Telomeres and telomerase in mesothelioma: Pathophysiology, biomarkers and emerging therapeutic strategies (Review)

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Abstract. Malignant mesothelioma (MM) is a rare but aggressive cancer linked to asbestos exposure and characterized by advanced-stage disease at presentation. Despite advances in treatment, prognosis remains abysmal, highlighting the imperative for the development of novel biomarkers and treatment approaches. Telomere biology plays a pivotal role in the tumorigenic process and has emerged as a key area in oncology research. Short telomeres have been associated with genomic instability, and substantially shorter telomere length (TL) has been identified in MM, showcasing the potential of TL in risk assessment, early detection, and disease progression monitoring. MM predominantly maintains TL through telomerase activity (TA), which in research has been identified in >90% of MM cases, underscoring the potential of TA as a biomarker in MM. Telomerase reverse transcriptase (TERT) polymorphisms may serve as valuable biomarkers, with research identifying associations between single nucleotide polymorphisms (SNPs) and the risk and prognosis of MM. Additionally, TERT promoter mutations have been associated with poor prognosis and advanced-stage disease, with the non-canonical functions of TERT hypothesized to contribute to the development of MM. TERT promoter mutations occur in ~12% of MM cases; C228T, C250T and A161C are the most common, while the distribution and frequency differ depending on histological subtype. Research reveals the promise of the various approaches therapeutically targeting telomerase, with favorable results in pre-clinical models and inconclusive findings in clinical trials. The present review examines the role of telomere biology in MM and its implications in diagnosis, prognosis, and therapy.

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1. Introduction

Malignant mesothelioma (MM) is a rare, but aggressive cancer which has been associated with asbestos exposure (1). MM is a malignancy of mesothelial surfaces, the most common locations being the pleura (65-70%) and the peritoneum (30%) (2). MM presents with different histological types, the most common type is epithelioid (~60%), followed by sarcomatoid (20%), and biphasic (20%), which has features of both epithelioid and sarcomatoid types (3).

MM often presents clinically with nonspecific symptoms that may lead to delayed diagnosis (4,5). In pleural mesothelioma, patients commonly experience dyspnea, chest pain, and persistent cough, which may mimic other pulmonary conditions (4). Peritoneal mesothelioma typically manifests with abdominal pain, ascites, anorexia, and weight loss (2). Diagnostic workup includes chest radiography and computed tomography scans, while definitive diagnosis requires histopathological examination of biopsy specimens (4,5). Early detection is challenging, highlighting the need for high clinical suspicion, especially in individuals with a history of asbestos exposure (4,6). Due to its insidious onset and nonspecific symptoms, MM is frequently diagnosed at advanced stages (6,7). The advanced stage at diagnosis results in a 5-year survival rate <5%, far lower compared with the average survival rate of all cancers, which lies at 62.7% (8,9). The median survival rates also vary depending on the type, with 19 months for epithelioid, 4 months for sarcomatoid, and 12 months for

biphasic (10). The abysmal prognosis of MM underlies the imperative for novel approaches.

Telomeres are the nucleoprotein complexes comprised of repetitive hexameric sequences, that cap eukaryotic chromosomes ensuring genomic stability by protecting chromosome ends from degradation and preventing end-to-end fusions (11-13). In human cells telomeres undergo progressive shortening with each cell division, leading to senescence or apoptosis once a critical length is reached. Telomerase activity (TA) maintains telomeres and is physiologically encountered in stem cells, germline cells, and certain somatic cells that require extensive proliferative capacity, but in most somatic cells TA is low or absent (14,15). Telomerase reactivation constitutes a crucial tumorigenic mechanism as it maintains telomeres, preventing cellular senescence and apoptosis (16). The relationship between telomere biology and the tumorigenic process is complex on multiple levels, and the precise mechanisms have yet to be fully elucidated (17-21). Clinical application faces several challenges, including limited cohort sizes, non-standardized methodologies, and variability in study results. Nevertheless, manifold aspects of telomere biology have been widely investigated as potential cancer biomarkers, and corollary to its ubiquitousness in malignancy it has emerged as a potential therapeutic target (19,21-23).

To the best of our knowledge, this is the first comprehensive review with regard to telomere biology in MM (17,24). The aim of the present review was to elucidate and contextualize the role of telomere biology in MM pathophysiology, and thoroughly examine the potential applications of telomeres and telomerase in MM diagnosis, prognosis and therapy.

2. Mechanisms of mesothelioma pathogenesis

It is widely recognized that the most significant risk factor for the development of MM is asbestos exposure, and although there have been strict regulations on asbestos use, MM incidence continues to rise (25). Amphiboles and crocidolite are the two main types of asbestos, with the former identified as having a greater role in MM development (26,27). Mesothelioma attributed to asbestos exposure has an average latency period of 40 years, and in some cases, it could reach 60-70 years (28). Asbestos exposure may lead to MM in 5-10% or up 25% of the highly exposed cases depending on the research conducted (28,29). Chronic stress may potentially increase risk as induced immune dysregulation, and the release of stress-related hormones such as norepinephrine may further exacerbate inflammatory responses, promoting tumor progression in an already inflamed microenvironment (30). Additionally, recent evidence suggests that tumors may modify not only the local microenvironment, but create conditions conducive to tumor progression by influencing systemic homeostasis through interactions with the neuroendocrine system (31). Other established factors which increase the risk of MM include exposure to ionizing radiation, radiotherapy, simian virus 40 (SV40) infection, and genetic mutations (Fig. 1) (32-35).

Asbestos fibers when inhaled lodge in the mesothelial lining of the lungs, pleura, peritoneum, or pericardium, inducing a chronic inflammatory response. The response is characterized by the recruitment of macrophages and the release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (36). The inflammatory response promotes the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), resulting in significant DNA damage in mesothelial cells (37,38).

In the unified theory for the development of cancer by Spandidos (39), damage to genes, such as point mutations, deletions, and chromosome translocations, represent key steps in cancer development, with the activation of class I (transform the phenotype of the cell directly) and II (act on the transformed phenotype of the cell indirectly, through class I and III oncogenes) oncogenes and the inactivation of class III (tumor suppressor genes) contributing to cancer progression. DNA damage in class III oncogenes, tumor suppressor genes is a crucial step is the pathogenesis of MM. Mutations of the class III oncogene, BRCA1-associated protein 1 (BAP1), are often associated with MM (40), as are mutations of the class III oncogene neurofibromatosis type 2 (NF2), which encodes merlin, a protein that regulates contact inhibition and cellular growth (39,41,42). Rat sarcoma virus (RAS) family oncogenes, classified as class I oncogenes, are also linked to tumorigenesis and stimulation of survival pathways in MM (39,43).

Oxidative stress caused by asbestos further contributes to tumorigenesis through the activation of signaling pathways related to mesothelial cell transformation. The activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway is associated with the transcription of genes that promote inflammation, cell proliferation, and inhibition of apoptosis (44,45). Another key signaling pathway is the Hippo pathway which controls cell proliferation and apoptosis. In MM, mutations of NF2 and leucyl-tRNA synthetase 1 (LARS1/2) may lead to Hippo signaling pathway dysregulation (42,46). Furthermore, the activation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mechanistic target of rapamycin (mTOR) pathway, which leads to inhibition of apoptotic signals, has been revealed to be associated with MM (47), as is the wingless-related integration site/β-catenin (Wnt/β-catenin) pathway, which causes increased cell proliferation and invasion (48,49). An essential aspect of contemporary research in MM pathogenesis involves epigenetic modifications. Histone modifications alter chromatin structure and gene expression patterns promoting tumorigenesis in mesothelial cells (50). The hypermethylation of tumor suppressor genes such as cyclin-dependent kinase inhibitor 2B ($p15^{INK4B}$), cyclin-dependent kinase inhibitor 2A ($p16^{INK4A}$), Ras association domain family member 1 (RASSF1A) and Ras association domain family 5 (NORE1A) has been correlated with the development of MM (51). Finally, non-coding RNAs, which regulate gene expression post-transcriptionally, have been associated with tumorigenic changes in mesothelial cells (52).

3. Current and emerging therapeutic strategies in mesothelioma

The treatment options for MM have evolved over the years, with the standard treatment modalities being surgery, chemotherapy, and radiation (53). Surgery is typically used for patients with overall good health and diagnosis at an early

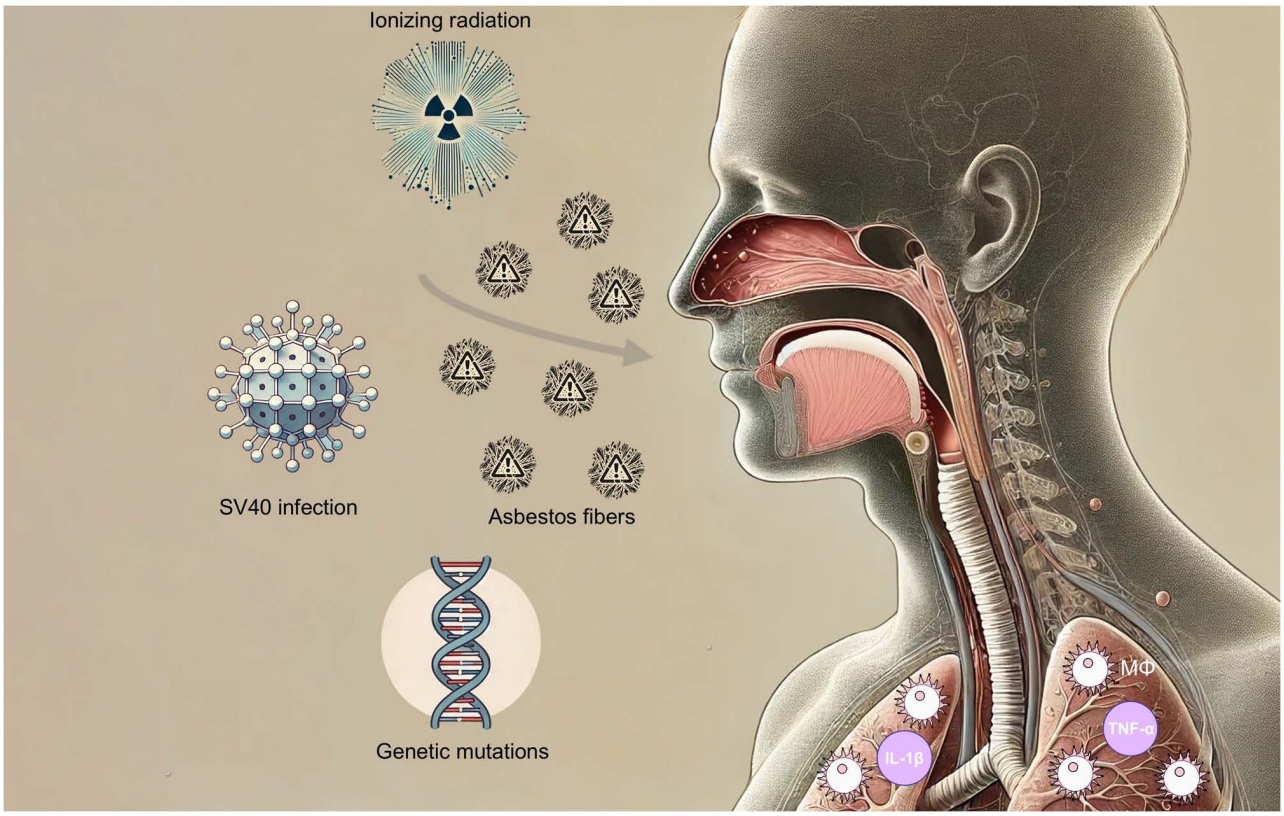

Figure 1. Mesothelioma risk factors. Asbestos has been established as the primary risk factor for mesothelioma, and other key risk factors contributing to the risk of mesothelioma include SV40 infection, ionizing radiation, and genetic mutations. Asbestos fibers, once inhaled, become lodged in the mesothelial lining of the lungs, pleura, peritoneum, or pericardium, triggering a chronic inflammatory response. This inflammatory response involves the recruitment of macrophages and the release of pro-inflammatory cytokines, including TNF-α and IL-1β. The inflamed microenvironment induced by asbestos contributes to tumorigenesis by activating further signaling pathways that promote mesothelial cell transformation.

stage, and is usually combined with other treatment modalities. In the last decades a shift has been observed from extra-pleural pneumonectomy (EPP) to pleurectomy decortication (PD) (54). In terms of systematic therapy, the standard chemotherapeutic regimen as per the EMPHACIS trial consists of a combination of pemetrexed and cisplatin (55). The evidence for second-line treatment options is limited (56). Chemotherapy is not curative, and is often used in a palliative manner (55,56). Radiation therapy is widely used in MM, although the evidence from clinical trials is limited (57). Newer radiotherapy protocols such as image guided radiotherapy, proton therapy, and stereotactic ablative radiotherapy, are still under investigation (58).

The rapid developments in therapeutic modalities targeting cancer have resulted in manifold emerging treatments for MM. Immunotherapy has been one of the most revolutionary developments in cancer treatments, consisting of several treatment modalities such as adoptive cell transfer (ACT) and immune checkpoint inhibitors (ICIs), which combat cancer by increasing the response of the immune system (59). Immunotherapies, including ICIs and chimeric antigen receptor T-cell therapy (CAR T-cell therapy), have shown promising results in MM (60,61). Recently the combination of nivolumab and ipilimumab was approved by the Food and Drug Administration (FDA) as a first-line treatment option for MM (62,63). In gene therapy, tools such as CRISPR-Cas9 are employed to edit genes related to carcinogenesis and tumor cell proliferation, showing potential for applications in MM (64-66). Oncolytic virus therapy employs genetically modified viruses that infect and kill cancer cells (67). Trials in other malignancies have demonstrated the efficacy and safety of agents, such as talimogene laherparepvec (T-VEC) (68). Finally, targeted therapies focus on the specific molecular profile of the tumor, providing a personalized medical approach (69). There are numerous trials which target different aspects of the molecular pathways which induce carcinogenesis or support tumor growth, such as vascular endothelial growth factor (VEGF) (70,71), platelet-derived growth factor (PDGF) (72,73), epidermal growth factor receptor (EGFR) (74,75), and telomerase (76).

4. Structure and function of telomeres and telomerase

Telomeres are complex nucleoprotein structures at the ends of eukaryotic chromosomes, which protect them from degradation and end-to-end fusions (77). Telomeres contain a repetitive hexameric nucleotide sequence (5'-TTAGGG-3') that forms a protective cap 10-15 kilobases (kb) long, and the cap prevents end-to-end fusions and chromosomal degradation (11-13). The shelterin complex protects the telomeres, regulates their length and structure, and regulates TA. The complex consists of six core proteins, namely telomeric repeat binding factor 1 (TRF1), telomeric repeat binding factor 2 (TRF2), TERF1-interacting nuclear factor 2 (TINF2), repressor activator protein 1 (RAP1), adrenocortical dysplasia protein homolog (TPP1), and protection of

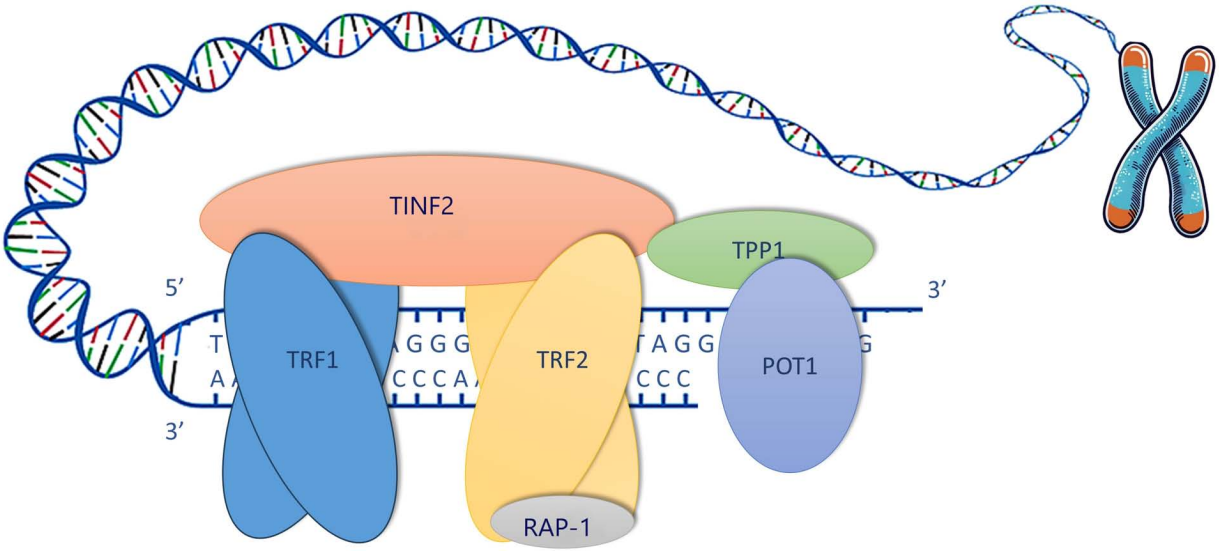

Figure 2. Telomere structure and shelterin complex. Telomeres are specialized nucleoprotein structures that cap the ends of eukaryotic chromosomes, Telomeres are composed of long regions consisting of repetitive hexameric sequences (5'-TTAGGG-3') that form a protective cap, typically 10-15 kilobases long in humans. The shelterin complex helps maintain telomere integrity, and is composed of six core proteins: TRF1, TRF2, TINF2, RAP1, TPP1, and POT1. TRF1 and TRF2 bind directly to the double-stranded regions of the telomeric DNA, while TINF2 and TPP1 contribute to stabilization. RAP1 binds exclusively to TRF2. The TINF2 subunit links TRF1 and TRF2 while interacting with the TPP1 subunit. The TPP1 subunit connects to the POT1 subunit forming a heterodimer which binds the 3' single-stranded overhang. TRF1, telomeric repeat binding factor 1; TRF2, telomeric repeat binding factor 2; TINF2, TERF1-interacting nuclear factor 2; RAP1, repressor activator protein 1; TPP1, adrenocortical dysplasia protein homolog; POT1, protection of telomeres 1.

telomeres 1 (POT1) (78). TRF1 and TRF2 bind directly to the telomeric DNA, while TINF2, RAP1 and TPP1 stabilize the complex. POT1 protects the single-stranded 3' overhang and forms a heterodimer with TPP1 that is essential for telomere elongation and capping (Fig. 2) (79). During cell division telomeres shorten by 30-200 bp during each cycle, as DNA polymerase cannot completely replicate the 3' ends (80). The progressive decrease in length limits the number of divisions a cell can undergo, and the phenomenon is known as the Hayflick limit (81). Decrease in telomere length (TL) in cells with intact cell cycle checkpoints results in senescence, and in cells with compromised checkpoints telomere crisis is induced and either telomere maintenance mechanisms (TMMs) are reactivated, or apoptotic pathways are activated (81,82). The vast majority of cells that necessitate increased replicative potential activate telomerase. A minority activates telomerase-independent mechanisms, denoted as alternative lengthening of telomeres (ALT) (83,84).

By adding telomeric repeats to the ends of chromosomes, telomerase maintains TL thus allowing continued cell division (85). Telomerase is a ribonucleoprotein enzyme that is responsible for the maintenance and lengthening of telomeres (86). Telomerase consists of two main components: A catalytic subunit, telomerase reverse transcriptase (TERT), and the RNA component, telomerase RNA component (TERC) (87). TERT, located on chromosome 5p15.33, functions as a reverse transcriptase which adds telomeric repeats based on an 11-nucleotide template region within TERC (88,89). TA is highly regulated to achieve proper telomere maintenance (24). TA is often found in stem cells, germline cells, and certain somatic cells that require extensive proliferative capacity, however in most somatic cells TA is low or absent (14,15).

5. Telomere biology in tumorigenesis

Telomere length and chromosomal instability. The associations between the elements of telomere biology and the tumorigenic process are complex and diverse (17-21). Among the more widely studied elements is TL, with research investigating the association between TL and cancer, and indicating that cancer is associated with shorter telomeres. The proposed mechanism involves short telomeres leading to genomic instability thus inducing chromosomal aberrations and carcinogenic mutations, while the reactivation of telomerase leads to maintenance of TL above the threshold triggering apoptosis or senescence (17,18,21,90-92). The available clinical research does not reveal a unanimous conclusion. A significant relationship between increased cancer risk and short TL was demonstrated in urological cancers, lung cancer and cancers of the digestive system (22,23,93). However, in studies of breast and colorectal cancers the association between risk and short TL was not significant (94,95) and in melanoma the association was reversed (96). As TL has been studied in various cancers and can be readily measured using peripheral leukocyte TL, it has been proposed as a potential biomarker for cancer risk and progression (97-99).

Telomerase reactivation in cancer cells and replicative immortalization. Telomerase elongates short telomeres, preventing cellular senescence and apoptosis related to multiple cell divisions. Therefore, TA constitutes a crucial step in the tumorigenic process (16). Among key factors in the tightly regulated TA is the TERT promoter. Mutations in the TERT promoter that result in upregulation of telomerase expression are frequently identified in cancer (20). TERT promoter mutations may create new transcription factor binding sites, such as for the E-twenty-six (ETS) family,

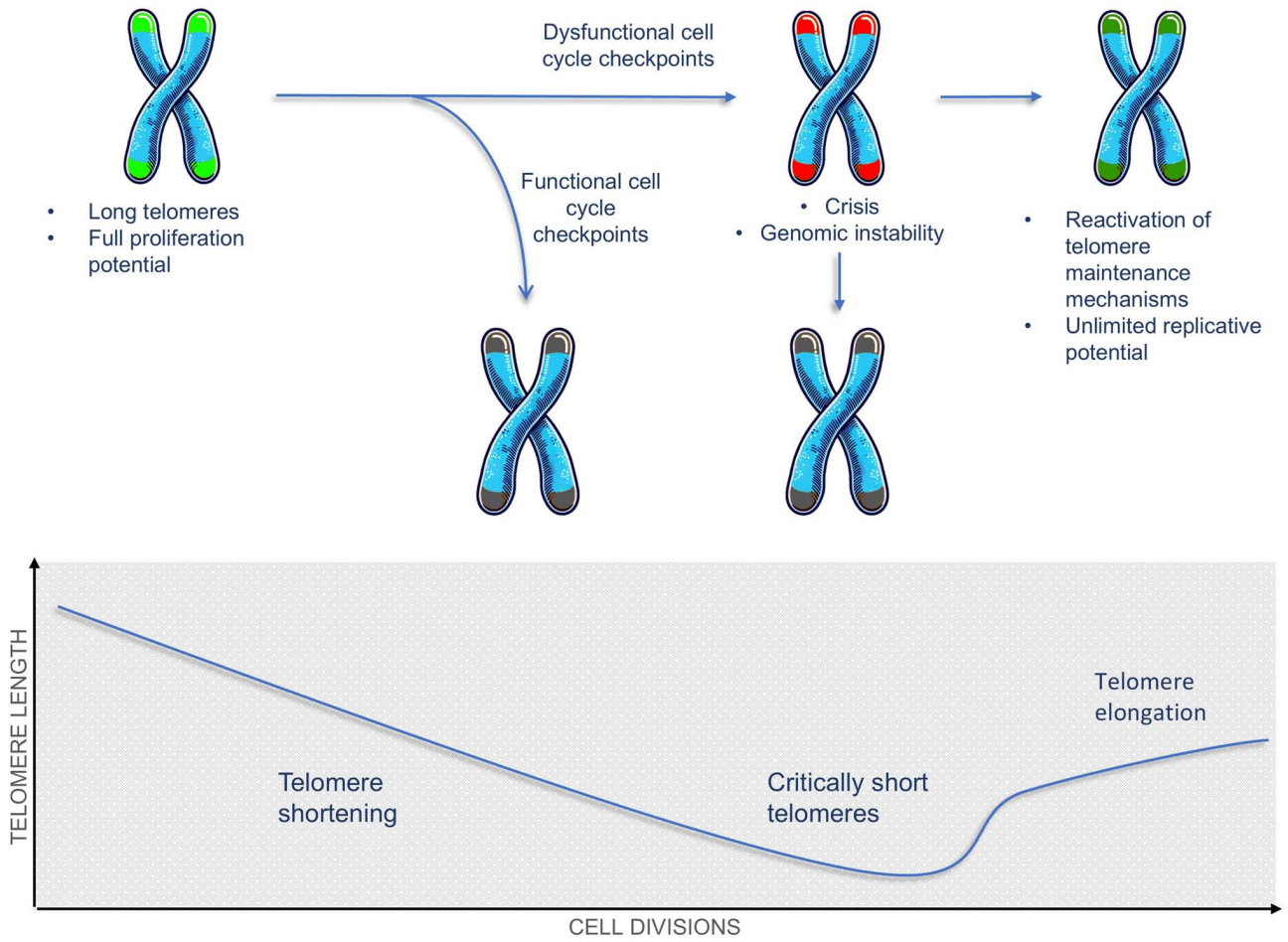

Figure 3. Phases of telomere length in the physiologic and the pathologic cell. i) Cells begin with long telomeres, and during each cell division telomeres progressively shorten due to the end-replication problem inherent in DNA replication. In most cells with intact cell cycle checkpoints critically short telomere length results in cellular senescence. In a cell cycle in which the checkpoints fail or are compromised, cells bypass senescence and enter a crisis phase characterized by chromosomal instability and genomic aberrations. In most cells the crisis is resolved upon the activation of apoptotic pathways. A subset of cells reactivates telomere maintenance mechanisms (telomerase activation or alternative lengthening of telomere mechanism), escaping the crisis and acquiring replicative immortality, a hallmark of cancer cells. ii) Telomere length naturally shortens with each cell replication until reaching a critical minimum. In malignant cells, telomere maintenance mechanisms are reactivated, resulting in telomere elongation and the preservation of telomere length despite further divisions, facilitating the unlimited cell division potential critical in tumorigenesis.

leading to an increase in TERT expression (21). The activation of telomerase in cancer is regulated at the transcriptional level by mesenchymal-epithelial transition factor (c-MET), nuclear factor kappa B p65 subunit (NF-κB p65), myelocytomatosis viral oncogene homolog (Myc), and specificity protein 1 (Sp1) (100,101). At the post-translational level phosphorylation, ubiquitination and sumoylation further modulate telomerase function and stability in cancer cells (101). Epigenetic modifications, such as TERT promoter methylation may cause telomerase reactivation in cancer (21). Telomerase reactivation has been found in ~90% of human cancers (19). It is considered a crucial factor in tumorigenesis as it can provide the cells with unlimited replicative potential, enabling cancer cell immortalization and preventing apoptosis and cellular senescence (Fig. 3) (15,19,102). TA constitutes a potential diagnostic or prognostic biomarker as it can be measured with high sensitivity using the telomeric repeat amplification protocol (TRAP) assay and is present in most cancers (19,103).

TERT promoter mutations in cancer. The mechanism of telomerase reactivation has not yet been fully elucidated, however according to numerous studies TERT promoter mutations enhancing TA, are frequently observed in cancer (20,104). The frequency of TERT promoter mutations varies between cancer types, with mutations identified in 59% of bladder cancers, 49% of central nervous system (CNS) cancers and 29% in melanoma (105). TERT has been found to participate in the maintenance of cancer stem cells enabling tumor growth and metastasis (106). Additionally, research indicates that TERT interacts with the oncogenic pathway Wnt/β-catenin further contributing to tumorigenesis (107). The TERT gene is studied as a biomarker for cancer diagnosis and prognosis (108). TERT promoter mutations, C228T and C250T, have been identified as the most common mutations found in multiple cancers (109,110). TERT expression levels can be measured in non-invasive ways, including circulating tumor cells (CTCs) and cell-free DNA (111). TERT mutations may act as prognostic biomarkers due to their association with tumor aggressiveness and poor prognosis (104,112), with research linking increased TERT expression with worse outcomes in patients with cancer (113).

Telomere biology as a therapeutic target. Aberrations in telomere biology have been found in the vast majority of cancers, thereby highlighting the need for their investigation as potential therapeutic targets (19). The direct inhibition of TA has been considered as a therapeutic target in oncology research. Imetelstat, an oligonucleotide that binds to the RNA template of telomerase, has emerged as a direct inhibitor of telomerase (114). In clinical trials for hematologic malignancies, imetelstat showed promising results, however, serious side effects, including myelosuppression, due to the inhibition of TA in other cells have been noted (115-117). In cancer telomere maintenance is achieved by mechanisms of telomerase reactivation, or by another mechanism called ALT (118). Ataxia telangiesctasia and Rad-3-related (ATR) inhibitors have been demonstrated as highly effective against cancer that rely on ALT for telomerase maintenance. There has been evidence of cancer cells switching to ALT following telomerase targeted therapies. Thus, targeting both telomerase and ALT could offer enhanced therapeutic results (119,120).

6. Telomere length as a biomarker in mesothelioma

The utilization of aspects of telomere biology as potential MM biomarkers has garnered significant attention, with the most commonly researched biomarkers being TL, TA, and mutations of TERT and its promoter (22,23,93,99,104,112, 113,121,122). Clinical application faces several challenges, including limited cohort sizes, non-standardized methodologies, and variability in study results. This review critically assesses the potential of TL, TA, and TERT mutations as biomarkers in MM.

One of the most widely investigated telomere biology-related biomarkers is TL, yet there are limited studies exploring the potential of TL as a biomarker in MM (22,23,93,123-127). Telomere shortening has been significantly associated with the pathogenesis of MM among individuals with asbestos exposure, with significantly longer telomeres in the mesothelial cells of pleural effusions of non-neoplastic disease compared with MM ($P<0.001$) (124). The result is in concordance with studies between patients with MM and individuals with pleural plaques, which found significantly shorter TL in patients with MM ($P<0.001$), and is consistent with research in other cancers demonstrating that telomeres in patients with cancer are shorter compared with healthy individuals (123,124,128-130). Additionally, a shorter TL was identified in asbestos-exposed individuals without MM compared with non-exposed individuals ($P=0.047$) (124). Aida *et al* (124) suggest a progressive shortening of TL, with the longest TL observed in non-exposed individuals, followed by shorter TL in asbestos-exposed individuals without MM, and the shortest TL in those diagnosed with MM, which may function as the basis for a potentially powerful biomarker, but due to the limited sample size further studies with larger cohorts are necessary to validate the findings.

The measurement of TL in mesothelial cells could be utilized as a predictive tool for assessing the risk of MM in asbestos-exposed individuals. A suggested method, accounting for the significantly long latency period, may be longitudinal monitoring of TL in pleural effusions in exposed individuals, which could facilitate improved risk assessment and early detection of MM (131). Moreover, assessment of TL could be utilized to aid in challenging cytological diagnosis, such as differentiating low grade MM from reactive atypia, where current methods, such as CDKN2A (p16) deletion analysis via fluorescence *in situ* hybridization (FISH) and BAP1 immunocytochemistry, sometimes yield inconclusive results (124,132-135).

In non-MM individuals, an association between shorter TL and expression of insulin-like growth factor II mRNA-binding protein 3 (IGF2BP3), cluster of differentiation 146 (CD146), epithelial membrane antigen (EMA), and glucose transporter 1 (GLUT1) has been suggested (124). While not specific to MM, they are more commonly expressed in MM compared with reactive mesothelial cells, thus indicating that their association with telomere shortening should be investigated in MM (124,136).

A notable finding is that older patients with MM had longer telomeres than younger patients (Spearman's $rho=0.370$; $P<0.001$), which contradicts the general association of telomere shortening with aging, and highlights the complex dynamics between aging and cancer in telomere biology (124,128-130,137-139). A potential explanation is that telomerase reactivation found in MM allows potentially longer TL in older patients compared with younger patients. The mechanism, of possibly increased telomerase reactivation in older patients with MM compared with younger patients with MM, has not yet been elucidated (124,126,140).

In assessing the relationship between TL and chemotherapy response, no significant correlation between TL and MM chemotherapy outcomes was found (123). This is consistent with prior research in breast cancer, where chemotherapy-induced telomere shortening was observed to be temporary (141). The impact of TL on progression-free survival (PFS) in patients with MM was also found to be not significant (123). This finding is consistent with studies in other cancers, but overall the findings regarding TL and survival outcomes are conflicting, necessitating the need for future cohort studies (123,142-144). Another limitation is the variability in measuring methodology across studies, employing quantitative polymerase chain reaction (qPCR) or quantitative FISH (Q-FISH) to measure TL (123,124).

Overall, the literature shows the potential of TL as a biomarker for MM. Studies have revealed shorter TL in patients with MM, suggesting utility in risk assessment, early detection, and as a tool in challenging diagnoses. However, the role of TL in therapeutic response and survival remains inconclusive.

7. Telomere maintenance mechanisms in mesothelioma

The majority of cells necessitating increased replicative potential activate telomerase, while a minority activates telomerase-independent TMMs, denoted as ALT (83,84). TA is primarily found in stem cells and germline cells, and is rarely detected in somatic cells, while telomerase reactivation has been found in 85-90% of human cancers, including MM, making it a promising cancer biomarker (15,19,145,146). The frequency of TA observed in MM ranged from 91 to 100%, and the activation of ALT was either absent or rare (126,145,147,148). Investigation of the basic mechanisms

of telomere maintenance in MM is crucial in ascertaining the role of TMMs, particularly TA, as a potential biomarker or therapeutic target in MM. The minimal or absent ALT in MM is in contrast with research suggesting that ALT is more frequently observed in mesenchymal tumors (146). Despite ALT being scarcely detected in pleural MMs, in diffuse malignant peritoneal mesothelioma (DMPM), ALT was identified in 18% of cases, suggesting distinct TMMs, which aligns with the differences in their clinical and molecular profiles (149,150).

To elucidate ALT activation in MM, normal mesothelial cells were transformed by SV40 resulting in an extended but finite proliferative capacity subsequently, without TMM activation until the cells have escaped crisis, suggesting that these viral oncogenes alone are insufficient to directly activate TMMs (126). Notably, in other studies SV40 infection directly induced TMMs in mesothelial cells, suggesting that differences in experimental approaches may influence TMM activation (34,151). Following additional genetic changes to result in immortalization, the study observed that the immortalization of mesothelial cells could result in either ALT or TA in pleural mesothelial cells derived from the same individual, showing activation of either ALT (MeT-4A) or telomerase (MeT-4D) after escaping crisis (84,126). It is noteworthy that while the *in vitro* findings suggest varied TMMs, analysis of TMMs in tumor samples indicates absence or minimal expression of ALT, and a high frequency of TA. The reactivation of TA in normal cells with long telomeres and intact checkpoints only extends cellular lifespan and additional tumorigenic mutations are required, which might explain the different TMM profiles, alternatively, these results may suggest selective pressure in the tumor microenvironment, potential differences in *in vitro* immortalization vs. tumorigenesis *in vivo*, or differences in experimental approaches (126,145,147,152,153). The current evidence firmly indicates that pleural MMs are overwhelmingly telomerase-positive and ALT-negative, but further studies directly investigating ALT activation in large cohorts are needed.

Assessing telomerase activity in mesothelioma. A critical aspect of TA detection, which may partially explain the variability in results between studies is the utilization of different measuring methodologies (148). The utilization of TA as a diagnostic tool necessitates accurate and standardized methodology; the discrepancies between the accuracy of TRAP *in situ*, and cell lysate-based TRAP enzyme-linked immunosorbent assay (ELISA) in measuring TA in pleural effusions are crucial. In the study by Hansson *et al* (148), the two methods are compared to determine the most reliable method (148). The TRAP *in situ* method found TA in 13 out of 14 malignant cases and 2 out of 2 equivocal cases exhibiting moderate to strong reactivity. Among benign effusions 5 out of 7 were negative, and in the other cases, only weak activity was observed, indicating the potential of TA as a diagnostic tool. However, the use of the cell lysate-based TRAP ELISA assay incurred significant overlap between malignant and benign cases. It is suggested that TRAP ELISA may lead to less accurate results compared with TRAP *in situ* (148,154).

A potential explanation for the reduced accuracy of the TRAP ELISA as a diagnostic tool is the presence of telomerase-positive non-malignant cells, such as lymphocytes which may express telomerase upon activation (155,156). The study observed that at least a fraction of the lymphocytes in effusions consistently exhibited weak or moderate reactivity, which aligns with prior findings that activated lymphocytes upregulate TA (155). The findings concur with studies of TA in benign effusions, particularly those rich in lymphocytes, such as those associated with tuberculosis (157-159). Although TRAP ELISA is easier to perform, it is less diagnostically reliable than TRAP *in situ*. It is suggested that the use of adjustments for non-malignant cells could potentially enhance TRAP ELISA diagnostic specificity (148). Currently, TRAP *in situ* is suggested as the preferred measurement technique due to its superior accuracy and ability to more accurately differentiate malignant cases from benign ones, leading to a more precise diagnosis (148).

Telomerase activity in mesothelioma diagnosis. In ascertaining the potential of TA as a biomarker in MM, a comprehensive review of the available data on TA is essential to determine its true diagnostic value in MM and is necessary to elucidate the variability in the published literature.

In the study by Dhaene *et al* (145), TA was identified in 91% of MMs and in both solitary fibrous tumors (SFTs) when examined using TRAP. TA was detected in all four human MM cell lines examined but not in normal mesothelial cells. False negatives, which could impact the assessment as a biomarker, were ruled out through successful amplification of internal control products in all samples. The number of MMs which showed no TA could potentially indicate alternative TMMs, however the study did not explicitly investigate the activation of ALT (145). The TA in SFTs could have indicated potentially, reduction in specificity, however the sample size of SFTs was too small to draw firm conclusions. Furthermore studies have demonstrated markedly low activity in SFTs with typical features and increased activity in atypical tumors, suggesting future studies could investigate TA as a biomarker in SFT for potential malignant transformation (160,161). As Dhaene *et al* (145) did not utilize a TRAP *in situ* assay, there was an inability to capture the heterogeneity of TA at the cellular level potentially overlooking intratumoral heterogeneity, and the accuracy of the result could be affected by false positives in the benign cases (145).

Further studies detected TA using a TRAP assay in all MM samples (126,147). Additionally, studies measured hTERT by employing immunohistochemistry (IHC) to detect TERT protein levels and *in situ* hybridization (ISH) to detect hTERT mRNA. hTERT expression was observed in 98.5% of MM cases by IHC and in all MM cases tested by ISH (147).

Notably, in the study by Au *et al* (126), in all MMs examined there was no detectable c-circle presence, indicating the absence of ALT activity. ALT activity was not explicitly found in most TA studies in MM. The absence of ALT in the aforementioned study (126), may be the result of the relatively small sample size in relation to the frequency, as studies measuring ALT in MM, have detected it in 3.57% of cases (1 out of 28 cases) (126,152).

The potential of TA as a diagnostic tool is strongly supported by the literature consistently finding a high frequency of TA in MM compared with non-malignant

Table I. Comparison of TMM frequencies between MM, DMPM and the average across all cancer types.

TMM	MM (refs.)	DMPM (refs.)	Cancer average (refs.)
Telomerase activity	91-100% (126,145,147,148)	63.6% (149)	85-90% (15,19,146)
Alternative lengthening of telomeres	0-3.5% (126,152)	18% (149)	10-15% (146,152)

TMM, telomere maintenance mechanism; MM, malignant mesothelioma; DMPM, diffuse malignant peritoneal mesothelioma.

cases (126,145,147,148). The use of a TRAP *in situ* assay in a cohort of diagnostically refractory effusions reported a diagnostic sensitivity for malignancy of 91% (162). Additionally, evidence suggests a strong correlation between TRAP *in situ* assay and hTERT IHC (162-164). Cakir *et al* (163) found that hTERT IHC detected MM with sensitivity and specificity of 68%.

The TRAP *in situ* assay demonstrated no strong nuclear TA in the effusions from patients with benign diseases (162). This finding suggests the potential high specificity of strong nuclear TA as a diagnostic biomarker in effusions. However, the statistical significance of strong TA as a malignancy marker reached only P=0.08, potentially due to the small sample size (162). It is suggested that TRAP *in situ* may also be used in distinguishing epithelial MMs from other malignancies, as epithelial MMs exhibited strong TA in all cases tested, in contrast to the variable TA found in other malignancies such as adenocarcinoma and squamous cell carcinoma (145,149,162,165), however future studies are necessary to examine the validity of this observation. Overall, TA shows promise as a diagnostic tool for MM, especially when traditional methods yield inconclusive results, with a sensitivity of 91%. Due to limited research quantifying the diagnostic capacity of TA, further studies are warranted to refine the diagnostic parameters and validate the biomarker clinically in MM.

Research investigating the potential of telomerase as a biomarker in DMPM has been limited compared with pleural mesothelioma. It was shown that TA is the predominant TMM in DMPM, observed in 63.6% of cases, while ALT was identified in 18% of cases (149). The frequency of TA was revealed to be significantly lower than that reported in pleural mesothelioma and ALT frequency was minimal in MM, indicating a potentially significant variation in telomere biology, and suggesting differences in application of TA as a biomarker as well as the need for distinct studies (126,145,147,148). The TA and ALT frequencies in DMPM also differed comparing the 85-90 and 10-15%, respectively, found on average in other cancers, indicating a reliance on ALT for DMPM telomere maintenance (15,146,152). These findings demonstrate the heterogeneity of TMMs in MM, where DMPM may exhibit a distinct telomere biology profile compared with pleural mesothelioma and other cancers (Table I).

Studies have yet to investigate the sensitivity and specificity of TA as a diagnostic biomarker in DMPM. The potential of TA as a diagnostic tool in DMPM is likely reduced due to the high frequency of alternative TMMs, however the pattern of TA and ALT differing from MM and other cancers may allow for the development of high sensitivity and specificity biomarkers (145,149,152). Investigation of TMMs as prognostic biomarkers found that TA^+ correlates with a poorer clinical outcome compared with TA^-, with 4-year relapse hazard ratio (HR) at 3.30 (95% confidence interval, 1.23-8.86; P=0.018) and a cancer-related death HR at 3.56 (95% confidence interval, 1.03-12.51; P=0.045), which is consistent with other research (166). ALT status did not significantly impact clinical outcomes in DMPM, although a trend towards increased survival is noted in ALT cases. In other studies, the prognostic value of ALT differed between cancer types, with improved outcomes in ALT glioblastomas and worse outcomes in ALT liposarcomas. This may be related to tumor-specific genetic alterations associated with ALT (167,168). An association between ALT and a younger age at diagnosis in DMPM is observed, which has also been indicated in glioblastoma multiforme (168). It is noteworthy that the study found the coexistence of both TMMs in two DMPM specimens, which has been reported in other tumor types, including osteosarcoma, liposarcoma, and glioblastoma multiforme, although it still remains controversial whether TA and ALT can coexist within the same tumor cell or if they represent distinct subpopulations within the tumor (167-172). Furthermore, a subset of DMPM (~14%) appeared to lack detectable TMMs, a possible interpretation being the presence of unidentified mechanisms or that an active TMM may occasionally not be necessary for tumor maintenance. The observations align with studies in other tumors (168,169,171), and there has been experimental research that indicates telomere maintenance is not always necessary for malignant transformation (173).

TMMs represent promising therapeutic targets in DMPM. Preclinical studies have shown that targeting these mechanisms can induce tumor cell senescence and apoptosis (174,175). Although no therapies specifically targeting ALT have been developed in MM, telomerase inhibitors are currently in clinical trials in MM (76). As TA frequency is lower and ALT is higher in DMPM compared with MM, potential treatments targeting ALT are likely to be effective in DMPM; this highlights the importance of ascertaining TMMs in patients with DMPM, and the need for the development of therapies targeting ALT (126,176).

8. TERT gene and TERT promoter mutations in mesothelioma

The TERT gene, encoding the catalytic subunit of telomerase, has been the focus of significant research in MM, especially the mutations of its promoter (123,125,177). TERT promoter mutations enhancing TA are frequently observed in cancer and have been studied as biomarkers in cancer (20,104,112). The TERT gene has also been studied as a biomarker for cancer

Table II. Association of TERT polymorphisms with risk, treatment response, and survival in MM.

SNP	Genotype	Risk of developing MM OR[a] (95% CI)	Chemotherapy response rate OR[b] (95% CI)	Progression-free survival HR[c] (95% CI)	Overall survival HR[d] (95% CI)
rs2736098	CC	Reference	Reference	Reference	Reference
	CT	1.49 (1.06-2.10)	1.20 (0.67-2.16)	1.31 (0.98-1.76)	1.02 (0.75-1.39)
	TT	0.78 (0.40-1.54)	1.27 (9.42-3.87)	1.46 (0.82-2.59)	0.94 (0.48-1.83)
	CT + TT	1.36 (0.98-1.88)	1.21 (0.69-2.14)	1.33 (1.00-1.77)	1.01 (0.75-1.36)
rs2736100	CC	Reference	Reference	Reference	Reference
	CA	0.71 (0.48-1.04)	0.85 (0.45-1.63)	0.83 (0.60-1.14)	0.88 (0.62-1.24)
	AA	0.56 (0.35-0.90)	1.16 (0.53-2.57)	0.68 (0.45-1.03)	0.86 (0.56-1.31)
	CA + AA	0.66 (0.46-0.95)	0.94 (0.51-1.71)	0.78 (0.57-1.06)	0.87 (0.63-1.21)
rs10069690	CC	Reference	Reference	Reference	Reference
	CT	1.41 (0.99-2.01)	2.08 (1.13-3.84)	1.06 (0.79-1.43)	1.13 (0.82-1.55)
	TT	2.22 (1.10-4.48)	1.85 (0.64-5.31)	0.81 (0.48-1.38)	0.74 (0.41-1.35)
	CT + TT	1.52 (1.08-2.12)	2.04 (1.13-3.67)	1.01 (0.76-1.33)	1.04 (0.77-1.41)

Adapted from Ref (123). [a]Adjustment for age. [b]Adjustment for weight loss and ECOG performance status. [c]Adjustment for smoking, asbestos exposure, weight loss, C-reactive protein, and histology type of MM. [d]Adjustment for asbestos exposure, ECOG performance status, C-reactive protein, and histology type of MM. TERT, telomerase reverse transcriptase; SNP, single nucleotide polymorphism; MM, malignant mesothelioma; OR, odds ratio; CI, confidence interval; HR, hazard ratio; ECOG, Eastern Cooperative Oncology Group.

diagnosis and prognosis, and it has been suggested that it may contribute to tumorigenesis beyond its telomere maintenance function (107,108).

TERT polymorphisms in the risk and prognosis of mesothelioma. Several single nucleotide polymorphisms (SNPs) have been investigated as risk and prognostic biomarkers in MM (Table II). A significant relationship between the rs2736098 T allele and an increased risk of MM has been observed, with the CT genotype having an odds ratio (OR) of 1.63 (95% CI, 1.20-2.21; P=0.002), and the CT + TT genotype exhibiting an OR of 1.46 (95% CI, 1.09-1.96; P=0.011) (123). The association of the CT genotype with increased risk is also demonstrated after adjusting for age (OR=1.49; 95% CI, 1.06-2.10; P=0.023), the polymorphism has been linked to lung and bladder cancers (178,179).

The carriers of two rs10069690 T alleles face a higher risk of MM, with the TT genotype showing an OR of 2.28 (95% CI, 1.24-4.22; P=0.008). After age adjustment, the OR for the TT genotype remained significant at 2.22 (95% CI, 1.10-4.48; P=0.026). This SNP has also been linked to increased risk of malignancy in numerous other types of cancer, including ovarian, lung, breast and thyroid cancers (180). By contrast, the rs2736100 A allele was associated with a decreased risk of MM, particularly after adjusting for age, with the AA genotype having an OR of 0.56 (95% CI, 0.35-0.90; P=0.017) and the CA + AA genotype showing an OR of 0.66 (95% CI, 0.46-0.95; P=0.026); polymorphisms in this allele have been associated with increased risk of thyroid cancer, bladder cancer, lung cancer, myeloproliferative neoplasms, glioma and acute myeloid leukemia (123,181).

TERT SNPs could potentially be predictive biomarkers in MM. The rs2736100 A allele was significantly associated with a lower risk of MM progression, with the AA genotype exhibiting an HR of 0.68 (95% CI, 0.47-0.98; P=0.038). However, in studies on other cancers, such as kidney cancer, multiple myeloma and glioma, it was associated with a poor prognosis (182-184). The confirmation of TERT SNPs as potential biomarkers of response to chemotherapy could critically improve outcomes in MM. The rs10069690 T allele was found to be associated with a favorable chemotherapy response in MM, with the CT genotype having an OR of 1.81 (95% CI, 1.03-3.17; P=0.039) and the CT + TT genotype exhibiting an OR of 1.72 (95% CI, 1.00-2.93; P=0.048). The associations became stronger after adjustment for weight loss and Eastern Cooperative Oncology Group (ECOG) performance status (CT genotype: OR=2.08; 95% CI, 1.13-3.84; P=0.019; CT + TT genotype: OR=2.04, 95% CI, 1.133.67; P=0.017). By contrast, in breast cancer and multiple myeloma the rs10069690 T allele was associated with poor outcomes (184,185).

Caution is suggested in interpreting these results due to the limited and sometimes contradictory evidence available, particularly in non-Caucasian populations, and the absence of high quality meta-analysis on the TERT polymorphisms as prognostic biomarkers in cancer (123,186). In a previous study there was no significant association revealed between any TERT polymorphisms and PFS in patients with MM (all P>0.05) (123).

These findings strongly suggest that TERT SNPs present valuable biomarkers in MM, with rs2736098 and rs10069690 significantly associated with increased risk, the latter also associated with improved chemotherapy response, and rs2736100 A with a decreased risk of disease and low risk of progression. The validation of the clinical utility of TERT SNPs in MM necessitates future larger, multi-ethnic cohort studies and meta-analyses to clarify the roles of these SNPs in risk and prognosis. Mechanistic studies may elucidate the biological basis of these associations in MM, allowing for potential targeted therapeutic interventions.

Methylation of the TERT promoter in mesothelioma. The utilization of real-time methylation-specific PCR (MSP) has been investigated as a diagnostic biomarker in MM, showing that DNA methylation is significantly associated with MM compared with reactive mesothelial proliferations (RM) (187). Methylation of the TERT promoter results in activating TA, compared with the usual downregulating function of methylation (188). While most of the promoter region has been shown to be methylated in other types of TERT-positive tumors and cell lines, TERT promoter methylation was infrequent in MM, observed in only one MM case (4%) and in none of the RM cases (187,189). This may be interpreted as the result of the specific CpG sites examined not being critical in MM as they are in other TERT-positive tumors (188,189). Due to the limited evidence, further research may be necessary to fully ascertain the diagnostic value of TERT methylation profiling in mesothelioma.

TERT promoter mutations in mesothelioma. The role of TERT mutations in MM may be critical, as TA is the predominant TMM (126,145). The frequency of TERT promoter mutations in MM has exhibited some variation, from 10.4 to 15%, the most commonly observed frequency being ~12%, and the mean non-weighted frequency being 12.25%, similar to thyroid follicular cell-derived carcinoma (10%), and smaller than the frequency observed in other cancers, 59% of bladder cancers, 49% of CNS cancers and 29% in melanoma (105,125,177,190,191).

The analysis of a mutational profile of 43 patients with MM in a Brazilian cohort identified TERT promoter mutations in 11.6% of the cases (191). In a study of 182 MM samples TERT promoter mutations were detected in 10.4% of MM cases (125), slightly lower compared with another study (177). In the most comprehensive study using 266 MM tumors (177), the TERT promoter was identified with a frequency of 12%, and was the third most frequently mutated locus in MM, highlighting its importance in the genetic landscape of this cancer (177,190,192). A study of 61 MM in culture and 71 frozen MM tumor samples, found an overall frequency of 15.2%, a frequency of 11.3% in MM tumor samples and 19.7% in MM cultures (190).

A potential limitation of the studies may be indicated by the lower rate of TERT promoter mutations in MM tumor samples compared with cell cultures. The observation could be due to reduced sensitivity in the frozen tumors samples, possibly caused by the presence of normal cells, however it aligns with TERT promoter frequencies in other research, which could potentially be interpreted as an indication of underestimation in other studies (177,190). Furthermore, the potential underestimation of mutation frequencies may be due to the inability of targeted sequencing to accurately detect large exon deletions, and by the contamination of tumor samples with normal cells, a common issue in next-generation sequencing studies (177,193). Another interpretation of the discrepancy between cell line and tumor frequencies could be that in cell cultures higher TA may confer a selective advantage.

The frequency of TERT promoter mutations has been revealed to be associated with the subtype of MM, with studies typically observing a higher frequency in MM of nonepithelioid histology (125,177,190). A previous study reported TERT promoter mutation frequency as significantly higher in nonepithelioid histology (22.2% vs. 5.5% in epithelioid MM; P<0.001) (125). Quetel *et al* (177) also found that TERT promoter mutations were more common in non-epithelioid MM, and identified them to be positively associated with the S score (representing the proportion of sarcomatoid-like molecular features in a tumor), and with the C2B molecular subtype, suggesting a potential subtype-specific role in MM pathogenesis (177). By contrast, the study by Campanella *et al* (191), including 43 patients with MM predominantly of the epithelioid subtype (88.4%), the rest (11.6%) classified as sarcomatoid, found mutations in only the epithelioid subtype, a potential explanation being random effects due to the relatively small sample size. There has yet to be a definitive explanation for the discrepancy, future studies are needed to further validate the findings of the majority of the investigations (125,177,191).

In the majority of studies the most frequently observed mutation in the TERT promoter in the MM population was C228T, also known as -124C>T, followed by A161C, also known as -57A>C (125,177,190). C228T was either the most or the second most commonly identified TERT promoter mutation in MM, concurring with studies in other cancers revealing it among the most common mutations (108,125,177). The frequency of the C250T mutation in the TERT promoter among patients with MM varied across studies. C250T was identified as the most common mutation in the study by Campanella *et al* (191), reported in 7% of all samples, while other studies have reported it as the third most common mutation, or, as in the case of Pirker *et al* (125), not identified it at all, despite it being among the most frequent TERT promoter mutations in cancer (108,125,177,191). Further studies may reconcile these differences. The C158A TERT promoter mutation has been associated with bladder carcinoma (194). The A161C TERT promoter mutation has been described as a causal high-penetrance germline mutation in a melanoma-prone family (104), and rarely as a somatic event, in melanoma and carcinoma of the bladder, suggesting the possibility of a high specificity biomarker for epithelioid MM (195,196).

The distribution of TERT promoter mutations has been revealed to differ between studies. In the study by Pirker *et al* (125), they were observed as 124C>T (C228T) (68.4%), -57A>C (A161C) (21%) and -146C>T (C250T) (10.5%), while Quetel *et al* (177) identified C228T, A161C and C158A in 81%, 16 and 3% of samples, respectively. The study by Tallet *et al* (190) identified only the C228T TERT promoter mutation, and Campanella *et al* (191) identified C250T and C228T present in 7 and 4% of the cases, respectively. The distribution of TERT promoter mutations also differed across histological subtypes, with the -124C>T mutation strongly prevalent in the non-epithelioid cases, while in epithelioid cases, the prevalence of the -124C>T mutation was equal with that of the rare -57A>C mutation (125,195,196). Overall, TERT promoter mutations, especially C228T and C250T, are significant contributors to telomere maintenance in MM, driving cancer cell proliferation through the stabilization of telomeres. The discrepancies in mutation frequencies across different studies, likely due to methodological limitations, highlight the need for larger and more precise studies. The evidence, particularly in non-epithelioid MM, underscores their potential as biomarkers (Fig. 4).

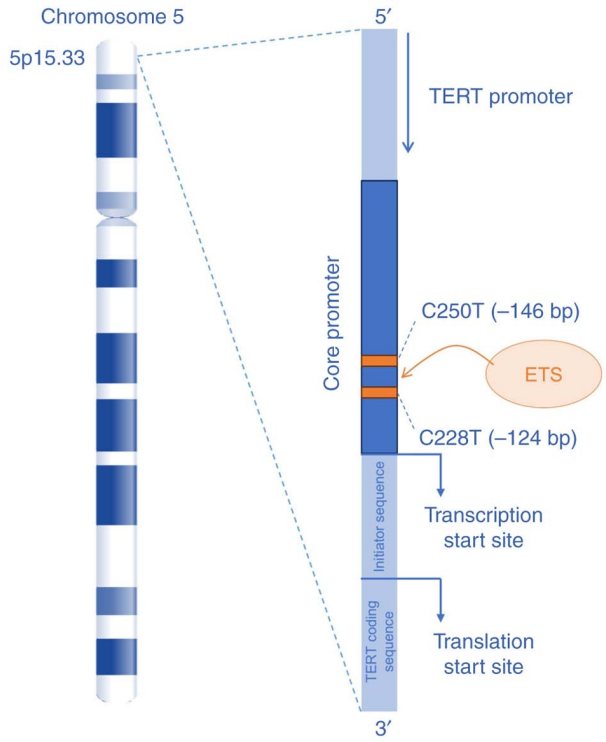

Figure 4. Role of TERT promoter mutations in mesothelioma. The TERT gene, located on chromosome 5p15.33, is essential for the proliferation of mesothelioma cells, as TA is the predominant telomere maintenance mechanism in MM. Mutations in the TERT promoter region, particularly C228T (-124C>T) and C250T (-146C>T), have been identified to contribute to telomere maintenance in MM by increasing TERT expression. These mutations create *de novo* binding sites for the ETS family transcription factors, upregulating TA. The numbering in the parenthesis reflects the distance from transcription start site of the TERT. TERT, telomerase reverse transcriptase; TA, telomerase activity; MM, malignant mesothelioma; ETS, E-twenty-six.

TERT promoter putations as prognostic biomarkers in mesothelioma. The current body of evidence indicates that TERT promoter mutations are associated with overall survival, disease stage, and response to therapy in MM (125,177). A significant association between TERT promoter mutations and overall survival was found in 266 patients with MM, with overall survival revealed to be lower in patients with TERT promoter mutations compared with wild type (P=0.0004) (177). The prognostic impact of TERT promoter mutations in MM is also demonstrated in the study by Pirker *et al* (125), identifying TERT promoter mutations as a strong and independent negative prognostic marker (P<0.0001) in two independent cohorts totaling 184 patients. The significance of these results persisted across demographic groups with potentially different therapeutic settings, including both nonepithelioid and epithelioid MMs, confirming the robustness of this prognostic marker (125,177). In the TERT promoter-mutated cases, median overall survival was significantly reduced, 262 vs. 469 days in wild-type cases (P<0.0001), and 353 vs. 459 days for patients with nonepithelioid MM and epithelioid MM, respectively (P=0.01) (125). The findings indicate that TERT promoter mutation status serves as an indicator of poor survival that may have greater prognostic significance than histological subtype (125). These findings highlight the potential value of TERT mutations as a prognostic biomarker of survival in MM, aligning with studies in other cancers. However, the number of studies is limited and the retrospective nature highlights the need for future prospective studies to confirm the prognostic value of TERT promoter mutations in MM survival (125,177,197).

The frequency of TERT promoter mutations has been significantly associated with MM tumor stage. A significantly higher frequency of TERT promoter mutations was observed in patients with stage IV tumors (28%) compared with patients with stage I/III tumors (13%) (P=0.007) (177). The findings align with the presence of TERT promoter mutations significantly associated with stage III/IV compared with stage I/II disease (P=0.002) (125). A similar association between TERT promoter mutation and advanced stage of disease has also been found in other cancers, denoting the potential of TERT promoter mutations as prognostic biomarkers (198).

The potential of TERT promoter mutations as biomarkers of therapeutic response remains underexplored in a clinical setting. In MM cell lines, TERT promoter mutations were significantly associated with a greater response to telomerase inhibition, suggesting a possible higher dependency on TA in MM with TERT promoter mutations (125). Further large cohort studies are necessary to validate the observations from MM cell lines.

Mechanisms underlying TERT promoter mutations in mesothelioma prognosis. The findings discussed in the previous sections strongly suggest that TERT promoter mutations serve as a useful prognostic marker in MM. However, while the association between TERT promoter mutations and poor overall survival is documented, the underlying mechanisms remain an area of active investigation. The potential of TERT promoter mutations as biomarkers is usually interpreted in the context of TERT function in TA. Telomere maintenance is critical for the proliferation of cancer cells, as TERT activation leads to TA, as well the stabilization of telomeres and the avoidance of replicative senescence (145,190). The mutations identified in the TERT promoter region were shown to create *de novo* ETS transcription factor binding sites, indicating that TERT promoter mutant tumors exhibited higher TERT mRNA expression than wild-type tumors (P=0.0015) (177,190,194). This upregulation of TA is crucial in the maintenance of TL, which in turn supports the unlimited proliferative potential of cancer cells (177). These findings underscore TERT promoter mutations as potential biomarkers for MM. However, Pirker *et al* (125) suggest that the prognostic value could also be corollary to noncanonical TERT functions, including effects on cancer cell motility, stemness, and therapy resistance (125,177,199,200). TERT promoter mutations have been associated with overexpression of TERT mRNA, (125). However the study by Pirker *et al* (125) identified no significant correlation between TERT promoter mutation status and TA (P=0.07) or TL (125), in contrast to research in other cancers (201).

Additionally, it has been shown that TERT promoter-mutated MM samples, which are associated with poor survival, displayed lower chromosomal instability compared with their wild-type counterparts, a finding contrary to previous studies linking high chromosomal instability with poor survival in MM (125,202). The mechanisms underlying this remain unclear, with one possible explanation being,

TERT promoter mutations resulting in early stabilization of telomeres and prevention of short telomere-induced chromosomal instability (203). The low chromosomal instability may also be attributed to the demonstrated mutual exclusion of TERT promoter and BAP1 mutations, which have been associated with high chromosomal instability (177,204-206). Loss of BAP1 has been revealed to be associated with improved survival outcomes in MM, and the mutual exclusion of BAP1 and TERT promoter mutations may also partially explain the poor prognosis observed in patients with TERT promoter mutations (125,207,208). Furthermore, TERT promoter mutations have also been significantly associated with deletions of the RNA binding fox-1 homolog 1 (RBFOX1), glutathione S-transferase θ1 (GSTT1) genes, and mutations in TNF receptor associated factor 7 (TRAF7), suggesting tumors with TERT promoter mutations may be prone to gene deletions and specific genomic alterations. The pattern of alterations may identify a particularly aggressive subset of MM, elucidating the association with poor prognosis (125,209). TERT promoter mutations were also strongly associated with increased *in vitro* immortalization potential in MM cells, indicating a link between TERT promoter mutations and high tumor aggressiveness, aligning with the poor prognosis (210-212).

Overall, TERT promoter mutations have emerged as potential independent prognostic indicators of survival in MM. The mechanisms through which TERT promoter mutations influence prognosis in MM have yet to be completely elucidated. With regard to genetic interactions, chromosomal stability, and noncanonical TERT functions are likely involved in addition to TA. The current evidence may be limited by the retrospective methodology of the studies, and sample sizes. Future prospective studies may confirm these observations, improving the accuracy of MM prognostication and clinical outcomes.

9. Therapeutic strategies targeting telomerase in mesothelioma

MM is characterized by rapid growth and abysmal prognosis corollary to MM being refractory to conventional treatment modalities such as chemotherapy, surgery, and radiotherapy (9,53). Telomere biology offers promising therapeutic options, which primarily focus on targeting telomerase. Telomerase is upregulated in >90% of MM cells while being absent in most non-malignant cells, making it an appealing therapeutic target, albeit with potential side effects corollary to affecting cells such as stem cells, activated lymphocytes and germline cells in which TA is present (126,145,147,148). In MM, oncolytic virotherapy and cancer vaccines have been investigated as telomerase-specific therapies that may provide additional therapeutic avenues beyond conventional chemotherapy and checkpoint inhibitors (76,213,214).

Oncolytic virotherapy targeting telomerase. Therapeutic modalities based on vectors, including virotherapy, are promising alternatives to conventional treatments. Intrapleural viral vector administration is appealing in MM, due to the anatomical localization of the tumor, which allows for locoregional delivery (215,216). Research, using replication-deficient adenoviral vectors to deliver suicide genes and pro-inflammatory cytokines, demonstrated safety and some clinical efficacy in generating antitumor immune responses, however due to the limited distribution of the non-replicative vectors they failed to achieve broad intratumoral penetration (217). The use of replication-selective, tumor-specific oncolytic viruses presents a potentially more efficient alternative (218,219).

Oncolytic virotherapy employs replication-selective viruses to target cancer cells, thereby inducing oncolysis through viral replication (220). The virus selectively infects cancer cells expressing specific cancer-associated markers, such as telomerase (218,220). The OBP-301 adenovirus, known as telomelysin, specifically targets telomerase-positive cells, which may also result in targeting non-malignant cells with TA, by utilizing the TERT promoter to drive the expression of the critical for replication viral genes, E1A and E1B (218,219,221). Viral replication leads to the production of new viral particles within the infected cancer cell, causing cell lysis and the release of viral material. The newly produced viruses infect neighbouring cancer cells, amplifying the oncolytic effect within the tumor microenvironment (222). Thus, OBP-301 may induce cell lysis of telomerase-positive MM cells, with minimal impact on normal, telomerase-negative cells, sparing healthy tissue (Fig. 5) (214,219,221,223).

The therapeutic efficacy of OBP-301 in MM has been explored *in vitro* and *in vivo* (214). OBP-301 was assessed in a panel of human MM cell lines, each characterized by distinct expression levels of the coxsackie and adenovirus receptor (CAR) and TERT (214). It was demonstrated that OBP-301 induced efficient, dose-dependent cell lysis in MM cell lines, with significant cytopathic effects observed within 3 days of infection (214). Furthermore, the use of a modified version of OBP-301, expressing green fluorescent protein (GFP) confirmed efficient viral replication and spread throughout tumor tissues, as evidenced by persistent fluorescence expression in neighboring tumor cells (214,218,222).

OBP-301 was also evaluated using an orthotopic pleural MM model that is based on the inoculation of H2452 cells into the thoracic cavity of athymic nu/nu mice (214). Intrathoracic delivery of OBP-301 resulted in a significant reduction in tumor dissemination and tumor burden compared with a replication-defective control adenovirus or phosphate-buffered saline (214). It was observed that a high dose of 10^8 plaque-forming units (PFU) of OBP-301 was required to achieve a significant antitumor effect, whereas a lower dose of 10^7 PFU showed no therapeutic benefit (214). The timing of administration affected efficacy, with delivery shortly after tumor inoculation being the most effective in reducing tumor weights and limiting dissemination. However, it was noted that treatment did not achieve complete eradication of established tumor nodules, suggesting the need for combination strategies to improve efficacy (214).

Heparinase-assisted dual virotherapy targeting telomerase in mesothelioma. A significant limitation to virotherapy efficacy in solid tumors is the physical barrier posed by the extracellular matrix (ECM), constituting a significant barrier to the distribution of viruses (214,224). The addition of a replication-defective adenovirus expressing the human heparinase gene (Ad-S/hep) may improve efficacy, as heparan sulphate has a significant role in limiting the viral spread in

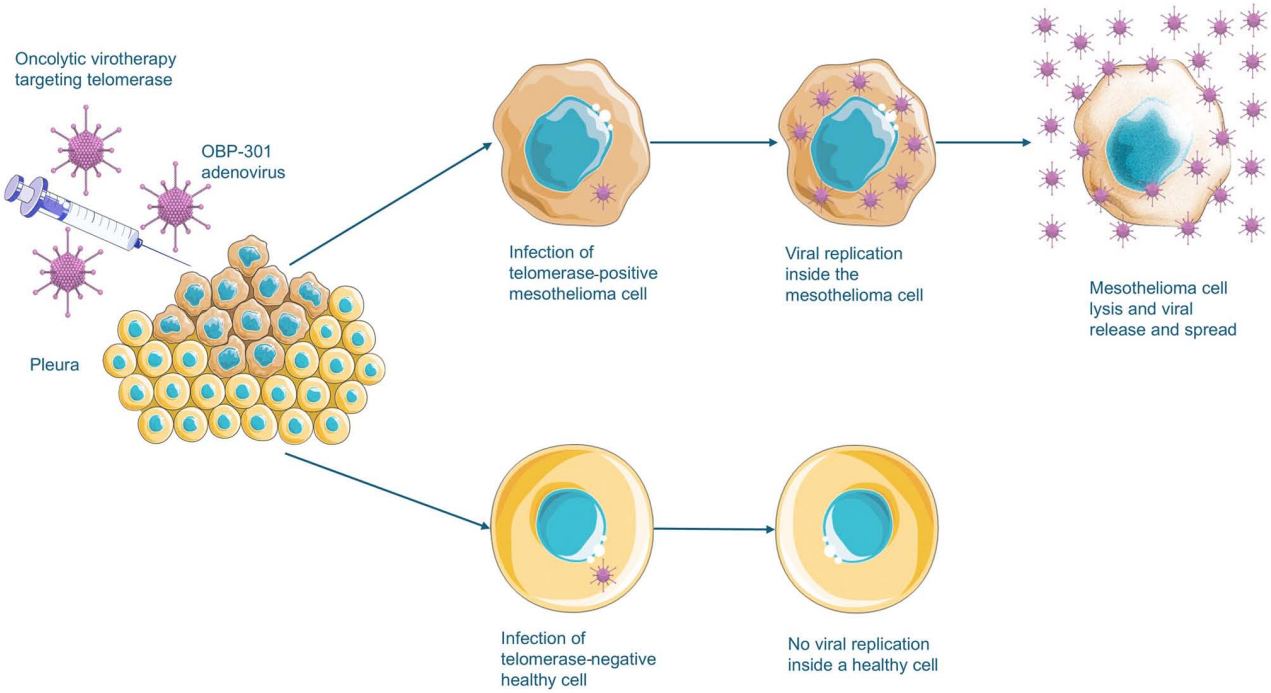

Figure 5. Oncolytic virotherapy targeting mesothelioma cells. OBP-301 (telomelysin), an oncolytic adenovirus, targets telomerase-positive cells by utilizing the TERT promoter to drive the expression of viral genes critical for replication. In MM, intrapleural administration is appealing as it allows for effective locoregional delivery. In most healthy tissues, excluding stem and germline cells, the virus does not replicate leaving these cells unharmed. In MM cells, viral replication leads to cell lysis and the release of new viral particles, that may then infect neighboring MM cells, amplifying the oncolytic effect within the tumor microenvironment. Parts of the figure were drawn by using images from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 4.0 (https://creativecommons.org/licenses/by/4.0/). TERT, telomerase reverse transcriptase; MM, malignant mesothelioma.

ECM, and heparinase degrades heparan sulphate, increasing the permeability of the ECM and facilitating deeper viral penetration (225,226).

The co-administration of OBP-301 and Ad-S/hep resulted in greater viral penetration into three-dimensional tumor spheroids, with GFP fluorescence detecting penetration in deeper tumor layers compared with OBP-301 alone (214,223). High-magnification confocal microscopy images showed that in the OBP-301 monotherapy treatment group viral distribution was limited to the surface layers of the spheroids, and in the co-administration with Ad-S/hep group there was greater uniform penetration and infection throughout the spheroid (214). *In vivo*, dual virotherapy significantly reduced tumor weights in an orthotopic MM model compared with OBP-301 monotherapy, resulting in a marked improvement in survival rates, with 71.4% of mice treated with the combination therapy remaining alive at 12 weeks, compared with only 14.3% with OBP-301 alone (214).

The findings highlight the potential of a telomerase-specific oncolytic adenovirus (OBP-301) in the treatment of MM, especially when combined with agents that enhance ECM permeability, such as heparinase-expressing adenovirus (214). These results concur with the findings in other cancers in which it was demonstrated that oncolytic viral therapy is enhanced by ECM modification (214,224). However, there are risks associated with ECM degradation, including an increased metastatic potential due to matrix remodelling. Studies have also found an association between matrix metalloproteinases (MMPs) and heparinase, and increased tumor invasion and metastasis (227,228). Additionally, the efficacy of OBP-301 was revealed to be dose-dependent, and high viral loads were necessary to achieve significant therapeutic outcomes, potentially leading to increased toxicity (214). Ongoing clinical trials are studying the safety and increased dosing of OBP-301 for refractory advanced liver cancer and evaluating the efficacy of OBP-301 combined with radiotherapy in patients with oesophageal cancer unfit for standard treatments (229,230). Clinical trials in patients with MM are needed to confirm and apply the findings of pre-clinical models with regard to the potential of telomerase-specific virotherapy both as a standalone treatment and in combination with other treatment modalities.

Telomerase vaccines in MM. Immunotherapy is a critical addition to the treatment landscape of MM, with the intra-tumor infiltration of $CD8^+$ T cells being associated with improved outcomes. However, numerous MM tumors are considered immunologically 'cold,' lacking the immune cell infiltration necessary to respond effectively to immunotherapy (231,232). ICI monotherapy using pembrolizumab, nivolumab, or avelumab has shown response rates ranging from 9.3 to 20%, indicating limited efficacy in MM (60,233,234).

The phase III CheckMate 743 trial showed an increased median survival for patients treated with a combination of nivolumab and ipilimumab compared with those treated with chemotherapy, with the median survival improving from 14.1 to 18.1 months (62). However, the survival benefit was largely restricted to patients with biphasic or sarcomatoid histology, while being limited in the epithelioid subtype. The

combination of nivolumab and ipilimumab has been approved by the FDA as a first-line treatment option for MM (62,63).

Response rates in MM are still moderate compared with those observed for ICIs in other cancers, potentially due to the immunologically 'cold' nature of numerous MM tumors. Thus, strategies that increase lymphocyte infiltration may be needed to improve patient outcomes (235-237). One approach is the combination of ICIs with therapies designed to increase immune cell infiltration, such as vaccines targeting tumor-associated antigens (232,238). Telomerase is an attractive target for therapeutic vaccines in MM, due to its high expression in MM cells, while being expressed minimally in most normal tissues, excluding stem cells, germline cells and activated lymphocytes (126,145,213).

Initial studies of telomerase vaccines have revealed vaccine-induced tumor-antigen-specific T-cell responses but did not confer an apparent clinical benefit (7-10,239-241). A potential explanation for this is that the induced T-cell responses were restricted by immune checkpoints. The emergence of ICIs has resulted in renewed interest in cancer vaccines. ICIs act in a complementary manner to vaccines, resulting in an increase in the induced T-cell response and suppression of immune checkpoints in the tumor microenvironment (242,243). Vaccines may generate *de novo* antitumor T-cell responses against the tumor of the patient, which has been identified as a fundamental aspect of cancer immunotherapy, shown to be necessary to invoke tumor regression in patients treated with pembrolizumab (244). Thus, cancer vaccines by complementing ICIs through *de novo* cancer-specific T-cell activation may amplify immune responses and enhance the efficacy of ICIs.

UV1 is a cancer vaccine targeting TERT, the catalytic subunit of telomerase, aiming to generate a strong immune response directed against the enzyme (76,245,246). The therapeutic cancer vaccine is designed to induce an immune response against telomerase, an ideal as it is nearly universally expressed in MM, and inhibiting it can selectively disrupt cancer cell proliferation while minimizing effects on healthy cells (245). By inducing telomerase-specific immunity, UV1 aims to disrupt MM cell proliferation without affecting most normal cells (245). The UV1 vaccine consists of long peptides derived from TERT, aimed at stimulating a $CD4^+$ T-helper type 1 (Th1) immune response. This response is characterized by the release of pro-inflammatory cytokines such as interferon (IFN)-γ, TNF-α, and interleukin-2 (IL-2), that lead to the recruitment and activation of cytotoxic $CD8^+$ T cells and other immune effector cells, creating an inflammatory tumor microenvironment, that target and kill telomerase-expressing cancer cells (cytotoxic $CD8^+$ T cells and other immune effector cells) (213,245). By inducing a strong $CD4^+$ response, UV1 aims to overcome the immunological 'cold' environment typically associated with MM, converting it into an immunologically active microenvironment that is more responsive to ICIs (236,245).

The programmed death-1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) pathways, that are targeted by nivolumab and ipilimumab respectively, serve as major immune checkpoints, differentially inhibiting $CD8^+$ and $CD4^+$ phenotypes, with research indicating that anti-CTLA4 and anti-PD-1 immune responses may be mediated through distinct cellular pathways (247-249). These findings highlight the mechanistic rationale for combining telomerase vaccines with both ipilimumab and nivolumab, which target CTLA-4 and PD-1 respectively, in order to maximize the potential therapeutic benefit (247-249). Furthermore, the evidence from clinical trials indicates higher and more rapid immune response rates in patients treated with UV1 combined with immunotherapy compared with UV1 monotherapy (250-252).

The hTERT peptide vaccine has demonstrated safety and effectiveness in multiple phase I/II trials in other malignancies, with common adverse events including pruritus, fatigue, and gastrointestinal symptoms, and serious adverse events being rare and primarily allergic in nature. In clinical trials in other cancers, UV1 induced vaccine-specific immune responses in 67-91% of patients, with survival improvement being correlated to the patient immunologically responding to the vaccine (250-252).

Clinical trials of telomerase vaccines in mesothelioma. The NIPU trial, an open-label, randomized, phase II clinical trial, investigates the efficacy of nivolumab and ipilimumab with or without UV1 vaccine as a second-line treatment in inoperable patients with MM, following platinum-based chemotherapy as first line treatment (76,245). A total of 118 patients were randomized to receive nivolumab and ipilimumab alone or in combination with UV1, with PFS as the primary endpoint, and evaluated using modified RECIST criteria (76,245).

The trial found that median PFS as determined by blinded independent central review (BICR) was 4.2 months in the vaccine arm and 4.7 months in the control arm, with an HR of 1.01 (80% CI, 0.751.36) (76). However, investigator-assessed PFS indicated a potential benefit in the UV1 arm, with a median PFS of 4.3 months in the vaccine arm and 2.9 months in the control arm leading to an HR of 0.60 (80% CI, 0.450.81); the benefit was particularly strong among patients with epithelioid tumors (P=0.005). The objective response rate (ORR) was 31% in the vaccine arm and 16% in the control arm. In a follow-up period of 17.3 months, the median OS was longer in the vaccine arm with an HR of 0.73 (80% CI, 0.53-1.0) (76). The safety profile of co-administration of UV1 with ipilimumab and nivolumab was comparable with that which has been observed in the control arm of the trial, with the adverse effects potentially specific to the telomerase vaccine being injection site reactions (14%), fatigue/asthenia (12%), pruritus (12%), and pyrexia (12%) (76).

The primary endpoint of the clinical trial, improvement of PFS as assessed by BICR with an 0.6 HR, was not achieved. However, secondary analysis demonstrated improvement in PFS in the vaccine arm in all MM histologies and particularly the epithelioid subtype, a discrepancy that may be corollary to the diagnostic challenges posed by MM (76,253). This observation suggests that OS may be considered as a more reliable endpoint for immunotherapy trials, which concurs with the observations in the CheckMate 743 study, where the HR for PFS was 1.00 but 0.74 for OS (62,76). The improvement in OS of 15.4 vs. 11.1 months (HR 0.73), although not statistically significant, alongside the BICR-assessed ORR being superior in the vaccine arm, and the improved PFS in the secondary analysis, collectively suggest that the UV1 vaccine may still offer clinical benefits. This being more potentially relevant

in patients with the epithelioid histology, which exhibited a greater favorable response to the combination therapy, suggesting the potential role of histological subtype as a factor in predicting treatment efficacy and the need for more precise patient stratification.

As the UV1 vaccine has shown promising results in other cancers, and the combination of nivolumab and ipilimumab despite being approved as first line treatments in MM, lacks response in most patients, further investigation through more targeted, histologically-stratified trials is crucial in ascertaining the potential of telomerase vaccines in MM (62, 76,241,250).

Imaging evaluating telomerase vaccine efficacy. To ascertain the treatment outcomes of telomerase-targeted therapies such as the UV1 vaccine, [18F]-fluorodeoxyglucose positron emission tomography/computed tomography [18F]-(FDG PET/CT) has emerged as a valuable tool for detecting tumor metabolic activity (254). The NIPU trial incorporated [18F]-FDG PET/CT scans at baseline and at week 5 to evaluate potential predictors of response, including metabolic tumor volume (MTV), total lesion glycolysis (TLG), and peak-standardized uptake value (SUVpeak) (254).

Patients who responded to treatment with the UV1 vaccine had significant reductions in TLG, SUVmax, and SUVpeak at week 5 compared with patients that did not respond to treatment. This demonstrates the ability of [18F]-FDG PET/CT to detect early metabolic changes that are related to treatment response, indicating its potential role as a non-invasive tool in monitoring telomerase-targeting treatment modalities (254). Studies in other types of cancer have found significant associations between FDG-PET/CT findings and overall survival, concurring that FDG-PET/CT may provide indicators of treatment success (254-256).

Additionally, [18F]-FDG PET/CT revealed insights into the biological effects of the UV1 vaccine, with significant reductions in the metabolic parameters, TLG, SUVmax, and SUVpeak, among responders (254). Metrics such as SUVmax have often been associated with poor outcomes in chemotherapy-treated MM, but this relationship did not hold true for patients treated with immunotherapy and the UV1 vaccine (254,257). It was found that high baseline SUV metrics were not indicative of poor outcomes, potentially reflecting the distinct mechanisms of action involved in immune-based therapies, where increased immune cell infiltration and activity may contribute to increased FDG uptake, potentially indicating the unique characteristics of immunotherapy compared with the direct cytotoxic effects observed in conventional chemotherapy (254,258).

The findings indicate the relevance of utilizing functional imaging to personalize treatment approaches for patients with MM receiving telomerase-targeted therapies, given its ability to detect metabolic changes prior to observable anatomical changes, highlighting its potential as a marker for vaccine efficacy, aiding in decisions pertaining to continuing or adjusting therapy (254). The utilization of [18F]-FDG PET/CT may also allow the exploration of differential responses across different MM histologies, aiding in identifying patients who may benefit the most from telomerase-targeting strategies (76,254).

10. Conclusion

Telomere biology plays a fundamental role in the development and progression of MM, holding significant potential for advancements in diagnosis, prognosis, and therapy. Telomere shortening is observed in patients with MM and may serve as a biomarker for early detection and risk assessment, especially in monitoring disease development in individuals exposed to asbestos. However, its role as a prognostic biomarker remains inconclusive. Studies in MM have demonstrated that TA is the predominant TMM, while being minimally expressed in most normal tissues, albeit with notable exceptions, thus constituting a promising biomarker for differentiating malignant from benign mesothelial cells, particularly when conventional methods yield inconclusive results. TRAP *in situ* has been identified as the method of choice for TA assessment, and has shown high sensitivity and specificity in distinguishing malignant from benign mesothelial lesions. TERT promoter mutations have been identified in ~12% of patients with MM and are more frequently observed in non-epithelioid histologies. They are associated with poor prognosis and advanced disease stage, with the most frequently observed mutations being C228T, C250T and A161C. Furthermore, TERT SNPs have been associated with increased risk of MM and have potential to act as prognostic biomarkers of chemotherapy response and disease progression. Telomerase has shown promise as a therapeutic target in both preclinical and clinical settings. Oncolytic virotherapy utilizing telomerase-specific adenoviruses combined with ECM-modifying heparinase-expressing adenoviruses, have demonstrated antitumoral effects in laboratory and animal models. Additionally, clinical trials of telomerase vaccines combined with ICIs have shown encouraging results particularly in patients with the epithelioid subtype; however, caution is needed in the interpretation of the findings. Future research should focus on further elucidating the pathophysiology of telomere biology in MM. The small cohort sizes in numerous studies highlight the need for larger prospective cohort studies with standardized methodologies and subtype-specific research to validate the potential of telomere biology as a biomarker, and for randomized clinical trials to examine and develop the therapeutic applications, leading to improvements in diagnosis, prognosis and survival.

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Authors' contributions

DAS, DA and VEG conceptualized the study. DA, VEG, and DAS made a substantial contribution to data interpretation and analysis. DA wrote and prepared the draft of the manuscript.

DAS and VEG supervised the study and provided critical revisions. All authors contributed to manuscript revision and approved the final version of the manuscript. Data authentication is not applicable.

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Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests.

Use of artificial intelligence tools

During the preparation of this work, an artificial intelligence-powered tool was used to proofread the manuscript and improve its readability. The manuscript was subsequently revised and edited. The authors take full responsibility for the final content of this manuscript. Certain elements within the figures were sketched using diffusion models.

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