

# Molecular markers and new diagnostic methods to differentiate malignant from benign mesothelial pleural proliferations: a literature review

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**Abstract:** Malignant pleural mesothelioma (MPM) is an aggressive tumor associated with asbestos exposure. Histopathological analysis of pleural tissues is the gold standard for diagnosis; however, it can be difficult to differentiate malignant from benign pleural lesions. The purpose of this review is to describe the most important biomarkers and new diagnostic tools suggested for this differential diagnosis. There are many studies concerning the separation between MPM and benign pleural proliferations from both pleural tissues or effusions; most of them are based on the evaluation of one or few biomarkers by immunohistochemistry (IHC) or enzyme-linked immunosorbent assays (ELISAs), whereas others focused on the identification of MPM signatures given by microRNA (miRNA) or gene expression profiles as well as on the combination of molecular data and classification algorithms. None of the reported biomarkers showed adequate diagnostic accuracy, except for *p16* [evaluated by fluorescent in situ hybridization (FISH)] and BAP1 (evaluated by IHC), both biomarkers are recommended by the International Mesothelioma Interest Group guidelines for histological and cytological diagnosis. BAP1 and *p16* showed a specificity of 100% in discerning malignant from benign lesions because they are exclusively unexpressed or deleted in MPM. However, their sensitivity, even when used together, is not completely sufficient, and absence of their alterations cannot confirm the benign nature of the lesion. Recently, the availability of new techniques and increasing knowledge regarding MPM genetics led to the definition of some molecular panels, including genes or miRNAs specifically deregulated in MPM, that are extremely valuable for differential diagnosis. Moreover, the development of classification algorithms is facilitating the application of molecular data for clinical practice. Data regarding new diagnostic tools and MPM signatures are absolutely promising; however, before their application in clinical practice, a prospective validation is necessary, as these approaches could surely improve the differential diagnosis between malignant and benign pleural lesions.

**Keywords:** Malignant pleural mesothelioma (MPM); benign pleural lesions; differential diagnosis; molecular markers

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## Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumor mainly caused by asbestos exposure, and it is largely unresponsive to therapies, with a median overall survival ranging from 12 to 18 months (1). Histologically, mesothelioma can be divided into three principal types: epithelioid, biphasic and sarcomatoid, whose incidences are 60%, 30% and 10%, respectively; patients with sarcomatoid and biphasic tumors have significantly poorer survival rates compared to patients with epithelioid tumors (2).

Diagnosis of MPM is primarily based on histopathological evaluation of pleural biopsies performed by thoracoscopy or open thoracotomy as well as on some immunohistochemistry (IHC) examinations. Pathological diagnosis of MPM may be challenging because of its composite epithelial/mesenchymal patterns, its phenotype varying from patient to patient and its property to mimic other cancers and mainly benign processes (2,3). Discernment between benign and malignant pleural lesions is one of the toughest diagnostic issues; indeed, it is often difficult to distinguish epithelioid MPM from benign reactive mesothelial hyperplasia (MH) and sarcomatoid MPM from benign fibrous pleurisy (3). Although morphological criteria for the differential diagnosis of benign and malignant mesothelial lesions have recently been revised (2,3), their application is frequently impossible, particularly on a small biopsied specimen hardly representative of the entire lesion, and the presence of neoplastic invasion is still the cornerstone used to assess the malignancy of a pleural proliferation (3). Moreover, most patients cannot undergo pleural biopsies, and cytological examinations on pleural effusions (PEs) alone rarely lead to a definitive diagnosis (2).

Over these years, several diagnostic biomarkers, including mRNA, DNA, microRNA (miRNA) and antibodies, have been investigated both on PEs and biopsies from patients with MPM or benign pleural proliferations; the newer techniques of molecular biology have been developed, the more the pathological diagnosis of MPM has evolved, according to the increasing knowledge of the MPM genetic landscape (3,4). To date, it is generally accepted that no single biomarker is absolutely sensitive for MPM; therefore, recent studies have focused on the analysis of biomarker combinations or panels and on the development of new diagnostic methods beyond the widely used ancillary tests such as IHC.

The purpose of this literature review is to provide an

overview concerning studies on MPM biomarkers and new diagnostic tools that could improve the pathological separation of malignant and benign pleural lesions.

## Data sources

Studies of interest were selected from PubMed using the following keywords and their combinations: malignant pleural mesothelioma, benign pleural proliferations, mesothelial hyperplasia, biomarkers, diagnosis and differential diagnosis. We considered only publications over the last 10 years written in the English language. Moreover, we selected papers that specifically concerned pathological differential diagnosis between malignant and benign pleural lesions; therefore, we did not take into account soluble serum and plasma biomarkers mainly applied in screening programs of asbestos exposed people.

For greater clarity, this review was divided into four main sections: diagnostic markers by IHC or fluorescent *in situ* hybridization (FISH), soluble markers in PEs, diagnostic miRNAs and new diagnostic tools (including diagnostic methods combining molecular data and computational analysis).

## Diagnostic markers by IHC or FISH

Several immunohistochemical markers have been reported to provide a positive or negative stain more frequently for malignant rather than benign mesothelial proliferations. In particular, some of them have been widely studied even if they have not been applied in clinical practice. Historical IHC markers for MPM include glucose transporter 1 (GLUT-1), desmin, epithelial membrane antigen (EMA), tumor protein p53 (p53), insulin like-growth factor II messenger RNA-binding protein 3 (IMP3), CD44 molecule (CD44) and melanoma cell adhesion molecule (MCAM, also known as CD146).

GLUT-1 is a member of the mammalian facilitative GLUT family of passive carriers that functions as an energy-independent system for the transport of glucose; it is a well-known marker of malignancy, increased in a variety of tumors (4). GLUT-1 has been investigated by many groups for MPM differential diagnosis, and it has continuously shown high specificity for MPM (90–100%), while its sensitivity values ranged from 21% to 85% (5–8). In 2007, Kato *et al.* performed GLUT-1 IHC analysis on formalin-fixed and paraffin-embedded (FFPE) tissues from 40 reactive mesothelial proliferation cases, 48 MPM cases

(mainly epithelioid and biphasic histotypes), and 58 lung carcinoma cases; they reported a positive stain in 100% of MPM cases, 96.5% of lung carcinoma and 0% of benign cases (9). However, according to the majority of available data, it appears that GLUT-1 is informative for malignancy only when positive because the absence of immunoreactivity does not exclude MPM diagnosis; and some authors revealed that benign lesions were positive for this marker (4,7). Furthermore, the evaluation of GLUT-1 requires particular attention because its staining of mesothelial cells could be confused with the staining of red cells (9).

Desmin is a muscle-specific class III intermediate filament whose homopolymers constitute a stable intracytoplasmic filamentous network that connects myofibrils to each other and to the plasma membrane. It is one of the few markers of benignity; its sensitivity ranges from 48% to 84%, and its specificity in some studies reaches 97% (6,10). Nevertheless, desmin stain is typical of a benign reaction, and a proportion of MPM (as high as 50%) has been reported to be positive as well; therefore, its efficacy in clinical practice is minimal (4).

EMA is a membrane-bound protein and a member of the mucin family, including O-glycosylated proteins that play an essential role in forming protective mucous barriers on epithelial surfaces and in intracellular signaling. EMA is known as a marker of malignancy, with sensitivity in discerning malignant and benign pleural lesions ranging from 41% to 79% and specificity from 88% to 100% (5,6). One of the major limitations for the utilization of EMA is that atypical MH may be positive for this marker; Churg *et al.*, in one of their studies, found approximately 30% of EMA positive benign lesions (11).

p53 is a tumor suppressor with a crucial role in cancer development; an accumulation of p53 in cell tumor nuclei has been suggested as supporting evidence of malignancy. Hasteh *et al.* found strong nuclear positivity for p53 in 2% (1 of 46) of benign and 47% (7 of 15) of malignant cases, and Churg *et al.* reported that, in a series of atypical mesothelial reactions, approximately 30% of patients displaying immunostaining greater than 10% were alive after 5 years after diagnosis. Therefore, p53 is not as beneficial as previously believed (10,11).

IMP3 is an oncofetal cytoplasmic protein expressed in fetal tissues, whose staining is present in many carcinomas, where it acts as an oncogene. Generally, IMP3 is a biomarker for tumor aggressiveness in many organ systems, and its expression correlates with a worse prognosis in human malignancies (12). It is not a beneficial marker for differentiating carcinomas arising in different organs;

however, it is a highly specific marker for malignant lesions. Therefore, it was suggested for the differentiation of MPM from reactive mesothelial proliferations (12). The presence of an IMP-3 protein is indicative of MPM with a sensitivity of 37% to 94%, regardless of subtype and location. However, some benign reactions, particularly for atypical hyperplasia, stain for this marker as well (5,6,11,13).

CD146 is a transmembrane glycoprotein belonging to the immunoglobulin superfamily; its increased expression is closely associated with an advanced stage of malignant melanoma, prostate and ovarian cancers (14). CD146 was tested on smear specimens of PEs from MPM and MH patients. Sato *et al.* found a sensitivity of 90–94% and a specificity of 100% because no reactive mesothelial case was positive for this marker (15). On the other hand, on FFPE tissues, Minato *et al.* found a sensitivity and specificity of 71% and 98%, respectively (6). In addition, Beije *et al.* performed CellSearch-based and flow cytometry-based assays using CD146 to identify circulating tumor cells (CTCs) from PEs and peripheral blood samples from 27 MPM patients (81% epithelioid MPM) and 22 control cases with PEs without MPM, confirming the malignancy of CD146-positive cells. Detection of MPM CTCs in PEs showed a poor specificity and a sensitivity of 48%, which, anyway, was higher than routine cytological analyses (16).

CD44 is a cell surface adhesion molecule involved in cell-cell and cell-matrix interactions. Furthermore, it is the major receptor for hyaluronate (HA). CD44 is involved in cancer progression, cell adhesion and cell migration. In cancer cells, CD44 interacts with hyaluronan-rich microenvironments modifying cell signaling pathways that trigger the ability of malignant cells to migrate, invade basement membranes and lodge at distant sites of the tumor (17). CD44 can be detected immunohistochemically in mesothelial tissues, confirming the presence of neoplastic cells, and it is more expressed in epithelioid rather than in sarcomatoid histotypes (18). However, it is a more reliable marker to distinguish MPM from pulmonary adenocarcinoma than MPM from MH (18,19). In a study by our group, CD44 expression was low in most (57.7%) mesothelioma samples and only in 11.5% of the MH samples (19).

Performance of these markers alone or in combination was compared both on PEs and tissues from patients with MPM and benign pleural diseases. Kuperman *et al.* performed, on PEs, a combined receiver operating characteristic (ROC) curve analysis and found a higher area under the curve (AUC) for GLUT-1 and EMA combination

(0.93) than for GLUT-1 (0.90) and EMA (0.82) used separately. They additionally reported an AUC of 0.84 for desmin (20). Chang *et al.* determined that the combination of IMP3/EMA, GLUT-1/EMA, and IMP3/GLUT-1 had a sensitivity of 66%, 53% and 45%, respectively (5). Minato *et al.* suggested IMP3/GLUT-1 as the most effective marker combination to differentiate malignant from benign pleural lesions; using the cut-off point of 30% for IMP3 IHC and 10% for GLUT1, their sensitivity for MPM was 100%, and the specificity of both was 95% (6).

In addition to the aforementioned markers, other proteins, mainly selected for their crucial role in cancerogenesis, have been investigated for MPM differential diagnosis.

Taheri *et al.* investigated certain IHC markers like marker of proliferation Ki-67 (Ki-67) and the restrictedly expressed proliferation-associated protein 86 kDa (repp86), reporting a sensitivity of 88% and 92% and a specificity of 92% and 94%, respectively, on pleural tissues (21). Ki-67 was additionally analyzed by Hasteh *et al.*, who found no significant difference between reactive MH and MPM cases (10), as well as by Kimura *et al.* on PEs, showing a sensitivity of 78% and a specificity of 79% (22). In the same study by Kimura, other proliferative markers were examined on PEs: minichromosome maintenance protein 7 (MCM7), geminin, and topoisomerase II alpha (Topo II  $\alpha$ ). All of these proteins showed high expression in MPM; their sensitivity and specificity were 100% and 100% for MCM7 (cut-off value 20.0%), 88% and 70% for geminin (cut-off value 4.5%), 88% and 92% for Topo II  $\alpha$  (cut-off value 11.0%), respectively (22).

$\beta$ -catenin has been reported as valuable for assisting in the differential diagnosis of mesothelial and spindle cell proliferations in the pleura. It has a well-defined role in the genesis of pleural conditions, including organizing pleuritis, MH and malignant mesothelioma; however, IHC analysis of  $\beta$ -catenin did not appear to be conclusive for separating benign from malignant mesothelial proliferations (23).

Our groups, by analyzing the expression of genes involved in extracellular matrix remodeling and cell adhesion, found that matrix metalloproteinase 14 (MMP14) and integrin alpha3 (ITGA3) could be potential IHC markers expressed higher in MPM than in MH (19).

Monocarboxylate transporters (MCTs) play an important role in cancer development because of their involvement in glycolysis regulation. In addition, their expression depends on a chaperone, CD147, whose high expression is typically associated with poor prognosis in cancer. Pinheiro *et al.* evaluated the immunoexpression of MCT1, MCT4 and

CD147 to differentiate tissue sections from 9 MPM and 11 reactive mesothelial proliferation tissues. MCT isoforms were not differentially expressed in benign and malignant types of cytological specimens, whereas CD147 was almost exclusively expressed in MPM, and it was able to distinguish these two proliferations with the same accuracy as GLUT-1 (24).

Additionally, Shen *et al.* reported an AUC of 0.91 and 0.80 for EMA and GLUT-1 and they analyzed also the diagnostic performance of X-linked inhibitor of apoptosis protein (XIAP), finding an AUC of 0.67 in the distinction between MPM and benign effusion (25).

Recent studies by Miyanaga and Guo, aiming to describe the molecular landscape of MPM, identified frequent alterations in the tumor suppressor genes neurofibromin 2 (*NF2*), large tumor suppressor kinase 1 and large tumor suppressor kinase 2 (*LATS1/2*) (26,27). Sheffield *et al.* tested the emerging biomarkers for the differential diagnosis between MPM and benign proliferations, and they determined that *NF2*, *LATS*, and *YAP/TAZ* immunohistochemical stains were not helpful in this context (28).

Finally, the latest International Mesothelioma Interest Group (IMIG) guidelines for histological and cytological diagnosis of MPM (3) suggested the analysis of two relatively new markers either on FFPE tissues from biopsies or on cytological specimens: the cyclin dependent kinase inhibitor 2A (*CDKN2A*) gene, better known as *p16*, by FISH, and the BRCA1 associated protein 1 (BAP1) by IHC (29,30).

*p16* is a cyclin-dependent kinase inhibitor, while BAP1 is a nuclear deubiquitinase targeting histones and the host cell factor 1 (HCF1) transcriptional cofactor. Both genes act as tumor suppressors (31,32). Both *p16* (locus 9p21) and BAP1 (locus 3p21.2) are frequently deleted in malignant lesions, and they have never been reported as altered in benign lesions. Therefore, they have 100% specificity for MPM; however, their sensitivity ranges between 43–93% and 61–67% for *p16* and BAP1, respectively (33–35). Combination of the two assays has been reported to increase sensitivity for MPM diagnosis up to 90% in some studies; however, specificity is always 100% (29,33,36). Moreover, BAP1 and *p16* have been specifically investigated for the discernment between sarcomatous and desmoplastic mesotheliomas from benign organizing pleuritis. Hwang *et al.* analyzed 20 sarcomatous and desmoplastic mesotheliomas, determining that BAP1 IHC was relatively insensitive in this context and that deletion of *p16* by FISH was considerably more sensitive; however, a proportion of cases remained in which *p16* was not deleted (37). BAP1 and *p16* examinations

do not allow the detection of all MPM cases, even when the combined assay approach is utilized, because the two markers are only deleted in a proportion of mesotheliomas. Therefore, failure to find their alterations does not assure the benign nature of a mesothelial process (4).

Furthermore, it was recently demonstrated by *in vitro* studies that BAP1 loss favors cell proliferation by the up-regulation of the enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) (38), a histone-lysine N-methyltransferase overexpressed in various cancers (38,39). In 2016, Shinozaki-Ushiku *et al.* investigated the combined utility of BAP1 and EZH2 in the differential diagnosis of malignant and benign mesothelial proliferations. Both BAP1 and EZH2 were analyzed by IHC in 32 MPM (23 epithelioid, 7 biphasic and 2 sarcomatoid cases) and 44 benign mesothelial proliferations (4 well-differentiated papillary mesotheliomas, 22 mesothelial inclusion cysts and 18 reactive MHs), and most samples were biopsies or surgically resected tissues, except for 8 cell blocks. They found BAP1 loss in 53% of MPM and EZH2 high expression (IHC positive nuclei 50–90% or >90%) in 66% of MPM. None of the benign lesions presented BAP1 loss or EZH2 high expression, considering the markers together had a diagnostic sensitivity of 90% and a specificity of 100% (40).

### Soluble markers in PEs

Soluble markers in PEs are interesting tools for rapid MPM diagnosis, and in this context, the most important ones include mesothelin, fibulin-3 and hyaluronan.

Mesothelin is a 40 kDa glycoprotein attached to the cell surface of mesothelioma, ovarian, pancreatic and other cancers. The *Mesothelin (MSLN)* gene encodes a precursor, which is processed in soluble mesothelin (also referred to as SMRP: soluble mesothelin related protein and C-ERC/mesothelin) and megakaryocyte potentiating factor (MPF) (also known as N-ERC/mesothelin). Elevated mesothelin levels in PEs, determined by enzyme-linked immunosorbent assays (ELISAs), are indicative of malignancy (41). Effusion mesothelin have specificity for malignancy in general; however, the sensitivity is low (60–70%) for the differential diagnosis of MPM, and its predictive value is approximately 75%. It was reported that negative results of mesothelin determinations do not exclude MPM, whereas positive test results have to be followed by further invasive diagnostic steps to diagnose MPM (42). Mesothelin has always been considered one of the most promising MPM biomarkers;

therefore, it has been tested by many groups alone or in combination with other soluble markers. For instance, Blanquart *et al.* evaluated the chemokine (C-C motif) ligand 2 (CCL2), galectin-3 (LGALS-3), the secretory leukocyte peptidase inhibitor (SLPI), soluble mesothelin and their combinations by ELISA assays in PEs. They found that the combination of SMRP/CCL2/galectin-3 greatly improved MPM diagnosis (AUC: 0.9680) when compared with biomarkers alone (43). Mundt *et al.* evaluated levels of HA, N-ERC/mesothelin, C-ERC/mesothelin, osteopontin, syndecan-1, syndecan-2, and thioredoxin using ELISA assays and high-performance liquid chromatography-mass spectrometry (HPLC) in PEs from a study cohort of 190 patients and a validation cohort of 375 subjects (including MPM, pleural metastases and benign diseases). They found that high levels of HA (odds ratio: 8.82), N-ERC/mesothelin (odds ratio: 4.81), C-ERC/mesothelin (odds ratio: 3.58) and syndecan-1 (odds ratio: 1.34) were significantly associated with malignant lesions. Moreover, they created a two-step model HA and N-ERC/mesothelin, improving the discernment between malignant and benign lesions, with an AUC of 0.99 in the model generation dataset and 0.83 in the validation dataset (44).

Fibulin-3 is a conserved member of the extracellular glycoprotein fibulin family, which appears to work better as a diagnostic biomarker in plasma rather than in PEs. Indeed, fibulin-3 was reported to be higher in PEs from MPM patients than patients with other diseases or benign controls; however, its levels were not statistically different (45). Recently, diagnostic performances of fibulin-3 and mesothelin were compared in patients with PEs from malignant mesothelioma, benign diseases and pleural metastases (33 patients with MPM, 64 with pleural benign lesions and 23 with non-MPM pleural metastases), and in contrast to SMRP levels, which were significantly higher in PE from MPM than other groups, levels of FBLN3 were similar in PE from MPM and PE from other pathologies (46). Therefore, FBLN3 detection in PE does not appear to be a useful biomarker for the diagnosis of MPM (46,47).

HA is an extracellular polysaccharide of connective tissue; it regulates different cellular activities such as cell migration, growth, differentiation and cell adhesion. In tumors, HA binding to CD44 evokes an interaction of CD44 with signaling receptors, and high levels of HA in PEs of MPM patients compared with non-mesothelioma fluids have suggested its diagnostic value (18). Analysis of HA expression in pleural fluids could increase sensitivity of cytological diagnosis from 48% to 71–91% (48); however,

its high levels do not appear specific for MPM because they can additionally occur in other malignant or benign reactive diseases, and low levels do not exclude MPM (49).

### Diagnostic miRNAs

miRNAs are short, non-coding RNAs that are post-transcriptional regulators in physiological and pathological processes. They regulate target mRNA molecules in a sequence-specific manner and are differentially expressed in many diseases, including cancer. Several studies reported that miRNAs are differentially expressed in specimens from MPM, asbestos-exposed, and healthy subjects, therefore showing their involvement in MPM biology and their potential role as biomarkers (50). In this review, we described only miRNAs that were differentially expressed between MPM and benign pleural lesions, whose expression profile from PEs or tumor tissues could be of diagnostic relevance.

Andersen *et al.* identified a four miRNA-classifier (miR-126, miR-143, miR-145, and miR-652) analyzed by retro-transcription polymerase chain reaction assays (RT-PCR) and capable of distinguishing MPM from reactive pleural proliferations with a sensitivity of 95%, a specificity of 93% and an overall accuracy of 94%. This classifier was validated on tissue samples from 40 MPM patients (preoperative diagnostic biopsy, surgical removed tumor tissues and surrounding reactive mesothelial proliferations), and it was found that chemotherapy reduced the differential expression of miRNAs. In addition, authors compared miRNA expression levels between biphasic and epithelioid histotypes, finding that biphasic MPM displayed significantly higher miR-126 and miR-145 and lower miR-193a-3p. Therefore, the differentiation pattern of different histological subtypes of MPM appeared to affect the levels of these three miRNAs. Furthermore, using IHC, they tested the expression of the amino acid transporter LAT1 and the oncogenic adaptor protein Crk-II, both reported as targets of miR-126. LAT1 expression inversely correlated with the expression of this miRNA; consequently, the authors suggested that a diagnostic assay that combined MPM-deregulated miRNAs and IHC detection of their related targeted gene products could potentially improve the differential diagnosis of benign pleural lesions and MPM (51).

In another study, Ak *et al.* analyzed miR-484, miR-320, let-7a and miR-125a-5p by RT-PCR assays on fresh frozen tissues from a total of 18 MPM and 6 benign asbestos-

related PE specimens. These miRNAs provided an AUC greater than 0.90 in MPM differential diagnoses. In detail, miR-484 had 100% sensitivity and specificity to differentiate MPM from benign asbestos related lesions; sensitivity and specificity for miR-320, let-7a, and miR-125a-5p were 78%, 100%; 94% and 83%; 89% and 100%, respectively. Within the MPM samples, there did not appear to be any significant miRNA expression differences among different histotypes nor between early stage (I–II) and late stage (III–IV) malignant diseases. In addition, through an integrated analysis examining miRNA-mRNA interactions, they found that multiple altered targets belonged to the Notch signaling pathway, providing again evidence that specific miRNAs and mRNAs may have diagnostic utility in differentiating patients with MPM from benign asbestos-related PEs (52).

Finally, Micolucci *et al.* recently performed a systemic review and qualitative meta-analysis to identify the most significant deregulated miRNAs in MPM. By examining all available data published until 2015, they identified two potential multimarker signatures: one composed of circulating miRNAs (miR-126-3p, miR-103a-3p, and miR-625-3p) and one composed of tissue miRNAs (miR-16-5p, miR-126-3p, miR-143-3p, miR-145-5p, miR-192-5p, miR-193a-3p, miR-200b-3p, miR-203a-3p, and miR-652-3p). The downregulation of miR-145-5p, miR-143-3p, miR-126-3p, miR-652-3p, and miR-16-5p and the upregulation of miR-625-3p, highlighted by the qualitative meta-analysis, agreed with the chromosomal instability and epigenetic modifications described in MPM. The most consistently described tissue miRNAs were additionally found to constitute a potential signature applicable for MPM differential diagnosis (50).

### New diagnostic tools

Currently, no tissue or soluble marker (including BAP1 and *p16*) has shown sufficient sensitivity to replace the evidence of invasion as the gold standard for differential diagnosis, particularly between epithelioid mesothelioma and reactive MH. The advancement in molecular biology techniques, such as microarray, next-generation sequencing or NanoString technologies, not only has shed light on the molecular landscape of MPM, thus suggesting new potential biomarkers, but also allowed the analysis of multiple markers from a low input of biological material. In the same way, the development of bioinformatics techniques has led to classification algorithms, which facilitated the application

of molecular data, such as gene expression, to cancer diagnostics. We found four papers from 2013 to 2016 regarding the combination of molecular and computational analysis to diagnose MPM and particularly to differentiate malignant and benign pleural lesions.

In 2013, De Rienzo *et al.* used a sequential combination of gene expression ratio tests based on the expression profile of 26 genes able to distinguish MPM from normal pleura and other thoracic malignancies, such as sarcomas, renal cell carcinoma and thymoma, achieving a sensitivity and specificity of 100% and 90% in the training set and of 92% and 97% in an independent test set, respectively. The gene signature was identified by an Illumina whole genome microarray analysis on 113 fresh frozen tissues from 39 MPM patients (24 epithelioid, 7 biphasic and 8 sarcomatoid), 7 normal pleural samples and other common thoracic malignancies and then validated by RT-PCR in a validation cohort of 170 samples, including 100 MPM tissues (63 epithelioid, 27 biphasic and 10 sarcomatoid), 12 normal pleural samples and 58 other tumors. In detail, they developed four gene ratio-based tests: one to distinguish MPM from normal pleura (*UBE2T*, *AGENCOURT\_14535501*, *MAGED1*, *ADCY4*, *PAK4*, and *MYH11*), a second to distinguish MPM from all sarcomas (*MSLN*, *TGFBR3*, *ANXA8*, *TCEAL7*, *KRT8*, and *PCDH18*), a third to distinguish MPM from renal cell carcinoma (*NFKBIZ*, *ARHGAP2*, *ARL6IP6*, *HPN*, and *LOC648293*), and a fourth to distinguish MPM from thymoma (*KRT18*, *PRSS16*, *RGS16*, and *BCL11A*) (53). In addition, the authors added to their analysis their previously validated diagnostic test for discerning MPM and lung adenocarcinoma (54). Additionally, a gene-ratio-based test was developed to differentiate epithelioid and sarcomatoid MPM (*CLDN15*, *LOC57228*, *ORF1-FL49*, and *NP*). Application of a sequential method allowed the authors to overcome limits due to the binary nature of individual gene expression ratios. Moreover, the gene ratio signature showed a sensitivity and specificity comparable or even better than those achieved by the more complex algorithm k-nearest neighbor (KNN) and linear discriminant analysis (53). One limitation of this study is that for microarray analysis, 0.75 µg of total RNA and 1 µg for RT-PCR were utilized; these amounts can be obtained from fresh frozen tissues, but they can be scarcely obtained from FFPE or cytological specimens. However, this gene signature deserves further validation, and the use of more recent techniques, requiring less RNA input, may facilitate its application.

In 2014, Parodi *et al.* used an innovative method of

supervised data analysis, the logic learning machine (LLM), to distinguish MPM from pleural metastases of other tumors and benign diseases associated with pleurisy. They utilized LLM to exploit the complex multivariate correlation between cytological examination results and the concentration of PEs of three well-known MPM markers: carcinoembryonic antigen (CEA) (typically down regulated in MPM, however highly expressed in other tumors like lung cancer), a soluble fragment of cytokeratin 19 (CYFRA 21-1, expressed by all histological types of lung cancers and by other malignancies) and SMRP. CEA and CYFRA 21-1 are typically utilized to differentiate PE from mesothelioma patients and from other malignancies (55). Parodi *et al.* analyzed 52 MPM, 62 pleural metastases and 55 benign disease PE samples. Their LLM model was able to classify malignant mesothelioma, pleural metastases from other tumors and benign pleural diseases, reporting a classification accuracy of 77.5%. LLM correctly classified 79% of mesothelioma, 66% of pleural metastases and 89% of benign diseases. Moreover, they demonstrated that LLM outperformed standard data mining techniques: decision tree, artificial neural network and KNN. CEA and CYFRA 21-1 are not the best markers reported for the differential diagnosis between malignant and benign pleural lesions, and analysis of new tumor markers is possibly necessary to improve this classification model (55).

In 2015, Tosun *et al.* developed a computer-aided diagnostic approach for MPM based on nuclear chromatin distribution from digital images of mesothelial cells in effusion cytology specimens using the KNN algorithm. They developed a computerized method to determine whether a set of nuclei of a patient was benign or malignant, and quantification of chromatin distribution was performed through optimal transport-based linear embedding for segmented nuclei together with the modified Fisher discriminant analysis. They analyzed 34 cases of PEs, 16 malignant mesothelioma cases and 18 benign lesions, confirmed by pleural biopsies obtaining a 100% accurate prediction. They provided evidence that nuclear structure of mesothelial cells alone may be sensitive enough to distinguish malignant and benign pleural lesions. However, this analysis required a trained pathologist for selecting mesothelial cells from the image field of view, and by initializing the segmentation procedure, the automatization of such procedures could enhance the introduction of this method in MPM clinical practice (56).

Finally, our group developed a diagnostic tool for the differential diagnosis of MPM and MH, which relied on

the expression analysis of 117 genes deregulated in MPM, utilizing the highly sensitive and innovative NanoString system and the uncorrelated shrunken centroid (USC) classification algorithm. In the first part of our study, we analyzed mRNA from FFPE tissues of 36 epithelioid MPM and 17 MH patients by NanoString, among which 25 epithelioid MPM and 15 MH samples were adequate for statistical analysis and became our training samples. By performing an unsupervised hierarchical cluster analysis based on the Pearson correlation, we determined that the entire panel was able to correctly group malignant and benign samples. Then, to make our approach directly reliable for clinical practice, we utilized the USC classification algorithm, which identified two classification models, including the most representative genes of MPM and MH. One model included 22 genes (*ASS1*, *BAP1*, *CAV1*, *CCNB1*, *CD44*, *CDH1*, *EGR3*, *FN1*, *ITGA3*, *KRT5*, *LAMA3*, *LGALS3*, *MICAL2*, *MMP9*, *MYH11*, *NME2*, *NMU*, *PAPPA*, *PECAM1*, *PKM*, *RAD21*, and *TGFBR2*), and the other model included 40 genes (*ASS1*, *BAP1*, *CAV1*, *CCNB1*, *CD44*, *CDH1*, *CDH11*, *COL4A2*, *CTNNA1*, *CXADR*, *EEF2*, *EGR3*, *EIF4G1*, *FANCI*, *FN1*, *GALNT7*, *GLI2*, *HEG1*, *IFITM1*, *ITGA3*, *KRT5*, *LAMA3*, *LGALS3*, *MAGED1*, *MICAL2*, *MMP9*, *MYH11*, *NME2*, *NMU*, *PAK4*, *PAPPA*, *PECAM1*, *PKM*, *PTGS2*, *RAD21*, *SDC1*, *SMARCA4*, *TGFBR2*, *TOP2A*, and *VEGFA*); both models were able to classify all training samples without any error (57). In the second part of our study, we utilized classification models to determine the diagnostic category of 14 pleural tissues (test samples) blindly analyzed, and all samples were correctly attributed to their diagnostic category (9 epithelioid MPM and 5 MH). The main advantages of our approach include a low RNA input required to analyze 117 genes (as low as 150 ng), which is obtainable from FFPE tissues, and the fact that the USC algorithm does not require a priori assumption; therefore, normalized NanoString data from all genes could be directly utilized for computational classification without any further manipulation of data. On the other hand, our system requires further validation on a larger number of samples to determine the best classification model and its positive and negative predictive values, and our system should additionally be tested on cytological specimens before introduction in clinical practice (57).

## Discussion

The aim of this review was to describe biomarkers and novel

approaches applicable to the differential diagnosis between malignant and benign pleural lesions. The best criterion to assess the malignancy of pleural lesions is still the presence of tumor invasion, which is not always easy to evaluate (2,3). Furthermore, some MPM patients are not eligible for pleural biopsy, and diagnosis has to be conducted on PEs, where diagnostic sensitivity is not satisfying (from 26% to 73%) (3).

Distinguishing reactive mesothelial cells from malignant ones is a difficult challenge for pathologists, and for this reason, during the last decades, many efforts have been focused on the identification of reliable diagnostic biomarkers.

In this context, we can mainly categorize the identification of reliable diagnostic biomarkers as the following: historical MPM biomarkers, including immunohistochemical ones (such as GLUT-1, p53, desmin, EMA, IMP-3) (5-13) and PE soluble ones analyzable by ELISA (such as mesothelin and fibulin-3) (41-47), which have been and are still extensively studied; emerging biomarkers recently introduced into clinical practice (*BAP1* analyzable by IHC and *p16* by FISH) (3); suggested MPM signatures based on miRNAs and mRNA expression panels (50-52); and new diagnostic tools based on molecular panels and classification algorithms (53,55-57).

Despite encouraging results reported in numerous studies, none of the IHC and PE soluble markers are highly sensitive for the separation of benign and malignant pleural lesions. Indeed, most of them, particularly the markers of malignancy, are informative only when positive and others present the same IHC stain or ELISA results for benign lesions as for MPM. In addition, there are no universally accepted cut-off values. We completely agree with Churg *et al.* in that these markers have a statistically differential expression when comparing benign and malignant lesion cohorts but they could not be useful in individual cases (4). To date, only *BAP1* and *p16* showed a specificity of 100% in discerning malignant from benign lesions, both in PEs and tissues (4). *BAP1* and *p16* markers appeared to be exclusively unexpressed or deleted in MPM, and their application in clinical practice is strongly suggested (3). However, *BAP1* and *p16* are not deleted or lost in all MPM tumors, and even when used together, negative results cannot confirm the benign nature of the lesions (31,36).

Thanks to genomic and transcriptomic studies, an increased number of genes have emerged as deregulated or altered in MPM (58), thus suggesting new biomarkers and diagnostic strategies. The latest research tendency to optimize MPM differential diagnosis is to define a

molecular signature primarily based on multiple genes or miRNA expression profiles. Indeed, MPM is an extremely heterogeneous tumor, and this possibly explains why neither single biomarkers nor a combination of a few biomarkers have ever reached adequate diagnostic sensitivity. Moreover, the advent of new molecular techniques not only allowed the analysis of multiple biomarkers with low input material but also overcame technical problems linked to IHC and FISH, like the quantity of biological material for the evaluation of a single marker and the need of well-trained pathologists for interpretation. Recently, the combination of molecular analysis by advanced molecular techniques and computational data, through classification algorithms, has acquired importance for MPM diagnosis.

Data concerning miRNAs and new diagnostic tools are absolutely promising, even if no molecular MPM signature can be translated in clinical practice without proper validation. Unfortunately, all reported studies in this review were retrospective and based on a low number of selected benign and malignant mesothelial proliferations, with the sarcomatoid histotype scarcely represented according to the rarity of this condition. Prospective validation regarding larger cohorts is warranted to accurately calculate positive and negative predictive values of suggested biomarkers and diagnostic tools as well as to estimate their actual cost-effectiveness.

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### Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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