating vascular endothelial growth factor (VEGF) and other pro-angiogenic molecules (**Figure 1**).

Hypoxic conditions lead to HIF-1α and HIF-2 transcription factor stabilization and activation, which in turn control VEGF mRNA production (12). VEGF can also be synthesized in response to nitric oxide (NO) production by the specific endothelial NO-synthase (eNOS) (13). The most extensively studied member of the VEGF family is VEGF-A, secretion of which can be up-regulated in tumor, including mesothelioma, primarily in response to hypoxic stimulus. VEGF-A exists as more than 20 splice isoforms, ranging from 121 to 206 kDa molecular weight; the $VEFG_{165}$ isoform is the most abundant tissue variant. Type B, C, D, E, or F members of the VEGF family have been less comprehensively studied. VEGFs are potent mitogen and survival factors for endothelial cells, signaling through binding to the two receptors, Flt-1 (VEGFR-1) and KDR (VEGFR-2). Activation of VEGFR-2 leads to auto-phosphorylation and downstream signaling through various pathways, such as phosphatidylinositol 3′-OH kinase/Akt. In pleural mesothelioma, VEGF also acts as a powerful mitogen for mesothelial cells themselves. Indeed, mesothelial cell lines secrete VEGF-A and VEGF-C and express both VEGF receptors Flt-1 (VEGF-R1) and KDR (VEGFR-2) (14–16). Thus, VEGF signaling can induce mesothelial cell growth in an autocrine fashion (16–18). This may explain why mesothelioma cells show exquisite sensitivity to anti-VEGF agents, in addition to the more canonical role of such agents in inhibiting neo-angiogenesis.

Other growth factors can also regulate migration, survival, and differentiation of endothelial cells, contributing to new vessel development. Factors from the large fibroblast growth factor (FGF) family (aFGFs and bFGGFs) (19, 20) are secreted by both stromal fibroblasts (including pericytes that stabilize new vessels) and tumor cells acting on the FGF receptor (FGFR) family (21). Tumor-associated macrophages, plus endothelial cells, express Tie receptors 1 and 2 for angiopoietins. Angiopoeitins are secreted by endothelial cells and pericytes, and are involved in endothelial cell migration via the process of endothelial tube formation. In addition, vascular cells express Ephrin B2 and B4 [found in mesothelioma (22)] from the ephrin family of tyrosine kinase trans-membrane receptors. These are localized in filopodia of tip endothelial cells that generate vascular spouts during vessel growth and formation (**Figure 2**). Other proteins expressed by endothelial cells or mesothelial tumor cells, such as TGFβ, EGF, angiogenin, IL-8, and platelet-derived growth factor (PDGF) could also contribute directly or indirectly to endothelial proliferation (23), migration, vessel formation, and stabilization. This complex process may be finely regulated by natural anti-angiogenic proteins such as thrombospondin (24), angiostatin, endostatin, and/or vasostatin (24, 25); these are mainly stocked in the extra-cellular stromal matrix as inactive precursors, and activated by proteolytic cleavage upon activation of matrix metalloproteinases (MMPs). Hence, angiogenesis was a clear rational target in mesothelioma.

MODULATING ANGIOGENESIS IN MESOTHELIOMA

Several targeted anti-angiogenic strategies have been used to treat various cancer types: anti-VEGF antibodies i.e., bevacizumab; various tyrosine kinase inhibitors; and other small-molecule inhibitors. Results of trials in mesothelioma have been mixed, as described below.

Bevacizumab

Background on Bevacizumab

Bevacizumab is an anti-VEGF recombinant humanized IgG1 antibody derived from the murine monoclonal antibody A4.6.1 (26). Bevacizumab neutralizes all isoforms of human VEGF, hampering the ability of VEGF to bind to VEGF receptors on the surface of endothelial or mesothelial cells, and inhibiting VEGF-induced proliferation of endothelial cells *in vitro* (26).

Preclinical inhibition of VEGF signaling by MAb also decreased tumor vascular permeability in human xenografts implanted into mice (27). These changes, linked to vascular network normalization (**Figure 3**), are thought to explain the antitumor effects of VEGF inhibitors which can inhibit tumor growth (28) and control micro-metastatic disease in tumor xenografts (29–32). Furthermore, an orthotopic murine xenograft mesothelioma model demonstrated synergy between pemetrexed and bevacizumab compared to the either treatment alone (33). In human studies, bevacizumab has a half-life of around 20 days, and is dosed by weight, 3-weekly, reaching steady state in around 100 days (34).

To our best knowledge there are not preclinical or clinical data about the topical use of bevacizumad, infused directly in the pleural space, although it could theoretically increase

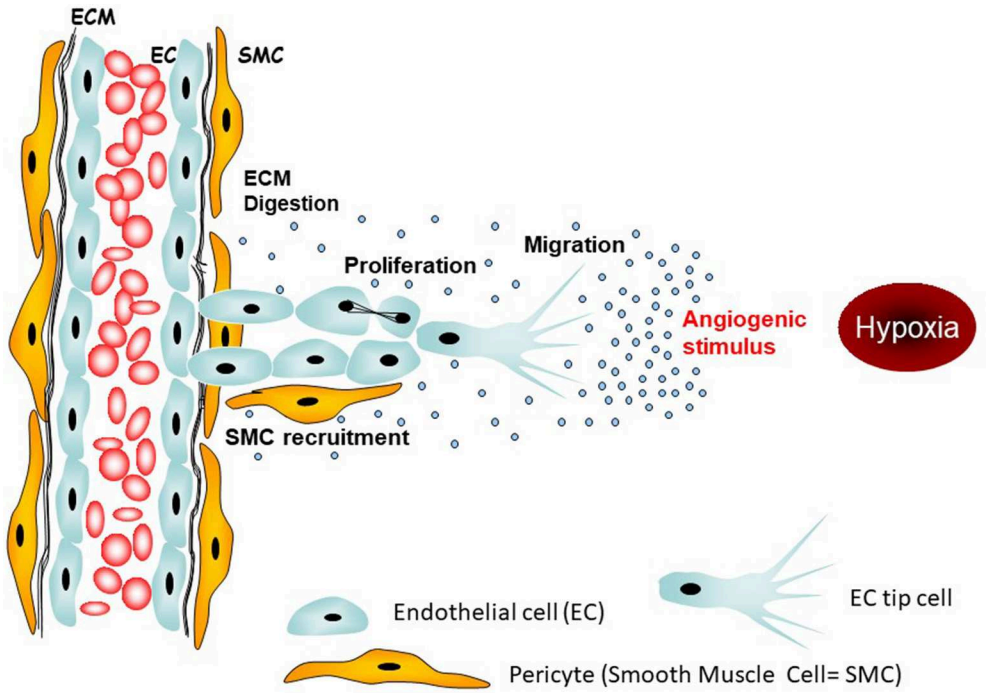

FIGURE 1 | Targetable initial steps of angiogenesis. The main angiogenic stimulus in tumors is hypoxia leading to activation of tip endothelial cells which tract neighboring endothelial cells toward the origin of the stimulus, i.e., the hypoxic region.

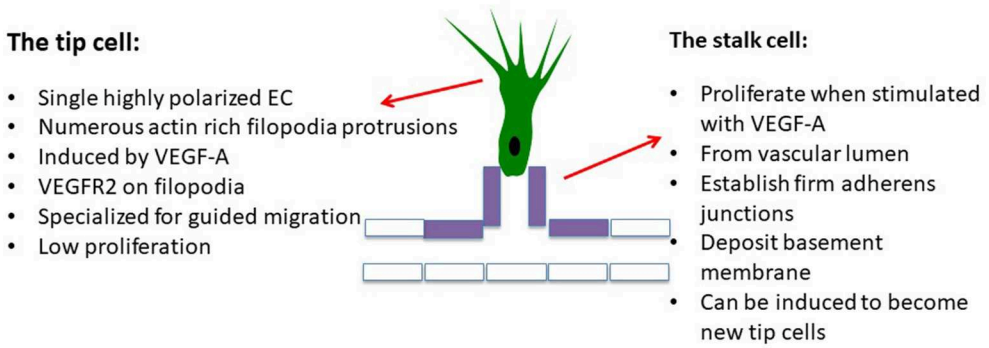

FIGURE 2 | Initial steps, the cell actors: the endothelial tip cell is the first endothelial cell reached by hypoxia-induced stimuli which differentiates into a polarized migrating cell, inhibiting the differentiation of neighbor cells, called "stalk cells," which passively follow the tip cell, attracting the cell monolayer in which cells adhere to each other via adherens junctions containing VE-cadherin. Stalk cells are still able to proliferate. VEGF-targeting agents are active on both tip and stalk cells, inhibiting both endothelial cell migration and proliferation.

mesothelial permeability and help chemotherapy diffusion and efficacy. Bevacizumab was the first anti-angiogenic molecule to be approved by the FDA in 2006, in combination with first-line platinum-based chemotherapy for metastatic non-squamous non-small cell lung cancer (NSCLC). Throughout the last decade, several anti-angiogenic agents have been assessed but none significantly improved survival outcomes, with the exception of nintedanib and ramucirumab in second-line therapy of NSCLC. Nevertheless, as they demonstrated only modest improvement, this did not convince some European countries to fund their reimbursement despite European Medicines Agency approval.

Bevacizumab Toxicities

Bevacizumab is generally well-tolerated. Adverse events ≥Gr3 include thromboembolism, hypertension, bleeding, proteinuria, and pulmonary hemorrhage. Meta-analyses demonstrate a bleeding risk of 0.7–0.9%, varying from grade 1–2 (epistaxis) to fatal hemorrhage events like haemoptysis, gastrointestinal bleeding, hematemesis, and cerebral hemorrhage (35–38), similar

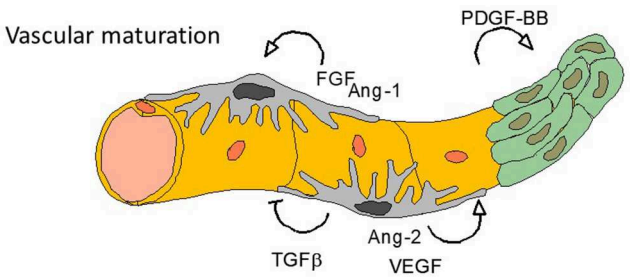

FIGURE 3 | Final steps—vasculogenesis. Tumor angiogenesis is characterized by an anarchic vasculogenesis with immature vessel structure. One of the effect of anti-angiogenic treatments (especially targeting VEGF or VEGFR) is to normalize the vessel cell architecture: all other targetable growth factors listed here are involved in such normalization process.

to reported in MPM (5). The risk of major bleeding in patients with advanced solid tumors is around 2.8% (95% CI 2.1–3.6) (35). Higher risks are observed in patients with NSCLC (RR 3.41, 95% CI 1.68–6.91), renal cell carcinoma (RR 6.37, 95% CI 1.43–28.33), and colorectal cancer (RR 9.11, 95% CI 1.70–48.79) who were receiving bevacizumab 5 mg/kg per week. Use of bevacizumab in squamous cell lung cancer is associated with a high incidence of significant pulmonary hemorrhage, linked to the central location of these tumors, and is currently contraindicated. An increased risk of arterial thromboembolism is also described with anti-angiogenesis therapy (39) while the risk of venous thromboembolism remains controversial with a meta-analysis suggesting no statistically significant increase for bevacizumab compared with control groups (10.9 vs. 9.8%, $p = 0{,}13$) (40).

As VEGF plays a key role in the maintenance of vascular homeostasis via the NO pathway, VEGF signaling inhibition is associated with arterial vasoconstriction and hypertension. In a large meta-analysis, the incidence of all-grade hypertension was significantly increased at 25.4% of cases (41, 42).

The incidence of proteinuria in patients treated with bevacizumab is 21–63%, but grade 3–4 proteinuria (>3.5 g of protein/24 h, or nephrotic syndrome) occurs in only 1–3% of cases (43). The combination of bevacizumab with chemotherapy significantly increasing the risk for high-grade proteinuria and nephrotic syndrome (43). Few studies *in vivo* have demonstrated that VEGF plays a major role in endothelial development and in repair of glomerular endothelial injury (44).

Bevacizumab is also associated with impaired wound healing (45), likely due to the critical role of VEGF in this process. Whilst the half-life of plasma bevacizumab is 20 days, its tissue half-life is 6 weeks, hence a minimum of 28 days (preferably 6–8 weeks) should elapse between major surgery and the previous dose of bevacizumab (46). Gastrointestinal perforation (GIP) and fistula formation are infrequent but potentially fatal (47).

Clinical Trials of Bevacizumab in Malignant Pleural Mesothelioma

The main results of the phase 2 trials assessing bevacizumab in mesothelioma patients are presented in **Table 2**. Jackman et al. evaluated bevacizumab with erlotinib in patients who had previously received chemotherapy (48). In this phase II, multicenter open-label study, 24 patients received erlotinib 150 mg daily and bevacizumab 15 mg/kg every 21 days. The trial did not achieve its primary endpoint and was discontinued.

The first multicenter, double-blind, placebo-controlled, randomized phase II trial of gemcitabine/cisplatin plus bevacizumab in 108 patients with previously untreated and unresectable mesothelioma was published in 2012 (49). Patients received gemcitabine (1,250 mg/m^2 days 1 and 8 every 21 days), cisplatin (75 mg/m^2 every 21 days), and either bevacizumab (15 mg/kg) or placebo every 21 days for six cycles, then bevacizumab or placebo every 21 days until progression. The addition of bevacizumab did not significantly improve progression-free survival PFS (6.9 vs. 6 months, $p = 0.88$) or OS (15.6 vs. 14.7 months, $p = 0.91$). There were no significant differences in toxicity. Besides a probably underpowered phase 2 trial, Kindler et al. attributed this disappointing result to a possible negative interaction between gemcitabine and bevacizumab. As shown in preclinical studies, gemcitabine does not mobilize endothelial cell progenitors or increase angiogenesis to the degree observed with taxanes. Another reason may be an unbalanced use of second-line pemetrexed, which was good activity in the second-line setting in patients who have not previously received this drug.

A third phase II study evaluated bevacizumab with carboplatin/pemetrexed as first-line therapy in MPM (50). Patients received pemetrexed 500 mg/m^2 with carboplatin [area under the plasma concentration–time curve (AUC) 5] plus bevacizumab 15 mg/kg every 21 days for six cycles, followed by maintenance bevacizumab (maximum 1 year). This study did not achieve its ambitious endpoint to show a 50% improvement in median PFS compared to pemetrexed/platinum (from 6 to 9 months), although a longer OS and more long-term survivors were observed in the experimental arm with median PFS (primary endpoint) and OS of 6.9 and 15.3 months, respectively. Treatment was generally well-tolerated, but bowel perforation was reported in 4% of patients, with three toxic deaths.

Finally, Dowell et al. evaluated bevacizumab combined with cisplatin/pemetrexed as first-line treatment in 53 patients with advanced, unresectable MPM (51). The primary objective of a 33% improvement in 6-month PFS with addition of bevacizumab was not met. Median PFS and OS were 6.9 and 14.9 months. Importantly, two fatal adverse events (4%) were possibly related to bevacizumab (one cerebrovascular accident and one small bowel obstruction and fistula).

The MAPS Trial

The phase II/III Mesothelioma Avastin Cisplatin Pemetrexed Study (MAPS) was initiated to assess the effect on survival of adding bevacizumab to standard of care chemotherapy as first-line treatment (5). In this large, well-powered, multicenter, randomized, controlled open-label trial, adding bevacizumab to pemetrexed/cisplatin improved both PFS and OS survival compared with pemetrexed/cisplatin alone. Four hundred and forty-eight eligible patients were randomized to receive cisplatin/pemetrexed with or without bevacizumab. Only

patients with measurable or evaluable lesions (e.g., pleural effusion) who were younger than 76 years were included, with fewer than 10% of performance status 2 patients. It should be emphasized that large biopsies *via* video-assisted thoracoscopic surgery (VATS) were performed in 85% of patients before chemotherapy, explaining the 10% low rate of recurrent pleural effusion, since thoracoscopy led to efficient pleurodesis.

After six cycles of chemotherapy, the bevacizumab group continued 3-weekly maintenance bevacizumab until progression or toxicity. The primary outcome was OS, and patients were stratified by mesothelioma histology, performance status, and smoking history. After a median follow-up of 39.4 months, patients who received bevacizumab demonstrated significant improvement in median PFS [9.2 vs. 7.3 months; adjusted hazard ratio (HR) = 0.61; $P < 0.0001$] and median OS (18.8 vs. 16.1 months; adjusted HR =0.75; $P = 0.0167$).

As expected, the bevacizumab group experienced more toxicities than the standard chemotherapy group (respectively, 71 vs. 62%). More patients treated with bevacizumab stopped treatment due to toxicity (24.3 vs. 13%), but more patients stopped the treatment due to disease progression in the control arm. Zalcman et al. described more grade 3 hypertension (22 vs. 0%), cardiovascular events (29 vs. 1%), and thrombotic events (6 vs. 1%) in the bevacizumab arm. However, these events were manageable, rarely led to treatment interruptions, and no grade 4 events were observed. Patients receiving bevacizumab experienced more hemorrhage, mainly easily manageable grade 1–2 epistaxis. Strikingly, no haemoptysis was reported. However, notably, patients in this trial were younger than 76 years, and a higher risk of bleeding has been reported in older patients. Only 5 (2.3%) arterial thromboembolic grade 3–4 (and no lower grade) events were observed; this rate was not significantly different between groups. There were more venous thromboembolism grade 2–4 events in the bevacizumab than control arm (12 vs. 3, $p = 0.02$) but the incidence of grade 4 events did not differ statistically between groups. The maximum proteinuria grade was 3 in only 3.2% of patients in the bevacizumab group and did not reduce bevacizumab dose-intensity. No GIP was observed and patients with a previous history of gastro-intestinal surgery were carefully screened before inclusion.

Notably all subgroups (by gender, age, Eastern Cooperative Oncology Group (ECOG) status, PS, or histological subtype) derived an OS benefit from bevacizumab. Patients receiving bevacizumab experienced less fatigue at 9 weeks as assessed by the QLQ-LC30 than control patients. Likewise, significantly more control group patients experienced deteriorating scores for general health on the LCSS-meso at 9 weeks than in the bevacizumab group. A longitudinal QoL study confirmed that not only was bevacizumab not associated with global QoL deterioration, it improved functional scores for two dimensions. There was a clinically significant prolongation of deterioration-free survival for pain scores at 2 months, although this was not statistically significant (HR = 0.85, 95% CI [0.69–1.03], $p = 0.097$). Bevacizumab also significantly delayed the time to deterioration for chemotherapy-related peripheral neuropathy (HR = 0.74, 95% CI [0.61–0.91], $p = 0.004$) (52). Despite these appealing positive results, probably because of the registrations of several bevacizumab biosimilars, the Company marketing bevacizumab took the decision not file the drug for mesothelioma patients, considered to represent a too much limited niche to justify the filing investments needed.

Anti-angiogenic Tyrosine Kinase Inhibitors

Anti-angiogenic Tyrosine Kinase Inhibitors as Single Agents

Interest in anti-angiogenesis in mesothelioma was first noted in the late 1990s, when tyrosine kinase inhibitors targeting these pathways became available. Agents including sunitinib, sorafenib, axitinib, cediranib, and others were tested in a series of single-arm phase II clinical trials, predominantly in the second-line setting, with most trials recruiting fewer than 70 participants. The mains results are presented in **Table 1** showing objective radiological response rates were mostly below 15% (54, 57, 59–61, 66). None of these agents proceeded to randomized phase III clinical trials. Notably, many of these agents targeted multiple pathways including not only VEGF receptor isoforms, but also several of the PDGF receptors (PDGFR), FLT4, and others. Despite the targeting of multiple tyrosine kinase receptors, these agents failed to generate meaningful anti-tumor activity against mesothelioma.

Anti-angiogenic Tyrosine Kinase Inhibitors in Combination

As a number of anti-angiogenic TKIs had demonstrated modest response rates in mesothelioma, some were trialed in combination with cisplatin/pemetrexed chemotherapy, with the hope that inducing vascular normalization would enhance chemotherapy efficacy. Sunitinib, sorafenib, cediranib, and nintedanib were tested in combination with platinum/pemetrexed (summarized in **Table 2**), and despite the completion of at least two well-conducted randomized trials, none of these agents demonstrated efficacy that will take them into clinical practice. Here, we will describe in more detail the most conclusive clinical trials incorporating these agents.

Nintedanib and the LUME-Meso Clinical Trials

Nintedanib is an oral angiokinase inhibitor which has multiple targets, including VEGFR1-3, FGFR1–3, PDGFRα/β, RET, Abl, FLT3, and Src (72). When a randomized phase II clinical trial in mesothelioma, LUME-Meso II, was initiated, nintedanib had already been demonstrated safe and tolerable in combination with chemotherapy (72) and a positive clinical trial had been completed in combination with docetaxel as second-line treatment for advanced NSCLC of adenocarcinoma histology (73). Preclinical studies suggested potential activity in mesothelioma (74). LUME-Meso II was initiated to assess the efficacy and safety of nintedanib in combination with cisplatin/pemetrexed (69). This study enrolled 87 participants with chemo-naïve unresectable MPM, ECOG performance status 0–1, and non-sarcomatoid disease histology. Patients were randomized 1:1, double blinded, to cisplatin/pemetrexed with nintedanib 200 mg b.d. or placebo, and nintedanib or placebo was subsequently continued as monotherapy until progression. The study primary endpoint was PFS. Results were released

TABLE 1 | Results from clinical trials of single agent anti-angiogenic and vascular targeting agents in mesothelioma.

Drug	Target	Study phase	Setting	No. of patients	Response rate	Survival (months)	References
Semaxanib	VEGFR, PDGFR	II	2nd line	9	PR 11%: SD NR	PFS NR; OS 12.4	(53)
Vatalanib	VEGF	II	1st line	47	PR 11%; SD 66%	PFS 4.1; OS 10	(54)
Thalidomide	Angiogenesis	II	1st line 2nd line	40	SD > 6 months: 27.5%	PFS NR; OS 7.6	(55)
NGR-h TNF	NGR-h TNF	II	2nd line	57	PR 2%; SD 44%	PFS 2.8; OS 12.1	(56)
Sunitinib	VEGFR, Flt-1, KDR, Flt-4, PDGFR	II	2nd line	53	PR 12%; SD 65%	PFS 3.5; OS: 7	(57)
Sorafenib	VEGFR, PDGFR, Raf-kinase	II	1st line 2nd line	50	PR 6%; SD 54%	PFS 3.6; OS 9.7	(58)
Cediranib	VEGF-2	II	2nd line	54	PR 9%; SD 34%	PFS 2.6; OS 9.5	(59)
Sunitinib	VEGFR, Flt-1, KDR, Flt-4, PDGFR	II	1st line 2nd line	18 17	PR 6%; SD 56% PR 0%; SD 65%	PFS 2.7; OS 6.7 PFS 2.8; OS 8.3	(60)
Cediranib	VEGFR-2	II	2nd line +	50	PR 10%; SD 34%	PFS 1.8; OS 4.4	(61)
B2P2M2: BNC 105	Vascular disrupting agent	II	2nd line +	30	PR 3%; SD 43%	PFS 1.5; OS 8.2	(62)
Sorafenib	VEGFR, PDGFR, Raf-kinase	II	2nd line	53	PR 6%; SD 56%	PFS 5.1; OS 9	(63)
Pazopanib	VEGFR-1,2,3; cKIT; PDGFR	II		34	PR 6%	PFS 4.2; OS 11.5	Clinicaltrials.gov
Vandetanib	VEGFR, EGFR, RET	II		66	PR 0%; SD 0%	PFS 1.4; OS 7.8	Clinicaltrials.gov
NVALT study: Thalidomide maintenance	Angiogenesis	III	Maintenance	222	Th: NR ASC: NR	Th: PFS 3.6; OS 10.6 ASC: PFS 3.5; OS 12.9	(64)
NGR010	NGR-hTNF, Vascular targeting	III	1st line	400	NGR: DCR 61% PI: DCR 47%	NGR: PFS 3.4; OS 8.5 PI: PFS 3.0; OS 8.0	(65)

NR, Not reported for mesothelioma patients; Clinicaltrials.gov, results extracted from clinicaltrials.gov but not published; Th, thalidomide arm; ASC, active supportive care; NGR, NGR-hTNF arm; PI, placebo arm.

after completion of the randomized phase II portion of the study, strongly favoring the nintedanib-containing arm, with a HR for PFS of 0.54 (95% CI, 0.33–0.87; $P = 0.010$). Although underpowered, OS also showed a trend to benefit with addition of nintedanib (HR, 0.77; 95% CI, 0.46–1.29; $P = 0.32$). Benefits appeared most marked in those patients with epithelioid disease, although patients with non-epithelioid disease only comprised 12% of the study population. The combination appeared safe and tolerable, albeit with a higher incidence of grade 3 neutropenia in the combination group.

These promising results triggered the expansion to a subsequent international confirmatory randomized phase III study, the LUME-Meso-III trial. The phase II observation of more apparent benefit in patients with epithelioid histology, although not paired with an explanatory biological rationale, led to this expansion study excluding those with any other histological subtype; other inclusion criteria remained similar. Patients received an identical treatment regimen to the previous study, including maintenance therapy, and PFS was again the primary endpoint, with a secondary endpoint of OS. The study had statistical power to detect a HR of 0.63 favoring the nintedanib arm (75). A total of 458 patients were randomized in a 1:1 ratio. Unfortunately, there was no difference in PFS between the two arms (HR = 1·01; 95% CI: 0·79–1·30; $p = 0·91$) with a median PFS of 6.8 months in the nintedanib arm and 7.0 months in the placebo arm. The HR for OS was 1·12 (95% CI: 0·79–1·58, $p = 0·538$), with a median survival of 14.4 months in the nintedanib arm and 16.1 months in the placebo arm; there were no new adverse safety signals (71).

Results of the double-blind randomized phase II study "NEMO" from the EORTC Lung Cancer Group, assessing Nintedanib as switch maintenance treatment for MPM patients after disease control obtained with first-line pemetrexed/cisplatin doublet, are still awaited for 2021.

Cediranib

Two early-phase clinical trials assessed the efficacy of the single agent VEGF-R tyrosine kinase inhibitor cediranib (AZD2171, Astra-Zeneca) in MPM in the second-line setting (59, 61). Cediranib was also more recently tested combined with pemetrexed/cisplatin as frontline therapy in chemo-naive patients in a phase 1 trial and subsequent randomized phase II trial (68, 70).

The phase 2 trial performed by the Southwest Oncology Group (SWOG) enrolled 54 patients (PS = 0–2) with proven MPM, 47 evaluable, after at least one line of platinum-based chemotherapy and measurable lesions by RECIST. Participants received single-agent cediranib 45 mg daily until progression or

TABLE 2 | Results from combination clinical trials of anti-angiogenic and vascular targeting agents in mesothelioma.

Drug	Combination	Target	Study phase	Setting	No. of patients	Response rate	Survival (months)	References
Thalidomide	Cisplatin	Angiogenesis	P2	1st line	16	PR 14%; SD 55%	PFS NA; OS 11	(67)
	Gemcitabine			2nd line	22	PR 6%; SD 50%	PFS NA; OS 11	
Bevacizumab	Carboplatin Pemetrexed	VEGF	P1/2	1st line	13	PR 33%	PFS 7.8	Clinicaltrials.gov
Bevacizumab	Cisplatin Pemetrexed	VEGF	P2	1st line	53	PR 40%; SD 35%	PFS 6.9; OS 14.8	(51)
Bevacizumab	Cisplatin	VEGF	RP2	1st line	53	PR 25%	PFS 6.9; OS 15.6	(49)
Placebo	Gemcitabine				55	PR 22%	PFS 6.0; OS 14.7	
Bevacizumab	Carboplatin Pemetrexed	VEGF	P2	1st line	76	PR 34%: SD 58%	PFS 6.9; OS 15.3	(50)
Axitinib	Pemetrexed	PDGFR	RP2	1st line	14	PR 36%; SD 43%	PFS 5.8; OS 18.9	(64)
–	Cisplatin	VEGFR-1,2,3; cKIT			11	PR 18%; SD 73%	PFS 8.3; OS 18.5	
Bevacizumab	Cisplatin	VEGF	P2/3	1st line	223	NR	PFS 9.2*; OS 18.8*	(5)
–	Pemetrexed				225	NR	PFS 7.3; OS 16.1	
Cedirinib	Pemetrexed Cisplatin	VEGF-2	P1	1st line	20	PR 24%; SD 66%	PFS 8.6; OS 16.2	(68)
Nintedanib	Cisplatin	VEGR 1,2,3; SRC; PDGFR; FGFR; ABL-Kinase	RP2	1st line	44	PR 57%	PFS 9.4*; OS 18.3	(69)
Placebo	Pemetrexed				43	PR 44%	PFS 5.7; OS 14.2	
Cediranib	Pemetrexed	VEGF-2	RP2	1st line	45	PR 50%	PFS 7.2; OS 10	(70)
Placebo	Cisplatin				47	PR 20%	PFS 5.6; OS 8.5	
Nintedanib	Cisplatin	VEGR 1,2,3; SRC; PDGFR; FGFR; ABL-Kinase	RP3	1st line	229	PR 45%	PFS 6.8; OS 14.4	(71)
Placebo	Pemetrexed				229	PR 43%	PFS 7.0; OS 16.1	

PR, partial response; SD, stable disease; PFS, progression free survival; OS, overall survival; RP2, Randomized phase II; NR, not reported.
** Denotes a result which was statistically significantly superior to the other study arm.*
Clinicaltrials.gov, results extracted from clinicaltrials.gov but not published.

toxicity (59). Median PFS was 2.6 months (95% CI: 1.74–3.68), and median OS 9.5 months (95% CI: 5.6–10.7), with 1-year survival of 36% (95% CI: 23–50%); subsequent lines of therapy or patient selection could have played a role in OS which would otherwise be considered acceptable in this disease. Six patients ceased treatment due to adverse events attributed to cediranib, and 43/47 patients had a dose reduction.

Modest activity was also reported in a multi-center phase II trial that accrued 51 unresectable, histologically-confirmed pre-treated MPM patients who received cediranib 45 mg daily (61). Due to toxicity, the starting dose was lowered to 30 mg/d after the 15 first patients. Modest ORR and SD rates are reported in **Table 2** and the study did not reach its primary endpoint. No responses were observed in patients with sarcomatoid or biphasic histology. Median PFS was only 1.8 months (95% CI: 0.1–14.2 mo.) and median OS 4.4 months (95% CI: 0.9–41.7 mo.), with 15% 1-year survival. The authors concluded that the limited activity and substantial toxicity did not support use of cediranib single-agent therapy for MPM.

The SWOG phase I study reported first-line therapy combination of cediranib (30 mg/d and 20 mg/d cohorts) with cisplatin/pemetrexed for 6 cycles, followed by maintenance cediranib (68). Twenty chemo-naïve patients with unresectable MPM were enrolled (seven in 30 mg/d cohort, 13 in 20 mg/d cohort). Median PFS was 12.8 months ($n = 17$; 95% CI: 6.9–17.2) by RECIST, and 8.6 months ($n = 19$; 95% CI: 6.1–10.9) using modified RECIST. For all patients, the disease control rate at 6 weeks was 90%, and median OS was 16.2 months (95% CI: 10.5–28.7). Therefore, cediranib combined with cisplatin/pemetrexed was considered to have a reasonable toxicity profile and promising preliminary efficacy—leading to the launching of the S0905 phase II trial which has recently reported (70). In this study, 92 patients with MPM (75% epithelioid, 25% biphasic, or sarcomatoid) were randomized in a 1:1 ratio to platinum/pemetrexed with either cediranib or placebo, followed by maintenance cediranib or placebo. The primary endpoint was PFS via RECIST 1.1. Whilst the addition of cediranib numerically improved PFS by RECIST 1.1 (HR 0.71; $p = 0.062$; 7.2 vs. 5.6 months) there was no significant difference in OS (10 vs. 8.5 months HR 0.88, $p = 0.28$). Toxicity was also problematic, with the addition of cediranib associated with more anorexia, dehydration, diarrhea, and weight loss. This combination is unlikely to move further forward.

Other Miscellaneous Vascular-Targeting and Vascular-Disrupting Agents

Other vascular-targeting agents have also been trialed in mesothelioma, including NGR-hTNF and BNC-105P.

NGR-hTNF is comprised of the N terminal of TNF fused with the C terminal of the tumor-homing peptide NGR (asparagine-glycine-arginine). It targets the aminopeptidase N/CD13 which is expressed on solid tumor endothelial cells, blocking development of new blood vessels, and demonstrating anti-tumor activity (76). An initial single agent phase II study in 43 patients with pre-treated mesothelioma showed manageable toxicity, disease control in 44% of patients (one experiencing PR), and a median PFS of 2.8 months in a cohort treated every 3 weeks. A subsequent 14-patient cohort was treated weekly, with 50% stable disease and median PFS of 3.0 months (56). In hindsight, this is consistent with or even lower than the PFS seen in best supportive care and does not indicate significant activity (77). Nevertheless, given that this agent had the potential to improve the activity of chemotherapy through enhancing penetration into tumor, the international randomized phase III NGR015 study was designed to assess the activity of NGR-hTNF or placebo in combination with investigator choice of management in 400 patients with pre-treated mesothelioma. This study used the weekly regimen of NGR-hTNF, and was partnered with any of gemcitabine, vinorelbine, doxorubicin, or best supportive care. The primary endpoint was OS, which was not different between the two groups (median 8.5 months in the NGR-hTNF group vs. 8.0 months in the placebo group) with a non-significant HR of 0.94 (65). Whilst *post-hoc* subgroup analyses suggested some benefit in those with a shorter prior treatment-free interval, it is unlikely that this agent will be further studied in mesothelioma.

The vascular disrupting agent BNC105P is a small-molecule tubulin polymerase inhibitor that is highly potent and selective for tumor blood vessels, and had preclinical and phase I activity in mesothelioma. This agent was investigated in a single-arm phase II clinical trial as second- or third-line treatment. With an ORR of 3% in 30 patients, and a median PFS of 1.5 months, again there was no evidence of activity (62).

BIOMARKERS OF ANTI-ANGIOGENIC AGENTS

Although there has been over a decade of intense investigation, there are still no clear, validated biomarkers which predict the efficacy of bevacizumab or other anti-angiogenics, either in MPM or in other cancers (78). In the MAPS trial, the prognostic or predictive effect of baseline serum VEGF concentrations were assessed by ELISA in the 372/448 (83%) of patients with available samples. The prognostic analysis based on VEGF assessed as a continuous variable showed that high VEGF concentrations were associated with worse PFS and OS. This was confirmed by bootstrap resampling, a smart statistical method for internal validation of biomarkers, VEGF significantly correlating with worse PFS in 891 (89%) of 1,000 theoretical samples generated by bootstrapping, and with OS in 979 (98%) of 1,000 bootstrapped samples, with high optimism corrected concordance index of 0.64 for PFS and 0.65 for OS. Similar results were obtained by dichotomization at the median value as a cut-off. However, the predictive analysis based on VEGF assessed as a continuous variable showed that the interaction between treatment group and VEGF concentration was not significant for PFS ($p = 0.60$) or OS ($p = 0.99$). An exploratory subgroup analysis according to baseline serum VEGF concentration dichotomized at the median value showed that patients with VEGF concentrations below (adjusted HR 0.56 [95% CI 0.41–0.77]; $p = 0.0004$) or above (0.59 [0.44–0.80]; $p = 0.0007$) the median derived similar benefit in PFS from bevacizumab.

In the group with baseline VEGF concentrations below the median, patients receiving bevacizumab derived a 5.2 months longer OS compared to the chemo-only group (median OS 23.7 vs. 18.5, respectively; adjusted HR 0.73 [0.52–1.03]; $p = 0.07$). Similar results were identified in the study of cisplatin/gemcitabine plus bevacizumab (49). In addition, patients with baseline VEGF concentrations above the median value derived a 2.3 month benefit if they received bevacizumab (15.7 vs. 13.4 months; adjusted HR 0.86 [0.63–1.19], $p = 0.37$). To summarize what is to date the largest prospective study of serum VEGF in MPM patients, high serum VEGF concentration was clearly a worse prognostic biomarker. Regardless, patients with either high or low serum VEGF benefited from bevacizumab—resulting in the conclusion that serum VEGF could not accurately predict a survival benefit upon bevacizumab treatment over chemotherapy-alone treatment. Other studies from the MAPS trial assessing biomarkers for their prognostic/predictive values are still to be presented and published, including baseline plasma concentrations of angiogenesis-regulating micro-RNAs, baseline serum amphireguline, VEGFR immunostaining tumor expression, and microvessel density on CD44 staining. However, no analysis of the effect of BAP1 mutations is available in this study and the influence of such molecular alterations on sensitivity to bevacizumab-containing triplet remains unknown.

There was also extensive investigation of angiogenesis-related biomarkers in the phase II LUME-Meso trial which added nintedanib to chemotherapy. Investigators explored a large panel of putative biomarkers including 58 angiogenic factors by multiplex immunoassay, as well as microvessel density on CD31 staining and germline variants of VEGF. When allowance was made for multiple testing, there were no significant associations with treatment outcome (79).

WHY DID BEVACIZUMAB SUCCEED AND NINTEDANIB FAIL?

Bevacizumab and nintedanib both underwent phase 3 studies in MPM using a very similar design, comparing combination with standard pemetrexed-based chemotherapy over the chemotherapy doublet alone. However, the former showed a significant OS advantage whilst the latter unfortunately resulted in a negative trial; their contradictory fates could derive from both biological and methodological causes.

Biologically, nintedanib concentrations of 20–100 nmol/L block VEGFR, with biochemical IC50 concentrations ranging from 13 to 34 nmol/L on the three VEGFR subtypes—resulting in significant inhibition of endothelial cells, pericytes, and smooth

muscle cells proliferation (80). However, such concentrations were shown to be insufficient to reduce survival of lung cancer cell lines, needing much higher concentrations of up to 10 μmol/L (81), above the nanomolar concentrations of most TKI inhibitors used in the clinics. Furthermore, nintedanib shows neither any *in vitro* anti-proliferative effect, nor sensitizes lung tumor cells to chemotherapy, whilst only altering *in vivo* tumor growth by decreasing microvessel density, pericyte coverage, and perfusion, resulting in increased tumor hypoxia (82). Thus, these findings support a purely anti-angiogenic effect for nintedanib, which proved insufficient for an anti-tumor effect in malignant mesothelioma. This suggests that beyond anti-angiogenesis, the inhibition of VEGF-VEGFR signaling pathway would likely work in MPM by inhibiting the autocrine cell growth loop, lacking in other cancer cells such as lung or pancreatic cancer, in which inhibition of VEGFR mainly functions *via* anti-angiogenesis. Of course, this hypothesis remains to be experimentally proven; but would explain a fundamental difference between bevacizumab, a high-affinity binding antibody to VEGF, and a TKI, admittedly efficient on endothelial cells at very low concentrations. Indeed, endothelial cells express a high density of VEGF receptors when compared with MPM cells, in which directly inhibiting the growth factor is needed to alter tumor cell survival. Meanwhile a higher dose of TKI would be needed to inhibit the autocrine loop. Possibly both would be required, because of a lower number of VEGF receptors, and a lower affinity of the receptor than the antibody for VEGF. In addition, nintedanib was recently shown to exert direct anti-tumor effect on tumor cells, but only those with oncogene addiction to growth factors receptors targeted by nintedanib, such as PDGFRα, FGFR2, FLT3, or RET (83).

The second possible reason for the difference in results between these phase 3 trials can perhaps also be found in a putative methodological pitfall of the nintedanib trial. The phase 3 trial had slightly different inclusion criteria compared to the positive nintedanib randomized phase 2—specifically, excluding sarcomatoid or biphasic MPM subtypes (15–20% of MPM). The sponsor claimed that the phase 2 study failed to show any effect in this subpopulation, contrary to the effect observed in the epithelioid subtype. Although it is unlikely that restricting the second study to epithelioid-only patients is the only reason for failure, the phase 2 trial was still not powered to detect any OS difference in the sarcomatoid and biphasic subgroup; a negative result cannot exclude an actual effect without sufficient power, while positivity could reflect a real effect or consist of a false positive result. As an example, the randomized bevacizumab phase II trial by Kindler et al. (49) was presented as negative (although the OS in the two arms were promising), whilst the French phase III was positive. Furthermore, in the phase III trial, bevacizumab's advantage in sarcomatoid and biphasic subtype was at least as strong as in the epithelioid subtype (if not stronger, since the HR was lower)—suggesting that the statistical interpretation by the Nintedanib trial sponsor may have been erroneous, and could have changed the fate of the Nintedanib phase 3 trial. Of course, we will never know the actual reason of such failure for Nintedanib, and we cannot exclude that there was a mix of biological and methodological reasons contributing to the final negative result. Extensive examination of the data, as well as biomarker studies, has failed to identify a subgroup that may derive benefit, or a reason for failure of LUME-Meso-III.

CURRENT RECOMMENDATIONS FOR USE OF ANTI-ANGIOGENIC STRATEGIES IN MESOTHELIOMA

Currently both ASCO (84, 85) and NCCN guidelines (86) suggest that a bevacizumab, pemetrexed and platinum triplet can be used as first-line treatment in PS 0–2 patients with mesothelioma not amenable to radical surgery, without cardiovascular contraindications to bevacizumab, provided there is reimbursement. The national French guidelines "AURA-MESOCLIN" also recommend this strategy although, officially, no reimbursement is assured in France. However, taking into account the small patient numbers (around 1,000 per year in France) and a strong consumer lobby group with occupational asbestos exposure, reimbursement has not been difficult to obtain. In other European countries reimbursement is more uncertain. In the USA insurance companies do reimburse bevacizumab; this not the case in the UK, Australia or Canada. The manufactures of bevacizumab have not submitted an FDA filing for this indication, and it is noted that bevacizumab biosimilar agents are becoming available. Whether biosimilar availability may open access to triplet therapy including a VEGF targeting antibody remains to be seen.

Indeed, a key issue for triplet therapy is cost, and the lack of cost-benefit based on the MAPS data. Most costs derive from direct drug cost rather than indirect toxicity costs, which are generally low grade and manageable. Thus, the cost-benefit varies internationally depending on the drug cost and health system structure in each location. Moreover, previous cost-effectiveness studies in NSCLC or colorectal cancer patients treated with bevacizumab reported conflicting results, likely because of health systems differences. Italian, Taiwanese and Korean studies supported cost-effectiveness, while the UK stated that use of bevacizumab could be associated with increased costs. Chinese and US studies were inconclusive, each with both positive and negative studies (87–93). However, a recent cost-effectiveness study from the IMpower 150 trial, using a Markov model, showed improved cost-effectiveness of an atezolizumab, bevacizumab, carboplatin, and paclitaxel (ABCP) combination over bevacizumab, carboplatin, and paclitaxel (BCP) and carboplatin and paclitaxel (CP) in the first-line treatment of patients with metastatic NSCLC (94). It is difficult to directly extrapolate to mesothelioma patients from NSCLC data, since people with mesothelioma are generally older, but conversely have fewer smoking induced comorbidities. Fewer comorbidities may reduce toxicity, which in turn might lower costs. The lower risk of hemorrhagic complications in the MAPS trial than in NSCLC bevacizumab trials supports this hypothesis.

Finally, because of the lack of any positive phase III studies, no anti-angiogenic TKI has reached the market, and their further development remains uncertain unless efficacy in combination with immune checkpoint inhibitors is demonstrated.

INCORPORATING ANTI-ANGIOGENICS INTO THE NEXT GENERATION OF CLINICAL TRIALS

The next generation of clinical trials in mesothelioma will be split into those that do and do not incorporate bevacizumab in the control arm. The US FDA has not mandated the inclusion of bevacizumab in future clinical trials. Not all patients are eligible for bevacizumab, and more liberal inclusion and exclusion criteria can be considered for trials that do not incorporate bevacizumab, potentially accelerating recruitment and broadening applicability. Bevacizumab is not appropriate for neoadjuvant studies due to impact on wound healing. Furthermore, bevacizumab is not routinely available and used in all jurisdictions, with cost limiting availability in Australia, the United Kingdom, and some parts of Europe.

Nevertheless, there is a strong rationale for testing combinations of chemotherapy, bevacizumab, and checkpoint blockade. VEGF favors tumor recruitment of myeloid-derived suppressor cells (MDSCs), which suppress both T-cell and dendritic cell function thus supporting tumor immune escape (95). VEGF also induces vasodilatation and increases inter-endothelial space, thus favoring extravasation of immune cells that could infiltrate tumor tissue (notably regulatory T cells that can inhibit tumor immune responses). Finally, VEGFR stimulation by its ligands can suppress LATS kinase, leading to nuclear translocation of the YAP transcriptional co-activator and its interaction with TEAD transcriptions factors. This complex activates transcription of several genes involved in the immune response, especially CXCL5, CCL2, PD-L1, CXCR4, and TNF. In parallel, YAP-TEAD activation leads to the transcription of genes involved in stemness such as ALDH1A3 and LGR5, potentially increasing tumor aggressiveness. Hence, the consequences of anti-VEGF therapies are to elicit immune responses through increasing T-cell trafficking into tumors (96, 97), reducing MDSC infiltration (98), reducing regulatory T cells (99), and increasing memory phenotype CD8+ and CD4+ T-cells.

Moreover, in NSCLC, combining atezolizumab with bevacizumab and chemotherapy was efficacious in the IMpower150 phase 3 trial comparing a carbo-paclitaxel-atezolizumab-bevacizumab quadruplet to the triplet therapy (minus atezolizumab) in non-SCC patients. Thus, three early-phase clinical trials are on-going looking for proof-of-concept. The PEMBIB phase Ib trial phase accrued 37 patients with MPM in 2nd or 3rd line setting who subsequently received pembrolizumab with the oral VEGFR TKI Nintedanib. There were no concerning safety signals, and efficacy results are awaited. An MD Anderson Cancer Center trial combined atezolizumab (1,200 mg IV) and bevacizumab (15 mg/kg IV, q21 days) in MPM patients in the same setting: 20 patients were accrued and results are still pending. Twenty patients with peritoneal mesothelioma were also recruited on this study, with results due early 2020. One possible driver to increase testing of combinations is the FDA registration of at least two bevacizumab biosimilars, with more to come, potentially leading to a decrease in drug costs of such combinations.

CONCLUSIONS

In conclusion, the addition of bevacizumab to combination chemotherapy remains an important option for selected patients with MPM, but widespread use as a worldwide standard of care is currently limited by registration and reimbursement considerations. No other antiangiogenic has shown benefit in this setting, and use of other agents should be confined to a clinical trial. This will result in the next generation of clinical trials being those that build on a two-drug combination, and those that build on the triplet combination, and may have the unintended effect of reducing the interpretability and applicability of some future studies. Nevertheless, as not all patients, and not all settings, are appropriate for anti-angiogenic therapy, moving forward to study combinations both with and without bevacizumab remains appropriate.

AUTHOR CONTRIBUTIONS

All the authors contributed to the manuscript concept, drafting, revision, and final approval.

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Immunotherapy in Malignant Pleural Mesothelioma

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The only registered systemic treatment for malignant pleural mesothelioma (MPM) is platinum based chemotherapy combined with pemetrexed, with or without bevacizumab. Immunotherapy did seem active in small phase II trials. In this review, we will highlight the most important immunotherapy-based research performed and put a focus on the future of MPM. PD-(L)1 inhibitors show response rates between 10 and 29% in phase II trials, with a wide range in progression free (PFS) and overall survival (OS). However, single agent pembrolizumab was not superior to chemotherapy (gemcitabine or vinorelbine) in the recent published PROMISE-Meso trial in pre-treated patients. In small studies with CTLA-4 inhibitors there is evidence for response in some patients, but it fails to show a better PFS and OS compared to best supportive care in a randomized study. A combination of PD-(L)1 inhibitor with CTLA-4 inhibitor seem to have a similar response as PD-(L)1 monotherapy. The first results of combining durvalumab (PD-L1 blocking) with cisplatin-pemetrexed in the first line are promising. Another immune treatment is Dendritic Cell (DC) immunotherapy, which is recently tested in mesothelioma, shows remarkable anti-tumor activity in three clinical studies. The value of single agent checkpoint inhibitors is limited in MPM. There is an urgent need for biomarkers to select the optimal candidates for immunotherapy among MPM patients in terms of efficacy and tolerance. Results of combination checkpoint inhibitors with chemotherapy are awaiting.

Keywords: immunotherapy, malignant pleural mesothelioma, angiogenesis inhibitors, PD-L1, dendritic cell therapy

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INTRODUCTION

Malignant pleural mesothelioma (MPM) is a rare, aggressive malignancy with limited treatment options. Surgery is controversial since only a minority of patients is fit enough to be a surgical candidate and a complete microscopic (and sometimes macroscopic) resection is not realistic. Therefore, the indication of surgery, within a multimodal strategy, has become stricter over the last years. At this time, the only registered systemic treatment is platinum-based chemotherapy combined with pemetrexed, with or without bevacizumab. Numerous phase I and II trials have been performed to make a step forward in the treatment of MPM. Immunotherapy seemed promising in small phase II trials. However, single agent pembrolizumab was not superior to chemotherapy (gemcitabine or vinorelbine) in the recent published PROMISE-Meso trial. Currently, we are awaiting the outcome of randomized phase III studies with immunotherapy in the first line. In this review, we will highlight the most important immunotherapy-based research performed and put a focus on the future of MPM.

PD-(L)1 BLOCKING

Several PD-(L)1 inhibitors have been tested in patients with progressive disease after first line chemotherapy. The KEYNOTE-028 phase I trial was the first study testing a PD-1 inhibitor (pembrolizumab) in 25 patients with a PD-L1 immunohistochemistry expression (IHC) ≥1%. The trial reported a response rate of 20%, a disease control rate (DCR) of 72% with a median duration of response of 12 months (1). Desai et al. reported similar results in 65 patients treated with pembrolizumab, in a unselected patient population (2). The response rate was 19%, a DCR of 47% and with a median progression free survival (mPFS) of 4.5 months (**Table 1**). Metaxas et al. reported the efficacy of this checkpoint inhibitor using real world data. In 93 patients they observed an objective response rate (ORR) of 18%. However, the mPFS was only 3.1 months with an OS of 7.2 months (3).

Single agent nivolumab has been tested in 2 single arm phase II trials and in the MAPS2 trial, a randomized, non-comparative phase II study of nivolumab and nivolumab-ipilimumab. All three studies showed activity with an ORR between 15 and 29% and a DCR between 44 and 68% (4, 5, 8). In one of the phase II trials (NivoMes), the mPFS was disappointing with only 2.6 months (5). The second study tested nivolumab monotherapy (MERIT) and showed a higher mPFS of 6.1 months (4). In the combination study of the MAPS-2, the nivolumab monotherapy reported a mPFS of 4.0 months (8). The study with avelumab, a PD-L1 blocker, showed less efficacy with a response rate of 9.4% in 53 patients and a mPFS of 3.9 months (6).

The first randomized study in patients with recurrent MPM has recently been presented at the ESMO congress 2019; ETOP PROMISE-meso, randomizes patients to chemotherapy (gemcitabine or vinorelbine) vs. pembrolizumab. The primary endpoint; PFS was not met with a median PFS for pembrolizumab of 2.5 (95% CI 2.1–4.2) vs. 3.4 months (2.2–4.3) in the chemo arm, HR = 1.06 [0.73–1.53], $p = 0.76$. Surprisingly, the response rate was significantly higher in the pembrolizumab arm (22%) compared to chemotherapy (6%; $p = 0.004$), despite an equal PFS. The median OS was 10.7 months for patients in the pembrolizumab arm vs. 11.7 months for chemotherapy, HR = 1.05 ([0.66–1.67]; $p = 0.85$). Forty-five patients out of the chemotherapy arm crossed over to pembrolizumab after progression on chemotherapy. Accounting for crossover yielded a similar OS result. Treatment-related adverse events were similar in both groups. (TrAE) grade ≥3 were experienced by 19% in the pembrolizumab arm vs. 24% chemotherapy arm (14).

The CONFIRM trial in UK is ongoing, in which 336 patients with progression after at least 2 treatment lines will be randomized to 12 months treatment with nivolumab or placebo (15). The primary endpoint is OS, with secondary endpoint i.e., quality of life (QoL). These trials will hopefully provide evidence of the potential benefit of the use of PD-1 blocking in the treatment of relapsed mesothelioma.

CTLA-4 INHIBITORS

To date, only three studies were performed with an anti-cytotoxic T lymphocyte antigen 4 (CTLA-4) inhibitor alone. Initially, the phase II trials MESOT-TREM-2008 (10) and MESOT-TREM-2012 (11) trial showed some promising results and a large randomized controlled trial (DETERMINE) was initiated (12). In both MESOT-TREM trials 29 patients with MPM were included and treated with tremelimumab. In the first trial from 2008, two patients had a partial response and 7 others achieved disease control.

In the 2008 study the treatment dosage was 15 mg/kg every 90 days. After a retrospective analysis of a study in melanoma with tremelimumab, it was suggested that the dosage of tremelimumab administered was to low (16). In the subsequent MESOT-TREM-2012 trial, patients were treated with tremelimumab 10 mg/kg every 4 weeks, and after 6 cycles every 12 weeks. The response rate was slightly better, with a PR of 4 patients and disease control with a total of 15 patients, when measured with immune RECIST criteria. However, in the 2008 study, the modified RECIST criteria were used and based on these criteria only 1 patient had a partial response and 11 in total achieved disease control in the 2012 study.

Based on the results of the MESO-TREM studies, a large randomized controlled trial (DETERMINE) with higher dosage of tremelimumab was performed. Five hundred seventy-one patients were included and randomized (2:1) to tremelimumab or placebo. There were no significant differences in response or survival between the two groups. In earlier performed studies with PD-L1 blockers, a better result was suggested in the non-epitheloid subtype. The DETERMINE study did not confirm this observation. Although there seems to be a trend in the sarcomatoid group in favor of tremelimumab, the number of patients are too small to detect a significant difference. To explain the difference between de MESOT-TREM and the DETERMINE studies, one may argue that the number of patients was too small in DETERMINE trial; There were only 3 patients with a sarcomatoid subtype in this study. As known this is a more aggressive subtype and therefor faster growing. Only two patients in the study had a partial response (12).

COMBINATION THERAPY

As seen in melanoma and NSCLC, there can be an additive or synergic effect when combining CTLA-4 with PD-(L1) checkpoint inhibitors. The non-comparative MAPS-II trial, randomizing patients between nivolumab alone or nivolumab with ipilimumab showed clinical activity in both arms with a DCR of 40 and 52%, an ORR of 19 vs. 28% and mPFS of 4.0 and 5.6 months respectively. The combination group had a slightly higher proportion of drug-related adverse events (93% with combination vs. 89% with monotherapy and 3 toxicity-related deaths (vs. none in the monotherapy group). In their study, the French investigators concluded that nivolumab monotherapy with or without ipilimumab provides a clinically meaningful response (8). Updated results showed a median OS of 11.9 months (6.7–17.4) in the nivolumab arm and 15.9 months (10.7–22.2) in the combination arm (17). The occurrence of hyper progression disease (HPD) was assessed by two formulae; Tumor Growth Rate (TGR) and Tumor Growth Kinetics (TGK). The TGK definition of HPD did impact OS after pooling data from

TABLE 1 | Overview of study results.

References	Agent	N	Line of treatment	DCR %	ORR %	mPFS *months*	mOS *months*	Response by PD-L1 status nr of pts and %	Response in subtypes nr of pts and %	Study type
Alley et al. (1)	Pembro	25	>1st	72 *RECIST 1.1*	20	5.4	18.0	All patients ≥1% PDL-1	Not reported	Ib
Desai et al. (2)	Pembro	65	2nd, 3rd	66 *RECIST 1.1*	19	4.5	11.5	<1%: 2/26 (7%) 1–49%: 4/16 (25%) >50%: 6/20 (31%)	E:8/50 (16%) B:1/10 (10%) S: 2/5 (40%)	II
Metaxes et al. (3)	Pembro	93	1st, 2nd, 3rd	48 *Unknown*	18	3.1	7.2	<5%: 5/45 (11%) 5–49% 5/12 (42%) ≥ 50%: 4/9 (44%)	E: 11/67 (16%) B+S: 6/25 (24%) NE: 1	RS
Okada et al. (4)	Nivo	34	2nd, 3rd	68 *mRECIST*	29	6.1	17.3	<1%: 1/12 (8%) ≥1%: 8/20 (40%) NE: 1/2 (50%)	E: 7/27 (26%) B:1/4 (25%) S: 2/3 (67%)	II
Quispel-Janssen et al. (5)	Nivo	34	2nd, 3rd	47 *m-iRECIST*	24	2.6	11.8	(PR+SD) 0%: 8/21 (38%) 1–5%: 2/3 (67%) 5–50%: 0/2 (0%) >50%: 1/1 (100%) NE: 2/7 (29%)	E: 7/28 (25%) B: 2/4 (50%) S: 0/2 (0%)	II
Hassen et al. (6)	Ave	53	>1st	58 *RECIST 1.1*	9 1 CR	4.1	10.7	<5%: 2/27 (7%) ≥5%: 3/16 (19%)	Not reported	1b
Disselhorst et al. (7)	Nivo + ipi	34	2nd, 3rd	67 *mRECIST*	38	6.2	NR (12.7–NR)	(PR+SD) 0: 6/19 (32%) ≥1%: 11/15 (73%) ≥50% 4/5 (80%)	Not reported	II
Scherpereel et al. (8)	Nivo vs Nivo + ipi	63 vs. 62	2nd, 3rd, 4th	N: 40 NI: 52 *mRECIST*	N: 17 NI: 30	N: 4.0 NI: 5.6	N: 11.9 NI: 15.9	N: < 1: 3/31 (10%) ≥1: 7/19 (37%) NE: 1/13 (8%) NI: <1: 9/27 (33%) ≥1: 7/22 (32%) NE: 3/13 (23%)	N: E:7/52 (13%) B+S: 4/11 (36%) NI: E: 15/53 (28%) B+S:3/9 (33%)	RA II
Calabro et al. (9)	Treme + durva	40	1st, 2nd	65 *mRECIST*	28	8.0	16.6	0%: 4/15 (27%) ≥1%: 7/23 (30%) NE: 2	E: 9/32 (28%) B+S:2/7 (29%)	II
Calabro et al. (10)	Treme	29	>1st	31 *RECIST*	7	6.2	10.7	Not reported	E:9/25 (36%) B: 0/1 S: 0/3	II
Calabro et al. (11)	Treme	29	2nd	52 *iRECIST* 38 *mRECIST*	14 *iRECIST* 3 *mRECIST*	6.2	11.3	Not reported	Not reported	II
Maio et al. (12)	Treme vs. placebo	571	>1st	T: 4.5 P: 1.1 *mRECIST*	T: 27.7 P: 21.7	T: 2.8 P: 2.7	T: 7.7 P: 7.3	Not reported	HR for survival event E: 0.95 (0.77-1.18) B: 1.04 (0.55-1.98) S: 0.68 (0.34-1.39)	RA IIb

(Continued)

TABLE 1 | Continued

References	Agent	N	Line of treatment	DCR %	ORR %	mPFS *months*	mOS *months*	Response by PD-L1 status nr of pts and %	Response in subtypes nr of pts and %	Study type
Nowak et al. (13)	Durva + chemo	54	1st	48 *mRECIST* 50 *iRECIST*	*mRECIST* 48% *iRECIST* 50%	6.9	Not reported	Not reported	Not reported	II
Popat et al. (14)	Pembro vs. chemo (gemcitabine or vinorelbine)	142	2nd	Pembro 45, chemo 38 *RECIST 1.1*	P:22 C: 6	P: 2.5 C: 3.4 HR: 1.06 (0.73–1.53)	P: 10.7 C: 11.7	Pembrolizumab <1% 3/19 (16%) ≥1%: 10/32 (31%) NE: 3/22(14%) Chemotherapy <1% 1/17 (6%) ≥1%: 3 /34 (9%) NE:0 /20 (0%)	HR for survival PD-L1 <1% 1.26 (p=0.57) HR for survival PD-L1 ≥1%: 1.06 (P=0.82)	RA III

Pembro, Pembrolizumab; Nivo, Nivolumab; Ipi, Ipilimumab; Treme, Tremelimubab; mRECIST, Modified RECIST criteria for malignant pleural mesothelioma; M-I-RECIST, Combination of modified RECIST and iRECIST; N, Nivolumab; NI, nivolumab + Ipilimumab; NE, not evaluable; NR, Not reached; E, epitheloid; B, biphasic; S, Sarcomatoid; RA, Randomized; RS, Retrospective; Ave, avelumab.

TABLE 2 | Hyper Progression Disease reported in the MAPS2 trial (17).

	Nivolumab	Nivolumab + Ipilimumab	Both treatment arm
TGR			
Number of patients with HPD	4	2	
OS			
With HPD	Mean 4.6 (0.9–7.8)	Mean 4.5 (0.5–8.6)	
Without HPD	Mean 4.0 (2.4–8.6)	Mean 5.8 (1.4–9.9)	
TGK			
Number of patients with HPD	7	4	
OS	1.6 (0.8–7.7)		
With HPD			
Without HPD	4.4 (2.4–10.8)		
TGK			
OS (months)			
With HPD (*N* = 11)			2.6 (0.8–7.7)
Disease control (*N* = 75)			23.1 (16.1–26.7)*
Progressive disease (*N* = 42)			5.5 (2.6–8.9)**

It is not reported in how many patients Hyper Progressive Disease (HPD) could be assessed.
**Hazard ratio (HR, disease control vs. HPD): 0.12 (0.06–0.25; P < 0.001).*
***HR (progressive disease vs. HPD): 0.37 (0.19–0.75; P = 0.006).*
HR for correlation of OS and TGR is not reported.
TGR, Tumor Growth Rate; TGK, Tumor Growth Kinetics.

both treatment arms. The was no significant correlation of HPD defined by TGR and OS (see **Table 2**).

The clinical activity of combination ipilimumab-nivolumab was also seen in the Dutch INITIATE trial with a response rate of 38% and a DCR of 68% at three months. However, the combination treatment was more toxic with 94% of patients experienced an adverse event. Most side effects were easily managed and no grade 5 toxicity was observed (7).

Tremelimumab, another CTLA-4 blocker was also tested with a PD-L1 blocker (durvalumab) in 40 patients (in first and second line) in the NIBIT trial. The ORR of 28% was comparable to the MAPS-2 trial with a DCR of 65%, a median PFS of 8.0 months and an OS of 16.6 months (9).

The combination of PD-1 blocking and chemotherapy is an effective first line treatment in NSCLC. The first results of combining durvalumab (PD-L1 blocking) with cisplatin-pemetrexed in the first line are hopeful. In the Australian DREAM study, a single arm phase II in 54 first line patients reported an ORR of 48% by mRECIST but a mPFS of 6.9 months only (13). The PFS at 6 months (PFS6) was 57% (90% CI 45–68%). An international world-wide phase III randomized study with this combination is planned, led by the USA and Australia.

At this moment multiple randomized studies are running or awaiting evaluation:

(1) The phase 3 Checkmate 743 study (NCT02899299) in which 600 patients have been randomized between cisplatin (or

carboplatin)-pemetrexed or nivolumab-ipilimumab as first-line treatment. First results are expected beginning of 2020;

(2) The IND-227 (NCT02784171) study has been initiated to determine the value of pembrolizumab in the first line. This randomized phase II part of this study had three treatment arms: single agent pembrolizumab, cisplatin/pemetrexed, or a combination of the three agents. In the ongoing phase III part, extended to Italy, France (IFCT) and UK, the patients are randomized between cisplatin (or carboplatin)-pemetrexed plus pembrolizumab vs. the same chemotherapy alone. The estimated primary completion date is August 2020;

(3) The ETOP BEAT-meso trial (NCT03762018) in which 320 patients will be randomized between platinum-pemetrexed-bevacizumab with or without atezolizumab. The primary endpoint is PFS. First results are expected Q4, 2024.

DENDRITIC CELL THERAPY

Dendritic Cell (DC) immunotherapy is tested in several cancers. In mesothelioma, there are three clinical studies with DCs showing remarkable anti-tumor activity. In the first study published in 2010, autologous monocyte-derived DCs loaded with autologous tumor cell lysate were given to 9 MPM patients. The DCs were administrated in three dosages of 50×10^6 DCs; twice intravenous and once intradermal. Three out of nine patients showed a partial response in the first 8 weeks. Two of these patients were treated shortly before start of DC treatment with chemotherapy. This might intervene with the result (18).

The second study published in 2016 (19), the same type of DCs were administered; this time in combination with cyclophosphamide, a drug inhibiting regulatory T-cells (20). Five postsurgical and 5 non-surgical MPM patients were treated. In one of the non-surgical patients, a partial response was found. Overall, 7 out of 10 patients lived longer than 24 months. The OS was promising with a mean survival of 37 months (19).

Since the process of obtaining proper autologous tumor cell lysates is very time consuming and patient reluctant to multiple pleural biopsies, an alternative source of antigens to pulse the DCs was investigated. DCs were pulsed by a spectrum of tumor associated antigens derived from allogeneic tumor lysate form human mesothelioma cell line cultures. These DCs were tested in 9 MPM patients including 5 subjects pretreated by chemotherapy. In these 9 patients, a partial response was established in 2 patients; one treatment-naïve patient and one pretreated patient, lasting 15 and 21 months. Disease control was described in all other patients, with a median overall survival higher than 22.8 months (21). To validate these promising results, a European (H2020) randomized phase II/III trial (DENIM) assessing DCs immunotherapy vs. best supportive care as maintenance treatment after standard first line chemotherapy is ongoing.

BIOMARKERS

Similar to NSCLC, melanoma and other cancers, biomarkers to predict the response (or toxicity) to treatment in patients, are a crucial issue. In MPM, PD-L1 is expressed in 40–60% of the tumors, mostly in patients with sarcomatoid histology. PD-L1 expression is a negative prognostic factor for overall response to standard care but not for PFS or OS. In a retrospective study, the PD-L1 positive patients exhibited a mOS of 5 months, while median survival in PD-L1 negative patients was 14.5 months (22), while other studies and trials results had discrepancies on this finding (23).

In several studies, PD-L1 expression was correlated with response to PD-L1 inhibitors, with or without CTLA-4 inhibitors. In the PD(L)-1 monotherapy (2–6) studies responses to PD-L1 >1% varied between 19 and 44%. Generally, PD-L1 negative tumors show responses up to 10%, with only one study reporting an ORR of 56%; although in a small group of 9 patients (5). In the studies combining PD-(L)1 inhibitors with CTLA-4 inhibitors, a correlation between response and PD-L1 positive expression on tumors was found. In these studies (7, 8, 13) PD-L1 > 1% showed a response rate of 23–73%. Patients with PD-L1 negative tumors showed an ORR of 27–33%. Interestingly, the study of Scherpereel et al. (8) showed that the PD-L1 negative tumors had a similar response compared to the PD-L1 positive tumors to the combination therapy.

A reason for PD-L1 IHC not to be a very reliable biomarker might be the immune environment of MPM. In multiple studies a relatively low number of $CD8^+$ tumor infiltrating lymphocytes (TIL) have been observed (24, 25). MPM is also known to have an increased suppressive immune environment, with a high amount of $CD4^+$, FOXP3, and $CD25^+RO^+$ TILs. Marcq et al. showed in MPM with low numbers of $CD8^+$TILs, that their function was either moderately or severely suppressed (26). A high number of CD8+ TILs on the other hand correlates with more tumor cell apoptosis, lower N-stage and higher overall survival (25, 27, 28). Higher numbers of $PD\text{-}L1^+CD8^+$TIL were found in sarcomatoid subtypes (26), which might explain the slightly better results in PD-(L)1 checkpoint inhibitor therapy. High $CD8^+$TILs is a prognostic biomarker (28), it is not clear if this can also be used as a predictive biomarker in checkpoint inhibitors.

CTLA-4 is expressed in a little more than half of the MPM tissues. In the study of Roncella et al. CTLA-4 expression was measured in tissue, serum and pleural effusion of 45 patients. CTLA-4 expression seems a favorable prognostic factor, but this was only statistically significant in pleural fluid with a dead-rate reduction of 60% when a cut-off at 67 pg/ml soluble CTLA-4 was applied. Whether a positive finding of CTLA-4 expression in MPM will have therapeutic implications has not been investigated yet (29).

In NSCLC, tumor mutational burden (TMB) is a suggested biomarker to predict the efficacy in immunotherapy, in particular for the ipilimumab-nivolumab combination. As MPM harbor a low average TMB (30), this is thought to be of little prognostic use. One of the newer findings indicate that chromothripsis; which is chromosome scattering followed by random chromosome rearrangement, occurs more often in MPM and cannot be identified with whole genome sequencing. It is believed that the large parts of spliced DNA will accumulate in the cytoplasm and give rise to neoantigens (31).

Other factors that might correlate with response to checkpoint inhibitors such as HLA class I genotype, foregut microbiome

composition are investigated but no results were reported yet (32).

DISCUSSION

The NCCN guidelines (2018) recommend nivolumab ± ipilimumab or pembrolizumab as subsequent systemic therapy (33). Most of the previous trials in MPM with immunotherapy show activity in a limited number of patients with low and manageable toxicity. As summarized in **Table 1**, the studies exhibited a large variation in outcome as measured by PFS and OS. This might be related to the relatively small size of most studies, and variations in pathology and study execution. These factors are possibly due to a patient selection bias, with different inclusion criteria (34). The only reported randomized trial, the PROMISE-meso trial, did show that pembrolizumab was not superior to chemotherapy in the second line in terms of PFS. Patients in both arms could cross-over to either pembrolizumab or chemotherapy after progression. It could imply that in daily practice both pembrolizumab and chemotherapy are effective, in selected groups of patients.

Response assessment in MPM is challenging. Modified RECIST (mRECIST) for pleural mesothelioma was developed in 2004. Recently, immune-based therapeutics (iRECIST) was published to stage solid tumors. In the previous described studies different RECIST criteria were used. This can be an explanation for the wide range in reported response rates (see **Table 1**). NIBIT-MESO used immune-related objective response (complete response or partial response) according to immune-related modified RECIST criteria in patients with pleural mesothelioma. They pointed out the importance of criteria for follow up. irRECIST is based on solid tumors, but does not take specific MPM response considerations into account. Therefor mRECIST 1.1 recommends adoption of irRECIST into mRECIST (35). More research is needed to assess the immune-related modified RECIST criteria.

Disease control rate (DCR) is a commonly used endpoint in MPM. However, this endpoint is subject to several forms of bias; the time points for DCR is inconsequent between studies. The DETERMINE trial measured DCR at $\geq$6 weeks after randomization (29%) (31), the KEYNOTE-028 reported DCR at 8 weeks (72%) (1), several studies at 12 weeks (5, 7, 8, 31)[38–67] while other studies did not specify at which time point DCR was measured (47–68%) (2–4, 9) (see **Table 1**). This leads to a time-to-event bias, making it hard to compare DCR between studies. By selecting the best patients, almost all small phase II trials recruit only performance status 0 or 1, there is a possibility that DCR is also a reflection of the tumor biology. We suggest that ORR is a better primary endpoint for future studies with immunotherapy in MPM, and reporting of the DCR as secondary endpoint at a pre specified time point.

The MAPS2 trial reported hyper progressive disease (HPD) due to immunotherapy, which raises questions. It was not reported how hyper progressive disease was measured. It is unclear if patients had 2 CT-scans without treatment before start of study-treatment, to be able to evaluate the growth rate. The subgroups were very small, ranging from 2 to 11 patients, and the relation between HPD and OS was not equal between the different definitions of HPD (17). It is not known if HPD is unique for immunotherapy. In the PROMISE-meso trial, also patients in the chemotherapy arm had an increase of up to 80% in tumor size at the first response evaluation (14).

To be able to distinguish which patient will benefit from immunotherapy and who will not, better biomarkers are urgently needed. As in NSCLC, PD-L1 positive patients, especially the non-epithelioid group, seem to have a better outcome compared to PD-L1 negative patients. Unfortunately, there is no validated clear-cut for the percentage of PD-L1 positive tumor cells, probably due to the heterogeneity of the tumor and other immunosuppressive and –activating factors such as tumor infiltrating lymphocytes, T-regs, inflammation, HLA class genotype, and microbiome composition. The need for better biomarkers is also high, to prevent costs and possible unnecessary complications due to immunotherapy.

Since malignant mesothelioma is a rare disease, selecting agents for large phase III trials should be based on impressive response rates of single agent phase II data and positive randomized phase II results. However, in MPM numbers of large phase II/III trials have been initiated based on very limited evidence; (e.g., the DETERMINE trial, the NVALT5 trial (thalidomide vs. best supportive care), the NGR015 trial (investigator choice plus NGR-hTNF or placebo), the VANTAGE-014 trial (vorinostat vs. placebo) and the COMMAND trial [maintenance defactinib or placebo)] (12, 36–39). Recommended endpoint for future RCT's in MPM would be to confirm an overall survival benefit with an HR of $\leq$ 0.7 and a gain of $\geq$3 months without a statistically significantly in grade 3–4 toxicities to preserve quality of life (40).

Although all patients eventually will experience a recurrence after first line chemotherapy, the standard of care (platinum-pemetrexed therapy) is effective with response rates around 45%, a median PFS of up to 7.3 months and a OS up to 16 months (41, 42). Results of the DREAM- study should be placed in perspective with a response rate of 48% and a PFS of 6.9 months (13).

In conclusion, immunotherapy seems to bring hope for a selected group of MPM patients but several crucial questions remain unanswered to date. Phase III randomized trials with clear primary end-points are on their way and will probably establish the role of immunotherapy in MPM. In addition, there is an urgent need for biomarkers to select the optimal candidates for immunotherapy among MPM patients in terms of efficacy and tolerance.

AUTHOR CONTRIBUTIONS

CG and FB performed a literature search, interpreted data, and wrote the manuscript. AS and PB supervised and contributed to the writing process.

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REVIEW
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Emerging Treatments for Malignant Pleural Mesothelioma: Where Are We Heading?

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Malignant pleural mesothelioma (MPM) is an uncommon but aggressive and treatment resistant neoplasm with low survival rates. In the last years we assisted to an exponential growth in the appreciation of mesothelioma pathobiology, leading several new treatments to be investigated both in the early stage of the disease and in the advanced setting. In particular, expectations are now high that immunotherapy will have a leading role in the next years. However, caution is required as results from phase II studies in MPM were often not replicated in larger, randomized, phase III trials. In this review, we describe the most promising emerging therapies for the treatment of MPM, discussing the biological rationale underlying their development as well as the issues surrounding clinical trial design and proper selection of patients for every treatment.

Keywords: Malignant mesothelioma, checkpoint inhibitors, immunotherapy, tumor-treating fields, dendritic cell therapy, mesothelin, anti-angiogenic, targeted therapy

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INTRODUCTION

Malignant pleural mesothelioma (MPM) is an uncommon and highly lethal cancer. The annual incidence of MPM ranges between 10 cases per million to 29 cases per million depending on the country and, because of the long latency period, the peak is expected in the 2020s (1) in high-income countries. In addition, according to WHO prediction (2), developing countries where asbestos is still used, are likely to face a new epidemic of asbestos-related diseases, including MPM.

MPM pathogenesis is peculiar, as the direct causal relationship between exposure to airborne asbestos particles and the development of MPM is well established (3). The chronic exposure to asbestos fibers, which may enter the lung periphery and the pleura, leads to chronic inflammation of the mesothelium which sustains the carcinogenic processes (4). Individuals with germline BRCA1 associated protein-1 (BAP1) mutations may be predisposed to MPM, since they may develop it without any apparent asbestos exposure (5). Recent biological and preclinical studies provided further insights into MPM carcinogenesis, revealing the importance of tumor suppressor gene inactivation, through several mechanisms (single nucleotide variants (SNVs), copy number losses, gene fusions, and splicing alterations). Tumor suppressor genes highly altered are cyclin-dependent kinase inhibitor 2A (CDKN2A, 60% of the cases), BAP1 (60% of the cases also in sporadic MPM), and neurofibromin 2 (NF2, 75% of the cases) (6–9).

The chronic inflammatory response to asbestos involved in the pathogenesis of MPM also causes a unique tumor environment. This microenvironment is mainly composed of immunosuppressive

cells [regulatory T cells, macrophages and myeloid-derived suppressor cells (MDSCs)] and the number of these cells as determined by immunohistochemistry (IHC) represents a negative prognostic factor (10, 11). On the other hand, immune-activating responses, such as the presence of $CD8^+$ T cells, are correlated with better outcome, although such links with prognosis are less important when compared with other cancer entities which are more immunogenic than MPM (12).

The management of MPM is complex and outcomes remain poor. For patients with early stage MPM the role of radical surgery is still a matter of debate and it should be considered only as part of a multimodal treatment (i.e., surgery combined with chemotherapy, radiotherapy, or both). Looking at unresectable MPM, no major breakthroughs have been made since the approval of antifolate and platinum combination chemotherapy (13, 14). Median overall survival (OS) time with standard first-line options is about 13 months, with the best outcome for the epithelioid MPM subtype (14). Second-line treatment scenario is even more disappointing. With the only exception of a repeated course of pemetrexed-based chemotherapy for previously responsive patients (15), limited options are available for relapsed MPM and new treatments are urgently needed.

Steps have been made toward a best appreciation of mesothelioma biology and have been essential to identify novel molecular therapeutic targets, representing the rationale for testing multiple targeted therapies in MPM (**Table 1**). Nevertheless, the potential to improve the potency and the specificity of the immune system, along with recent successes in other thoracic tumors, have attracted a growing interest in cancer immunotherapy. Continue efforts are necessary to further deepen our understanding of mesothelioma, taking into account biological and temporal heterogeneity of the disease in order to finally optimize the development of new treatment options in the context of well-designed clinical trials (**Figure 1**).

In this review, we describe last emerging therapies for mesothelioma, discussing the current status of knowledge in mesothelioma genetics and immune-biology, as well as the issues surrounding the conduction of high-quality trials in MPM and the selection of best patients for different treatments.

NEOADJUVANT/ADJUVANT SETTING

Due to the anatomy, microscopically radical (R0) resection is not achievable in mesothelioma surgery and the goal of mesothelioma surgery is macroscopic complete resection (R1). Surgery alone is not curative; it is usually performed with chemotherapy and/or radiation therapy and reserved to a subset of patients with early tumor stage, epithelioid histology and good performance status.

Therapeutic surgery in mesothelioma has historically involved either an extended pleurectomy-decortication (eP/D) or an extrapleural pneumonectomy (EPP) (16, 17). eP/D has been proven to offer better results in the context of multimodality treatment (18, 19), and although the benefit of systemic therapy has been shown only in the advanced/unresectable disease, it is common practice to give four cycles of cisplatin or carboplatin with pemetrexed as adjuvant or neoadjuvant therapy. Two ongoing trials, MARS 2 (NCT02040272) and EORTC1205-LCG (NCT02436733), are currently evaluating the usefulness, the feasibility and the best timing for the combined approach of surgery and chemotherapy.

In order to improve local control and ideally survival, radiotherapy can be given. New approaches of radical hemithoracic radiation using intensity-modulated techniques are being tested. Rimner et al. showed that hemithoracic intensity-modulated pleural radiation therapy (IMPRINT) after chemotherapy and P/D was safe in 27 MPM patients as part of a multimodality lung-sparing treatment, with an acceptable rate of radiation pneumonitis (20). Larger clinical trials are awaited to confirm the effectiveness of this approach.

Recently, intrapleural therapies have been reported with the aim of improving loco-regional control of the disease by spreading drugs directly on the tumor surface. Several techniques with different rationale have been used with promising results: hypertermic intrapleural chemotherapy, photodynamic therapy (PDT), intrapleural immunotherapies [interferons (IFNs) and interleukin-2 (IL-2)], and gene therapy (21). However, available evidences are mainly based on retrospective, small and single-institution studies and controlled randomized trials are required.

If given as neoadjuvant therapy, novel agents should have the ability to induce tumor shrinkage, increasing the possibility of a complete microscopic resection and ultimately prolonging overall survival while maintaining a good safety profile. Designing studies in this setting remains a challenging effort that requires multidisciplinary involvement (22). Nevertheless, the neoadjuvant setting provides the unique possibility to conduct translational research in the context of window-of-opportunity trials, acquiring valuable information from blood and tissue collection. For example, the focal adhesion kinase (FAK)-inhibitor defactinib showed immunomodulatory effects when administered pre-operatively in a phase II window of opportunity trial (23) with a good tolerability profile, an objective response rate of 13% and 67% of stable disease, thus not altering resectability or mortality compared to historical controls. Final trial data are expected for 2020.

This approach has also paved the way for testing the properties of immune check-point inhibitors (CIs). There are several ongoing neoadjuvant trials which aim to assess the immunomodulatory and pharmacodynamics effect of CIs, as monotherapy (NCT02707666), as combination of anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and anti-programmed cell death protein (PD-1) agents (NCT02592551, NCT03918252) and as combination of anti-programmed death-ligand 1 (PD-L1) with standard chemotherapy (NCT03228537).

By assessing translational surrogates of response, these trials may represent an opportunity to look into predictive biomarkers, improving selection of candidates to CIs-treatment.

CIs are also tested in the adjuvant setting (NCT02707666). From an immunological perspective, the main goal of combining surgery with adjuvant CIs is to reduce tumor induced immunosuppression (24). Increased tumor size correlates with major immune suppression and surgically shrinking tumor

TABLE 1 | Ongoing trials in malignant pleural mesothelioma patients (source: ClinicalTrials.gov).

Class	Treatment	Trial name/Identifier	Phase	Setting/Line of treatment	Single agent/Combined therapy	Estimated enrollment	Notes
Surgery	eP/D	NCT02040272 (MARS2)	III	Surgically resectable	Standard neoadjuvant chemotherapy before surgery	328	Multicentre randomized trial comparing eP/D vs. no surgery
	eP/D - chemotherapy	NCT02436733	II	Surgically resectable	Neoadjuvant or adjuvant chemotherapy	64	Chemotherapy before or after P/D in patients with early stage MPM
Radiotherapy	Accelerated hypofractionated radiotherapy with tomotherapy	NCT03269227	I	Adjuvant (after eP/D)	N/A	30	
	Hemitoracic intensity modulated radiation therapy (IMPRINT)	NCT00715611	II	Adjuvant	Adjuvant chemotherapy	81	After enrolling 45 patients, hemithoracic IMPRINT was safe and had an acceptable rate of pneumonia
	Short neoadjuvant hemithoracic intensity-modulated radiation therapy	NCT00797719	I	Neoadjuvant	Adjuvant chemotherapy (+/-)	100	
Chemotherapy	Mithramycin (continuous 24-hours infusion)	NCT02859415	I/II	Relapsed	Single agent	100	Mithramycin is an antineoplastic antibiotic that inhibits cancer stem cell signaling
Antiangiogenic agents	Nintedanib	NCT02863055	II	Maintenance treatment after chemotherapy	Single agent	116	
PARP inhibitors	Olaparib	NCT03531840	II	Relapsed	Single agent	40	Recruitment is not limited to patients with germline/somatic mutations in DNA repair genes
	Niraparib	NCT03207347	II	Relapsed	Single agent	57	
EZH2 inhibitors	Tazemetostat	NCT02875548	Extension (rollover)	Relapsed	N/A	300	In multiple solid tumors
Base-excision repair inhibitors	TRC-102	NCT02535312	I/II	First line/Relapsed	Cisplatin and pemetrexed or only pemetrexed	58	
PI3K inhibitors	IPI-549	NCT02637531	I	Relapsed	Nivolumab (+/-)	220	In multiple solid tumors
FAK inhibitors	Defactinib	NCT02004028	Window-of-opportunity	Neoadjuvant	Single agent	38	
		NCT02758587	I/II	Relapsed	Pembrolizumab	59	
	APG-2449	NCT03917043	I	Relapsed	Single agent	40	APG-2449 is a novel, oral, multi-targeted tyrosine kinase inhibitor, which inhibits FAK, ALK, and ROS1
BCR/ABL pathway	Bosutinib	NCT03023319	I	N/A	Pemetrexed	24	In multiple solid tumors
Arginine deprivation	ADI PEG 20	NCT02709512 (ATOMIC)	II/III	First line	Cisplatin and pemetrexed	386	Double-blind, randomized (standard chemotherapy in the control group); only patients with biphasic or sarcomatoid histology are eligible; ASS1-deficiency is not required for study entry

(Continued)

TABLE 1 | Continued

Class	Treatment	Trial name/Identifier	Phase	Setting/Line of treatment	Single agent/Combined therapy	Estimated enrollment	Notes
Arginase inhibitors	INCB001158	NCT02903914	I	Relapsed	Pembrolizumab	424	In multiple solid tumors
Anti-CD30	Brentuximab vedotin	NCT03007030	II	Any line	Single agent	55	CD30 positive MPM
MDM2 antagonists (p53 pathway)	ASTX295	NCT03975387	I	Relapsed	Single agent	135	In multiple solid tumors (p53 wild type)
DR5 agonists	INBRX-109	NCT03715933	I	Relapsed	Single agent	80	INBRX-109 is a multivalent agonist of DR5
Tie2 inhibitors	Rebastinib (DCC-2036)	NCT03717415	I	First line/Relapsed	Carboplatin	117	Rebastinib acts on Tie2, a tyrosine kinase receptor that is expressed on endothelial cells and pro-tumoral macrophages
Immune check-point inhibitors	Pembrolizumab	NCT02707666	Window-of-opportunity	Neoadjuvant	Adjuvant pemetrexed and cisplatin	15	
		NCT02784171	II/III	First line	Cisplatin and pemetrexed	126	Randomized trial with both cisplatin/pemetrexed and pembrolizumab alone (only in the phase II part) as active comparators
		NCT02959463	I	Adjuvant to radiotherapy	N/A	24	Primary goal is to determine the safety and tolerability of pembrolizumab administered after radiation therapy in patients with MPM who have not undergone EPP
		NCT03393858	II	Relapsed	DC-CIK immunotherapy combined with hyperthermia	40	
		NCT02628067 (KEYNOTE-158)	II	Relapsed	Single agent	1350	A trial of pembrolizumab (MK-3475) to evaluate predictive biomarkers in advanced cancers
	Nivolumab	NCT03063450 (CONFIRM)	III	Relapsed	Single agent	336	Double-blind, placebo controlled
		NCT03502746	II	Relapsed	Ramucirumab	35	
		NCT02834013	II	Relapsed	Ipilimumab	707	Anti-CTLA-4 and Anti-PD-1 combination in rare tumors
	MEDI4736	NCT02592551	Window-of-opportunity	Neoadjuvant	Tremelimumab (only 8 patients)	20	
	Atezolizumab	NCT03762018 (BEAT-meso)	III	First line	Bevacizumab and standard chemotherapy	320	Open-label, randomized (bevacizumab plus standard chemotherapy in the control group)
		NCT03074513	II	Relapsed	Bevacizumab	160	

(Continued)

TABLE 1 | Continued

Class	Treatment	Trial name/Identifier	Phase	Setting/Line of treatment	Single agent/Combined therapy	Estimated enrollment	Notes
		NCT03228537	I	Neoadjuvant	Cisplatin and Pemetrexed	28	Within 90 days after completion of surgery patients receive atezolizumab for up to 1 year
	Avelumab	NCT03399552	I	Adjuvant to radiotherapy (stereotactic body radiation therapy)	N/A	27	
	INCMGA00012	NCT03920839	I	First line	Cisplatin and pemetrexed	98	INCMGA00012 is a humanized IgG4 monoclonal antibody that targets human PD-1 and lacks antibody dependent cell-mediated cytotoxicity directed against effector lymphocytes
	XmAb20717	NCT03517488	I	Relapsed	Single agent	87	Phase I trial assessing the safety and tolerability of XmAb20717, a bispecific antibody that simultaneously targets immune checkpoint receptors PD-1 and CTLA-4, in multiple tumors
	Cosibelimab	NCT03212404	I	Relapsed	Single agent	500	In multiple solid tumors; CK-301 (cosibelimab) is a fully human monoclonal IgG1 antibody against PD-L1
	ABBV-181	NCT03000257	I	N/A	Single agent	221	In multiple solid tumors; ABBV-181 is an anti-PD1 monoclonal antibody
	TIM-3 inhibitor (INCAGN02390)	NCT03652077	I	Relapsed	Single agent	41	In multiple solid tumors
	LAG-3 inhibitor (INCAGN02385)	NCT03538028	I	Relapsed	Single agent	40	In multiple solid tumors
	GITR agonist (INCAGN01876)	NCT03126110	I/II	Relapsed	Nivolumab/Ipilimumab	285	In multiple solid tumors
	OX40 agonist (ABBV-368)	NCT03071757	I	Relapsed	Single agent/combination with anti-PD1 therapy	170	In multiple solid tumors
Mesothelin targeted therapy	Immunotoxin LMB-100	NCT03644550	II	Relapsed	Pembrolizumab	38	
		NCT04034238	I	Relapsed	Tofacitinib (inhibitor of Janus kinases)	45	
	Anetumab ravatansine	NCT03126630	I/II	Relapsed	Pembrolizumab	134	Open-label, randomized but not comparative (pembrolizumab alone in the non-experimental arm)
		NCT03926143	Extension (rollover)	Relapsed	N/A	20	
	Thorium-227 labeled antibody-chelator conjugate (BAY2287411)	NCT03507452	I	Relapsed	N/A	228	All tumors known to express mesothelin are eligible
Vaccines	Galinpepimut-S	NCT04040231	I	Relapsed	Nivolumab	10	

(Continued)

TABLE 1 | Continued

Class	Treatment	Trial name/Identifier	Phase	Setting/Line of treatment	Single agent/Combined therapy	Estimated enrollment	Notes
	Dendritic cell therapy (Mesopher)	NCT03610360 (DENIM)	II/III	Maintenance treatment after chemotherapy	Single agent	230	Dendritic cells are loaded with allogeneic tumor cell lysate (PheraLys)
		NCT02649829	I	Neoadjuvant	Standard concomitant chemotherapy and eP/D afterwards (in case of resectable disease)	20	Dendritic cells are loaded with the tumor antigen WT1
Adoptive cell therapy	iCasp9M28z CAR-T cells (targeting mesothelin)	NCT02414269	I	Relapsed	Cyclophosphamide prior to infusion +/- Pembrolizumab after infusion	66	After treating 20 patients, intrapleurally administered mesothelin-targeted CAR T cells were safe with encouraging antitumor activity
	TC-210 CAR-T cells (targeting mesothelin)	NCT03907852	I/II	Relapsed	Cyclophosphamide and fludarabine before treatment as lymphodepleting agents	70	
	CAR-T cells (targeting mesothelin)	NCT03638206	I	N/A	Cyclophosphamide and fludarabine	73	In multiple solid tumors
	TILs	NCT02414945	I/II	N/A	Cyclophosphamide and Fludarabine before treatment, low-dose IL-2 after cell infusion	10	
		NCT03935893	I	Relapsed	Cyclophosphamide and fludarabine	10	
Virotherapy	Intrapleural adenonovirus-deliveres interferon alpha-2b (rAd-IFN)	NCT03710876 (INFINITE)	III	Relapsed	Celecoxib and gemcitabine	300	Open-label, randomized with control group receiving only oral celecoxib plus intravenous gemcitabine
Other intrapleural therapies	Intrapleural Cryotherapy	NCT02464904	I	Neoadjuvant	N/A	15	
	Hyperthermic intraoperative chemotherapy (with pemetrexed and cisplatin)	NCT02838745	I	Adjuvant	N/A	36	
	Intracavitary cisplatin-fibrin localized chemotherapy	NCT01644994	I/II	Adjuvant	N/A	54	
	Intraoperative porfimer sodium -mediated photodynamic therapy	NCT02153229	II	Adjuvant	N/A	102	Open-label, randomized

N/A, data not available; ALK, anaplastic lymphoma kinase; ASSI, argininosuccinate synthase I; CAR, chimeric antigen receptor; CD30, cluster of differentiation 30; CTLA-4, cytotoxic T lymphocyte associated protein-4; DC-CIK, autologous dendritic cells-cytokine induced killer cell; DR5, death receptor 5; eP/D, extended pleurectomy and decortication; EPP, extrapleural pneumonectomy; EZH2, enhancer of zeste homolog 2; FAK, focal adhesion kinase; IgG, immunoglobulin G; MDM2, murine double minute 2; MPM, malignant pleural mesothelioma; PARP, poly ADP ribose polymerase; PD-1, programmed cell death-1; PI3K, phosphoinositide 3-kinase; ROS1, ROS proto-oncogene 1; TIE2, tyrosine kinase with immunoglobulin-like and EGF-like domains 1; WT1, Wilms' tumor.

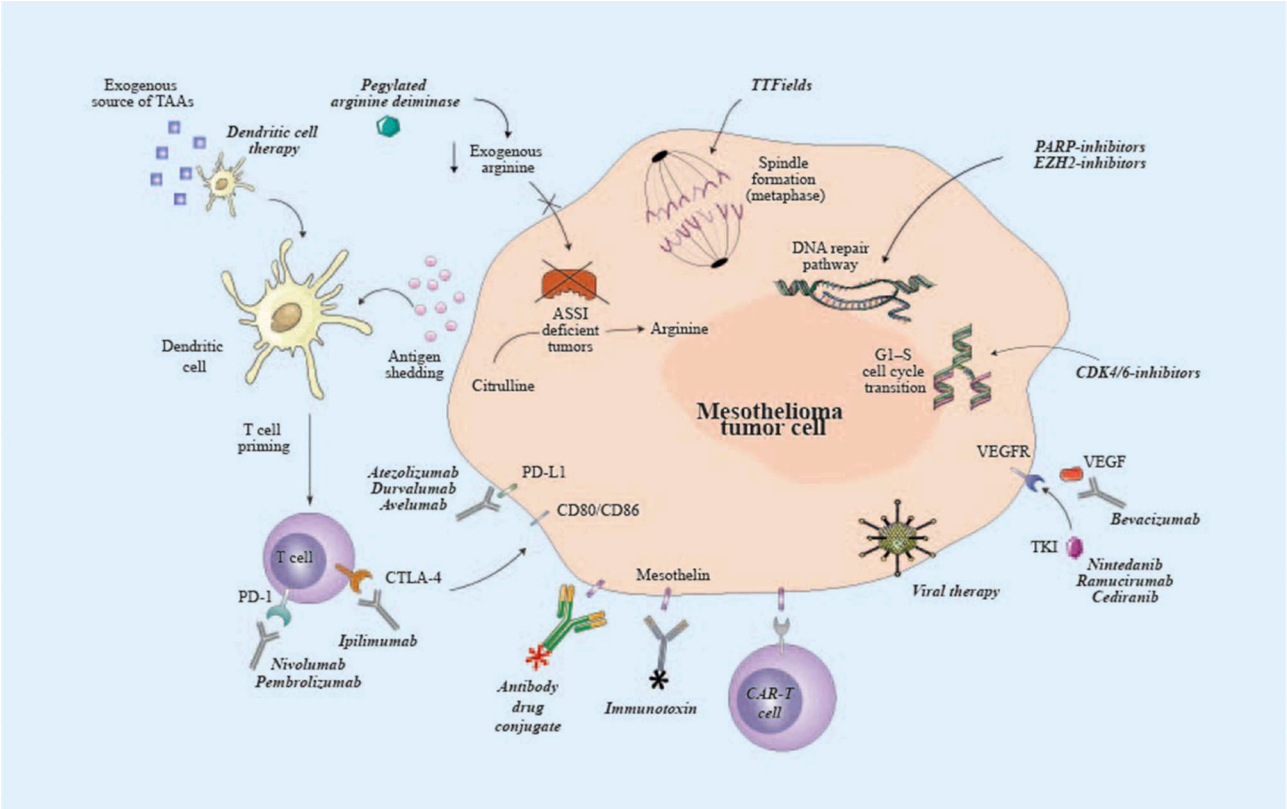

FIGURE 1 | Potential targets of emerging therapies for malignant pleural mesothelioma. ASSI, argininosuccinate synthase I; CAR, chimeric antigen receptor; CD80, cluster of differentiation 80; CD86, cluster of differentiation 86; CDK4/6, cyclin-dependent kinase 4/6; CTLA-4, cytotoxic T lymphocyte associated protein-4; EZH2, enhancer of zeste homolog 2; PARP, poly ADP ribose polymerase; PD-1, programmed cell death-1; PD-L1, programmed death ligand-1; TAAs, tumor-associated antigens; TKI, tyrosine kinase inhibitor; TTF, tumor-treating fields; VEGFR, vascular endothelial growth factor receptor.

size may potentially reduce immune inhibition and T-cell exhaustion (25).

Another approach to increase immune activation in the adjuvant setting is represented by vaccines, either protein, bacteria or cell-based. An adjuvant Wilms tumor 1 (WT1) vaccine (galinpepimut-S), given with granulocyte-macrophage colony-stimulating factor (GM-CSF) and an immunologic adjuvant called montanide ISA 51 UFCH in MPM patients whose tumors expressed WT1 at IHC, had completed combined multimodality therapy and had no evidence of disease, showed a median progression-free survival (PFS) of 10.1 months (95% CI 5.5–20.8 months) and a median OS of 22.8 months (95% CI 9.1–37.6 months) with a favorable safety profile (26). Galinpepimut-S is currently being tested in the advanced setting combined with CI-treatment (NCT04040231).

In peritoneal mesothelioma, the feasibility of administering dendritic cells pulsed with an allogenic tumor cell lysate after cytoreductive surgery and hyperthermic intraperitoneal chemotherapy (HIPEC) is being assessed in the ongoing MESOPEC trial (NTR7060) (27). Secondary objectives of the study are to assess the safety of dendritic cells and determine whether this adjuvant treatment may induce a specific immunological response against the tumor (27). Pre-clinical evidences showed that dendritic cell therapy leads to better outcome when dendritic cells are injected in murine models with lower tumor volume (28, 29). An efficient immune response is hampered by cytokines and regulatory T-cells induced by mesothelioma cells, showing that a low tumor load correlates with a better functioning immune system and higher anti-tumor responses. Giving dendritic cell therapy after surgically reducing tumor load might therefore improve response to therapy and clinical outcome.

To date, despite the neoadjuvant/adjuvant treatment represents a promising setting to test new therapeutic strategies, the global level of evidence is quite low and international guidelines (30) do not recommend either neoadjuvant or adjuvant radiotherapy/chemotherapy as standard options for resectable MPM.

UNRESECTABLE MESOTHELIOMA

Chemotherapy

There is no approved maintenance treatment for MPM patients who did not progress after first-line chemotherapy. NVALT19 was an open label, multicentric, randomized phase II trial, in which patients were assigned 1:1 to gemcitabine (1,250 mg/m2

day 1 and 8 of 3 weekly schedule) or best supportive care (BSC) after 4-6 cycles of first-line platinum-pemetrexed without progression. Data presented at the last ESMO conference showed an improvement in PFS (median 6.2 months vs. 3.2 months in the BSC arm [hazard ratio (HR) 0.42 (95% CI 0.28-0.63), $p < 0.0001$)], at the cost of an increased yet manageable toxicity (57% of patients experienced grade 3–4 adverse events vs. 13% in the BSC-arm, with neutropenia, nausea and lung infection being the most frequent) (31). Since post-study treatments and OS data were not reported, the reported improvement in PFS could be simply due to an anticipation of second-line therapy.

Lurbinectedin is a new molecule that binds to the DNA minor groove in regulatory regions, inhibiting the function of oncogenic transcription factors. It also modulates the transcriptional program of monocytes and TAMs, hampering cytokine production (32). Investigator tested the role of lurbinectedin in the context of relapsed MPM, where no approved therapy exists. Recent data from the SAKK 17/16 multi-center, single-arm phase II trial, showed activity of lurbinectedin. Median PFS and median OS were 4.1 months (95% CI 2.6-5.5) and 11.9 months (95% CI 9.2–14.7), respectively. Lurbinectedin also worked independently of histology or prior immunotherapy (32).

These data support evaluation of the both gemcitabine as switch maintenance and lurbinectedin as second-line strategy in larger, randomized, phase III trials.

The NovoTTF-100L represents another approach that has been recently investigated to improve the efficacy of chemotherapy. NovoTTF-100L is a portable Tumor Treating Fields (TTFields) delivery system. TTFields represent a non-invasive, regional treatment modality by which alternating electric fields (at a frequency of 150 kHz) are continuously administer to the local site to arrest tumor cancer cell division. In human mesothelioma cell cultures, combining TTFields with cisplatin or pemetrexed led to reduction in cell count, induction of apoptosis and reduced clonogenic potential (33). These alternating electric fields act by disrupting spindle formation during metaphase and blocking the localization of intracellular organelles during telophase.

Based on the results of the prospective, single-arm, phase II STELLAR trial, the NovoTTF-100L System was approved by U.S. FDA in combination with pemetrexed plus platinum-based chemotherapy for the first-line treatment of unresectable locally advanced or metastatic MPM. NovoTTF-100L was approved under Humanitarian Device Exemption, an approval process guaranteed by the U.S. FDA which, taking into consideration the urgent need to identify more effective treatments for rare disease (such as MPM), allows medical devices to be marketed without requiring evidence of effectiveness.

However, the STELLAR trial raised several issues that need to be addressed before implementing this strategy into daily practice. The 80 patients enrolled in the STELLAR trial (34) had a median OS of 18.2 months (95% CI 12.1-25.8), with 40.3% of partial responses and 97.2% of them obtaining a clinical benefit. Response rates were similar to the ones with standard chemotherapy but lasted longer by adding TTFields (median response duration was 5.7 months, ranging from 1.4 to 13 months). The rate of serious systemic adverse events remained the same when NovoTTF-100L was added to chemotherapy (either pemetrexed plus cisplatin or pemetrexed plus carboplatin, according to investigator choice). Expected TTFields-related skin toxicity was reported in 66% (53 patients) with only 5% of grade 3 skin toxicity. These results should be considered in context of the randomized phase III MAPS trial (35), in which bevacizumab added to pemetrexed and cisplatin significantly improved median OS compared to pemetrexed plus cisplatin alone (median OS 18.8 vs. 16.1 months, HR 0.77, $p = 0.0167$). The control arm of this trial performed 4 months better than the historical cohort analyzed by Ceresoli et al.—the landmark study by Vogelzang et al.—(14) and should be considered while discussing STELLAR data. Also PFS (7.6 months) and response (40%) were similar when compared to control groups in the MAPS and the recent LUME-meso trials (36). This fact, together with the potential sampling bias in single-arm studies and the effect of subsequent therapies, limits the interpretation of STELLAR data.

To date, TTFields represent one of many empirical approaches to MMP and further investigation of this approach in randomized trials is strongly encouraged.

Anti-angiogenic Agents

Activation of the vascular endothelial growth factor (VEGF) pathway, via its tyrosine kinase receptors, is crucial for mesothelioma cells growth (37), thus representing a rationale for antiangiogenic treatments in this neoplasm.

The addition of bevacizumab to pemetrexed and cisplatin chemotherapy as first-line treatment with bevacizumab maintenance therapy in patients who did not progress showed improved overall survival. However, bevacizumab remains currently unlicensed in this setting since the MAPS trial was not a registration trial (35). Moreover, results of Bevacizumab [an anti-VEGF monoclonal antibody (mAb)] as first-line option in combination with chemotherapy were not confirmed by other anti-angiogenic agents, such as the tyrosine-kinase inhibitors (TKIs) axitinib (an anti-VEGFR TKI), sorafenib (anti-VEGFR2/3, platelet-derived growth factor receptor (PDGFR) and rapidly accelerated fibrosarcoma (RAF)/c-KIT), or imatinib mesylate (targeting BCR-ABL, c-KIT, and PDGFR) (38–41).

Since the benefit in the phase 2 trial ($n = 87$ patients) (42) was higher in epithelioid MPM than in non-epithelioid subtypes, the multi-targeted anti-angiogenic kinase inhibitor, nintedanib (targeting VEGFR 1–3, PDGFR α or β, fibroblast growth factor receptor (FGFR) 1–3, SRC and ABL kinases pathways) was tested in conjunction with first-line cisplatin plus pemetrexed in a randomized phase III trial vs. placebo only in patients with epithelioid histology. However, among the 458 randomized patients, the previous phase II efficacy findings were not confirmed and PFS did not differ between the nintedanib group (median 6.8 months [95% CI 6.1–7.0)] and the placebo group (7.0 months (95% CI 6.7–7.2); HR 1.01 (95% CI 0.79–1.30), $p = 0.91$). The interim analysis of OS also showed no difference between groups (36).

Nintedanib is also being currently investigated as only maintenance treatment for patients non-progressive after first line chemotherapy (NCT02863055).

Cediranib, a VEGFR and PDGFR inhibitor, added to first-line platinum-based chemotherapy, improved PFS in a randomized phase II trial (43). Primary end-point of the trial was to detect a PFS difference (by RECIST version 1.1) at the 1-sided 0.10 level and it was met. PFS was significantly higher in MPM patients who received cisplatin-pemetrexed chemotherapy with cediranib followed by maintenance cediranib, compared to the ones receiving cisplatin-pemetrexed with placebo. HR was 0.69 (median PFS 7.2 vs. 5.6 months, $p = 0.096$). However, PFS was not different by modified RECIST and no significant difference in OS was reported. As with bevacizumab, cediranib is not approved as first-line treatment combined with chemotherapy.

Ramucirumab is a monoclonal antibody that binds the extracellular domain of human VEGFR-2. Due to VEGF-R2 expression on macrophages, ramucirumab also inhibits macrophages and their infiltration into mesothelioma microenvironment, thereby decreasing tumor growth and proliferation (44). One-hundred sixty-four patients are planned to be randomized in a multicenter, double-blind, placebo-controlled phase II trial comparing gemcitabine with or without ramucirumab in the second-line setting [NCT03560973 (RAMES)], whose completion is expected for 2020.

Targeted Therapies

New studies have recently provided a comprehensive genomic profiling of mesothelioma. Genomic analysis may help in detecting actionable alterations and developing more tailored and effective therapies for MPM patients (6). Tumor suppressor inactivation (loss-of-function) represents one of the most frequent mutational events in this tumor. In addition, multiple studies have pointed out frequent copy gains and copy losses involving different portions of the genome (6, 7, 45–48).

Carriers of inherited loss-of-function mutations in BAP1 are predisposed to mesothelioma (5, 45, 49, 50). BAP1 encodes a deubiquitinase enzyme, a member of the ubiquitin carboxy (C)-terminal hydrolase (UCH) family, involved in different cellular pathways among which the cell cycle, cellular differentiation, cell death, metabolism, and the DNA damage response (51). In particular, BAP1 is thought to bind to the breast cancer type 1 susceptibility protein (BRCA1) and the BRCA1-associated RING domain protein 1 (BARD1) and enhance their tumor suppressor function (52). Besides germline mutations, recent analysis of the BAP1 locus by targeted next-generation sequencing identified homozygous inactivating mutations in approximately 60% of patients (53). This implies that the role of BAP1 in defective DNA repair and homologous recombination might be therapeutically exploited in a large number of MPM.

In a recent paper, among 385 patients treated with platinum chemotherapy, median OS was increased for MPM patients who had inherited mutations in DNA repair and/or other tumor suppressor genes (54). This is consistent with what already observed in ovarian and breast cancer patients with inherited mutations in BRCA1 and BRCA2 (55–58). Conversely, BAP1 mutant mesothelioma cell lines resulted significantly less sensitive than BAP1 wild type cells to gemcitabine (59). In addition, the role of somatic BAP1 expression in MPM patients receiving chemotherapy still represents a matter of debate, with retrospective studies showing contradictory evidences (60, 61).

By inducing synthetic lethality of alternate DNA repair pathways, poly-ADP ribose polymerase (PARP) inhibitors have proved to be able to cause cell death in cell lines with loss of function of BAP1. This observation suggests that patients with mutations in BAP1 and in DNA repair genes might also benefit from treatment with PARP inhibitors (62). An enrolling clinical trial in MPM patients is examining the relationship between patient genotype and response to the PARP inhibitor olaparib (NCT03531840). Another PARP inhibitor, niraparib, is being tested in patients with BAP1 and other DNA damage response (DDR) pathway deficient neoplasms including mesothelioma (NCT03207347).

BAP1 inactivation also works as a putative epigenetic regulator involved in the polycomb repressive complex 2 (PRC2) and enhancer of zeste-homolog 2 (EZH2) pathway. Mesotheliomas with BAP1 loss proved to be responsive to EZH2 inhibition *in vitro* and *in vivo* (63). EZH inhibition may then represent a promising strategy, with tazemetostat showing a promising disease control rate of 51% at 12 weeks in a multicenter phase 2 trial (64).

CDKN2A is a tumor suppressor gene frequently inactivated in mesothelioma. CDKN2A encodes the ADP-ribosylation factor (ARF, also known as p14) and INK4A (also known as p16) via alternative reading frames (65). By inhibiting cyclin–dependent kinase 4 (CDK4) and CDK6, INK4A decelerates the G1–S cell cycle transition. Small molecules CDK4 and CDK6 inhibitors induce apoptosis in CDKN2A-mutated tumors (66–69) and MPM cell lines viability was inhibited in a dose-dependent manner by the CDK4/CDK6 inhibitor abemaciclib (70). Combined with radiotherapy, this agent also completely suppressed tumor growth in a mouse model of MPM (70). These finding led to the investigation of abemaciclib in p16INK4A negative MPM patients [NCT03654833 (MiST)].

The hepatocyte growth factor (HGF), by binding to the MET receptor and activating its downstream target PI3K has been shown to enhance MPM cell proliferation, migration and invasiveness. Therefore, this pathway represents a compelling therapeutic target in this disease (71). However, the modest response rate observed in the early phase trials assessing agents targeting this pathway (72), indicates that combination regimens with other classes of antitumor agents with a sufficiently wide therapeutic window, will be necessary.

The enzyme argininosuccinate synthetase 1 (ASS1) leads to arginine biosynthesis from citrulline and is epigenetically suppressed in a high proportion of mesothelioma cell lines (73). Loss of ASS1 renders mesothelioma cells addicted to exogenous arginine (74), and this defect may be therapeutically exploited by pegylated arginine deiminase (ADI–PEG20), which works by clearing circulating arginine (73). Non-epithelioid (biphasic and sarcomatoid) MPM subtypes are characterized by a 75% rate of ASS1 loss and disease control rate (DCR) of this subgroup resulted 94% in the TRAP Phase I trial (75) of ADI-PEG 20 combined with 1st-line pemetrexed and cisplatin chemotherapy. Results from the randomized, placebo-controlled, double-blind phase 2/3 global

ATOMIC-meso trial (NCT02709512) in non-epithelioid MPM are awaited.

In conclusion, despite our improved understanding of the biology of MPM, response to targeted therapies is hampered by intra-tumor heterogeneity and it is still unclear whether most of the actionable mutations constitute clonal or sub-clonal driver events. Longitudinal prospective studies, such as the TRACERx study in lung cancer (76), aiming at elucidating mechanism of resistance to treatment, are still missing in MPM. Properly designed clinical trials, which stratify patients for predictive biomarkers, are warranted. To this regard, patients enrolled in the MiST trial (NCT03654833) are currently offered a specific study treatment (either the parp-inhibitor rucaparib, the CDK4/6 inhibitor abemaciclib, the combination of the PD-1 inhibitor pembrolizumab and the AXL inhibitor bemcentinib or the combination of the PD-L1 inhibitor atezolizumab and the anti-angiogenic agent bevacizumab) determined by the results of the molecular panel testing of their diagnostic tumor block. The ones who exhibit positive testing in more than one biomarker, will potentially be eligible for a subsequent protocol upon disease progression. This trial design is aimed at providing a more tailored approach for MPM patients.

Mesothelin Targeted Therapies

Mesothelin (MSLN) is a glycoprotein with high expression in epithelioid mesothelioma and low expression in normal tissues, thereby it represents an attractive target for several therapies. A phase II trial comparing amatuximab (an anti-MSLN chimeric monoclonal antibody) plus first-line chemotherapy vs. chemotherapy alone was prematurely stopped in January 2017, not because of unacceptable toxicity but because of business reasons (NCT02357147).

According to a public announcement, anetumab ravtansine (an antibody-drug conjugate made by combining a human anti-MSLN antibody and the maytansinoid tubulin inhibitor DM4) also failed to improve PFS compared to vinorelbine in a randomized phase II trial for patients progressing after first-line (NCT02610140) (77).

CRS-207 is a live, attenuated, non-virulent, Listeria monocytogenes (LADD) encoding human MSLN. After receiving two priming infusions of CRS-207, followed by pemetrexed/cisplatin chemotherapy, and CRS-207 booster infusions in a phase Ib trial, 89% (31/35) of patients had disease control; one complete response (3%) and 19 partial responses (54%) were reported. Reduction of tumor size was also observed post-CRS-207 infusion prior to chemotherapy in 11 patients and no treatment-related serious adverse events or deaths were observed. These results suggested that combining CRS-207 with traditional chemotherapy might potentially result in increased anti-tumor activity (78). However, after a phase II trial had showed no clinical activity of the combination of CRS-207 with PD-1 inhibition (NCT03175172), clinical development of this therapy was discontinued.

LMB-100 is a next generation immunotoxin against MSLN that consists of a humanized fragment of the anti-MSLN Fab bound to a de-immunized Pseudomonas exotoxin (PE). This PE-fusion protein has been engineered to decrease its immunogenicity. A Phase I, open-label study to investigate the safety, pharmacokinetics, and activity of LMB-100 in relapsed MPM patients is planned to complete accrual this year (NCT02798536).

Evaluating new combinations of MSLN directed therapies with checkpoint inhibitors and integrating MSLN targeting into new approaches such as adoptive T cell transfer might constitute the next step in the field, as first results have been promising (79).

Immunotherapy

Immune Checkpoint Inhibitors

The immune system is known to play a key role in MPM. Immune suppression locally induced by the tumor is high (80). Survival of patients with MPM is longer when tumors are highly infiltrated by cytotoxic $CD8^+$ T cells (tumor-infiltrating lymphocytes), whereas PD-L1 expression is associated with shorter survival (median OS 5.0 in patients who are PDL1-positive vs. 14·5 months PDL1-negative patients; $p < 0.0001$) (81, 82). Due to their ability to restore the capacity of immune system to counterattack tumor growth, CIs (directed toward CTLA4, PD1, PDL1 or their combinations) started to be investigated in MPM patients. A large randomized phase IIb trial, assessing tremelimumab, an anti-CTLA4 mAb, vs. placebo in a second or third-line setting did not show superiority of the immunotherapy in terms of OS (83). Looking at agents targeting the PD-1/PD-L1 pathway, interesting results were reported in the first early phase trials with overall response rates (ORR) ranging from 9 to 29% in patients previously treated with chemotherapy (84).

As shown in other types of cancer (85), combining CTLA-4 and PD-(L)1 mAb might further improve outcomes. In a single-center, single-arm, phase II trial (INITIATE) (86), the combination of ipilimumab and nivolumab for the treatment of recurrent MPM was assessed. Of the 34 patients evaluated for radiological response at 12 weeks, ten (29%) patients were partial responder and 13 (38%) had stable disease; adverse events were quite frequent (94% of patients) with 12 (34%) patients reporting grade 3 toxicity. Another randomized, non-comparative, open-label, phase 2 trial (MAPS2), conducted in 21 hospitals in France (87), met its primary endpoint of DCR after randomization in the first 108 patients. This trial aimed to assess the anti-PD1 mAb alone (nivolumab) or in combination with anti-CTLA4 (ipilimumab) mAb in MPM patients who progressed to first-line chemotherapy. Twenty-four (DCR 44%) of 54 patients treated with nivolumab and 27 (DCR 50%) of 54 patients treated with nivolumab plus ipilimumab achieved disease control at 12 weeks. Objective responses were ten (19%) with nivolumab and 15 (28%) with nivolumab plus ipilimumab. Again, the safety profile was consistent with previous data on the combination. To note, three (5%) treatment-related death were reported with the combination (one fulminant hepatitis, one encephalitis, and one acute kidney failure).

These findings confirm the promising activity of both single and double check-point blockade in MPM patients who have relapsed. However, data presented at 2019 ESMO conference from the European Thoracic Oncology Platform (ETOP 9-15) PROMISE-meso randomized phase III trial (NCT02991482) comparing PD-1 inhibition with pembrolizumab to institutional

choice single agent CT (gemcitabine or vinorelbine) as second-line treatment failed to show superiority of PD-1 treatment (88). Nearly four times more patients responded to immunotherapy (ORRs were 22% with pembrolizumab vs. 6% in CT, $p = 0.004$), but these responses were not translated into delayed progression or improved survival (median PFS was 2.5 months (95% CI 2.1–4.2) with pembrolizumab and 3.4 months (95% CI 2.2-4.3) with chemotherapy, HR 1.06 (95% CI 0.73–1.53), $p = 0.76$). In this study long-term responders to pembrolizumab were also found, again underlining the importance of understanding which patients should receive this treatment instead of chemotherapy (88). Data from another randomized trial comparing nivolumab vs. placebo in patients pre-treated with at least two lines of chemotherapy [NCT03063450 (CONFIRM)], are also warranted in order to select the best strategy. At the current time, results from the MAPS2 trial supported the National Comprehensive Cancer Network (NCCN) panel decision to introduce either nivolumab or nivolumab plus ipilimumab as treatment options in relapsed MPM patients and nivolumab was approved in Japan as second-line treatment after results from a multicenter, open-label, single-arm, Japanese phase II study in MPM (MERIT) were reported, with ten (29%) patients showing an objective response (89).

Similar to other cancers, there might be a subgroup of MPM patients who might obtain a larger benefit from CIs, but relevant biomarkers have not been determined yet. Tumor PD-L1 IHC expression (with a cut-off of 1%) was correlated to ORR in both groups of MAPS-2 trial (nivolumab alone or nivolumab combined with ipilimumab) (87) but resulted in a better OS only in the nivolumab group. These correlations were not consistent in another phase II trial with nivolumab (90) and, although PD-L1 status may be associated with sensitivity to CIs, also patients with low PD-L1 expression benefit from this treatment, with a reported ORR of 11.1% (91). Intra-patient heterogeneity, different cut-points for PD-L1 positivity and lack of assay standardization also prevent PD-L1 from being used as the only selection criteria for CIs-treatment in MPM. This should lead researchers to investigate other tumor and patients' characteristics (histological subtype, performance status, blood-derived tests) to get an upfront identification of patients who are likely to respond to CIs and integration of multiple parameters (infiltration of CD8 and other subpopulations of T-cells (92), genomic signatures, specific mutations, expression of different checkpoint inhibitors) beyond PD-L1 status will be crucial.

To improve response rate to CIs in MPM patients, two options may be pursued. The first one is to move CIs toward the first-line setting, where the reinvigoration of the immune system may be stronger and more efficient, and to combine them with chemotherapy, similar to what happened in non-small cell lung cancer. Results of the addition of the PD-L1 inhibitor durvalumab to cisplatin and pemetrexed were presented in form of an abstract at the 2018 World Conference on Lung Cancer (93), showing a PFS of 6.2 months with a 48% ORR in the context of a non-randomized phase II trial—ORR is 41.3% with first-line chemotherapy alone, as historically reported (14). In the United States, a similar phase II trial investigating durvalumab (MEDI4736) in combination with chemotherapy for first-line treatment of MPM is currently in the analysis phase (NCT02899195). The addition of either pembrolizumab (NCT02784171) or nivolumab (in a Japanese population) (94) to chemotherapy is also being studied. The combination of ipilimumab and nivolumab is being compared with the cytotoxic chemotherapy standard in the first-line setting as well, with about 600 patients expected to be enrolled in a phase III trial (95).

The second option may be to combine CIs with either different immune-modulatory molecules, targeted therapies, antiangiogenic agents, or radiotherapy. Additional co-inhibitory and co-stimulatory molecules such as T-cell immunoglobulin and mucin-domain containing-3 (TIM3, also known as HAVCR2), lymphocyte activation gene 3 (LAG3) and inducible T cell co-stimulator (ICOS) are being investigated in mesothelioma (96–98). Inhibiting FAK together with PD-1, may enhance immune cell-associated antitumor cytotoxicity *in vivo*, which is hampered by expression of PD-L1 (99) and this represented the rationale for a phase I/IIa currently ongoing (NCT02758587). Similarly, in addition to the direct anti-tumor effects, pegylated arginine deiminase (ADI-PEG 20) may boost tumor immune surveillance and might be a good primer for an additional anti-tumor immune therapy (100), raising the question whether combining ADI-PEG 20 with PD-1/PD-L1 blockers may further enhance these drugs' anti-tumor efficacy (101).

Early phase trials also assessed the combination of anti-PD1/PDL1 agents and MSLN-directed therapies (in MSLN-positive patients). After results from a pre-clinical murine lung tumor model (CT26hMeso) demonstrated anti-PD1 enhanced LADD-induced tumor response (102), a phase 2 single-arm study of CRS-207 with pembrolizumab in relapsed MPM was started but no responses were showed, and the study was discontinued (102). Two other phase 2 trials (NCT03644550, NCT03126630) assessing the combination of pembrolizumab with the anti-MSLN Immunotoxin LMB-100 and with the antibody-drug conjugate anetumab ravtansine are currently enrolling patients, with the latter one also randomizing patients to pembrolizumab alone as active comparator.

Growing evidence that pro-angiogenesis factors have immunosuppressive activity has led researchers to evaluate the potentially synergistic combination of antiangiogenic agents and immunotherapy also in the treatment of MPM. VEGF signaling has been shown to attenuate the immune antitumor response by either influencing lymphocyte trafficking across endothelia to the tumor or directly inducing inhibitory immune cell subsets (103). Several trials are aiming to address whether the combination of CIs and antiangiogenic agents (either mAbs as bevacizumab and ramcirumab or TKIs as nintedanib) is able to improve outcomes in MPM patients (NCT03762018, NCT02856425, NCT03502746).

Finally, similarly to certain types of chemotherapy, radiotherapy can be exploited for its ability to cause immunogenic cell death (ICD), thus priming the release of damage-associated molecular patterns (DAMPs) and tumor-associated antigens (TAAs) and inducing a systemic anti-tumor immune response, that may be further enhanced by PD-1 (pembrolizumab) or PD-L1 (avelumab) blockade (NCT02959463, NCT03399552).

Vaccines

Vaccines represent another way to boost the immune system activation against the tumor. Both protein, vector and cell-based vaccines have been tested in MPM.

Galinpepimut-S is a WT-1 synthetic peptide vaccine made out of molecules similar to those in the WT1 protein. After a phase II trial confirmed vaccine's safety when administered in the adjuvant setting, researchers' efforts are currently directed toward the assessment of the combination of galinpepimut-S and nivolumab (NCT04040231). It has been hypothesized that the negative influence of tumor microenvironment factors on the immune response might be mitigated by nivolumab, thus providing the opportunity for the reinvigorated immune cells, specifically sensitized against WT1 by the vaccine, to invade and destroy cancerous growth deposits.

Dendritic cells are antigen-presenting cells that present tumor-associated antigens (TAAs) to the immune system by trafficking from tumors to lymph nodes. They are essential in priming proliferation and activation of $CD8^+$ cytotoxic T–lymphocytes and $CD4^+$ helper T-lymphocytes resulting in a potent and specific anti-tumor response (104). Dendritic cell function is hampered in cancer patients by tumor-derived soluble factors that suppress their immune-stimulatory ability (105, 106). However, dendritic cells can be generated in large amounts *ex vivo* and loaded with TAAs, prompting their recent usage as cancer vaccines in several neoplasms, including MPM. Several sources of tumor antigens (mRNA, peptides, proteins or whole tumor cell lysate) can be used to load DCs (107). Because TAAs are difficult to identify in mesothelioma (thus excluding peptides as best source), and adequate tumor tissue is rarely obtained from mesothelioma patients (108, 109), an allogenic tumor lysate has been developed (110). Results from a first-in-human clinical trial involving nine MPM (non-progressive after at least 4 cycles of chemotherapy) showed that this approach is safe (no dose-limiting toxicities were established) and led to radiological responses and promising survival data, with median PFS of 8.8 months and median OS not reached (110). A large multicentric phase II/III randomized trial with allogeneic-lysate pulsed dendritic cell immunotherapy as maintenance treatment after platinum-based chemotherapy is currently enrolling in Europe [NCT03610360 (DENIM)] (111).

T Cell Therapies

Another promising cell-based strategy in mesothelioma is represented by adoptive T cell therapy. Data from a phase I trial investigating chimeric antigen receptor (CAR) T cell therapy targeted to the MSLN protein in 19 MPM patients progressed following standard platinum-based chemotherapy were recently reported (79). A single-dose of second-generation CD28-costimulated MSLN-CAR T cells with the Icaspase-9 safety gene (IcasM28z) was given intrapleurally (as recommended by previous observations in murine models, in which intrapleural administration vastly outperformed intravenous infusion) (112) with or without cyclophosphamide preconditioning. No evidence of on-target, off-tumor or therapy related toxicity was seen, and CAR T-cell persistence was associated with decreased levels of serum soluble MSLN-related peptide (SMRP) levels (>50% compared to pretreatment) and evidence of tumor response. Of the 14 patients who received anti-PD1 agents, off-protocol, after the CAR T-cell therapy, 2 achieved a complete metabolic response, 5 obtained a partial response, and 4 had stable disease. Combining anti-PD1 therapy with CAR T cells is also supported by prior preclinical data showing that CAR T cells become functionally exhausted in the presence of a large tumor burden and that anti-PD-1 therapy can reactivate these exhausted cells (113).

Virotherapy

Oncolytic viral therapy represented in the last decades an emerging field of immunotherapy and a promising experimental strategy. Viruses can act by infecting cancer cells and leading to cell lysis after replication. This renders tumor-associated and viral antigens recognizable to the immune system, thus triggering antitumor immune responses (viroimmunotherapy) (114, 115). Oncolytic viruses need also to be tumor selective, and although malignant cell-specific oncolysis naturally occurs because of the impairment of the type I interferon pathway in many tumor cells, viruses may be engineered in order to increase their selectivity. Viruses may be used also for gene therapy, thereby therapeutically changing the infected tumor cells by gene transfer (116).

The pleural location and the peculiar pattern of growth (mostly localized), which provide access to direct intratumoral injection of virus, make MPM an ideal candidate for assessing the efficacy of oncolysis (116). The safety of virotherapy has been assessed and some clinical response have been reported (114). Among the many viral vectors that have been investigated, the recombinant replication incompetent adenoviral (ADV) vector encoding human interferon-α (IFNα, a naturally-occurring protein with anti-cancer properties) administered "*in situ*" (intrapleurally) with celecoxib (to reduce the number of immunosuppressive MDSCs) before chemotherapy, was well tolerated and appeared to improve overall survival rates (117). Combinations of virotherapy with CIs, chemotherapy, and radiation are expected to further boost the effects on antitumor immunity and represent the object of ongoing trials (118–120), such as the phase III INFINITE trial (NCT03710876), in which about 300 patients will receive gemcitabine and celecoxib with or without the ADV-delivered IFNα-2b (rAd-IFN).

CONCLUSION

In the past two decades there was limited success in the development of novel therapies for MPM. Multiple biases in the design of clinical trials and the peculiar biological features of MPM were most probably responsible for delaying the discovery of effective therapeutic agents. Most of the previous trials attempted to readapt drugs that succeeded in other cancer types to MPM. However, they were either too small or not stratified for predictive biomarkers. Results from phase II studies were often not replicated in larger, randomized, phase III trials, pointing out that well controlled trials with appropriate size and duration are crucial to confirm the efficacy of a new agent (121).

In the last few years, mesothelioma genetics, epigenetics, and the tumor microenvironment (especially immune-biology) have been studied more deeply and this knowledge has started to be properly applied to discover new therapies. In particular, expectations are now high that CIs and other immunotherapies will have a leading role in the future therapeutic armamentarium of MPM. Noteworthy, scientific evidence supporting the use of CIs in MPM are still incomplete, mainly based on non-randomized studies with surrogate end-points and they have not been always replicated in the real-life context. Because of the risk of cumulative toxicities and of the high cost of these drugs (especially of combinations), validated biomarkers are urgently needed to select MPM patients who may benefit from immunotherapies. Since the "one-size fits all" approach is not recommended for immunotherapy and MPM and the efficacy of CIs is still to be established in a larger population, there is still a need for new treatments in MPM and the implementation of other targeted agents is eagerly awaited.

Only a close collaboration between medical centers and industry may lead to the conduction of well-designed, biomarker-driven clinical trials. New trials should always include translational and quality of life components, in order to clarify the molecular basis of response or progression to treatments and to finally improve the degree of reliability of the possible benefit of new therapies for MPM.

AUTHOR CONTRIBUTIONS

LC and JA wrote the manuscript and generated the figure and table. RH and DS contributed to the revisions of the manuscript. All authors approved the manuscript for publication.

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REVIEW
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The Biology of Malignant Mesothelioma and the Relevance of Preclinical Models

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Malignant mesothelioma (MM), especially its more frequent form, malignant pleural mesothelioma (MPM), is a devastating thoracic cancer with limited therapeutic options. Recently, clinical trials that used immunotherapy strategies have yielded promising results, but the benefits are restricted to a limited number of patients. To develop new therapeutic strategies and define predictors of treatment response to existing therapy, better knowledge of the cellular and molecular mechanisms of MM tumors and sound preclinical models are needed. This review aims to provide an overview of our present knowledge and issues on both subjects. MM shows a complex pattern of molecular changes, including genetic, chromosomic, and epigenetic alterations. MM is also a heterogeneous cancer. The recently described molecular classifications for MPM could better consider inter-tumor heterogeneity, while histo-molecular gradients are an interesting way to consider both intra- and inter-tumor heterogeneities. Classical preclinical models are based on use of MM cell lines in culture or implanted in rodents, i.e., xenografts in immunosuppressed mice or isografts in syngeneic rodents to assess the anti-tumor immune response. Recent developments are tumoroids, patient-derived xenografts (PDX), xenografts in humanized mice, and genetically modified mice (GEM) that carry mutations identified in human MM tumor cells. Multicellular tumor spheroids are an interesting *in vitro* model to reduce animal experimentation; they are more accessible than tumoroids. They could be relevant, especially if they are co-cultured with stromal and immune cells to partially reproduce the human microenvironment. Even if preclinical models have allowed for major advances, they show several limitations: (i) the anatomical and biological tumor microenvironments are incompletely reproduced; (ii) the intra-tumor heterogeneity and immunological contexts are not fully reconstructed; and (iii) the inter-tumor heterogeneity is insufficiently considered. Given that these limitations vary according to the models, preclinical models must be carefully selected depending on the objectives of the experiments. New approaches, such as organ-on-a-chip technologies or *in silico* biological systems, should be explored in MM research. More pertinent cell models, based on our knowledge on mesothelial carcinogenesis and considering MM heterogeneity, need to be developed. These endeavors are mandatory to implement efficient precision medicine for MM.

Keywords: thoracic cancer, mesothelioma, molecular characteristics, tumor heterogeneity, preclinical models, cell models, animal models

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INTRODUCTION

The therapeutic options for malignant mesothelioma (MM) are limited, especially for the most common form of mesothelioma, malignant pleural mesothelioma (MPM). Current MPM chemotherapy is based on intravenous injections of pemetrexed (PMTX) and cisplatin or carboplatin. Recently, this basic treatment has been improved by the addition of vascular endothelial growth factor (VEGF) antibodies (bevacizumab), where the overall survival of patients receiving PMTX, cisplatin, and bevacizumab was significantly enhanced (MAPS study) (1). Furthermore, immunotherapy-based strategies are currently becoming attractive therapeutic options, and several clinical trials have recently been performed. A phase II study using monoclonal antibodies against cytotoxic T-lymphocyte antigen 4 (CTLA4; tremelimumab) in patients showing progression of the disease after first-line treatment yielded encouraging results, but it was performed in a small number of patients (2). Another phase II study (DETERMINE) investigated the effect of tremelimumab in patients whose disease had progressed after one or two systemic treatments. There were no benefits, but the safety profile was acceptable (3). A more recent phase 2 study (IFCT-1501 MAPS2) reported the use of immune control checkpoint inhibitors, programmed cell death protein 1 (PD-1; nivolumab) and cytotoxic T-lymphocyte-associated protein 4 (CTLA4; ipilimumab), alone or in combination. The results showed objective anti-tumor responses and a significant increase in survival without progression and global survival (4). Clinical trials for cell-based immunotherapy using dendritic cells or chimeric antigen receptor T (CAR-T) cells have also yielded promising results (5–10).

In the future, new clinical trials will be developed that utilize novel anti-cancer compounds or immunological modulators in association with chemotherapies or in combination with immunological approaches. The efficiency of current treatments are dependent on the integrity of metabolic pathways and DNA repair mechanisms that account for resistance mechanisms. Overall, therapy improvements require better knowledge of the state of the cell regulatory pathways. In addition, immunotherapies need sound knowledge about the immunological status of the tumor. To date, molecular data are not ordinarily used to assist therapeutic decisions, and thus there is an urgent need for their use in translational medicine. To reach these goals, two different fields must be investigated: (i) the cellular and molecular status of MM tumors, regarding mutations, alterations in regulatory pathways, and the microenvironment landscape, and (ii) the methodology of preclinical assays to soundly test specific anti-tumor agents. The aim of this review is to provide an update on our present knowledge and issues on these subjects and to provide perspectives for advancements in MM treatment.

Abbreviations: 2D, two dimensional; 3D, three dimensional; CDX, cell-derived xenograft; CGH, comparative genomic hybridization; GEM, genetically modified mice; hom, homozygous; htz, heterozygous; IPl, intra-pleural; IPe, intra-peritoneal; luc, firefly luciferase; MCTS, multicellular tumor spheroids; MM, malignant mesothelioma; MPM, malignant pleural mesothelioma; MPeM, malignant peritoneal mesothelioma; MRI, magnetic resonance imaging; NGS, next generation sequencing; NSG, NOD-scid IL2Rγnull; PDX, patient-derived xenografts; PMTX, pemetrexed; SC, subcutaneous; SNP, single nucleotide polymorphism; SPECT, single photon emission computed tomography; TSG, tumor suppressor genes.

THE BIOLOGY OF MALIGNANT MESOTHELIOMA

Malignant mesothelioma are heterogeneous tumors that show a complex pattern of molecular changes, including genetic, chromosomic, and epigenetic alterations, all of which should be considered to model this pathology. Of all the MM types defined by tumor location, MPM has the best described molecular alterations and heterogeneity, and thus we will focus on it. Notably, recent integrative multi-omics analysis as well as next generation sequencing (NGS) studies on malignant peritoneal mesothelioma (MPeM) showed similarities to MPM in terms of molecular alterations, even though some alterations, such as *ALK* rearrangement, are only found in MPeM (11–13).

Molecular Alterations

Recent NGS studies identified a low mutation burden in MPM compared to other adult solid tumors (14). However, this mutation burden could be underestimated by classical NGS analyses, which focus on the detection of changes at the nucleotide level. Early karyotyping analyses and molecular cytogenetic techniques, such as comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) arrays, showed that MPM is characterized by numerous chromosomal abnormalities, including abundant numeric and structural chromosome changes and recurrent alterations in specific chromosome regions (15). More recently, a combination of high-density array-CGH with targeted NGS demonstrated the presence of chromothripsis in the 3p21 region, which includes the *BAP1* gene (16). Chromothripsis and also chromoplexy were confirmed on several other chromosome regions in MPM using mate-pair sequencing (17). These numerous inter- or intra-chromosomal rearrangements may result in the disruption of tumor suppressor genes (TSG) as well as the amplification of oncogenes or fusion genes that can drive carcinogenesis.

The mutated genes in MPM are essentially TSG that are inactivated by several mechanisms, including single nucleotide variants, copy number losses, gene fusions, and splicing alterations (14, 18). The only recurrent oncogenic mutation was identified in the promoter of *TERT*, which encodes telomerase, the essential enzyme that maintains the length of the telomeres (19). The most frequently altered TSG are *CDKN2A*, *BAP1*, and *NF2*, and to a lesser extent *TP53*, *SETD2*, and *LATS2*. All of the other mutated genes show <3% somatic mutation (14, 18). Germline mutations that predispose to MPM were first identified in *BAP1*, but two recent studies also highlighted germline mutations in several other genes that are less common than in *BAP1*. They are mainly involved in cell-cycle, chromatin regulation and DNA repair (20–24). Up to 7% of MPM patients may have germline mutations, but experimental validations

are needed to confirm that some of these genes are MPM susceptibility genes (like *BAP1*).

The epigenomic landscape of MPM has also been investigated, albeit to a lesser extent. A microarray-based methylome analysis demonstrated that MPM has specific patterns of gene methylation compared to normal pleura or other tumors (25, 26). The contribution of DNA methylation to mesothelial carcinogenesis has been clearly established, notably by the downregulation of TSG expression (27). The mechanisms for epigenetic regulation in MPM were principally studied in the context of *BAP1* inactivation; they highlighted the role of polycomb repressive complex 2 (PRC2) and histone methyltransferase (28). Other studies also emphasized the involvement of non-coding RNA such as micro-RNA (miRNA) or long non-coding RNA (lncRNA), both of which are deregulated in MPM, in carcinogenesis (29–31).

Altogether, these molecular alterations lead to changes in gene expression and deregulation of several biomolecular pathways, including signaling pathways such as Hippo or the PI3K/AKT/mTOR pathways, the cell cycle and apoptosis, among others (32). The implication for therapy from all these molecular changes has been recently reviewed (33).

Mesothelioma Heterogeneity

Like most adult solid tumors, MM is a heterogeneous cancer with high variability among patients. Hence, the development of experimental models must consider this heterogeneity. Histology defines three major types of MM: epithelioid, the most frequent histological subtype; sarcomatoid, with the worst prognosis; and biphasic, which is a mixture of the two previous morphologies. Histological subtypes within these three types have been defined (34). The histological classification only partially captures the tumor heterogeneity observed at both the molecular and clinical levels (35). Large-scale omics and NGS studies have demonstrated MPM heterogeneity at the molecular level that goes beyond the histological classification (14, 18, 36). The first MPM molecular classification, related to histological types and survival, proposed two tumor subtypes by clustering transcriptomic data (36). A new subtype with a poor prognosis and characterized by a double mutation in the TSG *NF2* and *LATS2*, both of which are involved in the Hippo signaling pathway, was identified by coupling genetic and transcriptomic analysis (37). Other studies have proposed classifications into four subtypes that are also related to prognosis and partially to genetic alterations (14, 18, 38). Interestingly, a meta-analysis that compared the subtypes obtained by clustering from several transcriptomic data sets showed that only the most extreme subtypes, which represent the "pure" epithelioid and sarcomatoid phenotypes, are found in all datasets. These findings suggest that intermediate subtypes might only reflect divisions of a continuum (38, 39). Based on these results, histo-molecular gradients obtained by a signal deconvolution method on transcriptomic data were proposed to consider MPM inter-tumor heterogeneity as well as intra-tumor heterogeneity. These histo-molecular gradients determine the variable proportion of epithelioid and sarcomatoid tumor cell contingents in tumor samples. They also have a strong prognostic value and may be of interest for guiding therapeutic strategies (38, 39). Another recent publication further sustained that MPM heterogeneity is better described by a continuum (40).

Intra-tumor heterogeneity is still partially described in MPM, in part due to the use of omics approaches only in bulk tumor samples. MPM is likely a polyclonal tumor that comprises multiple subclones with variable cellular prevalence (41, 42). To better define the polyclonal tumor origin and understand the tumor evolution of mesothelioma, further studies are required in a larger number of tumor samples. Several studies also highlighted the presence of cancer stem cells in MPM (35). In MPM, heterogeneity is not limited to tumor cells; the tumor microenvironment is also distinct from one patient to another in terms of type and number of stromal and immune cells that infiltrate the tumors (43). Immune signatures are linked to the patients' outcome (44). Spatial heterogeneity of the somatic mutations of cancer cells, as well as the immune microenvironment, was highlighted by studying tumor samples at different anatomic sites (45). In this complex context, the use of the emergent "single cell" approaches will be helpful in providing an accurate characterization of tumor and stromal cell heterogeneity and should contribute to a breakthrough in knowledge about intra-tumor heterogeneity. Besides the inter- and intra-tumor heterogeneity of tumor cells, the evolutionary features of tumors need to be considered to establish a classification that is clinically relevant (46).

PRECLINICAL MODELS

In this section, we will focus on preclinical models that are useful for chemotherapy, targeted therapy, or immunotherapy rather than for surgery or radiotherapy, even though those therapies have a place in the treatment of patients. The efficiency of anti-cancer compounds to treat MM patients has been tested using large variety of so-called preclinical MM models. These systems are based on use of human or mammalian MM samples, i.e., xenografts in immunosuppressed mice or isografts in syngeneic rodents. Multiple combinations have been developed based on the nature of the malignant sample (cells or tumor tissue), the recipient (rats or mice), the anti-cancer agent (anti-cancer drug, lytic virus, therapeutic cells sur as dendritic or CAR-T cells, etc.), the agent vector (if any), the method to implant tumor cells, and the analytical method. These models do not exactly reproduce human MM, but they are surrogates for a proof of concept. Preclinical model options are synthetized in **Figures 1**, **2**, and the main points are described below:

(i) Samples and recipients (**Figure 1**): Several MM samples are used for preclinical studies. Tumor fragments, pleural liquid, and ascites can be collected from patients. Commercial MM cell lines are available from different companies, but primary MM cell lines are a better model, as extensively discussed in the *in vitro* models section (see below). Cell models in culture mostly comprise two dimensional (2D) MM cells or three dimensional (3D) multicellular tumor spheroids (MCTS). These cell models are generally monoculture, but new developments include the introduction of stromal and immune cells to better

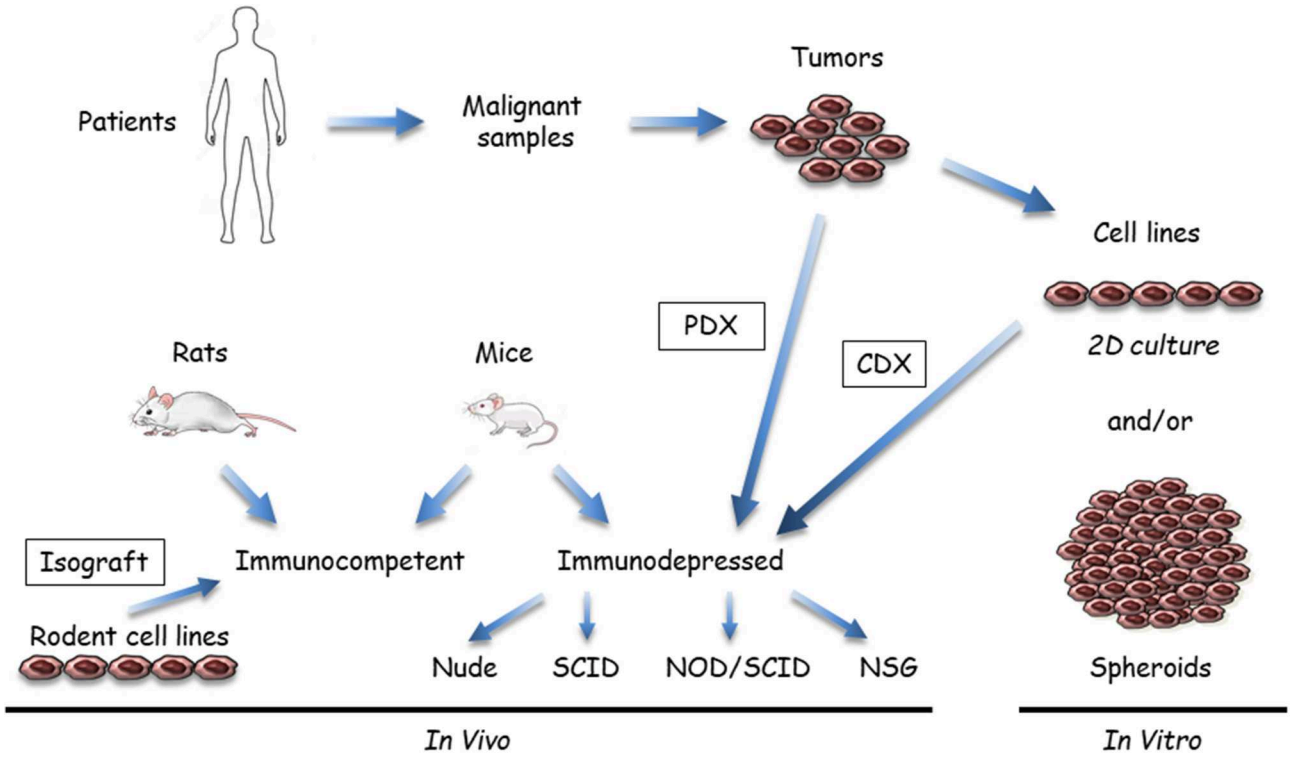

FIGURE 1 | Available malignant mesothelioma (MM) preclinical models that use tumor samples or cell lines. Malignant samples are tumor fragments or more often MM cells obtained after tissue dissociation; these samples are used *in vitro* or transplanted/inoculated in immunosuppressed or immuno-compatible rodents, almost exclusively mice. *In vitro*, two-dimensional (2D) cultures or three dimensional (3D) spheroids can be grown from tumor tissue samples. *In vivo*, MM tumor cells in culture are inoculated either subcutaneously or orthotopically (intracavitary, in the pleura or peritoneum) to generate cell-derived xenograft (CDX) or isograft. Tumor fragments can be also engrafted into immunosuppressed mice (patient-derived xenograft [PDX]). Immunocompetent models mainly comprise syngeneic rodent models. Human immunocompetent models can be obtained using NOD-*scid IL2Rγ*^{null} (NSG) mice.

recapitulate the tumor microenvironment. *In vivo* models are based on the injection of MM cells in subcutaneous (SC) or orthotopic (intrapleural [IPl] or intraperitoneal [IPe]) sites in relevant rodents, mainly mice. Fresh MM tissue samples can be also grown as tumoroids (tumor-derived organoids) in culture or xenografted in immunosuppressed mice as patient-derived xenografts (PDX). Regarding the heterogeneity of human MM, it is important to work with well-characterized MM, particularly when drugs have been designed to target a single protein or a specific pathway. With our developing knowledge of the MM biology, it appears that multiple samples would have to be used. Furthermore, MM classification according to data arising from multi-omic studies (12, 14, 18, 36, 38) might help to define key alterations representative of molecular subtypes of MM and limit the studies on representative samples.

(ii) Anti-cancer compounds (**Figure 2**): These compounds are intended for chemotherapy, target therapy, immunotherapy, gene therapy, or oncovirotherapy because MM is a compartmentalized tumor with accessibility for *in vivo* local delivery (47, 48). They are used alone or in combination with other compounds, and as a single molecule or vectorized. Preclinical studies on the chemotherapeutic agent PMTX illustrated this diversity. The effects of PMTX have been investigated in association with several anti-tumor agents (anti-tubulin, gemcitabine, cisplatin, anti-thymidylate synthase, RNA interference [RNAi] embedded in liposomes, miRNA expressed in adenovirus vector, etc.) to determine a potential synergistic effect (49–55). Liposomal PMTX formulations have been tested in an orthotopic mouse model (56). Due to the diversity of the assays, it is difficult to compare their predictability.

(iii) Analytical methods (**Figure 2**): The endpoints for *in vitro* assays comprise the determination of cell proliferation, cell death, motility and invasive properties, and spheroid state (morphology and volume). *In vivo* tumor analyses involve macroscopic and microscopic observations and evaluation of immune infiltration and angiogenesis. The key point is to monitor tumor evolution, especially for orthotropic tumor grafts. Different analytical methods have been developed for *in situ* tumor visualization. Firefly luciferase (*luc*)-engineered cells can be detected by a non-invasive bioluminescence imaging method, as in rats injected with *luc*-MM cells in the pleural cavity (57). However, data have shown that magnetic resonance imaging (MRI) is a more reliable method for MPM tumor burden measurement compared to bioluminescence (57). Computed tomography scanning may be also of interest, as shown with a lung cancer cell line in mice (58). Tumor lesions and the localization of

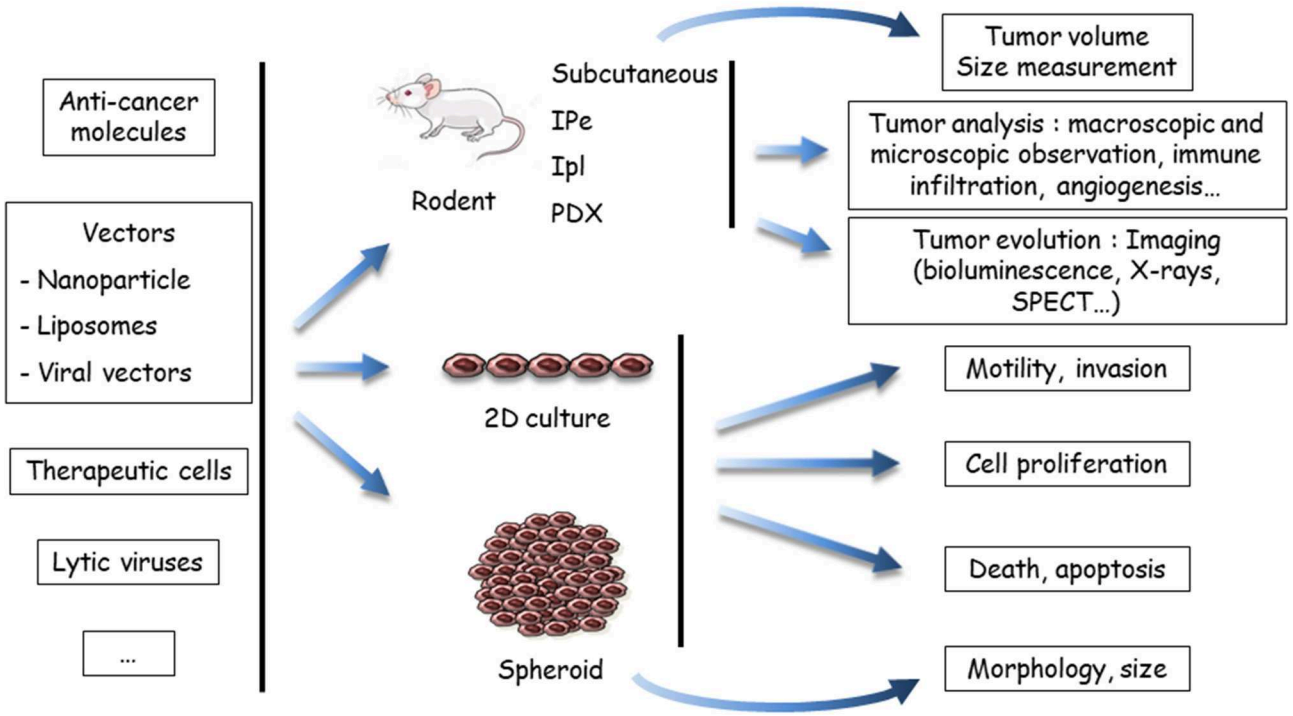

FIGURE 2 | Evaluation of treatments using preclinical models of malignant mesothelioma (MM). Anti-cancer agents are delivered to the MM models, either in a native, or vectorized form (in nanoparticles, liposomes, or viruses). MM cells may be labeled or engineered to allow one to determine tumor growth by imaging methods, mostly bioluminescence. To determine the host response, the analytical method depends on the MM model. *In vivo* endpoints comprise measuring the tumor volume, quantifying and identifying tumor cells in the pleural effusion or ascites (if produced), or measuring others parameters (angiogenesis, immune infiltration, etc.). *In vitro*, the endpoints are cell viability, type of death, proliferation, motility, invasion, morphology, and volume (spheroids). Both cell and mouse models have been applied to test the efficiency of drugs in mono- or multimodality chemotherapies, immunotherapies and target therapies, oncovirotherapies, or cell and gene therapies. SPECT, single photon emission computed tomography; IPe, intra-pleural; Ipl: intra-peritoneal; PDX, patient-derived xenograft.

epidermal growth factor receptor (HER) were visualized with single photon emission computed tomography (SPECT) and MRI in an orthotopic MM model with radiolabeled specific antibodies (59). Bioluminescence of *luc*-expressed MM remains the most common strategy to monitor tumor development in orthotopic models. These *in vivo* imaging methods require specific equipment and facilities and, for bioluminescence detection, the genetic modification of tumor cells with the *luc* gene. The introduction of an exogenous gene might have an impact on cell mechanisms and immune response.

In the following subsections, we detail the present and ongoing models, with a focus on their interest, limitations, and impacts to assess emerging therapies. The advantages and limitations of the mesothelioma preclinical models are presented in **Table 1**.

In vitro Models

MM cell lines have been widely used to study MM pathogenesis and evaluate the activity of numerous anti-cancer agents. The first MM cell lines were established in 1982 from the abdominal fluid of a patient (60). In 1987, a MM cell line was established from a surgical sample of malignant pleura, namely H-Meso-1 (61). Since that study, numerous cell lines have been established from samples of patients by different groups to constitute local biocollection. Some of these collections have been extensively characterized (19, 36, 37, 62–66), as well as 21 cell lines in the Genomics of Drug Sensitivity in Cancer (GDSC) database (https://www.cancerrxgene.org/celllines). MPM cell lines present common characteristics with regard to tumors and might lead to the identification of new biomarkers (62, 67, 68). One study discussed the limits of these cell models. The authors found strong molecular differences between primary and commercial cell lines (67), mainly due to a high number of divisions after their establishment, and thus an increased risk of new karyotypic changes. These models remain interesting for screening and preliminary investigations. Primary tumor cells represent an intriguing alternative because they share similar molecular characteristic with the primary tumor, even though they show a reduction of subclonal diversity (15, 42, 67). However, the necessity to perform studies before 6–10 passages limits the number of experiments. The most appropriate strategy would probably be to conduct large screening studies on cancer cell lines and then confirm the findings with primary cancer cells. The results obtained with cell lines should be confirmed on samples from patients (if applicable).

TABLE 1 | Advantages and limitations of the different models of mesothelioma.

Model	Advantages	Limits
Monolayer cells	Easy to obtain Suitable for high-throughput screening	Clonal selection in culture Response to therapies is poorly representative No microenvironment
Multicellular tumor spheroids (MCTS)	Easy to obtain Three-dimensional structure constraints Suitable for high-throughput screening Common features with tumors *in situ*	Not heterogeneous Partial and artificial microenvironment
Cell-derived xenograft (CDX)	Source of tumor cells easy to obtain (cell lines in culture) Useful for evaluation of targeted strategies	Not heterogeneous No microenvironment Immunodeficient context Response to therapies is poorly representative
Patient-derived xenograft (PDX)	Tumors with characteristics of the of patients (heterogeneity, microenvironment) Representative response to therapies	Availability of the tumor Time to obtain the tumor Not suitable for immunotherapy evaluation
Humanized model (NSG)	Human immune system Evaluation of immunotherapy possible	Time to obtain tumor Cost Graft vs. host response

In vivo Models

The *in vivo* models that use wild type rodents and genetically modified mice (GEM) are summarized in **Figure 3**. GEM have been generated to obtain "spontaneous" MM, without exposure to asbestos fibers, by heterozygous (htz) or homozygous (hom) conditional mutation of *Ink4a* and/or *Nf2* and/or *Trp53,* or by IPl/IPe injection of Ad*Cre* to mimic the human condition (69). In this system, the rate of MPM is dependent on the type of inactivated genes; high rates occur with at least two hom genes, including *Trp53*. Survival generally exceeds 30–50 weeks, although shorter survivals occur in a few situations with hom/hom combinations (70). Similar results were recently reported by inactivating *Ink4a*, *Nf2,* and *Bap1* (71). The generated MM express a similar morphology to human MM, with a proportion of each histological type depending on the modified genes.

MM have also been generated in several types of GEM exposed to asbestos fibers IPe injections. Experimental cancers induced in animals by the responsible carcinogen better reflect the natural history of these cancers. They are particularly relevant for the coupled asbestos-MM condition, given that the large majority of human MM cases are linked to asbestos exposure. With regard to conditional mutant mice, it takes several months to more than 1 year before the development of a MM. While the morphological features are reproduced, the sarcomatoid MM subtype most frequently forms, contrary to what is observed in humans (70).

These sophisticated models have been mainly used for mechanistic purposes, with a focus on the molecular mechanism of MM formation or mechanism of action of asbestos fibers. According to our knowledge, GEM with mutated genes that are relevant to human MM have not been used to test the effects of drugs. However, models of colorectal, non-small-cell lung, and pancreatic cancers have been used to predict therapeutic responses (72, 73). Although these models are physiologically different from humans, GEM mice form tumors that carry relevant gene changes, show histological similarities, and should allow one to perform tests in an immunocompetent environment. However, there are several biological and technical pitfalls. For instance, the tumor evolution can differ among mice, with the possible occurrence of metastases, other types of tumors may be generated, and the physiological differences between mice and human may bias the predictive value of the assays. Otherwise, the complexity of these models makes it difficult to produce homogeneous data from a rather small number of mice, to detect the tumor without autopsy, to follow its evolution, and to determine the right time of its development to establish the planed protocol.

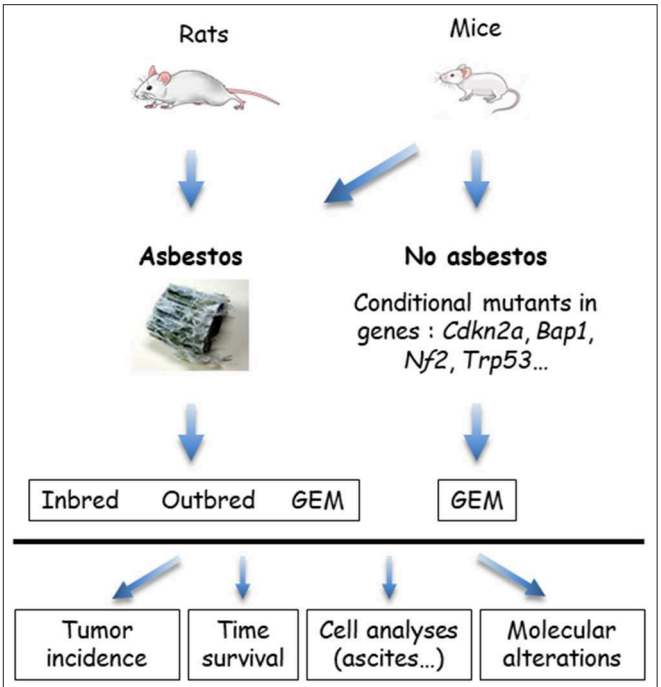

FIGURE 3 | *In vivo* models of malignant mesothelioma (MM) in asbestos-treated rodents and genetically modified mice (GEM). MM can be obtained from wild type (WT) rats or mice, exposed to asbestos, or GEM. Asbestos-induced MM models are generated by the injection of asbestos fibers intracavitary, in the pleura or peritoneum, mostly peritoneal in mice. Conditional mutant mice are obtained by engineering the major genes altered in MM. Asbestos-recipient animals and GEM may be investigated for tumor incidence, survival, quantitative and qualitative analyses of cells in ascites or pleural fluid, and molecular alterations in tumors.

Specific *In vivo* Models for Immune Therapies

Recent successes were obtained with the use of immune checkpoint inhibitors in clinical trials (2, 4, 74–76). However, the response rate remains limited and, therefore, the objectives are now to extend the benefit of these approaches to a large number of patients. Preclinical studies performed in appropriate *in vivo* models are mandatory to obtain relevant results and achieve

TABLE 2 | The main immunocompetent rodent models of mesothelioma.

Rodent strain	Cell lines	References
C57Bl/6 mice	AK7	(77)
	AE17	(78)
Balb/C mice	AB1	(79)
	AB12	
CBA/J mice	AC29	(79)
Fischer F344 rats	IL45	(80)
	M5-T2, F5-T1, F4-T2, M5-T1	(81) (82)

this objective. The first criterion is the presence of a completely functional immune system, a factor that excludes xenograft models that use human tumor cells. Several immunocompetent models of MM have been developed in rodents using cell lines obtained after inoculation of asbestos fibers in the peritoneal cavity (**Table 2**).

C57Bl/6 mice models with murine MM cell lines have been extensively used to evaluate immunotherapeutic approaches. The most utilized cell lines are AE17 and AK7 (77, 78). These cells have been modified, including exogenous expression of Ova as a neo-antigen, to increase their immunogenicity and evaluate a strategy to improve the anti-tumor immune response (78). Models of MM on the Balb/C genetic background, using AB1 and AB12 cells lines, are also available. CBA/J mice injected with AC29 cells can also be used as an immunocompetent model of MM; however, they have been exploited less than the other previously cited models (79). The main injection route to induce MM is IPe. Immunocompetent IPl models of MM have also been developed, but they are not frequently used due to the procedure required to access to pleural space. In both cases, tumor development is monitored by the previously mentioned imaging methods. SC injections are also used to overcome practical concerns, such as measurement of the tumor size and intra-tumor injection of therapeutics, but SC location is far from the pathophysiological context.

A MM model in Fischer F344 rats following IPl injection of IL45 cell line has also been described (80). With regard to rodent models, IL45 cells expressing *luc* was used to improve the monitoring of tumor development (83). Recently, models of MPeM in Fischer F344 rats have been developed (81). This effort generated several MM cell lines with distinct aggressiveness. Depending on the cell lines used, the immune infiltrate was different (with or without lymphocyte and/or macrophage infiltration of the tumors) (82). Therefore, the efficacy of immunotherapy approaches could be evaluated using this model in the appropriate immune context.

Xenograft PDX and Humanized Models

The previously mentioned rodent models allow one to evaluate therapeutic strategies in a living organism with several constraints: elimination, diffusion in the tissue, bio-distribution, and toxicity. These models were particularly used for the evaluation of target therapies using inhibitors of histone deacetylases or signal pathways such as Hippo or focal adhesion kinase (FAK) (84–86), anti-mesothelin or anti-podoplanin antibodies (87–92), or CAR-T cells (5, 93, 94). However, they showed major limitations: (i) differences compared to humans in the immune system and metabolism of chemotherapeutic agents for syngeneic rodent models; and (ii) the use of a human cell line to induce tumors, which does not reflect human intra-tumor heterogeneity, in the context of immunodeficient mice (xenografts). In order to improve the relevance of rodent models, PDX models were developed and they implies implanting a tumor fragment from a patient into an immunodeficient mouse. For MM, the implantation was only heterotopic. Mouse strains with different levels of immunodeficiency can be used depending on the objective of the experiment (Nude, SCID, NOD-SCID, or NOD-*scid IL2Rγ*^{null} [NSG]) (95). The first description of PDX models of MM was in 1980 (96). Tumors from three patients were transplanted into nude mice but only two tumors grew. Recently, SC implantation of tumors from 50 patients with MPM was evaluated in nude mice (97). This methodology maintains the heterogeneity of the tumor and its microenvironment at least during the first generation. However, the limitations include (i) a high proportion (60%) of MPM do not grow as PDX; (ii) the tumor microenvironment is replaced by murine cells over generations; and (iii) the immune context is modified, which is not suitable for evaluation of immunotherapeutic strategies (95, 97). PDX also requires access to tumor samples, which is not easy in the case of a relatively rare cancer as MM.

The use of a humanized mouse model of MM might be a good alternative to study the anti-tumor immune response. In these models, the mouse immune system is replaced by a human immune system. NSG mice are used for this research; they are deficient in the interleukin 2 receptor gamma subunit (IL-2Rγ) that is involved in differentiation and function of many hematopoietic cells (98). This feature confers a great advantage to study immunotherapy strategies in an environment that closely resembles human patients. However, these models present some limits, including the cost, the time to obtain NSG mice reconstituted with a human immune system, the risk of an incomplete differentiation of haematopoietic stem cells, and the graft vs. host reaction, which could limit the duration of the experiments.

Spheroid Models

In order to overcome some defaults of the existing *in vivo* models, 3D tumor spheroids, positioned between 2D cell culture and animal models, have been developed (99). MCTS involves culturing tumor cells in non-adherent conditions to obtain well-rounded cellular structures after 48–72 h. This culture mode has been applied to MM cell lines. The 3D organization of cells induces major changes in gene expression compared to 2D culture (100, 101). Indeed, some pathways involved in resistance to cell death are differentially regulated in monolayer and MCTS, and thus these models better mimic resistance to treatment compared with monolayer cells (64, 102–106). These models also reproduce the diffusion constraint of therapeutic molecules, such as antibodies or nanovectors (106–109). The 3D structures also share common features with tumors from patients (101). This aspect has been notably demonstrated in the field of autophagy (110–112). Indeed, resistance to treatment is associated with autophagy in MCTS and tumors *in situ*.

The main weakness of the current 3D models is the absence of cells from the microenvironment. An alternative to MCTS is the use of tumoroids, which include tumor cells and infiltrated cells (99, 113). However, these models require access to fresh surgical MM samples. Co-cultures serve as an alternative. MCTS of non-small-cell lung cancer, pancreatic, and breast cancer tumor cells supplemented with fibroblasts and/or macrophages have been described (114–116). The tumor-associated macrophages (TAM) obtained in these models present similar characteristics to those observed in tumors, namely increased resistance to treatment and an improved cytokine environment. These aspects are crucial for immunology studies. MCTS that include different cell types might constitute interesting tools for preliminary studies. They are achieved by combining stromal and immune cells issued from cell lines or isolated from different donors. However, although their relevance needs to be confirmed, MCTS reproduce a partial human microenvironment that is completely absent from cell-derived xenograft.

CONCLUSION

Multiple classical preclinical models of cancer have been applied, and new ones are under development, to test the potential effect of anti-cancer drugs on human MM. Each available model has benefits and limits (**Table 1**), and they must be selected depending on the objectives of the experiment. Overall, these models incompletely reproduce human MM, given that they do not consider the anatomical or biological tumor microenvironment or the intra-tumor heterogeneity of the tumors. The immunological context is not fully reconstructed, even with humanized mice. Three-dimensional spheroid cultures that have been developed as *in vitro* systems, and co-cultures with immune and stromal cells should be considered to improve the relevance of these models. Inter-tumor heterogeneity is also insufficiently studied because most models proceed with MM cell lines or tumors not always characterized at the molecular level, especially concerning the mutation burden and chromosomal abnormalities of the tumor cells. These models remain surrogates; however, they are of paramount importance in translational research and this encourage new developments to improve their predictability. Among recent developments are PDX models and the generation of GEM that carry mutations identified in human MM tumor cells. These approaches may be useful, but PDX and GEM models in general are complex and have limitations in the immune environment and animal cost. Their application in the context of MM heterogeneity will require the use of multiple cell lines according to their molecular profile. To achieve sound results with significant statistical value, including kinetics and dose-effect relationships, a large number of animals would also be needed, unless solid ancillary results are available. Besides economic issues, the 3R rules (Replacement, Reduction, and Refinemen) on the use of animals in scientific procedures are recommended. The identification of biomarkers to follow tumor evolution in response to anti-cancer drugs is of importance to limit the number of animals (117). Lower attrition rates for oncology drugs would be obtained with more predictive models (72, 104, 117, 118). Consequently, it is necessary to develop alternatives for replacement, robust and reproducible bases for reduction, and the use of advanced technologies for refinement (119).

Appropriately designed and analyzed preclinical assays are required (72), with the aim to identify new anti-cancer compounds for MM and novel biomarkers for sensitivity or resistance, which are essential to predict the tumor response. Although animal models are considered to be the most relevant, the development of sophisticated *in vitro* multicellular models should be encouraged. The continuing increase in the knowledge about mesothelial carcinogenesis will permit the use of more pertinent cell models that represent the MM tumor. New approaches not yet used in MM should be explored, including organ-on-a-chip technologies or *in silico* biological systems using computational modeling and machine learning (120, 121). Powerful technological tools should allow researchers to establish models with MM cells that grow in a more accurate tumor microenvironment, and possible *in situ* molecular analyses of tumor cells. The use of well-characterized tumor cells, classified in subgroups of molecular classifications or characterized by histo-molecular gradients, is particularly important regarding the molecular heterogeneity of human MM. This endeavor should allow researchers to obtain representative results of a given type of tumors.

The ongoing preclinical models should be improved with regard to precision, reproducibility, and predictivity, and the results should be supported by different approaches. Some standardization might be helpful. The use of existing consortia and/or the development of new consortia will allow the inclusion of more tumor samples in studies and increase the number of relevant cell models. These factors will enable researchers to adequately cover mesothelioma heterogeneity and be able to afford the high costs of new technologies. Some authors have recommended improving the reliability of preclinical cancer studies by using detailed information on the experimental methodology, different approaches, the publication of negative data, and better dialogue between physicians and scientists (122, 123). These factors are particularly important within the actual context of precision medicine, which implements complex methodologies and multidisciplinary investigations and has a high cost.

AUTHOR CONTRIBUTIONS

M-CJ and CB prepared the figures. CB, M-CJ, and DJ wrote the review.

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MINI REVIEW
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Cellular Immunotherapy and Locoregional Administration of CAR T-Cells in Malignant Pleural Mesothelioma

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Malignant pleural mesothelioma (MPM) is a treatment recalcitrant tumor with a poor overall survival (OS). Current approved treatment consists of first line chemotherapy that only modestly increases OS, illustrating the desperate need for other treatment options in MPM. Unfortunately, clinical studies that investigate the effectivity of checkpoint inhibitor (CI) treatment failed to improve clinical outcome over current applied therapies. In general, MPM is characterized as an immunological cold tumor with low T-cell infiltration, which could explain the disappointing results of clinical trials investigating CI treatment in MPM. Currently, many other therapeutic approaches, such as cellular therapies and cancer vaccines are investigated that could induce a tumor-specific immune response and increase of the number of tumor-infiltrating lymphocytes. In this review we will discuss these novel treatment approaches for MPM.

Keywords: mesothelioma, cancer vaccines, dendritic cell therapy, CAR-T cell therapy, immunotherapy

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INTRODUCTION

Malignant pleural mesothelioma (MPM) is a lethal cancer with limited treatment options (1–3). Current first-line treatment, consisting of platinum/antifolate combination therapy, leads to a median overall survival (OS) of 9–2 months (4). The addition of Bevacizumab to first-line treatment increased OS by 2.7 months and is now the accepted standard therapy in France (5, 6). Since then, no new treatments that could improve the outcome for MPM were reported. Immunotherapies, aiming at the activation of the immune system by blocking inhibitory checkpoint receptors, called checkpoint inhibitor (CI) treatment have drastically improved OS for non-small cell lung cancer and melanoma patients (7). So far, CI treatment has been promising for a small group of MPM patients in phase I/II trials, with response rates between 9 and 29% (8–17). However, unfortunately the DETERMINE phase IIb trial failed to show superiority of anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (Tremelimumab) over placebo in a second or third-line setting for MPM (18). Moreover, in the PROMISE-meso trial, blockade of programmed cell death protein 1 (PD1) failed to prolong progression free survival (PFS) or OS compared to second-line chemotherapy (gemcitabine/vinerolbine) treatment (19). Combination treatment of monoclonal antibodies (mAbs) targeting PD1 or PD1 ligand (PD-L1) with anti-CTLA4 mAb seems to be more effective than CI monotherapy in MPM (10, 20, 21).

The results of the ongoing Checkmate 753 phase III trial are awaited (NCT02899299), where Nivolumab (PD1 blockade) and Ipilimumab (CTLA-4 blockade) are combined as first line therapy in unresectable MPM and compared to first-line chemotherapy consisting of pemetrexed and cisplatin or carboplatin (22). As CI treatment, especially anti-PD(L)1 mAb, reinvigorates T-cells, the low number of tumor-infiltrating T-cells (TILs) in MPM might explain the relatively low response rates found in clinical trials investigating anti-PD1/PD-L1 treatment (23). Tumors with high numbers of TILs respond better to CIs (24). In MPM, dendritic cells (DCs) are reduced in both their numbers and their functionality, which could explain the low numbers of TILs (25). Induction of tumor-specific T-cells that infiltrate tumor and kill tumor cells upon antigen recognition by secretion of perforins, granzymes and death ligands, such as Fas and TRAIL could improve clinical outcomes (26, 27). Cancer vaccines and DC-therapy can induce activation and proliferation of tumor specific T-cells. Additionally, chimeric antigen receptor (CAR) T-cells, specific for a tumor antigen, can be used to target specific tumor antigens directly. Recent developments in therapies initiating a tumor directed immune response, such as cancer vaccines, DC-therapy and CAR T-cell therapy in a clinical setting in MPM will be discussed in this review (**Figure 1**).

CANCER VACCINES

Cancer vaccines can be made of tumor lysate, single or multiple peptides, viruses, or attenuated bacteria. The purpose of vaccinating cancer patients is to elicit a tumor-specific type 1-polarized T-cell response, leading to clinical benefit for the patient. Immunostimulatory adjuvants, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and toll-like receptor (TLR) ligands are often combined with cancer vaccines, to attract and activate antigen presenting cells (APC) that will take up the cancer vaccines (28). Certain adjuvants, such as Montanide, protect the peptides in the cancer vaccine and create a depot for slow antigen release that attracts lymphocytes and DCs, therefore called depot adjuvants (29). For MPM, Wilms Tumor 1 (WT-1) peptide-based vaccine, Galinpepimut-S and CRS-207 are the most thoroughly evaluated cancer vaccines and will be discussed in more detail.

WT-1 CANCER VACCINES

WT-1 is a protein expressed on almost all (97%) MPM cells with a variable distribution and intensity and serves as an immunohistochemical marker for MPM diagnosis, making WT-1 an appropriate target for immunotherapy (30). The cancer vaccine, Galinpepimut-S consist of four WT-1 peptides of different lengths that can be presented in both MHC class I and II molecules, permitting the activation of both $CD4^+$ and $CD8^+$ T-cells (31). Treatment with Galinpepimut-S was investigated in a randomized phase II study in MPM patients with positive (> 10%) WT-1 expression. Herein, Galinpepimut-S was administrated with adjuvants (GM-CSF and Montanide) and compared to placebo, in which only the adjuvants were administered. Unfortunately, the study was closed after inclusion of 41 patients due to futility of the placebo treatment and a non-significant increase in median OS (4, 5 months) and median PFS (2, 8 months) for Galinpepimut-S treated patients, as compared to the placebo arm (31). In July 2019, a clinical trial which investigates the combined treatment of Galinpepimut-S with nivolumab in patients with WT-1 expressing MPM (NCT04040231) has started.

CRS-207

CRS-207 is a live-attenuated listeria-encoding human mesothelin (MSLN) vaccine. APCs will phagocytose the Listeria bacteria in CRS-207, leading to release of MSLN, that is subsequently presented by APCs to T-cells in the lymph nodes, thereby inducing an MSLN-specific immune response. MSLN is expressed in 90% of epithelioid MPM patients, which comprises up to 80% of all MPM patients (32). MSLN is not expressed in most sarcomatoid MPMs and only minimally in biphasic MPM. MSLN has low expression on non-malignant cells, making it an attractive target for immunotherapy (32, 33). In a phase Ib trial, treatment-naïve MPM patients received 2 CRS-207 doses, followed by 6 cycles of pemetrexed/cisplatin and CRS-207 booster infusions (34). The disease control rate was 89%, with 1 complete response (CR) and 19 partial responses (PR) in 35 evaluable patients. Unfortunate, the median OS was 14.7 months, which is comparable to OS observed after standard chemotherapy treatment (34, 35). Additional trials were initiated with CRS-207 in combination with pembrolizumab (Keytruda), chemotherapy and GM-CSF transfected tumor cell vaccine (GVAX) (NCT 01675765, NCT03175172, NCT02243371), and results are awaited (36). Unfortunately, the Keytruda trial has been halted because of insufficient clinical activity (NCT03175172).

In conclusion, despite careful selection of adjuvants and antigenic targets of cancer vaccines applied in MPM, therapeutic success or induction of a clinically detectable cytolytic immune response has not yet been shown (37). Combining cancer vaccines specifically with agents that target the immunosuppressive tumor microenvironment (TME) might improve clinical outcome. Clinical trials investigating these combination therapies are currently investigated and results are awaited.

DC-THERAPY

DCs are low in numbers and are impaired in functionality in MPM patients (25). Moreover, the TME in MPM causes immunosuppression through secretion of immunosuppressive cytokines and expression of inhibitory molecules by tumor cells and immune cells again affecting DC mediated T-cell activation (38–41). To circumvent the immunosuppressive TME, DCs can be activated and loaded with selected tumor associated antigens

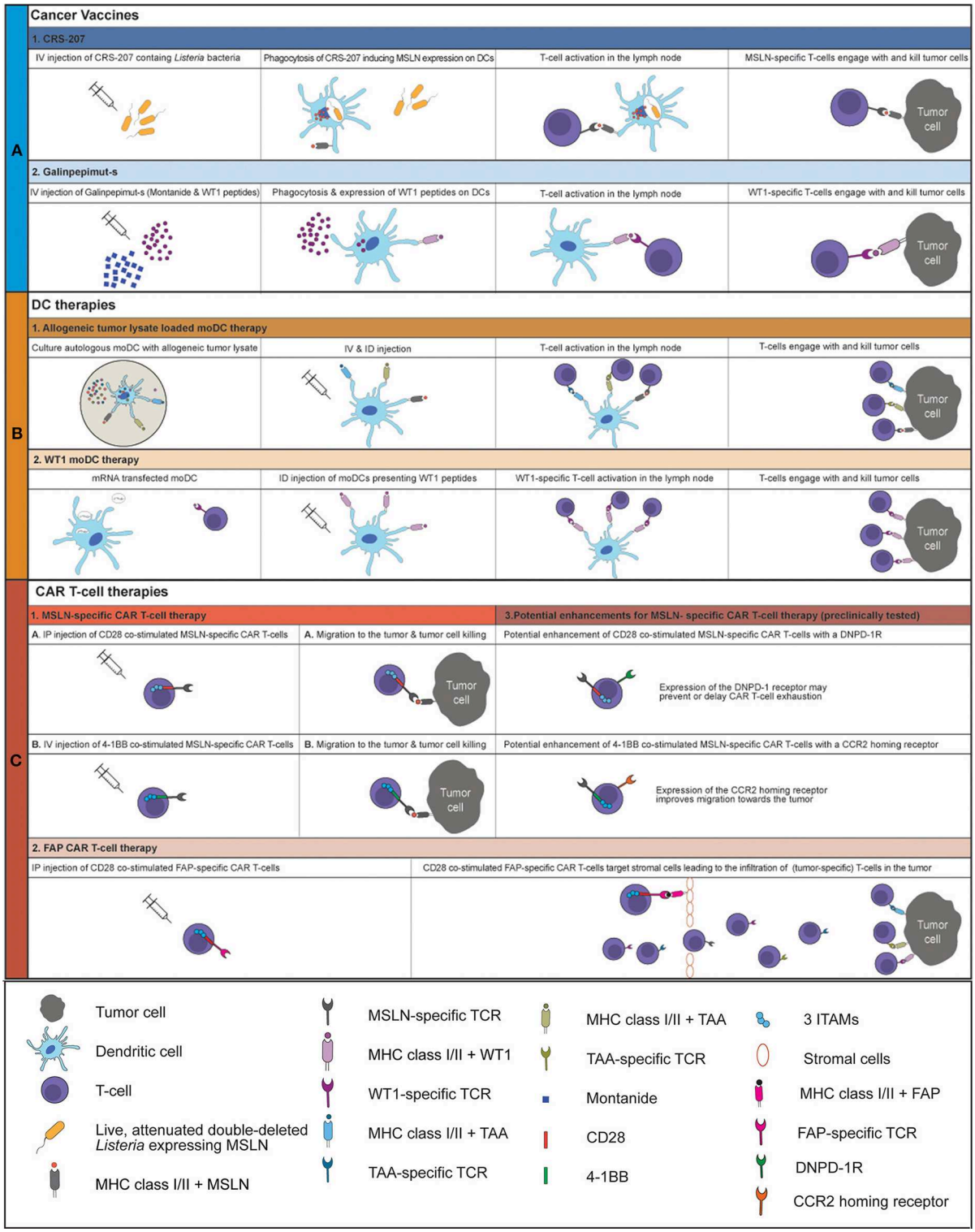

FIGURE 1 | Overview of current clinically tested cancer vaccines and cellular therapies for MPM An overview of the working mechanism of CRS-207 (A1), Galinpepimut-s (A2), allogeneic tumor lysate loaded moDC therapy (B1), WT1 moDC therapy (B2), MSLN-specific CD28 co-stimulated CAR T-cell therapy (C1A), MSLN-specific 4-1BB co-stimulated CAR T-cell therapy (C1B) and FAP CAR T-cell therapy (C2). The potential enhancements of MSLN-specific CAR T-cell therapy are displayed in C3. IV, intravenous; ID, intradermal; IP, intrapleural; MSLN, mesothelin; moDC, monocyte-derived dendritic cell; MHC, major histocompatibility complex; TCR, T-cell receptor; WT1, Wilms Tumor 1 protein; TAA, tumor-associated antigen; ITAM, Immunoreceptor tyrosine-based activation motif; DNPD-1R, dominant negative PD1 receptor; CCR2, CC chemokine receptor 2; FAP, fibroblast activation protein.

(TAAs) or whole tumor lysate *in vitro*. DC-therapy has been developed in three generations. In first generation DC-therapy, monocytes isolated from peripheral blood were cultured with GM-CSF and interleukin (IL) 4, leading to the differentiation into immature monocyte-derived DCs (moDC) (42). These immature moDCs were loaded with TAAs or tumor lysate and reinjected without any further activating stimulation into the patient. Second-generation DC-therapy, additionally stimulated the generated moDCs *in vitro* with a maturation/activation cocktail, consisting of cytokines and immune stimulants, such as poly IC, TLR ligands and prostaglandin E2 (40–42). Second generation DC-therapy is currently used in various clinical trials. Response rates for second-generation DC-therapy in melanoma, prostate cancer, malignant glioma and renal cell carcinoma vary from 8 to 15% with an increase in OS of ∼20% (42, 43). In contrary, an overall response rate of 7.1% was found in studies investigating first-generation DC-therapy in various malignancies, but mainly melanoma (44). Next-generation DC-therapy, aims at using naturally occurring DCs (nDC) that are purified directly from peripheral blood, *in vitro* loaded TAAs or tumor lysate and activated, and used for DC-therapy. The benefits of using nDCs are a shortened culture-time and lower manufacturing costs. It is also thought that DC-therapy containing nDCs will improve response rates, however this still has to be confirmed in clinical trials (42, 45, 46). DCs can be classically loaded with proteins during culture but TAAs can also be presented via RNA transfection methods or cancer cell-DC fusion (45, 47). The type of antigen source can vary from specific TAAs to complete tumor lysates. Analysis of 173 clinical trials in a wide variety of tumors showed that active immunotherapy using tumor-lysate (ORR 8.1%) was clinically more effective than peptide-based therapies (ORR 3.6%) (48), indicating that vaccinating with a broad range of tumor-associated proteins prohibits escape by the tumor and supports the hypothesis of immunoediting (**Box 1**).

BOX 1 | Immunoediting.

Immunoediting is a term that describes the balance between the prevention of tumor establishment through surveillance by the immune system and tumor cell growth when tumor cells escape from immunosurveillance (49–51).

Immunoediting by malignant cells contains three phases: elimination, equilibrium, and escape:

Elimination: cancer cells are eliminated by the innate and adaptive immune system.

Equilibrium: mutations and adaptations occur in certain cancer cells, leading to escape from the immune system of these cancer cells. During this phase, these mutated/adapted cancer cells will decrease antigen expression and become resistant to the immune system, whereas non-mutated cancer cells will be eliminated by the immune system, thereby increasing the frequency of mutated/adapted cancer cells. This process can take several years (52).

Escape: mutated/adapted cancer cells will proliferate and cause tumor outgrowth that can no longer be hampered or controlled by the immune system (53).

DC-THERAPY IN MPM

Two types of second-generation DC-therapy have been tested in clinical trials in MPM patients. Autologous moDCs transfected with messenger RNA (mRNA) encoding for WT1 and autologous moDCs loaded with autologous/allogeneic tumor lysate.

WT1-Targeted DC-Therapy

MoDCs transfected with WT1 encoding mRNA have resulted in promising clinical responses in MPM patients, but also in other malignancies. Prolonged stabilization of disease was noted in MPM patients, with OS (from start of chemotherapy) of 35.7 months (54, 55). This study was followed up by a phase I/II trial (MESODEC) in which treatment-naïve patients received WT1-targeting DC-therapy during chemotherapy, followed by pleurectomy/decortication (P/D) in the case of a resectable tumor (NCT02649829). The primary objective of this trial (recruiting since 2017 and enrolling 20 patients) is to assess the feasibility of WT1-targeting DC-therapy in combination with chemotherapy.

Tumor Lysate Loaded DC-Therapy

Two clinical trials that applied DC-therapy that consists of autologous moDCs loaded with autologous tumor lysate have been reported in MPM (56, 57). In the first Phase I clinical trial, ten MPM patients were treated with at least 3 biweekly DC vaccinations. Tumor lysate was prepared from single cell suspensions of tumor cell lines generated from tumor tissue and/or pleural effusions. Three patients had a PR, one had stable disease (SD) and six had progressive disease (PD). Median OS from time of diagnosis was 19 months (57). To improve the efficacy of DC-therapy in a sequential trial, ten MPM patients were treated with a combination of moDCs loaded with autologous tumor lysate and low-dose cyclophosphamide treatment, a chemotherapy that at low concentration specifically targets regulatory T-cells (Tregs) that favor anti-tumor immune responses (40, 58–60). At first radiological evaluation after treatment, one patient had a CR, four had SD and two had PD. Radiological response assessment was impossible in three patients as they had received additional P/D (56). Grade III/IV toxicities did not occur. Moreover, cyclophosphamide treatment indeed selectively depleted Tregs and the frequency of naïve Tregs prior to treatment was positively correlated to OS (61). Two patients were still alive 6 years after diagnosis.

Unfortunately, using autologous tumor material as a source for tumor lysate is not feasible for a large number of patients in a phase II trial, because of the varying quality and/or lack of tumor material. 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 (62). To create allogeneic tumor lysate for clinical trials, cell lines were generated of pleural fluid of 5 MPM patients with different histological subtypes and varying antigen expression. An allogeneic tumor lysate was derived from these cell lines that contained a broad spectrum of TAAs. Two out of nine MPM patients treated with allogeneic tumor lysate-loaded moDCs (MesoPher) in a phase I dose-escalation trial had a PR and two

patients are still alive 4 years after start of treatment. Grade III/IV toxicities were not reported (63). This phase I clinical study is followed up by an international, randomized, open-label, multicenter phase III trial (DENIM-trial), that will evaluate the efficacy of autologous moDCs loaded with allogeneic tumor lysate in MPM patients. Recruitment started in June 2018 and the first results are expected in 2021 (64). An overview of finished and ongoing clinical trials investigating DC-therapy in MPM is provided in **Table 1**.

Combination Treatment DC-Therapy

Multiple reviews have discussed strategies to combine DC-therapy with other therapeutic agents, such as low-dose chemotherapy to deplete specific immune cell subsets, radiotherapy to induce an abscopal effect or therapies that target specific immune cell subtypes or enzymes (40, 41). CI-treatment is thought to not only complement DC-therapy but work synergistically with DC-therapy. Mice treated with DC-therapy had more tumor-specific $CD8^+$ TILs than mice treated with placebo (65). Moreover, most of these TILs expressed high levels of PD1 on the cell surface, indicating their susceptibility for reinvigoration by CI treatment (65). The increase of TILs induced by DC-therapy may improve the current response rates of CI-treatment in MPM. Moreover, TILs induced by DC-therapy, that are hampered by inhibitory signaling may be reinvigorated. Based on this rationale, nine MPM patients who received autologous DC therapy in our center were sequentially treated with CIs. Three patients had a PR, five had SD and the median OS was 17.5 months from start of CI treatment (66). This data suggests a synergistic effect between DC-therapy and CIs in MPM that warrants further research.

CAR T-CELL THERAPY

The hypothesis for adoptive T-cell therapy is to introduce tumor-specific T-cells that directly target the tumor cells. The first step toward CAR T-cell therapy was the use of autologous TILs that were expanded *in vitro* and reinjected after one dose of cyclophosphamide and in combination with IL-2 to treat metastatic melanoma. Objective regression was observed in 11 out of 20 patients with a mean response duration of 5.6 months (2–13 months) (67). Unfortunately, the reproducibility and quality of these TILs could not be guaranteed due to interpatient differences of TILs (68). To avoid the need of TILs, T-cells can be genetically modified to express a T-cell receptor (TCR) that targets tumor-specific antigens. Although promising radiological responses were observed using these transgenic TCR T-cells, clinical use was still restricted to (Human Leukocyte Antigen A2) HLA-A2 patients (69). In an effort to enhance the efficacy of transgenic TCR T-cells and make target-antigen recognition independent of (Major Histocompatibility Complex) MHC, a CAR instead of a TCR was developed (70). A CAR classically consists of an extracellular part with an antigen-recognition domain, a transmembrane domain and an intracellular domain that contains three immune receptor tyrosine-based activation motifs (ITAMs). CAR constructs are transfected into (autologous) T-cells via mRNA or viral

TABLE 1 | Ongoing and completed trials for dendritic cell therapy in mesothelioma.

NCT nr.	Study type	Antigen	Type of DC	Additional therapy	Current status	Delivery method	Cancer type	*n*	Outcome	References
NCT00280982	Phase 1	Autologous tumor lysate	Autologous moDC	None	Completed	i.v./i.d.	MPM	10	Pos tAE: moderate fever, no grade III/IV tox 3PR, 1SD, 6PD	(57)
NCT01241682	Phase 1	Autologous tumor lysate	Autologous moDC	Cyclo-phosphamide	Completed	i.v./i.d.	MPM	10	Pos tAE: moderate fever, no grade III/IV tox 7/10 patients with an OS $\geq$ 24 months	(56)
NCT02395679	Phase 1	Allogeneic tumor lysate	Autologous moDC	None	Completed	i.v./i.d.	MPM	9	Pos tAE: moderate fever, no grade III/IV tox Median PFS 8.8 months, 2PR, 7SD	(62)
NCT01291420	Phase 1	WT-1	autologous moDC	None	Completed	i.d.	MPM	10	Pos tAE; mild skin reactions, no grade III/IV tox 18-month survival rate: 75%	(54)
NCT03610360	Phase 3	Allogeneic tumor lysate	AUTOLOGOUS MODC	NONE	Recruiting	i.v./i.d.	MPM	230	-	
NCT02649829	Phase 1/2	WT-1	Autologous DC	First-line chemotherapy optional P/D	Recruiting	i.d.	MPM	20	-	
NCT03546426	Phase 1b	Autologous tumor homogenate	Autologous moDC	Pembrolizu-mab, IL-2	Not yet recruiting	i.d.	PD-L1 negative MPM	18	-	

WT-1, Wilms Tumor 1; MPM, malignant pleural mesothelioma; n, expected number of patients; tAE, treatment related adverse event; i.d., intradermal; i.v., intravenous; pos, positive; BOR, best overall response; PD, progressive disease; tox, toxicity; SD, stable disease; CR, complete response; PR, partial response; OS, overall survival; PFS, progression-free survival; P/D, pleurectomy/decortication; moDC; monocyte-derived dendritic cell; DC, dendritic cell.

transduction (71). Historically, five generations of CAR T-cell therapy are distinguished. The most crucial adjustments that separate different generations concern the characteristics of the intracellular domain, which can contain, apart from the three ITAMs, one or two co-stimulatory molecules, such as CD28 or 4-1BB, and an inducible expression cassette for a protein, as IL-12 or a cytokine receptor, such as IL-2R (72, 73). Currently, two second generation CAR T-cell therapies targeting CD19 have been approved for the treatment of hematological malignancies (74). Although the clinical outcomes for CAR T-cell therapy in treatment-resistant hematological malignancies are impressive with complete response rates varying from 40 to 60%, these responses are not found for solid tumors. Also, CAR T-cell therapy induces severe treatment-related toxicities varying from 49 to 73% (75–77). Cytokine release syndrome (CRS) and neurological events are the most frequent severe treatment-related adverse events. CRS results from an immense release of cytokines from immunotherapy-targeted immune cells and cancer cells. The severity of CRS is dependent on the dosage of CAR T-cells, amount of tumor burden and level of IL-6. Blocking the IL-6 receptor with tocilizumab or neutralizing IL-6 through binding with a mAb siltuximab reduces CRS severity (74). The mechanism driving neurotoxicity, CAR T-cell Related Encephalopathy Syndrome (CRES), is still unknown. Locoregional admission of CAR T-cell therapy could reduce toxicity, however for hematological malignancies this is not an option.

Challenges for CAR T-Cell Therapy in Solid Tumors

CAR T-cell therapy encounters many challenges in solid tumors, such as migration of the CAR T-cells to the tumor, infiltration into the tumor, survival within the immunosuppressive TME as well as the lack of specific targetable tumor-specific antigens (78, 79). In B-cell driven malignancies, CD19 is a perfect target because it is expressed on all tumor cells (80, 81). Finding the perfect tumor-specific antigen to target in solid tumors is challenging due to heterogeneous expression of these tumor antigens. The lack of specific tumor antigens can also lead to severe "on target, off tumor" toxicity, caused by destruction of non-malignant cells expressing the antigen CAR T-cells are directed against (79). To migrate to and infiltrate the TME, CAR T-cells need to be equipped with appropriate tumor homing chemokine receptors and tumor endothelium degrading enzymes. Additionally, chemokines can be injected into the tumor that attract CAR T-cells. Another possibility to circumvent migration difficulties and even avoid development of systemic toxicities is locoregional administration of CAR T-cell therapy, but this is technically not achievable for all solid tumors. The stromal cells that are associated with nearly all epithelioid solid tumors form a physical barrier and severely hamper immune cell infiltration (79). A promising approach to attack the stromal component of the TME, is the development of CAR T-cells targeting (fibroblast activation protein) FAP which is expressed on various stromal cell types (82). Targeting the stromal cells by the FAP-specific CAR T-cells will allow and lead to infiltration of the tumor by TILs. Furthermore, as the target is expressed on non-malignant cells and not the malignant cells, this also reduces the risk of immunoediting and tumor escape. The immunosuppressive environment generated by the TME also affects the cytolytic activity of CAR T-cells and leads to CAR T-cell exhaustion. Secretion of inflammatory cytokines by CAR T-cells could counteract this immunosuppressive environment. Another possibility to directly circumvent exhaustion is to combine CAR T-cell therapy with CI treatment. Recently, CAR T-cells have been genetically modified with silenced PD-(L)1 coinhibitory signaling by the expression of a dominant negative PD1 receptor (DNPD-1R) that lacks an intracellular signaling domain. Although many challenges remain in the treatment of solid tumors with CAR T-cell therapy, current understanding and recent developments show great potential. Many of these new approaches are currently investigated in MPM.

Systemic and Locoregional CAR-T Cell Therapy in MPM

The choice of targetable tumor-antigen is crucial in the development of CAR-T cell therapy for MPM. Several tumor-antigen targets, such as MSLN, WT-1, FAP and the antigens of the ErbB family are evaluated for their applicability for CAR T-cell therapy in MPM. CAR T-cells targeting MSLN, FAP or WT-1 are already investigated in clinical trials, summarized in **Table 2**. Second generation CD28 FAP CAR T-cells have been evaluated in a phase I trial. Patients with metastatic MPM treated with these CAR T-cells developed no treatment related toxicities. Radiological responses were not reported, but 2 out of 3 patients were still alive with a median follow up of 18 months. Recently, Haas et al. showed that treatment with second generation, 4-1BB MSLN CAR T-cells as monotherapy or in combination with low-dose cyclophosphamide was well-tolerated in patients with MPM, ovarian carcinoma and pancreatic ductal carcinoma (84). One case of dose limiting toxicity (grade 4 sepsis) was reported without the use of cyclophosphamide. No radiological responses were seen and 11 out of 15 patients had SD as best overall response. Moreover, the persistence of CAR T-cells in the peripheral blood was <28 days after injection. Apart from the known hurdles for CAR T-cell therapy in solid tumors, a potential reason for the minimal persistence and clinical efficacy might be a consequence of the murine-derived CAR that was used. A new phase 1 trial has started evaluating a fully human CAR T-cell (**Table 1**, NCT03054298). CAR T-cells targeting the ErbB family antigens, T1E28z CAR T-cells showed promising results both *in vitro* and in mouse models, which needs to be validated in a clinical studies (86–88).

Currently methods to improve migration to the tumor site are heavily studied in mouse models. Herein, MSLN CAR T-cells that expressed a tumor homing chemokine receptor CCR2 showed improved tumor infiltration (89). Moreover, in an orthotopic mouse model of MPM, migration toward the tumor was circumvented by intra-pleural administration of second generation, CD28-costimulated MSLN CAR T-cells and led to a larger reduction of pleural an metastatic

TABLE 2 | Ongoing and completed trials for T-cell therapy in mesothelioma.

NCT nr.	Study type	Antigen	Stimulatory signal	Additional therapy	Current status	Delivery method	Cancer type	*n*	Outcome	References
NCT01722149	Phase 1	FAP	CD28	Neoadjuvant chemotherapy	Completed, no results posted	i.p.	MPM	3*	Pos: tAE: none	(82)
NCT01355965	Phase 1	MSLN	4-1BB	ns	Completed, no results posted	i.v.	MPM, pancreatic cancer	18*	Pos: (only reported outcomes of 2 patients): tAE: none	(83)
NCT01583686	Phase 1/2	MSLN	ns	Fludarabine, cyclophosphamide, aldesleukin	Terminated	i.v.	MSLN expressing tumors	15*	Terminated due to slow accrual (14/15 patients had a BOR of PD)	-
NCT02159716	Phase 1	MSLN	4-1BB	Cyclophosphamide	Completed, no results posted	i.v.	MPM, pancreatic cancer and ovarian cancer	15*	Pos: tAE: 1 grade IV tox 11SD, 4PD	(84)
NCT02580747	Phase 1	MSLN	ns	ns	Unknown	ns	MSLN expressing tumors	20	-	
NCT02930993	Phase 1	MSLN	ns	Cyclophosphamide	Unknown	i.v.	MSLN expressing tumors	20	-	
NCT03638206	Phase 1	MSLN	ns	Fludarabine, cyclophosphamide	Recruiting	ns	MPM	ns	-	
NCT03054298	Phase 1	MSLN	ns	cyclophosphamide	Recruiting	i.v./i.p.	MSLN expressing tumors	30	-	
NCT02408016	Phase 1	WT-1	ns	Cyclophosphamide, surgery IL-2	Active, not recruiting	i.v.	MPM/NSCLC	20	-	
NCT03615313	Phase 1/2	MSLN	PD-1 excreting CAR T cells	Fludarabine, cyclophosphamide	Recruiting	i.v.	MSLN expressing tumors	50	-	
NCT02414269	Phase 1/2	MSLN	CD28	Cyclophosphamide, pembrolizumab	Recruiting	i.p.	MPM	179 21***	Pos: tAE: no grade III/IV tox 2 CR, 5 PR, 4 SD, 10 PD	(85)
NCT03907852	Phase 1/2	MSLN	TRuC (novel T cell engenering platform)	Cyclophosphamide, pembrolizumab, fudarabine	Recruiting	ns	MSLN expressing tumors	70	-	
NCT03925893	Phase 2	-	TIL	Fludarabine, cyclophosphamide, aldesleukin	Recruiting	i.v.	Solid tumors	10	-	
NCT02414945	Phase 1/2	-	TIL	Fludarabine, cyclophosphmide, IL-2	Recruiting	i.v.	MPM	10	-	

*FAP, fibroblast activation protein; MSLN, mesothelin; WT-1, Wilms Tumor 1; MPM, malignant pleural mesothelioma; n, expected number of patients; *actual enrollment; ***21 patients were enrolled in the phase I trial which was reported at the AACR 2019; TRuC, T Cell Receptor Fusion Constructs; ns, not specified; TILs, tumor infiltrating lymphocytes; tAE, treatment related adverse event; i.p., intrapleural; i.v., intravenous; pos, positive; BOR, best overall response; PD, progressive disease; tox, toxicity; SD, stable disease; CR, complete response; PR, partial response.*

tumor load as compared to intravenous administration (90). Moreover, the intra-pleural treatment dose was 30-fold lower than the intravenous administered dose and elicited no grade III/IV toxicities.

In a clinical setting, no'on-target, off-tumor' effects were seen when 21 patients with malignant pleural disease were treated with CD28-costimulated MSLN CAR-T cells intrapleurally (85, 90, 91). In this study 19 out of 21 patients had MPM, of whom 13 were subsequently treated with pembrolizumab (anti-PD1). In total two patients had a CR, five had PR and four had SD as best overall response (85). Just as for DC therapy, Combining CAR T-cell therapy with anti-PD1 treatment showed promising clinical results. In a MPM mouse model, combined treatment of anti-PD1 mAb with CAR T-cell therapy improved treatment efficacy. CAR T-cell exhaustion can also be prevented by genetically modifying the CAR T-cells to express a dominant negative PD1 receptor (DNPD-1R) that lacks an intracellular signaling domain, avoiding the need for CI treatment and their related toxicities (92). A trial with CAR T-cells with a DNPD1R is expected to start in 2020 (93).

CONCLUSIONS

MPM remains a treatment-recalcitrant tumor with few registered treatment options. CI treatment failed to improve clinical outcome which might correlate with the low number of TILs in MPM. Cancer vaccines, DC-therapy and CAR T-cell therapy all induce a tumor directed immune response and increase the number of tumor-specific T-cells. Both cellular therapies and cancer vaccines face many challenges such as, migration of therapy-induced T-cells to the tumor, infiltration into the tumor, survival within the immunosuppressive TME and finding an optimal targeting approach. Improvement of cancer vaccines and cellular therapies and multimodal approaches that circumvent and overcome these difficulties should be investigated thoroughly. As both cancer vaccines and cellular therapies aim to induce infiltration of tumor-specific T cells into the TME, CI treatment serves as an ideal therapeutic option to block inhibitory signaling and reinvigorate TILs leading to enhancement of both treatments. In conclusion, additional research is needed to investigate and compare effectivity of cancer vaccines and cellular therapies for a cold tumor like MPM. Evaluating and influencing characteristics of the TME in MPM that withhold T-cell infiltration or impair cytotoxic T-cell function, is warranted to create a holistic treatment approach.

AUTHOR CONTRIBUTIONS

RB drafted and wrote the paper and contributed to the conception of the work. JA and HV contributed to the conception of the work and substantively revised the manuscript. All authors approved the submitted version.

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