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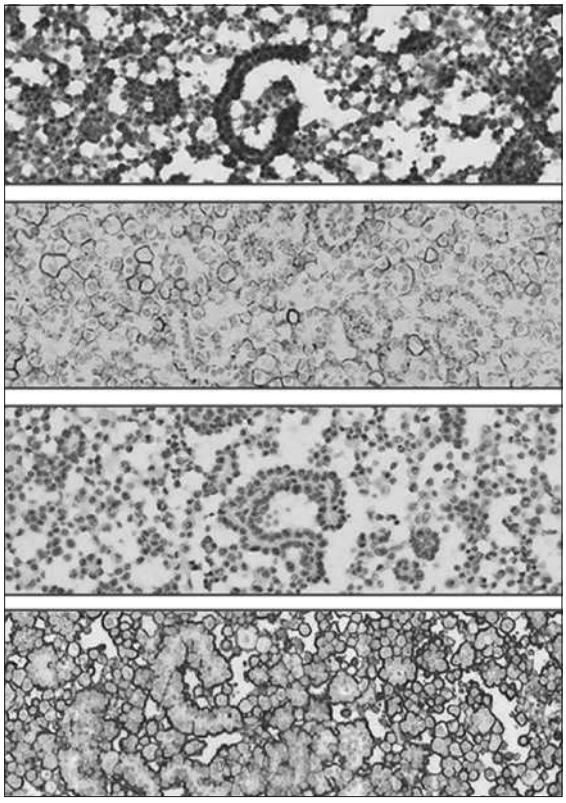
PATHOLOGICA

Journal of the Italian Society of Anatomic Pathology and Diagnostic Cytopathology,
Italian Division of the International Academy of Pathology



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SIAPEC-IAP
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Divisione Italiana della International Academy of Pathology

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PACINI EDITORE MEDICINA

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The journal welcomes contributions concerned with experimental morphology, ultrastructural research, immunocytochemical analysis, and molecular biology. Reports of work in other fields relevant to the understanding of human pathology may be submitted as well all papers on the application of new methods and techniques in pathology. The official language of the journal is Italian. Articles from foreign authors will be published in English.

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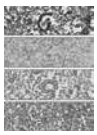

Front cover: Immunohistochemical evaluation on cell block sections consistent with a diagnosis of MPM: calretinin (A), podoplanin (B), TTF1 (C) and EMA (D) (page 15).

PATHOLOGICA 2018;110:12-28

GIPP (Gruppo Italiano di Studio di Patologia Pleuropolmonare) • Review

What's new in mesothelioma

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Key words

Malignant pleural mesothelioma • Cytology • Pleuritis • Prognostic and predictive biomarkers • BAP1 • c-MET • EMT • PD-L1 • CD9 • Immunohistochemistry • Familial cancer • Cancer predisposition syndrome • TPDS

Summary

Malignant pleural mesothelioma is a neoplasm characterized by a very poor prognosis and medico-legal implications. Diagnosis, prognosis and therapy are often challenging and include several issues. Cytological diagnosis is frequently the first step of the diagnostic process, and although its sensitivity may be somewhat lower, diagnostic criteria should be taken into account. When effusion cytology is inconclusive for the diagnosis, tissue biopsies should be taken. Even if the morphologic criteria for deciding whether a mesothelial proliferation is a benign or a malignant process have been defined, the separation of benign from malignant mesothelial proliferation is often a difficult problem for the pathologist, particularly on small biopsies. Thirdly, when the diagnosis is made, despite many efforts have been made to identify possible new biomarkers for early diagnosis, prognostic stratification and also predictive tools should be defined. Nowadays, the main prognostic parameter is still represented by the histological subtype, having the epithelioid MPM a better outcome than the sarcomatoid or biphasic MPM. A nuclear grading system have been also proposed to stratify patient outcome. Reliable predictive biomarkers are still lacking in MPM and a personalized therapeutic concept is eagerly needed. Mesothelioma occurs mostly as sporadic cancer and the main risk factor is asbestos exposure, but it also occurs among blood relatives suggesting possible increased genetic susceptibility besides shared exposures. Recently the study of genetic predisposition syndrome raised new aspect in the occurrence of mesothelioma cases.
This review summarize these most important issues.

Cytopathologic diagnosis of malignant pleural mesothelioma

Practical guidelines and recommendations

The guidelines for the diagnosis of malignant pleural mesothelioma (MPM) have been developed by various groups of expert pathologists [1 2]. More recently, a group of cytopathologists involved in the International Mesothelioma Interest Group (IMIG) and the International Academy of Cytology (IAC) and with an interest in the field contributed to compile the Guidelines for Cytopathologic Diagnosis of Malignant Mesothelioma [3-5]. The present section describes consensus opinions on how cytology together with adjuvant analyses can be used to establish a reliable diagnosis of MPM, based on the recently developed guidelines and on reference material including peer-reviewed publications [6] and textbooks.
The cytological diagnosis of a MPM is as reliable as that based on histopathology, although the sensitivity with cytology may be somewhat lower. Therefore, cytology with its ancillary possibilities should be considered as an accepted method for diagnosis of this malignancy, particularly with the first effusion. When effusion cytology is inconclusive for this diagnosis, tissue biopsies should be taken, the two techniques complementing each other. Some of the effusions do not display diagnostic features, but the negative finding does not exclude a diagnosis of MPM. The main obstacles for the diagnosis are (i) low content of diagnostic cells due to bleeding or inflammation, or recurrent effusions and repeated thoracentesis, (ii) when the tumor is a sarcomatoid MPM or it is dominated by a sarcomatoid component (mixed MPM); (iii) lacking experience of the cytopathologist and unaware-

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Tab. I. Protocol for processing effusions.

Fluid is collected with anticoagulant (EDTA 1 mg/mL or heparin 3 units/ml of fluid) and transported fresh to the laboratory. Addition of fixative is not recommended
Gross examination: volume, color, viscosity to be noted. Bloodstained effusions require hemolysis*
Any clot should be fixed in formalin
Centrifuge the whole sample to concentrate cells and separate the supernatant from the cell deposit
If the sediment is scarce prepare cytospins.
If the sediment is abundant prepare both wet-fixed and air-dried direct smears.
Stain with Papanicolaou and May-Grünwald-Giemsa.
Prepare cytospins and/or direct smears for ancillary staining techniques.
It is strongly recommended to process the residual cell deposit.
Routinely add formalin to the prepare cell-block.
Sediment can be stored at -80 °C as dry cell pellet or as vital cells in DMSO.
Supernatant aliquots can be stored at -80 °C.
After centrifugation add 5-10 ml of working solution to the sediment. Let incubate for 10 minutes in regriferator at 4 °C, and rinse cells repeatedly with isotonic saline.

*Hemolysis solution (to be kept refrigerated at 4 C°): 1 gr potassium carbonate + 8.3 gr di ammonium chloride + 0.037 gr EDTA in 1 liter of distilled water.

ness of this diagnostic possibility. Past or synchronous extraserosal cancer does not negate a diagnosis of MPM. Cytological samples are the first samples to examine in the diagnostic work-up of patients with MPM given that up to 90% of patients present with a serous effusion, regardless of histologic subtype (epithelioid, mixed, and sarcomatoid). Effusions are caused by increased permeability and reduced absorption of serous fluid trough lymphatics for the growth of multiple small tumor nodules on the serosal surface that ultimately may exfoliate into the serosal space. In early stages, serous effusions are voluminous (massive effusions) and re-accumulate repeatedly (recurrent effusions).

The whole effusion sample with anticoagulant should be submitted to the laboratory. A protocol for processing effusions is shown in Table I. Gross examination of samples should be noted. Fluids may be pale yellow, viscous for the high content of hyaluronic acid (Fig. 1A), bloodstained or overtly hemorrhagic. The cellularity is variable. Some effusions may contain sparse cells, others may be hypercellular. Centrifugation usually provides an adequate cell deposit (Figs. 1B and C) to prepare direct smears for staining with Papanicolaou (PAP) and May-Grünewald-Giemsa (MGG). Cytospins are prepared when the sediment is very scarce (Fig. 1D). The residual deposit and/or any formed clot can be processed for cell blocks (by previous fixation with formalin). Further aliquots can be stored for molecular studies. The acellular supernatant aliquot can be stored for mesothelin estimations or other biochemical testing [7].

The diagnosis includes two decisions:

(i) the establishment of a malignant effusion and (ii) defining the mesothelial origin of these malignant cells. The nuclear atypia may be evident and the malignancy uncontested, while the mesothelial phenotype can be less obvious or *viceversa* the nuclear atypia may be slight and the malignancy should be proven, while the mesothelial phenotype is obvious. Moreover, the diagnostic process can be very difficult due to the morphological variability from case to case.

Fig. 1. Pleural effusions after centrifugation: viscous fluid due to the high concentration of hyaluronic acid (A); blood-stained sediment (B); greyish-white optimal sediment (C); scarce blood-stained sediment (D).

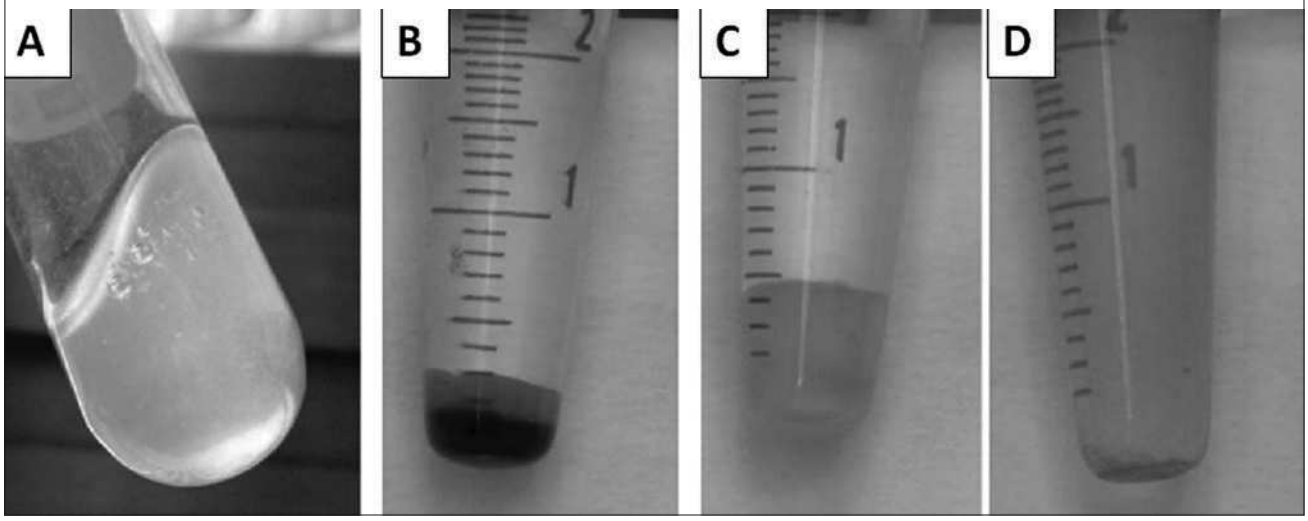


Fig. 2. Hypercellular smear (A). At higher magnification (B) note large, medium and small aggregates of mesothelial-looking cells, with peripheral shedding of orangiophilic cells (inset).

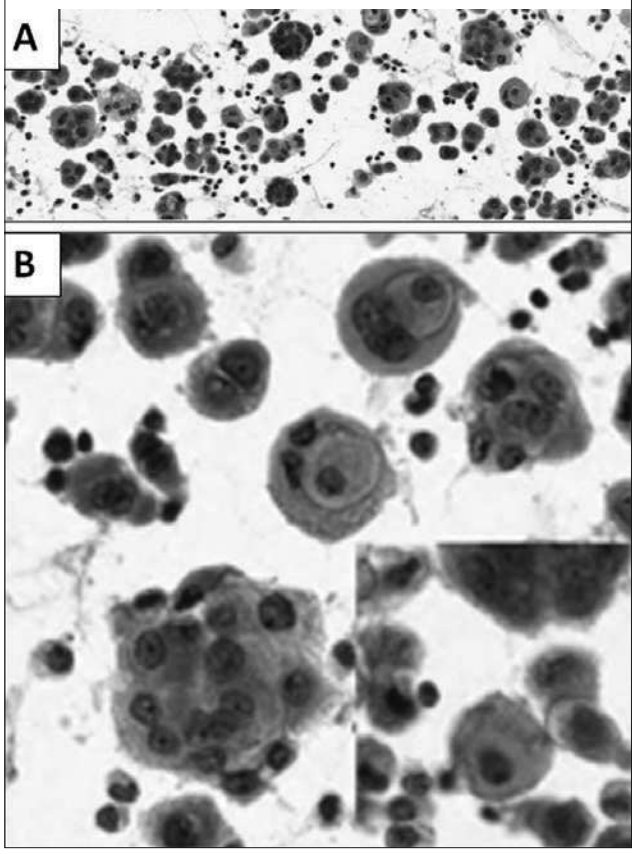
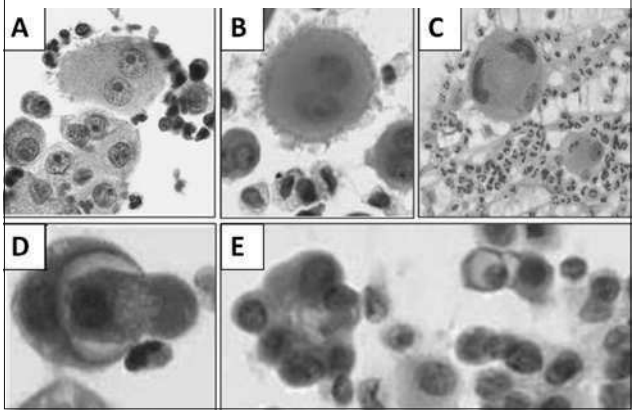


Fig. 3. Large mesotheliomatous cells with single macronucleoli are surrounded by lymphocytes and macrophages (A). Note the striking beard of microvilli at the cell periphery and the double staining of the cytoplasm (B). Multinucleated giant mesotheliomatous cell in a background of neutrophils (C). Fat granules dissolved out by alcohol leaving microvacuolation of the cytoplasm (D). A large cell surrounds a smaller one with a protrusion of the cytoplasm (E). Mesotheliomatous cells with bland morphology and orangiophilic squamous-like cells (F).



Pleural samples can be divided into three groups:

1. malignant effusions on cyto-architectural grounds;
2. effusions that require some form of ancillary testing to establish malignancy;
3. non-diagnostic effusions (minimal cell shedding, i.e., almost all sarcomatoid MPMs, and those epithelioid MPMs that do not have malignant cytomorphology, and that are negative for ancillary tests of malignancy).

The "typical" MPM cytological pattern is characterized by highly cellular samples (Fig. 2A), often including large, medium and small aggregates of mesothelial-looking cells (Fig. 2B), with peripheral shedding of cell balls of different size. All cells look alike (no evidence of two cell populations) and are either single, in duplets or in spherical (smooth surface) and berry-like clusters (scalloped surface) (Fig. 2B). In the background, which can be clean or dirty for inflammation and fibrin, there are small pyknotic orangiophilic squamous-like cells, an under-recognized and useful morphological feature [8] (Fig. 2B, inset).

Mesothelioma cells are significantly larger (5-10 times) than normal mesothelial cells. Nuclei are centrally located, are usually bland but show very prominent eosinophilic nucleoli (Fig. 3A). Cells may be bi-nucleated (Fig. 3B) or multinucleated (Fig. 3C). The cytoplasm is dense and abundant. In Pap-stained smears, mesothelioma cells show different staining in central and peripheral cytoplasm (a two-tone staining) and the "blebbing" with cytoplasmic protrusions at the plasma membrane (Fig. 3B). The cytoplasm often contains very small vacuoles that represent ethanol-extracted lipids that are best seen in MGG stained smears (Fig. 3D). A suggestive finding that establishes the mesothelial origin is the occurrence of intercellular slits or 'windows' and cell-engulfment (Fig. 3E). Mesothelioma cells frequently show bland morphology and this is why they sometimes get missed (Fig. 3F).

Factors leading to diagnostic pitfalls are low content of diagnostic cells also due to bleeding (Fig. 4A), heavy inflammation in the background (Fig. 4B) mainly granulocytes and macrophages; malignant cells are hidden within fibrin and intermingled with inflammatory cells (Fig. 4B, inset).

Atypical cytomorphological variants are not rare: a not choesive pattern (Fig. 5A) characterized by a striking numbers of large cells [9]; lymphocyte-rich pattern (Fig. 5B) and poorly differentiated cell pattern (Fig. 5C).

A diagnosis of MPM should be supported by immunocytochemistry as ancillary technique to demonstrate mesothelial lineage of tumor cells and mesothelioma cells *versus* reactive mesothelium. This can be done on cell blocks (reliable and reproducible) as well as on smear/cytospin preparations. It is widely recommended to use a panel of at least four antibodies, two in favor and two against MPM to demonstrate/exclude the mesothelial lineage of a tumor cell population (Fig. 6). Antibodies to be included in the battery are Calretinin, Podoplanin (D2-40), WT1 and Cytokeratin 5/6 (positive markers) and CEA, BerEp4, MOC31, and CD15 (negative markers). The use of antibodies indicating primary site are also often helpful. Thus, reactivity to TTF1 and Napsin A, common in lung adenocarcinomas will exclude a mesothelioma.

Fig. 4. Heavy blood contaminated smear (A). Smear dominated by necrotic material and inflammatory cells (B); at higher magnification note a very large cell neoplastic cell (B inset).

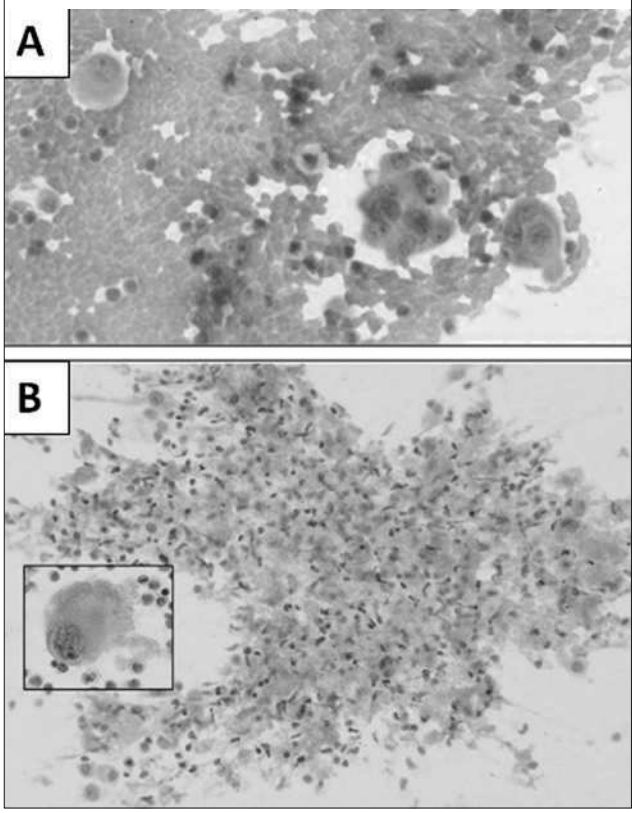


Fig. 5. Not cohesive pattern characterized by single large mesothelial looking neoplastic cells (A). Tumor cells are infrequent and intermingled with numerous lymphocytes (B). Very large tumor cells showing the double color of the cytoplasm and the covering of microvilli and blebs at the cell periphery (C).

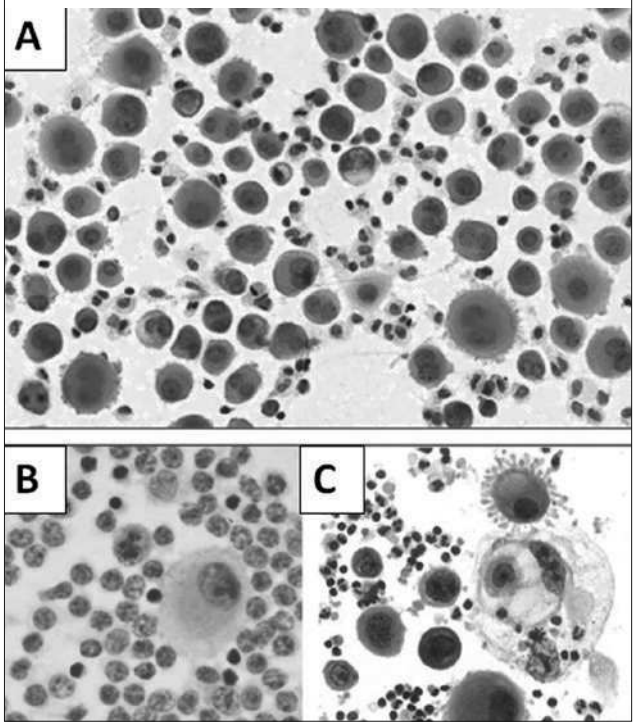


Fig. 6. Immunohistochemical evaluation on cell block sections consistent with a diagnosis of MPM: calretinin (A), podoplanin (B), TTF1 (C) and EMA (D).

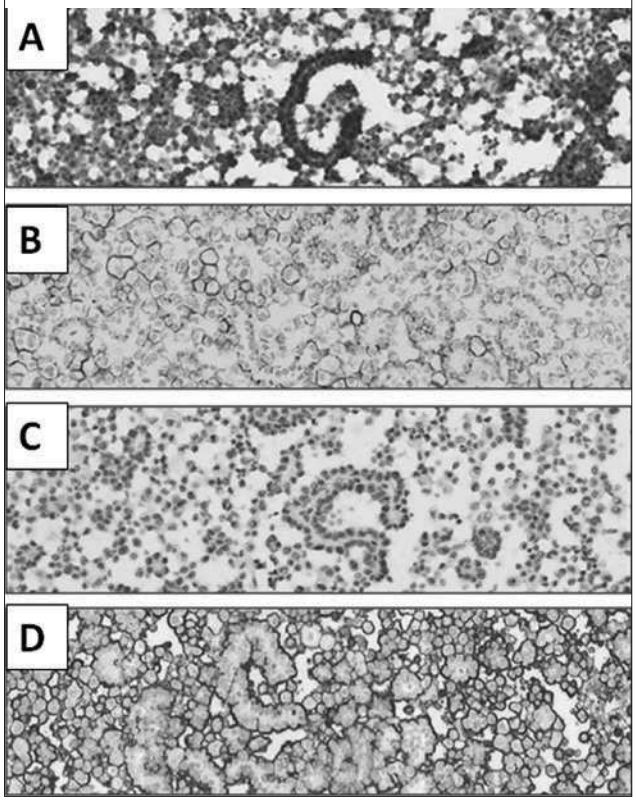
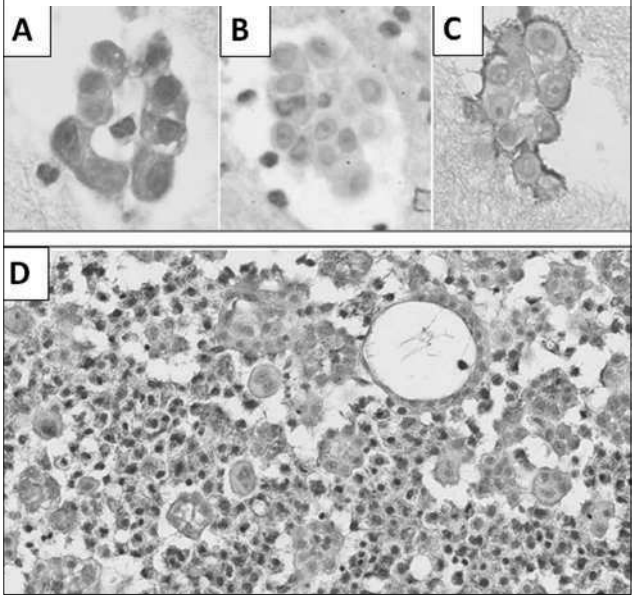


Fig. 7. Immunohistochemical evaluation for MPM vs reactive mesothelium: calretinin (A), desmin (B), EMA (C) and BAP1 (D). BAP1 stained sections from a pleural effusion associated with mesothelioma. All the neoplastic cells show completely negative staining for BAP1. Positive nuclear staining in not neoplastic and inflammatory cells is noted and acts as an internal positive control.



When the mesothelial lineage is demonstrated with Calretinin (Fig. 7A), it is difficult to distinguish reactive mesothelial cells from malignant ones. Absence of Desmin immunoreactivity is a strong indicator of malignancy (Fig. 7B). EMA with accentuated reactivity at the cell membrane is often used to support the diagnosis of MPM (Fig. 7C). The use of Desmin/EMA double stains has been recommended [10]. Loss of expression of BAP1 is a useful adjunct (Fig.7D), which strongly supports the diagnosis of MPM in effusion cytology [11]. However, interpretation of BAP1 immunohistochemistry on cell block may be difficult and that convincing positive staining in not neoplastic cells is required before atypical cells are considered negative BAP1 loss is not a sensitive test as it occurs in only half of all MPM and cannot be used to exclude the diagnosis

Optional ancillary studies that have been recommended (but not usually available) are electron microscopy, Fluorescent In Situ Hybridization (FISH) analysis of ploidy (a homozygous deletion of the 9p21) and ELISA (i.e for soluble mesothelin).

The two main differential diagnoses to MPM are adenocarcinoma and benign, reactive mesothelial cells.

MESOTHELIOMA *VS* ADENOCARCINOMA

Knowledge of prior cancer should not exclude a cytologic diagnosis of MPM. When malignancy is established the main question is whether malignant cells are from MPM or from metastatic cancer (adenocarcinoma is the more frequent type) or other malignancies that can mimic MPM. The first characteristic to be considered is the architecture (the arrangement of neoplastic cells) that can be in groups or as single cells. Cells in metastatic groups are usually haphazardly arranged. By contrast in mesotheliomatous groups, neoplastic cells are monotonously arranged. In adenocarcinoma individual cell morphology shows nuclear irregularity (size, shape) and multiple nucleoli (marked atypia). These features are not the rule in MPM. Nuclei are centrally located in MPM while show polarity in adenocarcinomas. Immunoistochemistry is the basis in the differential diagnosis. Although the literature suggests at least two positive markers for mesothelioma and at least two positive markers for carcinoma, most pathologists usually do use more antibodies, or less [12]. Gender and age of the patient and site of the effusion are issues in selecting antibodies.

MESOTHELIOMA *VS* REACTIVE MESOTHELIUM

Reactive mesothelial cells are seen in a variety of systemic diseases (LES, rheumatoid arthritis) and local conditions (pneumonia, lung infarct). Effusions may also occur after radiotherapy or chemotherapy. Benign effusions do not recur whereas MPM do recur. Therefore, a clinical based approach to fluid cytology is mandatory. Mesothelial cellularity is variable in both conditions. Inflammatory cells as well cannot be used as criterion to distinguish the two entities. Reactive mesothelial hyperplasia is usualy characterized by monolayers of flattened mesothelial cells (MPM is are characterized by tridimensional balls); however, few papillary groups may be formed; nucleoli may become prominent. Mitoses may be plentiful in reactive conditions as opposed to few in MPM but clearcut atypical mitoses favor malignancy. It would be useful to mention cytologic atypia of mesothelial cells in the final cytologic report with a recommendation for follow up and cytologic re-evaluation for possible recurrent effusions. Cytologic atypia can be present in organizing benign effusions. Necrosis (abundant) in the background is usually a sign of malignancy.

CONCLUSIONS

- Features favouring reactive mesothelium: clusters may be present but not as tight as spheres.
- Features favouring adenocarcinoma: clusters of pleomorphic cells with obvious atypia.
- Features favouring mesothelioma: morules and dischoesive cells with mild/moderate atypia Be careful to underestimate low cellular effusions containing atypical mesothelial cells or high cellular effusions containing bland mesothelial cells with a morular pattern.
- It should be to considered that an inflammatory background may obscure a scant number of mesotheliomatous cells.
- It is better to report effusions devoid of mesothelial cells as non-diagnostic instead of negative.
- In the final cytologic report it would be useful to mention cytologic atypia of mesothelial cells with a recommendation for follow-up and cytologic re-evaluation for possible recurrent effusions.

Separation of benign and malignant mesothelial proliferations

The separation of benign from malignant mesothelial proliferation is crucial to patient menagement, but it is often a difficult problem for the pathologist.

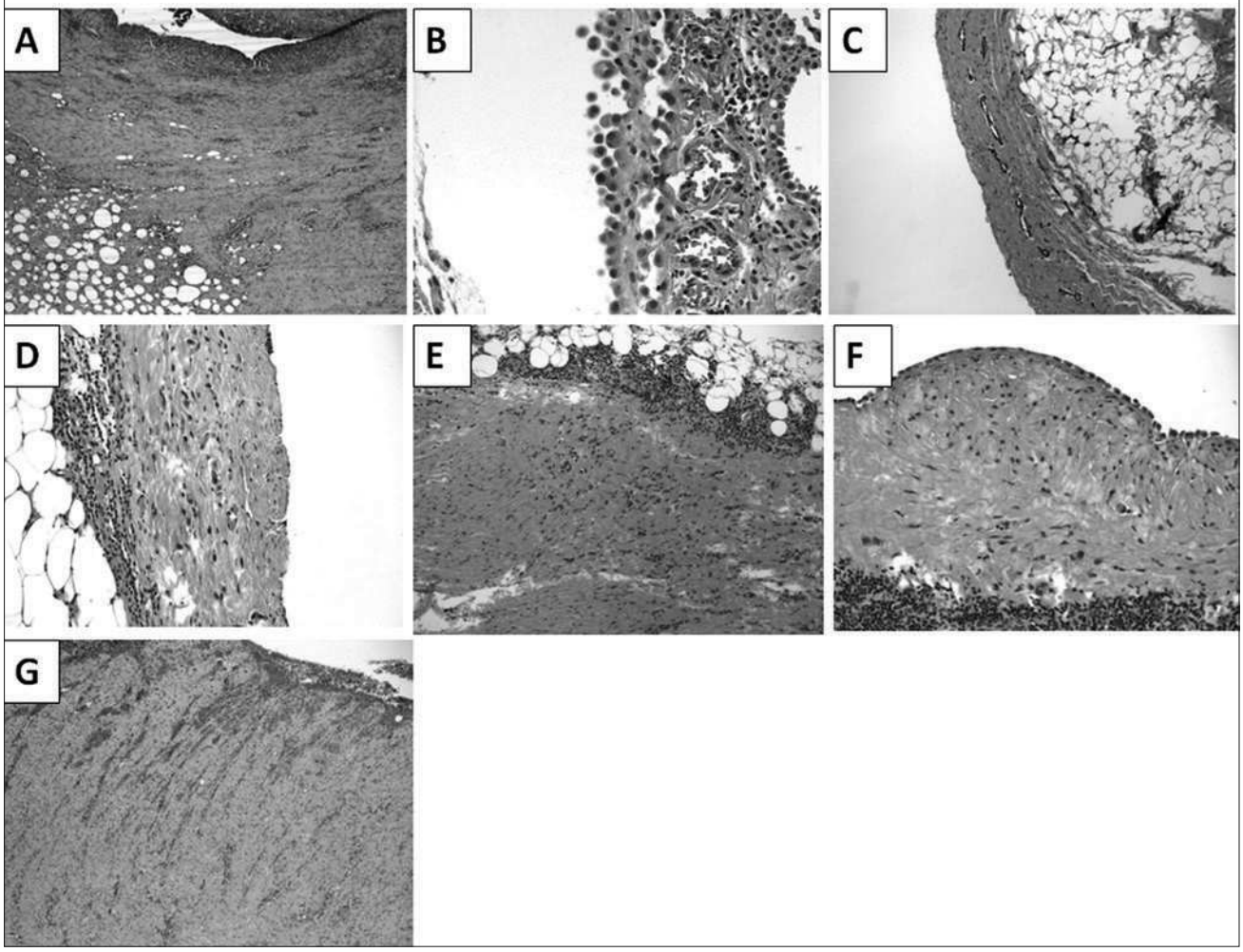
The morphologic criteria for deciding whether a mesothelial proliferation is a benign or a malignant process have been defined [13-15]. However, in the routinely diagnosis, their application is often challenging, particularly on small biopsies.

Benign, reactive pleural lesions usually presents a combinations of mesothelial hyperplasia and organizing pleuritis showing different morphologic appearance to be consider in distinguishing from epithelioid or sarcomatoid mesotelioma.

MESOTHELIAL PROLIFERATIONS *VS* EPITHELIOID MESOTHELIOMA

Histologic features suggesting a benign mesothelial proliferation include an increased cellularity confined to the pleural surface; “zonation” – progressive loss of cellularity and increasing fibrosis from the surface to the chest wall; entrapment of mesothelial cells in areas of organization, restricted to the submesothelial fibrous layer. Increased cellularity throughout the pleura; nodular espansion of stroma; desmoplasia and atypical mitosis are

Fig. 8. Mesothelial hyperplasia with high cellularity limited to the surface of the pleura. A loss of cellularity is seen from the surface to the chest wall (A). Visceral pleura with atypical mesothelial hyperplasia: there is an increased number of mesothelial on the pleural surface with some atipia. Entrapped mesothelial cells with nuclear atipia are seen beneath the surface simulating invasion (B). Entrapped mesothelial cells showing a tubular pattern arranged in a parallelly to the pleural surface. No cellular atipia is seen (C). Epithelioid Mesothelioma. Atypical mesothelial cells are distributed throughout the pleura (D). Epithelioid Mesothelioma. Cellularity throughout the pleura without orientation is diagnostic for mesotelioma (E). Nodular desmoplasia and atypical cells throughout the pleura are features suggestive for mesothelioma (F). G. Fibrous pleuritis showing decreasing cellularity away from the pleural surface and long capillaries perpendicular to the surface.



features suggestive of malignancy. The deeper invasion of the stroma remains the best criterion for diagnosing malignant mesothelioma [14-16] (Fig. 8 A-G).

Organizing/fibous pleuritis

Organizing/fibrous pleuritis is associated with "zonation", not seen in sarcomatoid/desmoplastic mesotelioma. Long capillaries oriented perpendicular to the surface of the pleura; inflammatory necrosis; orientation of cellularity toward the pleural surface are features suggestive of benignity [16]. Stromal invasion; extensive storiform, disorganized growth pattern; sarcomatous foci anywhere in the lesion; bland necrosis support the diagnosis of sarcomatoid/desmoplastica mesothelioma. Both benign and malignant spindle cell proliferations are pa-keratin – positive, but CK7 is less express in reactive spindle cell proliferations compared to malignant sarcomatoid mesothelioma [17].

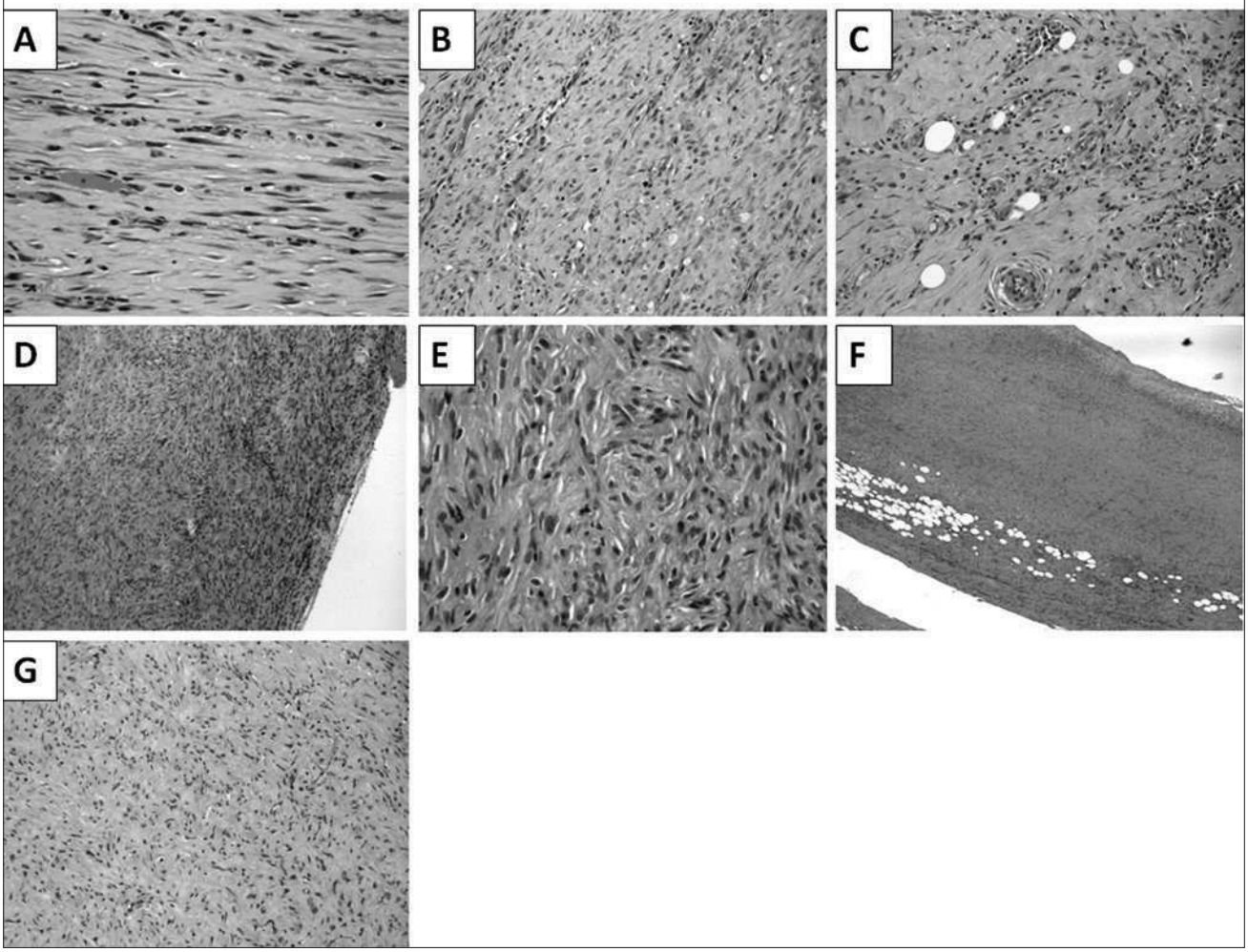
A possible diagnostic pitfall is the "fake fat" phenomenon in organizing pleuritis tha represent a traction artifact caused by biopsy. "Fake fat" presents as round or elongated spaces in fibrotic tissue, aligned parallel to the pleural surface with mesothelial cells in between, mimicking fat invasion. In difficult cases, immunostining for S-100 and/or calretinin may be helpful to distinguish "Fake fat", negative to these antibodies, from true fat that is positive to both molecules [18] (Fig. 9 A-G).

Role of immunohistochemistry

In the recent past, some reports suggested that p53 and EMA are positive only in malignant mesothelaial proliferations and other articles indicated desmin, commonly expressed in benign reactions, as a stain that provides separation from benign and malignant lesions of the pleura [19 20].

More recently glucose transporter 1 (GLUT-1) and insu-

Fig. 9. Fibrous pleuritis with increased cellularity. The spindle cells are regularly distributed and separated by mature collagen (A). Other example of fibrous pleuritis: spindle cells with plump nuclei and vessels are arranged perpendicularly to the surface. No mitosis or necrosis are seen (B). "Fake fat" phenomenon: round spaces in fibrotic tissue. They are negative for S-100 protein (C). Sarcomatous mesotelioma. It is tipically composed by spindle cells arranged in short storiform fascicles (D). Sarcomatous mesotelioma. Atypical spindle cells and mitotic figures (E). Desmoplastic mesotelioma: bland spindle cells proliferation without zonation. The cells are arranged in short fascicles and throughout the pleura, invading the adipose tissue (F). Desmoplastic mesothelioma: atipical cells with angolate nuclei and inapparent cytoplasm arranged in short fascicles with a patternless pattern (G).



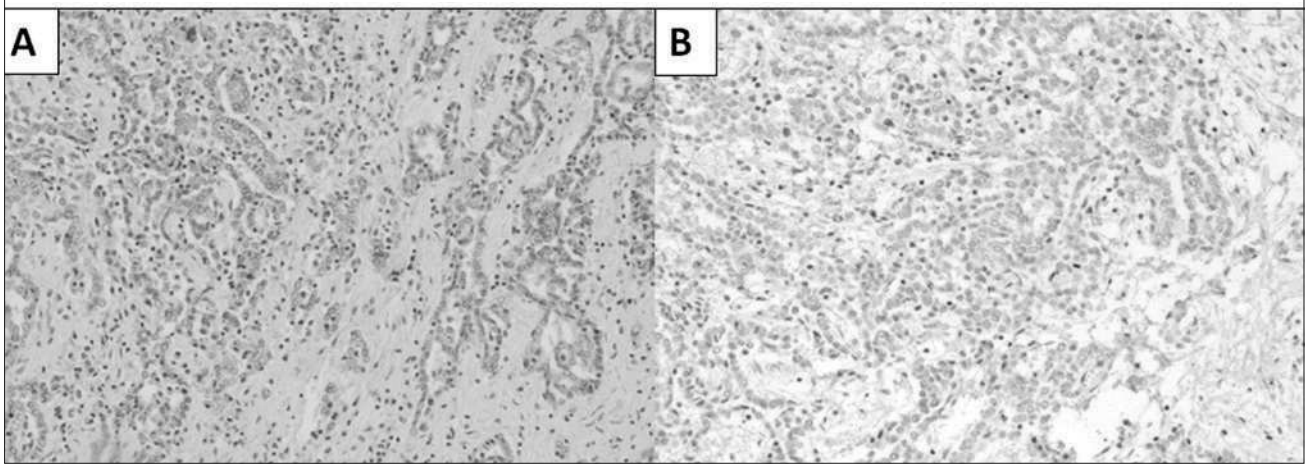
lin-like growth factor II messenger RNA-binding protein 3 (IMP-3) have been reported to have utility in differentiating benign and maligant mesothelial proliferations. GLUT-1 and IMP-3 show positivity in mesothelioma and negativity in reactive process, but this claim is refuted in other studies. Recent studies suggest that, even in combination, all these markers offer a limited help in this setting [21-23].

A limited value seems to have also the expression of proliferation markers such as Ki67 and or Repp86, a membrane of microtubules-associated protein that identifies e nuclear proliferation-specific protein expressed during the cell cycle [24].

Molecular studies have demonstrated that mesotheliomas commonly show deletion of the 9p21 region [25], resulting in loss of cicli-dependent kinase inhibitor 2 (CDKN 2, p16) and somatic mutation of BRCA-associated protein-1 (BAP-1). Recent studies have shown that loss of p16 gene, detected by FISH and the loss of BAP-1, detected by immunohistochemistry are potentially usefull in distinguishing benign, reactive mesothelial lesions from malignant mesotelioma [26 27]. A number of studies have clearly demonstrated that the majority of mesothelioma are positive for the p16 gene deletion, whereas none of the benign/reactive cases are positive for the deletion (100% specificity) in tissue sections and in effusion cytology specimens. For pleural epithelial mesotheliomas, sensitivity ranges from approximately 45 to 85%. In some reports sarcomatous mesotheliomas fare better, with deletion reported in up to 100% of cases, while other reports a lower expression of p16-deletion in sarcomatous mesotheliomas [28-30].

Recent studies have been demonstrated that BAP1 protein, detected by immunohistochemistry or by FISH, is frequently lost in a large proportion of epithelioid or biphasic mesotheliomas with a sensitivity higher than

Fig. 10. BAP1immunohistochemistry in an epithelioid MPM. The MPM neoplastic cells are negative for BAP1, while inflammatory not neoplastic cells retain BAP1 positivity. (A: H&E, B: IHC).



60%. In this contest, the use of BAP1 immunostain can be useful in the differential dignosis between benign and malignant mesothelial proliferations. More controversial is the utility of BAP1 immunostain in differential diagnosis between fibrous pleuritis and sarcomatoid mesotelioma in which BAP1 loss has been observed in less than 20% of cases [27].

Finally, a recent study has demonstrated that a proportion of mesotheliomas exhibit also loss of neurofibromin 2 (NF2) and large tumor suppressor kinase 2 (LATS1/2) both detected by FISH that should be useful in differentiating benign form malignant lesions of the pleura. This has not been confirmed by immunohistochemistry studies [31].

Up to now, the combined use of p16 FISH and BAP-1 immunoistochemistry probably represents the best approach in differentiating benign from malignant mesothelial proliferations expecially in problematic cases in which the stromal invasion is not clearly demonstrated. Much more controversial is the role of these markers in differential diagnosis between fibrous pleuritis and sarcomatoid/desmoplastic mesotheliomas.

Prognostic and therapeutic-predictive biomarkers

Prognostic Biomarkers

BRCA1-Associated Protein 1 (BAP1)

BAP1 gene is located at chromosome 3p21.1 and encodes BRCA1-associated protein 1 (BAP1) [32]. It is involved in regulation of DNA transcription, cellular growth, regulation of cell cycle, response to DNA damage and chromatin dynamics including chromatin remodeling [33 34]. As other chromatin remodelers, it has been considered a tumor suppressor as its bi-allelic inactivating mutations or deletions have been found in a wide range of tumor types [35 36]. Recently a BAP1-germline mutation syndrome has been described and is associated with an increased risk of cancers like cutaneous and uveal melanoma, cutaneous basal cell carcinoma, renal cell carcinoma and malignant mesothelioma [37]. Notably, the mutational status of BAP1 can be easily investigated with immunohistochemistry (IHC). Indeed most mutations in BAP1 result in a truncated protein, prone to degradation, thus BAP1 gene mutations are highly associated with loss of protein expression and thus with a negative IHC. The most important application of *BAP1* as biomarker is as a diagnostic immunohistochemical tool, since it allows to better distinguish between malignant (loss of *BAP1*) and benign (presence) mesothelial proliferations [27].

BAP1 mutated mesotheliomas represent a peculiar subgroup of this tumor type, with an onset at a younger age and a more common epithelioid morphology than *BAP1*-wild type mesotheliomas [38] (Fig. 10). Interestingly, a recent meta-analysis suggests a possible protective role of *BAP1* mutation in mesothelioma, but nowadays there are too few studies on this topic to have definitive indications [35]. Furthermore, Baumann et al. showed in multivariate analysis that *BAP1*-germline mutated mesothelioma patients had a 7-fold increased long-term survival than *BAP1*-wild type patients, suggesting that *BAP1* mutation status may ameliorate the prognosis in the case of germ-line predisposition [39].

Receptor tyrosine kinases (RTK) and other cell membrane proteins

c-MET is an important RTK, commonly overexpressed in cancer, also including MPM [40]. In a collaborative study by the MESOPATH group, a higher c-MET staining intensity and its localization to the membrane, compared to co-expression at the membrane and cytoplasm, or exclusively cytoplasmic localization, were associated with longer overall survival [41].

Pinato et al. recently investigated in MPM the protein expression of Axl by IHC; it is a RTK which mediates

cell survival and epithelial-to-mesenchymal transition (EMT). A higher Axl expression has been significantly associated with epithelioid histology and longer overall survival [42]. Another recent promising biomarker in MPM is Syndecan-1, a member of the Syndecan family; as most important functions, it mediates adhesion, cytoskeletal organization, and cellular proliferation [40]. Syndecan-1 was overexpressed in epithelioid compared to sarcomatoid MPM, and its presence resulted associated with longer overall survival [43].

Several other surface biomarkers have been studied for their prognostic role in MPM. For example, Alifano et al. studied by IHC the expression of neurotensin (NTS), a regulator of intestinal motility, smooth muscle activity and epithelial proliferation. Its expression was associated with poor overall survival [44]. The expression by IHC of another protein, CD9, was significantly related to younger patient age, epithelioid histology and better differentiation. CD9 is a member of tetraspanins, a family of membrane glycoproteins which may have tumor-promoting or -suppressing effects, and which are involved in adhesion, invasion, metastasis and angiogenesis. Its expression was associated with longer overall survival [45 46].

Another promising biomarker has been described among aquaporin family, a family of transmembrane channels physiologically involved in water transport, also involved in tumor progression in various malignancies. Aquaporin 1 was associated with significantly longer overall survival [47]. A negative prognostic biomarker, linked with poorer prognosis, is caveolin-1. It is a protein involved in endosomal transport, adhesion and signaling pathways. Its expression has been studied peri-tumoral stromal cells of epithelioid MPM [48].

Epithelial-to-mesenchymal transition (EMT)

EMT is a physiological process during embryogenesis, crucial for formation of the different germ cell layers. Pathological EMT in the context of cancer occurs when tumor cells lose their epithelial features, acquiring certain mesenchymal properties that promote extra-cellular matrix invasion and distant metastasis [40 49]. Fassina et al. analyzed 109 MPM, of which 74 were pleural, for the expression of some of the molecules involved in EMT, like cadherins, matrix metalloproteinase and the E-cadherin suppressors Snail, Slug, Twist, Zeb1 and Zeb2, using IHC and quantitative PCR (qPCR). They described that patients with epithelioid MPM who had higher E-cadherin expression had longer survival [50].

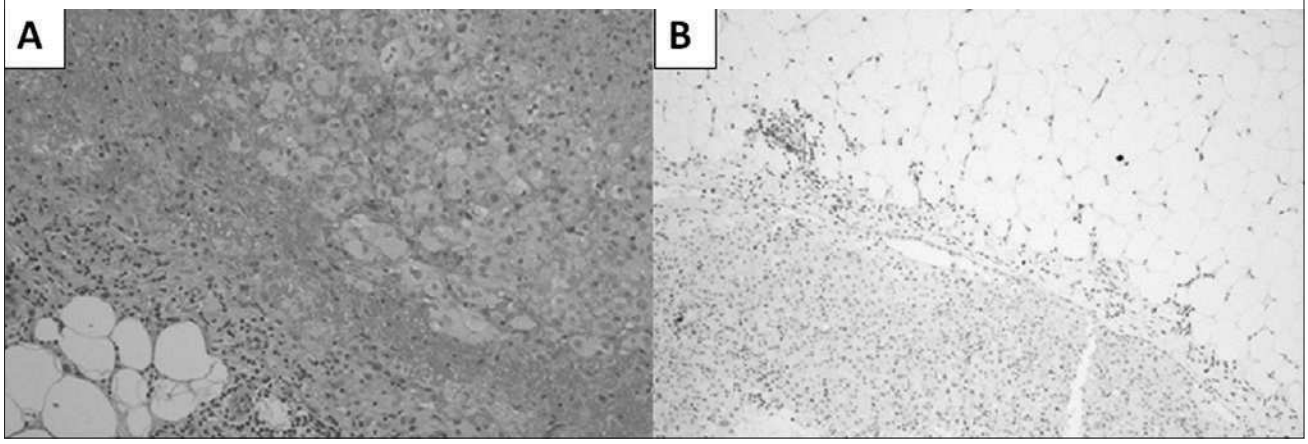
In another study, Snail expression was associated with longer overall survival [51]. A different finding was described by Kobayashi et al., that showed significantly poorer survival for patients with Snail-expressing tumors [52].

Other important biomarkers

Cell cycle dysregulation is a critical aspect of all malignancies; cyclins are a group of proteins that is fundamental in cell cycle control, interacting with the cyclin-dependent kinases (CDKs), another family of protein which in turn regulate the activity of specific transcription factors. The CDKs are also regulated by CDK inhibitors including p14/ARF, p16/INK4A and p21/Cip/WAF1 already demonstrated in MPM with variable expression [53]. In general the expression of CDK inhibitors is often lost in most aggressive tumors, correlating with worse survival. The CDKN2A gene encodes the p14/ARF and p16/INK4A proteins. A recent work of Jennings et al. shows that MPM with intermediate or high p16/INK4A expression had a significantly better post-diagnosis survival than MPM with lost p16 expression [54]. Those patients with sustained p16/ INK4A expression who received chemotherapy also had a better survival than those treated patients whose tumors had lost p16/INK4A expression (log-rank Po0.001); the Authors conclude that a sustained p16/INK4A expression predicts better post-diagnosis survival in MPM and also better survival following chemotherapy (Fig. 11) [54].

Another important biomarker in MPM is certainly rep-

Fig. 11. p16 immunohistochemistry in a representative malignant pleural mesothelioma case. In this case of MPM the neoplastic cells showed a diffuse and intense positivity for p16, which seems to correlated to a significantly better post-diagnosis survival than MPM with lost p16 expression. (A: H&E, B: IHC).



resented by E3 ubiquitin ligase, also known as MDM2, a fundamental regulator of P53 stability and activity [55]. Overexpression of MDM2 in some tumors including lung, breast, colon, stomach and hepatocellular carcinomas can lead to a loss of P53 regulatory function by increased proteasomal degradation of P53 [55 56]. Notably, approximately 20% of all MPM show strong nuclear MDM2 expression, restricted to epitheloid MPM or the epitheloid component of biphasic MPM; these MDM2-positive MPM show significantly decreased overall survival [57]. The physiological inhibitor of MDM2 is P14/ARF; loss of P14/ARF activity may have a similar effect as loss of P53 [58]. P14/ARF is recognized as a tumor suppressor inducing cell cycle arrest in a both P53-dependent and independent manner [59 60]. Notably, a recent study indicates that MDM2 mRNA and protein expression correlated highly significantly with overall and progression-free survival in MPM, showing a poor prognosis for patients with elevated MDM2 expression [59]. In such study, MDM2 has been indicated as a prognostic and predictive marker for a platin-pemetrexed therapy of patients with MPM; at the same time, downregulation of P14/ARF expression seems to contribute to MDM2-overexpression-mediated P53 inactivation in such patients [59].

Lastly, two markers useful in diagnosis have been investigated on the basis of their prognostic significance in a recent immunohistochemical study: Wilms' tumor antigen (WT1) expression was associated with longer overall survival, whereas calretinin expression was unrelated to survival [61].

Biomarkers from high-throughput sequencing

In a near future, high-throughput methodology will be more and more in use for helping diagnosis and prognostication of several tumors, following the model of a so called "next-generation histopathologic diagnosis" [62]. Gordon et al. identified a prognostic profile of 46 genes, of which the ratios of 4 genes (KIAA0097, GD1A1, L6-related EST, CTHBP) were found to provide the most accurate prognostic information in MPM [63].

A subsequent study from this group led to identification of an 8-gene set, including 4 genes associated with good outcome (EST DKFZp586J2118, CD9, DLG5, C3) and 4 related to poor outcome (KIAA1199, 2 copies of CD24, THBD) in MPM [64 65]. Notably, López-Ríos et al performed gene expression array analysis of 99 MPM from a cohort in which advanced stage, sarcomatous histology and the presence of p16/CDKN2A deletions resulted as independent poor prognostic parameters in multivariate analysis [66].

The main prognostic factors in MPM are summarized in Table II.

Predictive biomarkers

Nowadays, for MPM the first line treatment is represented by pemetrexed-platinum [67]. The main target of pemetrexed is thymidylate synthase (TS), a protein encoded by the gene TYMS; recent studies have shown a significant negative impact of elevated tumor TS as well as TYMS on both response and survival [68 69]. Furthermore, as already discussed in the prognostic factor, MDM2 is an important biomarker in MPM; notably, it is not only a good prognostic factor but also a useful predictive marker for a platin–pemetrexed therapy of patients with MPMs [59].

A recent study, moreover, indicates that the expression of peculiar DNA repair markers, like nuclear ribonucleotide reductase M1 (NRRM1) and excision repair cross complementation group 1 (ERCC1), is as independent prognosticators for freedom from recurrence of MPM in patients undergoing induction chemotherapy followed by extrapleural pneumonectomy [70].

Vinorelbine is an antitubuline often used in the second-line treatment of MPM; increased survival in patients treated with vinorelbine-cisplatin correlated with com-

Tab. II. Main prognostic biomarkers in malignant pleural mesothelioma.

Biomarkers	Significance	Role
BAP-1	Better prognosis*	Chromatin remodeler
c-MET	Better prognosis if overexpressed	Receptor tyrosine kinases
Axl	Better prognosis if espresse	Receptor tyrosine kinases
Syndecan-1	Better prognosis if overexpressed	transmembrane heparan sulfate proteoglycan
Neurotensin	Worse prognosis if espresse	Regulator of intestinal motility, smooth muscle activity and epithelial proliferation
CD9	Better prognosis if espresse	membrane glycoproteins
Aquaporin 1	Better prognosis if espresse	transmembrane channels
Caveolin-1	Worse prognosis	Protein involved in endosomal transport, adhesion and signaling pathways
E-Cadherin	Better prognosis	EMT factor
Snail	Equivocal data	EMT factor
p16/INK4A	Better prognosis with intermediate or high expression	CDK inhibitors
MDM2	Worse prognosis if expressed	Regulator of P53 stability and activity
WT1	Better prognosis if expressed	Transcription factor

*The diagnostic power of BAP1 has already well established; at the same time its prognostic significance in mesothelioma has not been definitively accepted, although its mutation seems to ameliorate the prognosis [35].

bined low ERCC1 and the beta-tubuline class III protein in tumor [71].

Recent reports indicate that MPM is an immunogenic tumor which induces immune recognition, infiltration of immune cells and death mediated by autoimmunity [72-74]; moreover, clinical studies show that lymphocyte invasion influences prognosis in MPM [73 75]. About this topic, one of the most important novelty in cancer therapy is now represented by the mechanism involving the programmed cell death-1 (PD-1) and its ligand (programmed cell death-1 ligand-1, PDL-1) [76]. The programmed cell death (PD-1/PD-L1) pathway plays a fundamental role in limiting the activity of T cells in peripheral tissues at the time of an inflammatory response to infection and in limiting autoimmunity. Notably, in tumors this pathway controls the tumor immune escape; PD-1 receptor is a negative regulator of T-lymphocyte and has a role as a co-inhibitory receptor to prevent off target immune activation [77-80]. PD-1 binds to PD-L1, the predominant mediator of immunosuppression; binding of PD-L1 to its receptor PD-1 inhibits proliferation of activated T cells in peripheral tissues leading to "T-cell exhaustion", a T cell hyporeactive condition [79].

In a recent study about this pathway in MPM, PD-L1 was expressed in 20% of MPM; PD-L1 negative patients had a significantly better prognosis than the positive patients. The effect of PD-L1 status on prognosis was indistinctive of the histology [76]. Pembrolizumab is an anti- Programmed Death 1 (PD-1) antibody approved for selected cases of melanoma, non-small cell lung cancer and investigated in malignant pleural mesothelioma [81 82]. It disrupts the engagement of PD-1 with its ligands and impedes inhibitory signals leading to recognition of tumor cells by cytotoxic T cells. Preliminary results of a single arm trial with 25 pretreated PD-L1- positive mesothelioma patients demonstrated a surprising response rate of 28%, a disease control rate of 76% including durable response rates, and a progression free survival rate at 6 months of 49% [81 82].

The immunotherapy and the sequencing-based targeted therapy seem to represent the future in the battle against cancer, above all for those with poor prognosis, of which MPM is one of the most deadly.

A nuclear grading system for mesothelioma

Despite aggressive therapy, the prognosis of diffuse malignant pleural mesothelioma (MPM) remains poor with a median survival of 9-12 months, that can be related to the paucity of prognostic factors in stratifying patients for therapy and clinical outcomes [83]. In fact, an improved prognostic stratification is mandatory to optimize treatment strategies and to include patients in specific clinical trials. To date, epithelioid histology is a strong prognostic factor in MPM and confers a better prognosis compared with biphasic and sarcomatoid histology [84 85]. Only a few studies proposed grading systems on the basis of morphology and some interesting results were obtained. A preliminary study performed on a large case series (232 epithelioid malignant mesothelioma) proposed a grading system using nuclear atypia and mitotic count [83]. In this study, the following features were evaluated: nuclear atypia, nuclear/cytoplasmic ratio, chromatin pattern, intranuclear inclusion, prominence of nucleoli, mitotic count and atypical mitoses. In univariate analysis different aspects correlated with a poor survival but multivariate analysis showed that nuclear atypia and mitotic count were the only independent prognostic factors. These two factors were used to develop a three-tier nuclear grade score. The resulting nuclear grade stratified patients into three distinct prognostic groups: grade I (n = 107, median overall survival = 28 months), grade II (n = 91, 14 months), and grade III (n = 34, 5 months). Moreover, nuclear grade was associated with the time of recurrence and its prognostic value was confirmed by MIB1 labeling index: it correlated with mitotic count, nuclear atypia and stratified time to recurrence and overall survival. Methodological details are described below. Nuclear features and level of mitoses have been recently correlated with overall survival in malignant peritoneal mesothelioma with epithelioid subtype [86]. The authors studied 46 cases and considered a 2-tier system incorporating the nuclear atypia score and the mitotic score to stratify cases: the low-grade tier included cases with a total sum of 3 or less, while the high-grade tier cases with a total sum of 4 to 6 (6 is the maximum sum). Although not statistically significant, the low-grade tier had a higher progression-free survival with a median of 4.7 years and 65% at 5 years, when compared with the high-grade tier with a median of 1.9 years and 35% at 5 years.

Methodological details

Evaluation of nuclear features: nuclear atypia, nuclear/cytoplasmic (N/C) ratio, chromatin pattern, intranuclear inclusion, prominence of nucleoli, mitotic count, and atypical mitoses. The presence of lymphatic and vascular invasion was also recorded if at least one tumor cell cluster was visible within an endothelial lined lymphatic vessel or vein, respectively.

- Nuclear features were evaluated using high-power-field (HPF) at × 400 magnification (0.237 mm^2 field of view).
- Mitoses were evaluated in 50 HPF areas (11.85 mm^2), with the highest mitotic activity identified after scanning through all tumor slides and counted as an average of mitotic figures per 10 HPF.
- Nuclear atypia was recorded only if it consisted of > 5% of the entire tumor area and was evaluated in the area with the highest degree of atypia (nuclear size and irregularity).

It was graded as follows:

- mild atypia: uniform nuclei in size and shape;
- moderate atypia: nuclei in intermediate size between mild and severe, with slight;
- irregularity in shape;

- severe atypia: bizarre, enlarged nuclei of varied sizes, with some nuclei at least twice as large as others.
- N/C ratio was graded as:
  - low: < 1/3 nucleus-to-cytoplasm area;
  - intermediate: 1/3-2/3;
  - high: > 2/3.
- Chromatin pattern was graded as homogeneous, fine granular, or coarse granular.
- Prominence of nucleoli was evaluated using as reference nearby red blood cells, which measured approximately 7 μm, and graded as the following three categories by the measurement of predominant size:
  - indistinct: inconspicuous or very small;
  - distinct: < 3 μm;
  - large: ≥ 3 μm.
- Intranuclear inclusions were determined as present or absent by examining 10-50 HPF, depending on the number of available tumor slides for each case.
- Identification of mitotic figures from pyknotic cells: absence of a nuclear membrane or a central clear zone, presence of hairy rather than triangular or spiky projections that reflected a mitotic spindle and cytoplasmic basophilia rather than eosinophilia (areas of necrosis and prominent stromal fibrosis or inflammation were avoided whenever possible). Tumors were graded into the following three groups by mitotic count number using optimal cutoff values associated with the difference in overall survival:
  - low: 0-1/10 HPF;
  - intermediate: 2-4/10 HPF;
  - high: ≥ 5/10 HPF.
- Atypical mitoses were defined as the presence of abnormal chromosome spread, tripolar or quadripolar forms, circular, or indescribably bizarre forms.

Nuclear grading in epithelioid mesothelioma provides a simple, pratical and cost-effective prognostic tool that better stratifies clinical outcome than currentlyu available clinicopathological factors. Multicentric larger studies are desirable to confirm the value and prognostic significance of this grading system.

Familial mesothelioma

Malignant mesothelioma (MM) occurs mostly as sporadic cancer and the main risk factor is asbestos exposure. MM also occurs among blood relatives suggesting possible increased genetic susceptibility besides shared exposures. The occurrence of MM cases within families has been reported in numerous studies in the period 1968-2016 [87-110] for a total of 159 clusters (at October 2016) from different countries worldwide, mainly from Italy, Australia and United States. According to the relationship of affected individuals, families are characterized by multiple MM in sibling pairs or more siblings (57%, horizontal pattern) and in parent-offspring pairs (43%, vertical pattern). The male-to-female ratio in familial MM is lower than sporadic MM (1.5:1 *vs* 2.5:1 [111]) and mean age at diagnosis in familial MM is much lower than sporadic MM (53 years *vs* 69 [111]). In most familial clusters there is a history of asbestos exposure: 54% occupational, 24% occupational/household, 18% household/environmental, 4% no exposure. Pathology details regarding histology and immunohistochemistry are not always reported in the articles, nevertheless, significant differences with respect to sporadic MM do not emerge. When reported, a history of cancer is positive in about 56% of clusters.

The proportion between familial and sporadic MM is not known. Only two recent studies have made an effort to calculate the familial risk of pleural MM, showing significant risk in blood relatives: 1.9 in the cohort of Wittenoom, Western Australia [108] and 3.88 and 12.37 in parent and sibling, respectively, in Sweden [109].

Recently, a new tumor predisposition syndrome (TPDS) has been described (OMIM #614327) caused by a heterozygous germline mutation in the BRCA-associated protein 1 (BAP1) gene on chromosome 3p21. Individuals carrying heterozygous BAP1 mutations are at high-risk for the development of a variety of tumors, including benign melanocytic tumors as well as several malignant tumors, including uveal melanoma, cutaneous melanoma and also MM [90 112] (TPDS malignancies).

The frequency of BAP1 germline mutations in familial MM is not known. So far, 53 families have been analyzed for germline BAP1 mutations [89-91 93 94 97-100 102-104 105 107 110 106 113] (Tab. III). In the literature, there are significant differences between wild type and mutated BAP1 families in terms of TPDS malignancies and asbestos exposure history. As a matter of fact, most clusters with BAP1 germline mutations have typical BAP1-TPDS malignancies and are heterogeneously linked to asbestos exposure [90 94 107], instead, most clusters without germline BAP1 mutation have asbestos exposure and a family history of malignancies other than those typical of TPDS [110 106 113].

Prior to BAP1-TPDS, other cancer predisposing syndromes have been reported in association with MM, including Li-Fraumeni syndrome due to TP53 [114], neurofibromatosis due to NF2 [115] and CDKN2A [106] germline mutations. Interestingly, all these genes that are tumor suppressor genes are often mutated or deleted at somatic level in sporadic MM, with a different frequency being BAP1 the most frequently mutated gene using direct sequencing: BAP1 23%, NF2 19%, TP53 8% [116 117]. Using multiple molecular techniques, the proportion of BAP1 mutations rises up to 60% [118]. Considering the high proportion of MM somatically mutated for BAP1, immunohistochemistry to reveal the expression of BAP1 protein is helpful in the diagnosis of MM both in histology and cytology samples [27 119 120]. However, an immunohistochemistry retention of BAP1 protein does not exclude MM because not all MM show a biallelic BAP1 mutation.

As far as familial MM is concerned, if a suspicion of BAP1 syndrome emerge, the search of germline mutation should be performed on peripheral blood [95]. Immunohistochemistry is not useful to reveal germline BAP1

Tab. III. Families with multiple cases of malignant mesothelioma analyzed for *BAP1* germline mutations.

	Families (n = 53)	BAP1 unmutated (n = 27)	Reference	BAP1 mutated (n = 6)	Reference
History of cancer					
Common TPDS [a] cancer	21	1	Popova, et al. 2013	20	Testa, et al. 2011, Carbone, et al 2012, Abdel-Rahman, et al. 2011, Wiesner, et al. 2012, Wadt 2012, Popova 2013,Pilarski 2014, Rai 2015, Klebe 2015, Wadt 2015, Cheung 2015, de la Fouchardière, et al. 2015, Ohar, et al. 2015, Carbone, et al. 2015
Other cancer [b]	19	16	Popova, et al. 2013, Betti, et al. 2014, Cheung 2015a, Ascoli, et al. 2016, Betti, et al. 2016	3	Popova, et al. 2013, Betti, et al. 2014, Ohar 2015
Negative	5	2	Betti, et al. 2014, Popova, et al. 2013, Ascoli, et al. 2016	3	Ribeiro, et al. 2013, Popova, et al. 2013, Betti, et al. 2014,
Missing data	8	8	Sneddon, et al. 2015	0	-
Asbestos Exposure					
Yes	29	22	Betti, et al. 2014, Sneddon, et al. 2015, Cheung, et al. 2015a, Ascoli, et al. 2016, Betti, et al 2016	7	Testa 2011, Carbone 2012, Betti, et al. 2014, Klebe, et al. 2015, Cheung, et al. 2015b, Ohar, et al. 2015
No	3	0	-	3	Wiesner, et al. 2012, Ribeiro, et al. 2013, Carbone, et al. 2015
Missing data	21	5	Popova, et al. 2013, Ascoli, et al. 2016, Betti et al 2016	16	Abdel-Rahman, et al. 2011, Wadt, et al. 2012, Popova, et al. 2013, Pilarski, et al. 2014, Rai, et al. 2015, Wadt, et al. 2015, de la Fouchardière, et al. 2015

[a] TPDS, tumor predisposition syndrome (OMIM, #614327); [b] Leukemia, prostate, lung, thyroid, breast, larynx, colon, stomach, pancreas (BAP1 unmutated families); Breast, salivary gland, stomach, throat (BAP1 mutated families).

Tab. IV. BAP1 immunohistochemistry in tumor tissues from malignant mesothelioma cases occurring in 20 out of 53 families analyzed for *BAP1* germline mutations.

BAP1 immunohistochemistry	N. of families	BAP1 germline sequencing	
		Unmutated	Mutated
Positive	4	3	1
Negative	16	7	9
Totale	20	10	10

mutations: if one allele only is mutated at germline level and the other is wild-type, the latter allele can encode a detectable protein by immunohistochemistry [103]. In 20 out of 53 clusters that have been analyzed at molecular level, BAP1 immunohistochemistry was performed [89-91 101 103-107 110 113 121]. Table IV shows data on BAP1 immunohistochemistry in tumour tissues from MM cases analyzed for BAP1 germline mutations. Immunohistochemistry is not always in line with molecular results; for example, BAP1 immunohistochemistry may be positive in association with a germline mutation [103]. When a patient affected by MM has a family history of MM or/and family history of TPDS malignancies, it would be necessary to search for potential BAP1 germline alterations by molecular studies, as direct sequencing.

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