Thesis

Is the comparison between different BAP1 antibodies possible in pleural mesothelioma?

submitted by

Andreas Wolkerstorfer

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Research Prof. Priv.-Doz. Dr. med. univ. Dr. rer. nat. Luka Brcic

and

Univ. FÄ Priv.-Doz. Dr. med. univ. et scient. med. Iva Brcic

Graz, 1st of September 2023

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Graz, 1st of September 2023 Andreas Wolkerstorfer m.p.

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Zusammenfassung

Das Pleuramesotheliom ist ein seltener und aggressive maligner Tumor der mesothelialen Zellen, die die parietale und viszerale Pleura bilden. Seine pathophysiologische Entwicklung, histomorphologische Merkmale und molekulare Muster als potenzieller Ansatz für neue Behandlungsmodalitäten wurden in den letzten Jahrzehnten intensiv erforscht. Leider bleiben die derzeitigen therapeutischen Möglichkeiten in ihrer Wirksamkeit begrenzt. Die Prognose des Pleuramesothelioms bleibt weiterhin eine der schlechtesten aller Krebserkrankungen, mit einem medianen Überleben (bei unbehandelten Patienten/innen) von meist nur wenigen Monaten. Dies ist hauptsächlich auf den späten Beginn und der allgemeinen Natur der assoziierten Symptome zurückzuführen, kombiniert mit der langen Latenzzeit der Krankheit und ihrem aggressiven invasiven Wachstum.

Die meisten Fälle von Pleuramesotheliom stehen im Zusammenhang mit der Exposition gegenüber Asbest, das in der zweiten Hälfte des 20. Jahrhunderts in großem Umfang auf Baustellen und zu Isolationszwecken verwendet wurde. Die genauen molekularpathologischen Mechanismen, die zu dieser Krebserkrankung führen, sind nicht vollständig geklärt und Gegenstand laufender Forschung. Eine der am häufigsten auftretenden Mutationen beim Pleuramesotheliom ist die Dysfunktion von BAP1. BAP1 ist ein Deubiquitinase-Protein. Es handelt sich um ein Tumorsuppressorgen und spielt bei einem mutationsbedingtem Funktionsverlust eine gewichtige Rolle im Wachstum maligner Tumore. Das Vorhandensein seiner Expression hat daher erhebliche Auswirkungen auf den Verlauf und die langfristige Prognose der Erkrankung. Der Verlust von BAP1 wurde als sicheres Zeichen für Malignität nachgewiesen und zeigt sich daher sehr nützlich bei der Diagnose des Mesothelioms.

Unser Ziel war es, einen Vergleich verschiedener BAP1-Antikörper-Klone in einer einzigen Kohorte von Mesotheliomen durchzuführen. Wir untersuchten eine Patientengruppe von 58 Fällen mit Pleuramesotheliom (43 männlich, 15 weiblich), einschließlich aller wichtigen histologischen Subtypen. Nur einer von drei getesteten Antikörpern (Klon C-4) zeigte verwertbare Ergebnisse in unserer Kohorte, sodass ein Vergleich verschiedener Klone nicht möglich war. Dieser BAP1-Klon zeigte jedoch eine zuverlässige Färbung, mit einer Verteilung von BAP1-Expression/Verlust in unserer Kohorte, die mit den verfügbaren Daten in der Literatur übereinstimmt.

Abstract

Pleural mesothelioma is a rare and aggressive malignant neoplasm of mesothelial cells, that make up the parietal and visceral pleura. Its pathophysiological development, histomorphological features, and molecular patterns as potential approaches for new treatment modalities have been the subject of intensive research over the last decades. Unfortunately, current therapeutical options remain limited in their effectiveness. The prognosis of pleural mesothelioma is and has always been among the worst of all malignancies, with a median overall survival (in untreated patients) of only a few months in most cases. This can mainly be attributed to the late onset and general nature of associated symptoms, combined with the long latency of the disease itself and its aggressive invasive growth.

Most cases of pleural mesothelioma are associated with exposure to asbestos, which was heavily used in construction sites and for isolation purposes around the second half of the 20th century. However, the exact pathological mechanisms leading to this malignancy are not fully understood and remain subject to further research, as many molecular aberrations play a role in its development. One of the most prominent associated mutations in pleural mesothelioma is the dysfunction of BAP1. BAP1 is a deubiquitinating protein. It is a tumor suppressor gene and accordingly plays an important role in tumor growth if loss of function is caused by genetic mutations. Its expression, therefore, has major consequences on the outcome and long-term prognosis of a patient. Loss of BAP1 has been proven to be a definitive sign of malignancy and hence is very useful in diagnosing mesothelioma.

Our goal was to perform a comparison of different BAP1 clones in a single cohort of mesothelioma. We examined a patient collective of 58 cases of pleural mesothelioma (43 male, 15 female), including all major histological subtypes. Only one of three tested antibodies (clone C-4) showed usable results in our cohort, so comparison of different clones was not possible. This BAP1 clone showed reliable staining, with a distribution of BAP1 expression/loss in our cohort which is in concordance with available data in the literature.

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Abbreviations

AR *Androgen receptor*

BPM *Biphasic pleural mesothelioma*

CD *Cluster of differentiation*

CDKN2A *Cyclin-dependent kinase inhibitor 2A*

CEA *Carcinoembryonic antigen*

CK *Cytokeratin*

CT *Computed tomography*

CTLA *Cytotoxic T-lymphocyte associated protein*

DNA *Deoxyribonucleic acid*

DPM *Diffuse pleural mesothelioma*

ECOG *Eastern Cooperative Oncology Group*

EPD *Extended pleurectomy decortication*

EPM *Epithelioid pleural mesothelioma*

EPP *Extrapleural pneumonectomy*

ER *Estrogen receptor*

ERS *European Respiratory Society*

ESTS *European Society of Thoracic Surgeons*

ETOP *European Thoracic Oncology Platform*

EURACAN *European Reference Network on Rare Adult Cancers*

EZH2 *Enhancer of ceste homolog 2*

FDG *Flurordeoxyglucose*

FISH *Fluorescence in situ hybridization*

G_1-Phase *Gap 1 phase*

GCDFP15 *Gross cystic disease fluid protein 15*

HCF1 *Host cell factor C1*

HEG1 *Heart development protein with EGF-like domains 1*

HMB45 *Human melanoma black 45*

IASLC *International Association for the study of Lung Cancer*

ICI *Immune checkpoint inhibitor*

IHC *Immunohistochemistry*

LTD *Limited*

M *Metastasis*

MAPS *Mesothelioma Avastin Cisplatin Pemetrexed Study*

MARS *Mesothelioma and radical surgery*

MERLIN *Moesin-Ezrin-Radixin-Like Protein*

mOS *Median overall survival*

MRI *Magnetic resonance imaging*

mRNA *messenger ribonucleic acid*

MTAP *Methylthioadenosine phosphorylase*

N *Nodes*

NCCN *National Comprehensive Cancer Network*

NF *Neurofibromatosis*

OS *Overall survival*

P/D *Pleurectomy and decortication*

PD *Programmed cell death*

PET *Positron emission tomography*

PFS *Progression-free survival*

PM *Pleural mesothelioma*

PR *Progesterone receptor*

PR-DUB *Polycomb repressive deubiquitinase complex*

PSA *Prostate-specific antigen*

RB *Retinoblastoma protein*

ref. *Reference*

RMH *Reactive mesothelial hyperplasia*

R-Point *Restriction point*

S-Phase *Synthesis phase*

SPM *Sarcomatoid pleural mesothelioma*

T *Tumor*

TMA *Tissue microarray*

TNM *Tumor-Nodes-Metastasis*

U.S. *United States*

UK *United Kingdom*

VEGF *Vascular endothelial growth factor*

WDPMT *Well-differentiated papillary mesothelial tumor of the pleura*

WHO *World Health Organisation*

WT1 *Wilms-Tumorsuppressorgene 1*

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1 Pleural Mesothelioma and other mesothelial tumors

1.1 Pleural mesothelioma - an overview

Pleural mesothelioma is an aggressive cancerous transformation of simple squamous epithelium (mesothelium), which as well covers most of the surface of the human body's cavities, as it encloses most internal organs to a certain degree. Accordingly, mesothelioma can form in various places of the human organism depending on where said epithelium is present (1, 2).However, 85% of mesothelioma in males and 73% of mesothelioma cases in females develop from either the parietal or visceral layer of the pleura, hence being called pleural mesothelioma (PM) (3). PM is amongst the types of cancer with the worst prognosis. Median survival rates, without therapy, are hovering between 8-14 months after diagnosis. Said survival rates have a strong dependency on the histologic subtype of PM, with sarcomatoid PM having the worst (~4 months), epithelioid PM the most favorable prognosis (~13 months) and biphasic/mixed PM ranking somewhere in between (dependent on its histologic composition of the other two subtypes) (4, 5). Although there are several causes known to trigger the genesis of PM (e.g., germline mutations, radiation, or mineral fibers) the most common cause is exposition to asbestos, which accounts for about 80% of all PM cases (6, 7).

1.1.1 Epidemiology

Pleural mesothelioma is a rare tumor. In the mid to late 2010s, around 30.000-35.000 cases worldwide were reported annually, with a yearly 25.000-30.000 associated deaths giving a clear sign of the tumor´s low survival rate. The incidence of the disease is particularly interesting, because of its clear relation to the historical and present use of asbestos (8). Data must be looked at critically, because there is a large unreported number of asbestos-associated cases, especially in strongly populated countries without a ban on asbestos and poor disease-reporting systems, like China or India. The latency time of mesothelioma development after asbestos exposure can be more than four decades. A former miner or construction site worker, who had strong occupational contact with asbestos in the 1980s, could be perfectly healthy today, but might still develop cancer, caused by this very exposure, dating 40 years back. The typical occupations in which asbestos is used (mining, construction, mechanics, shipbuilding, and other highly physical jobs) explain why around 80% of the patients are male; most of those jobs were traditionally male professions (9). The ratio between male and female patients varies greatly in different regions. This can be attributed to

worldwide differences in socioeconomic history regarding work environment and legislative, and therefore different amounts of occupational exposure. Other factors, like naturally occurring asbestos exposure, might also play a role but are hard to detect (10). The connection between asbestos and mesothelioma can be illustrated exceptionally well in countries, that banned the use of asbestos in the mid-1990s after its harmfulness was put on greater display. Those countries, mostly located in the western world, experienced rising numbers of pleural mesothelioma until the mid-2010s before they started to decline. Those cases were the cancerous manifestation of the asbestos-exposed workers 30-40 years earlier. Case numbers within a country are reported to rise for around two decades after the introduction of the asbestos ban, and after that time they start slowly to decline. This information has strategical value for some developing countries, which only recently implemented a ban on asbestos, like Brazil in 2018 (11-13). Throughout history, there have been three peaks in the number of mesothelioma cases, each associated with the predominant type of asbestos exposure at a specific time. The first wave dates back to the extensive and unprotected mining of asbestos, the second one to its use in construction sites, shipyards, insulation, railways, and other common usages during the 20th century, and the third one to the demolitions and repairs of buildings that contain asbestos (14, 15). Pleural mesothelioma today is much rarer in younger patients. This is not surprising, considering the long latency of the disease, paired with the historically relatively recent regulation of asbestos and the fact that overall work safety has improved, also in countries with more liberal laws on the material itself. Among others, these measurements have lowered the incidence rate of pleural mesothelioma per 100.000 in under-50-year-old Europeans to ≤0,6 in males and ≤0,4 in females respectively. In the age group of 80 and above, people that have been in their working age during the high time of asbestos, the incidence in Europe is 22,8/100.000 in males and 3,4/100.000 in females during the same time (16, 17).

1.1.2 Clinical Symptoms

Due to the extended latency and slow development of the disease, in most patients, initial symptoms occur decades after asbestos exposure. Hardly any patients are diagnosed with pleural mesothelioma in an asymptomatic stage of the disease. If so, it is usually due to incidental findings in other surgical or radiological interventions and assessments. Patients that do not show symptoms at the point of diagnosis are

reported to have a better survival rate (18). Pleural mesothelioma usually presents with unspecific symptoms, that can be explained by its location. Initial manifestations normally include dyspnea/breathlessness and dry coughing, caused by pleural effusion, which is prominent in 70% of patients. After thoracic drainage, these symptoms can resolve in many cases. Pleural mesothelioma can cause contraction of the chest wall, due to pleural thickening and adhesions. This can be underlying to unilateral chest pain, another common symptom. It often includes compression of intercostal nerves either by enhanced musculoskeletal tension or from tumor growth itself. Involvement of other nervous structures, like the brachial plexus in apical pleural mesothelioma, can further cause paresthesia, pain, or motoric deficiency in the corresponding body parts. In addition to its musculoskeletal and neuropathic causes, pain in more advanced stages is often caused by the destruction of adjacent tissue, for instance in chest wall invasion or pathological rib fractures. In advanced stages of tumor growth, additional symptoms that reflect its location can be present. Among other manifestations, patients can present with ascites, if the tumor affects venous return flow or invades the abdominal cavity; neurologic symptoms will occur if it invades the spinal cord; thrombosis, vena cava syndrome, or cardiac symptoms could occur. If the tumor compresses, invades, or destroys lymphatic vessels, swelling and/or palpable lymphadenopathy can be present (19). More general symptoms, that indicate advanced stages of pleural mesothelioma, include the notorious B-Symptoms (fever, night sweats, and weight loss), cachexia, and fatigue. In some cases, mesothelioma can mimic pneumonia or can present with unusual symptoms like recurrent pneumothoraxes (20). If the neoplasm has spread, symptoms associated with the location of metastasis can be present (21).

1.2 Histology

The histologic classification of mesothelial tumors is important for prognostic and therapeutical reasons. First, we have to distinguish between benign and malignant tumors, and malignant tumors (mesothelioma) should be further subclassified according to the current WHO classification and recommendations from international expert group. This has a major effect not only on the therapeutical approach and the expected survival rate but also on the quality of life a patient can expect after diagnosis (22). For example, patients with diffuse pleural mesothelioma of the sarcomatoid subtype do not show the same benefit from a surgical approach as the patients with

the epithelioid subtype and will be therefore subjected to different therapeutical strategies and goals (23). The last classification update by the WHO in 2021 contains improvements by implementing straightforward criteria for diagnosing and classification of mesothelioma (24). However, it is still expected among mesothelioma specialists that even more detailed subclassifications (even molecular classifications) might be necessary in the future to further adapt and develop therapy options which are very limited today (22, 25). The following paragraphs offer an overview of the main histologic types and subtypes of mesothelioma. The exact classification of the tumor has been continuously refined but remains subject to improvement. Addressing this, it was agreed upon by field experts in the 2019 EURACAN/IASLC proposal, that, among other things, classification of different histological patterns as well as cytological and molecular features, need to become more detailed to advance clinical handling of mesothelioma (22). Almost all recommendations have been integrated into the latest, WHO 2021, classification of mesothelioma.

1.2.1 Benign and preinvasive mesothelial tumors

1.2.1.1 Mesothelial hyperplasia

Mesothelial hyperplasia is the non-cancerous proliferation of mesothelial cells. It is often referred to as “benign reactive mesothelial proliferation” because most of the time, it is the mesothelium´s response to external causes, like inflammatory diseases, trauma, or infection. This can go hand in hand with morphological abnormalities. Although it is usually a rather harmless incidental finding, mesothelial hyperplasia can show a very similar histological appearance to PM. Especially when there is florid inflammation of surrounding tissue, it can be challenging to differentiate them. Histologically mesothelial hyperplasia does not have a specific distinct pattern that would enable definite differentiation from malignant neoplasms. Under the microscope, its architecture can show a wide variety of features, including papillary, tubular, tubulopapillary, or solid patterns. All of those also occur in pleural mesothelioma (26). Tissue invasion, as a certain sign of malignancy, is the most reliable criterion for differentiation, but there have been reported isolated cases where it was mimicked by mesothelial proliferation (27). This might happen, for example, in organizing pleuritis, where mesothelial cells can get embedded and “trapped” within proliferating fibrous tissue and submesothelial cells, which may imitate invasion. It is rare and not a true invasion but can be deceiving for the untrained eye (28). Cytological features may be

helpful, as mesothelial hyperplasia normally does not show major changes in its cellular features (like pleomorphic nuclei, hyperchromatic, deformed, giant, or anaplastic cells). If those characteristics are extensively present, it points towards malignancy. On the other hand, pleural mesothelioma can be cytologically bland and does not necessarily show these cytological abnormalities. It can be well differentiated and show only minimal atypical changes (like prominent nuclei, increased mitotic rate, or psammomatous calcifications), which can also be found in benign mesothelial (reactive) proliferations. If the histological evaluation of potential malignancy remains unclear, the most reliable option is immunohistochemical staining (see 1.4) and molecular examinations like FISH for possible gene losses (e.g., deletion of *CDKN2A* or *NF2*, both signs for malignancy) (29, 30).

1.2.1.2 Mesothelioma in situ

Only a few cases of mesothelioma in situ have been reported so far. It is characterized by a superficial proliferation of a single layer of neoplastic mesothelial cells on a serosal surface. Furthermore, it is defined as the absence of any invasion into its surrounding structures (31). To make a reliable diagnosis of mesothelioma in situ, it is crucial to collect extensive biopsy samples of around 100-200mm^2 from different areas of the pleural surface, to make sure that there is no invasion. Smaller samples and cytology are not suitable, since they cannot reliably show if any violation of the pleura's structural integrity has taken place. The layer of neoplastic cells shows only small cellular atypia, like prominent nuclei, and is usually cuboidal or flat in shape. There have been reports of small nodules and papillary structures, which both can show higher cellular atypia, but also do not show signs of invasion. (32-34). Furthermore, radiologically and clinically (during surgery) there should not be any obvious changes in pleura.

1.2.1.3 Well-differentiated papillary mesothelial tumor of the pleura

This uncommon pleural neoplasm shows stromal papillae that have a fibrovascular or myxoid core. They are covered by a single layer of well-differentiated mesothelial cells. No invasion is present in well-differentiated papillary mesothelial tumor of the pleura (WDPMT), which should be proven by histological examination of a completely embedded tumor resection. Rare findings within the papillae include foamy macrophages, calcification ("psammoma-bodies"), and hyalinized areas. WDPMT can develop from either the visceral or the parietal pleura in a single, branched formation

or in multiple smaller nodes, which typically do not exceed 10mm in size. The superficial mesothelial cells, covering the neoplasm, are cuboidal or flat in shape. Their nuclei are bland with almost no signs of mitosis or inflammation throughout the tumor (35, 36). Some papillae can grow very dense, which can trick the untrained observer into thinking, that invasion may be present. In uncertain cases, keratin staining can help in the presentation of the exact structure of the tumor (37).

1.2.1.4 Adenomatoid tumor of the pleura

This benign neoplasm is one of the rarest pleural tumors, with under twenty described cases in English literature, and is usually an incidental finding during radiological examinations, surgery, or autopsies. Most adenomatoid tumors occur along the genital tract and not the pleural surfaces, the reason for this remains unknown (38). It is a nodular pleural elevation that sometimes invades nearby soft tissue. It can show a variety of architectural patterns like papillae, tubules, glandular or vascular-looking structures, vacuoles, basophilic foci, or lymphoid aggregations (38, 39). Nuclei are usually without any atypia; epithelioid cells are scarce. It is important to sample the entire tumor, to exclude a possible epithelioid mesothelioma with adenomatoid histological appearance (40, 41).

1.2.2 Diffuse pleural mesothelioma (DPM)

The 2021 WHO classification of tumors of the pleura and pericardium separates DPM into its three main types epithelioid, sarcomatoid and biphasic mesothelioma. They each have specific architectural characteristics, which are used to differentiate them, not only from one another but also from other diseases that mesothelioma can mimic. Histopathological features can overlap between the individual types, which is why their demarcation can be unsharp and some share common morphological attributes. The importance of precise classification lies in the consequences for the patient, as the subtypes differ in their prognosis and the therapeutical strategies that are applied to them (22, 42).

1.2.2.1 Epithelioid pleural mesothelioma (EPM)

At around 70-80%, the majority of PM is attributed to the epithelioid type. With the commodity of its invasive spread originating from the pleural surface, EPM can present with a broad variety of histological features, which are described below (see Figure 1). It is not limited to just one of those but usually shows two or multiple patterns within

the same tumor. This heterogeneity can be used as a distinction from other malignant tumors occurring (or metastasizing) in pleura. Also, the prognosis of EPM is closely related to those morphologic characteristics, which makes accurate classification important. Trabecular and tubulopapillary patterns, two of the most prevalent in EPM, are reported by Kadota *et al*. (42) to be associated with the most favorable outcome. On the other end of the spectrum, cases of EPM with pronounced pleomorphism show median survival rates that are much shorter and more like those of sarcomatoid pleural mesothelioma (42, 43). Precise characterization of EPM is also important to distinguish it from other types of cancerous neoplasms, that might mimic its histological or cytological cellular appearance (for example clear cells or small cells, which can appear in EPM, but can mislead the observer to think about metastatic clear cell renal carcinoma or small cell lung carcinoma) (44).

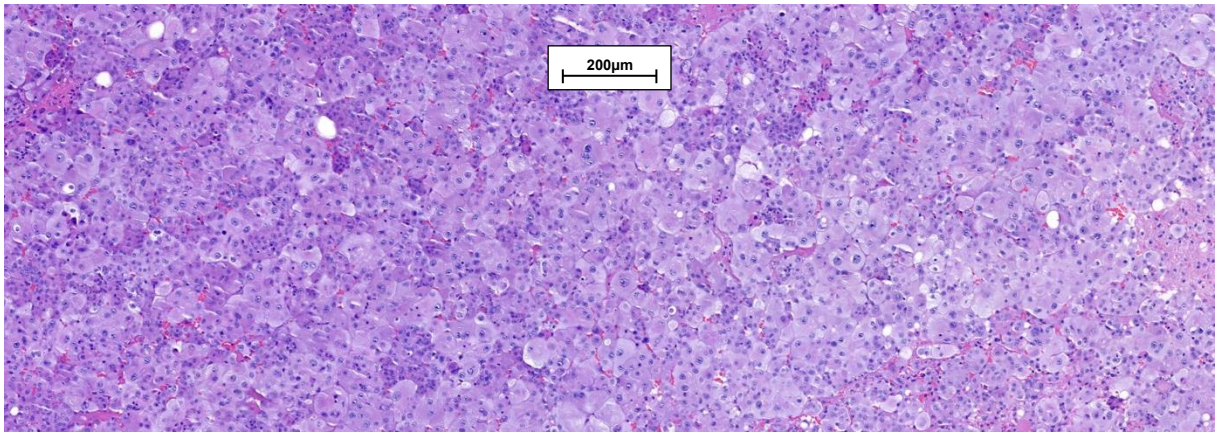

Figure 1: Cell block with cells of epithelioid mesothelioma, deciduoid type cells

Tubulopapillary pattern

Tubules are small pipe-shaped epithelial recesses, covered by a single layer of epithelium/mesothelium. Papillae are finger-like protuberances consisting of a fibrovascular stroma, and covered with (benign or malignant) epithelium. Both tubules and papillae are present in varying proportions, forming a tubulopapillary histologic pattern. When looking at individual cells, their appearance ranges from flat to cuboid or polygonal. Tubulopapillary EPM must be distinguished from its micropapillary relative, which has a far poorer prognosis (22, 42).

Trabecular pattern

This pattern shows a relatively orderly appearance, composed of thin cords with a

width of only a few or even just one line of cells, that infiltrate the surrounding tissue. They usually are homogenously arranged and uniform-looking cells. The trabecular pattern has been reported by Kadota *et al.* to have the most favorable survival rate of the EPM subtypes at 24,9 months (42).

Micropapillary pattern

The micropapillary histological appearance of EPM consists of small, clustered cell aggregates, without central fibrovascular stroma. It is associated with a poorer survival rate than its tubulopapillary counterpart (42).

Adenomatoid/Microcystic pattern

Adenomatoid/Microcystic EPMs show histological similarities to glandular structures and adenomatoid neoplasms. They are composed of flat to cuboidal epithelium forming small acinar structures, and focal also cribriform pattern (22).

Solid pattern

If no specific cellular features are found and there are no signs of extensive pleomorphism, and we have tumor cells growing in large solid sheets, we are dealing with the solid subtype. Its cells usually show a round or polygonal appearance and are arranged in clusters or layers (42). If it is found in more than 50% of tumor mass, it is also a sign of poor prognosis.

Pleomorphic cytologic features

If at least 10% of a tumor's cells show highly atypical cellular features (very large nuclei, multiple nuclei, hyperchromatic nuclei, usually accompanied by high mitotic rate) this tumor will be characterized as a mesothelioma with pleomorphic features. Based on the predominant morphology it can be classified as epithelioid, biphasic, or sarcomatoid. EPMs with pleomorphic features have the worst prognosis of all epithelial subtypes with the overall survival at only around 8,1 months, which is close to the poor survival rates of sarcomatoid and biphasic mesothelioma. They are heavily associated with lymphatic (44%) as well as vascular invasion (59%), making them a very aggressive subtype (42, 45).

Lymphohistiocytoid cytologic features

The rare lymphohistiocytoid subtype is often misinterpreted as lymphoma or lymphoepithelial carcinoma, due to the excessive lymphocytic infiltrate and histiocytoid morphology. It is characterized by the presence of polygonal tumor cells, similar to

histiocytes, with dens inflammatory infiltrate. Lymphocytes are almost exclusively CD8+ T-lymphocytes. Those lymphocytes can conceal further histiocytoid features with their dense infiltrate (46-49). These features can be found in all 3 major mesothelioma types. In sarcomatoid mesothelioma, lymphohistiocytoid features correlate to a favorable prognosis.

1.2.2.2 Sarcomatoid pleural mesothelioma (SPM)

If most mesothelioma cells are elongated cells that are more than 2 times in length than in width, they are classified as sarcomatoid (see Figure 2). Additional morphologic features, as described in the following paragraphs, can be present. The cells can be organized in bundles or show no signs of organization at all. Necrosis is often present in sarcomatoid mesothelioma. Nuclei can range from relatively bland over prominent, to highly anaplastic with higher mitosis number. The sarcomatoid subtype makes up around 10% of all mesotheliomas (42, 50, 51). Sarcomatoid type mesothelioma sometimes contains areas that do not correspond to the cells of origin, like chondrosarcoma-, osteosarcoma-, or rhabdomyosarcoma parts, which might include osteoclast-like giant cells and must be differentiated from metaplasia. If those patterns are present, the term “with heterologous elements” should be added to the classification of sarcomatoid mesothelioma (52, 53).

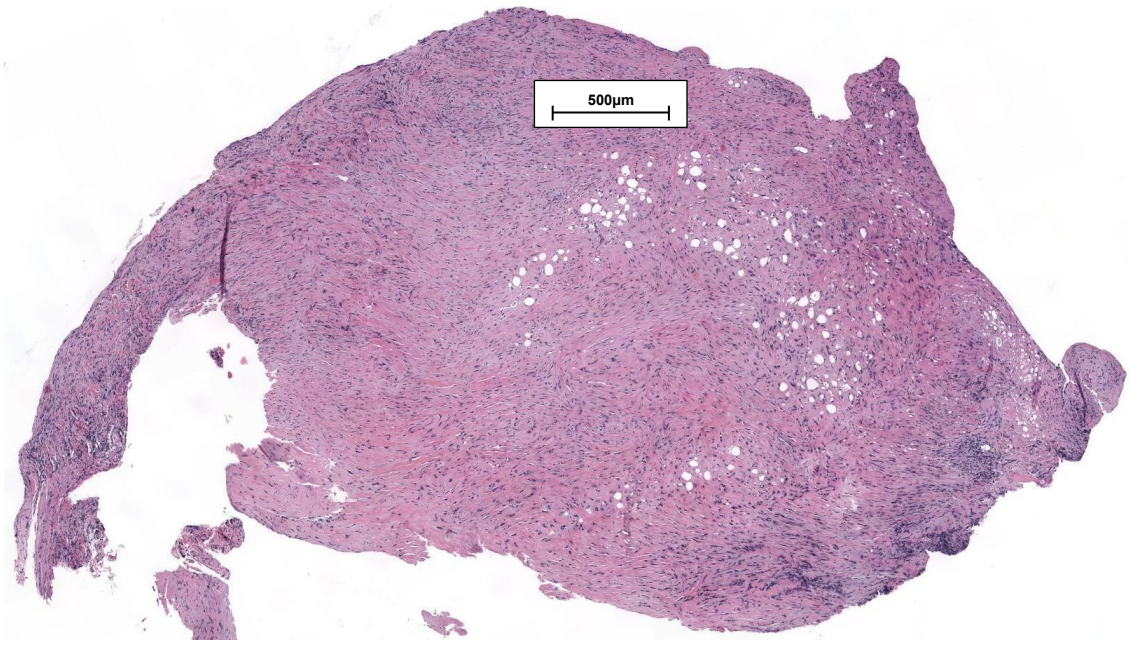

Figure 2: Histologic presentation of sarcomatoid mesothelioma

Transitional features

This type of mesothelioma cell is best described as cells morphologically in between epithelioid and sarcomatoid cells growing in sheets. Reticulin stain can help in their detection, since each transitional cell will have a positive reticulin reaction around, in contrast to cell clusters surrounded by the positive staining in epithelioid mesothelioma. Survival rates of the transitional subtype are close to sarcomatoid ones, which is why in the 2021 WHO classification it is classified as a sarcomatoid type (22, 54, 55).

Pleomorphic cytologic features

As previously mentioned, pleomorphic features might also be present in the sarcomatoid type of mesothelioma (42, 45).

Lymphohistiocytoid cytologic features

Analogous to pleomorphic features, both the epithelial and sarcomatoid as well as biphasic type of mesothelioma can present with lymphohistiocytoid features (see 1.2.2.1) (46-49). In sarcomatoid mesothelioma, it is a sign of a better prognosis.

Desmoplastic mesothelioma

This is a pattern where malignant spindle cells are arranged in a dense hyaline-rich stroma in seemingly unorganized patterns and show only minimal atypical features. Desmoplastic mesothelioma is hypocellular, and this pattern must be present in ≥50% of the tumor (56-58). Only in larger resection specimens can this diagnosis be made with confidence. If the pattern is found in a small biopsy, the term “with desmoplastic features” should be used (22).

1.2.2.3 Biphasic pleural mesothelioma (BPM)

Biphasic mesotheliomas make up around 10-30% of all mesotheliomas (59). They are characterized by epithelioid and sarcomatoid mesothelioma parts In one tumor. The fraction of those two types can vary, but each sarcomatoid and epithelioid mesothelioma must be represented in at least 10% of a tumor to classify it as biphasic. Small biopsies can be labeled as “biphasic” as soon as they contain cells of the epithelioid and sarcomatoid type (22). However, it still is important to specify the percentage of sarcomatoid cells found in any specimen, even smaller ones. The reason for this is that as the fraction of sarcomatoid type cells in a biphasic mesothelioma rises, the overall survival rate drops drastically. This has great effects on the prognosis and management of the disease (60).

1.2.3 Localized pleural mesothelioma

The rare entity of localized pleural mesothelioma grows as a confined mass (61). In contrast, to diffuse pleural mesothelioma, it shows no signs – histologic or clinical – of diffuse spreading to other parts of the serosal surface, hence forming a subcategory on its own. However, histologic classification is the same. It can be also divided into epithelioid, sarcomatoid, and biphasic types, which again contain the same potential histologic features, which are described in 1.2.2 (62-64).

1.3 Cytology

Early diagnosis of PM proves to be the most essential, yet most challenging factor of enabling effective therapeutical measurements. This is due to the neoplasm's slow growth and therefore late clinical manifestation (65, 66). It has been shown, that the median onset of symptoms starts around three to four decades after asbestos exposure, with less than 1% of patients showing any signs earlier than 15 years after their initial contact with asbestos and some even being diagnosed well over half a century afterward (67, 68). Due to the importance of fast diagnosis, cytology has been proven as a useful tool in this field. As in the diagnostic process of many other types of malignant neoplasms, the usage of cytology in PM has been continuously increasing over the last decades. Effusion is the most examined material since it is present as an early clinical sign in most cases of PM. It is relatively easy to get hold of it via pleural puncture and therefore especially beneficial to patients whose physical state does not allow the usage of invasive techniques, like surgical biopsy (69). Also, cytology can usually be performed much earlier since it is not as laborious, but, when done thoroughly, still has the same positive predictive value of malignancy as histology. Against the 2018 guidelines of the American Society of Clinical Oncology, which recommend cytological examinations as a screening method that should always be followed up by biopsy, it is a common cytopathological practice to diagnose mesothelioma, based on the cytology of pleural effusion in combination with ancillary studies (mostly IHC of BAP1, FISH for homozygous loss of CDKN2A, and IHC of MTAP as a surrogate marker for CDKN2A) (70). This approach is also supported by the 2021 WHO classification update of tumors of the lung and pleura (71). Furthermore, it not only minimizes delay for early therapeutical measurements and increases patients' life expectancy, but also lowers their morbidity and comes with a much lower risk of iatrogenic tumor spreading (72, 73). With all its potential benefits, the accuracy of

cytology heavily depends on the experience of the examiner. Kaur *et al*. (74) have reported cytological sensitivity overall to be 73% in PM, most comparable studies show similar results ranging around the 75% mark. This value though finds itself within a large spectrum, mainly depending on the histologic subtype. To diagnose mesothelioma, based on cytology of pleural effusion, the suspected tumor must be epithelioid or biphasic type. This is because epithelioid type shed tumor cells in pleural fluid, resulting in the high sensitivity of this method. On the other hand, sensitivity for the sarcomatoid type is around 20%. Sarcomatoid cells tend to shed much less into pleural fluid due to their higher intercellular cohesion. The sensitivity of cytology in biphasic PM can therefore be put in direct positive relation to the epithelial fraction of the neoplasm. In contrast to its mediocre sensitivity, the specificity of cytological examinations of suspected mesothelioma shows impressive results. If ancillary studies report the loss of BAP1 in IHC and deletion of CDKN2A in FISH in cytological probes of pleural effusion, multiple studies have shown specificity to be up to 100% (29, 75). In all cases where no malignant mesothelial cells can be proved by cytological methods, and morphological or clinical appearance indicates mesothelioma, biopsy must be performed to further confirm the result (74, 76).

1.3.1 Challenges and limitations of cytological examinations

As mentioned above, examination of pleural effusion only has diagnostic value for mesotheliomas with at least a partial epithelioid component. There is a variety of malignant neoplasms and metastasis from other primaries, that can present with similar characteristics as found in PM. The most common differential diagnosis is carcinoma of the lung, but great morphological similarity can, among others, also be found in other epithelioid neoplasms (especially vascular ones), melanoma, epithelioid sarcoma and sometimes, in less typical cases, even lymphoma or desmoplastic round cell tumors. These pitfalls occur due to the very heterogenic appearance of pleural mesothelioma, with cells ranging from well-differentiated to highly anaplastic and pleomorphic (see 1.2.2.1). The main tool to distinguish mesothelioma from morphologically similar tumors is immunohistochemistry (see 1.4). If cytological examination of pleural effusion shows signs of malignancy and no clear tumor can be seen in radiological assessments, this can indicate the presence of mesothelioma in situ. Mesothelioma in situ will have a loss of nuclear expression of BAP1 and usually shows deletion of CDKN2A by FISH. Both results enable differentiation from benign

proliferations, which never show loss or deletion of these markers. The diagnosis of mesothelioma in situ, is defined by the lack of any invasion of surrounding tissue and should therefore not be made by cytology of pleural effusion alone. The 2021 WHO classification states, that surgical specimens must be taken to exclude potential invasion and diagnose mesothelioma in situ (22, 32).

1.3.2 Morphology of malignant mesothelial cells in pleural effusion

Morphological assessment, cell shape, structure, and composition can give a general idea of a tumor´s harmful potential. Malignant mesothelial cells, to a varying degree, show typical features that can be used for distinction from normal mesothelium. Mesothelioma cells in pleural effusion will be present in high numbers, so it will be a very densely populated effusion. Mesothelioma cells can be in small groups, sometimes even with fibrous cores. “Cell-in-cell” is a common phenomenon, and points to mesothelioma. Cellular outlines can appear lobulated and look similar to cauliflower. Universal signs of atypia, like pronounced or anaplastic hyperchromatic nuclei, with prominent nucleoli, changed nucleus to cytoplasm ratio, and atypical mitoses, can be found, but are neither specific for pleural mesothelioma, nor can they exclude reactive lesions. Rare features in mesothelioma cells include parakeratosis, pyknosis, or orangeophilia, but again, none of these are specific to mesothelioma (77). Matsumoto *et al.* further reported that loss of CDKN2A does have an impact on cell morphology, as they show more multinucleation, bigger, raspberry-shaped groups of giant-sized cells, and recurrent cell-in-cell engulfment (78). However, for a distinct and accurate diagnosis of pleural mesothelioma, it is essential to combine cytomorphological assessment with additional, already mentioned examinations (like immunohistochemistry, FISH, biopsy, and radiographic imaging), adjusted to the individual case (79-81).

1.4 Immunohistochemistry

The reliable diagnosis and exact classification of pleural mesothelioma depend heavily on immunohistochemistry. Immunohistochemistry comes into play in separating mesothelial neoplasms from non-mesothelial processes of different origins. If a mesothelial process is confirmed, it further enables the distinction of malignant from benign lesions. There is no exclusive marker that will identify malignant mesothelial cells. Because of that, for the diagnosis, it is always necessary to have 2 positive

mesothelial markers and 2 negative carcinoma markers. A used panel of antibodies depends on which tumor is in the differential diagnosis (82, 83).

1.4.1 Assessing mesothelial origin

If, through any method, a pleural proliferation is discovered, immunohistochemistry is the primary modality to find out if it is of mesothelial origin. For this purpose, the 2021 WHO report has included the recommendation to use a panel of at least two mesothelial and two epithelial markers, which would be found in carcinomas (83). The most used markers that stain positive in mesothelium are calretinin (see Figure 3), WT1 (see Figure 4), D2-40 (see Figure 5), and CK5/6. On the other side of the spectrum typical carcinoma markers are MOC31, BerEP4, B72.3, CEA (see Figure 6), BG8 or CD15 (83-85). Only recently claudin4, a marker found in epithelial tight junctions, has been identified by multiple studies as the best marker for epithelial differentiation (86). Other niche markers are technically available in some areas, for instance, the mesothelial marker HEG1, which is used in Japan, but rarely plays a role in the rest of the world (87).

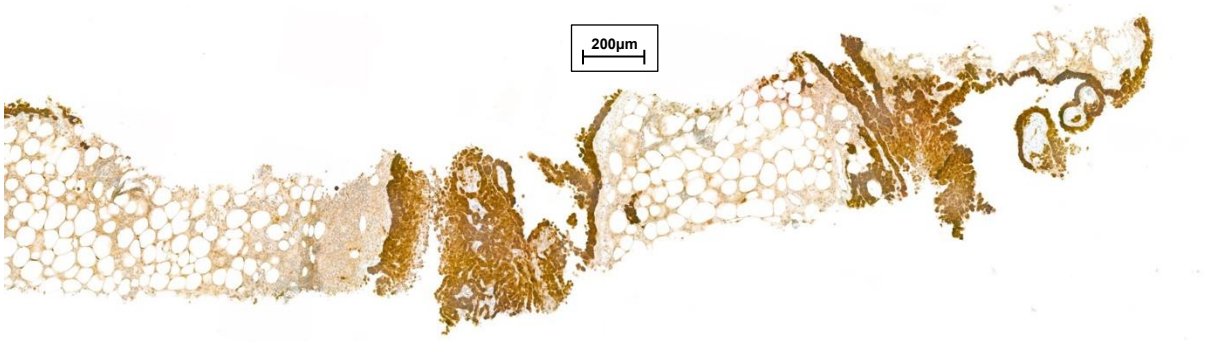

Figure 3: Positive staining reaction with calretinin (nuclear and cytoplasmic) in all tumor cells

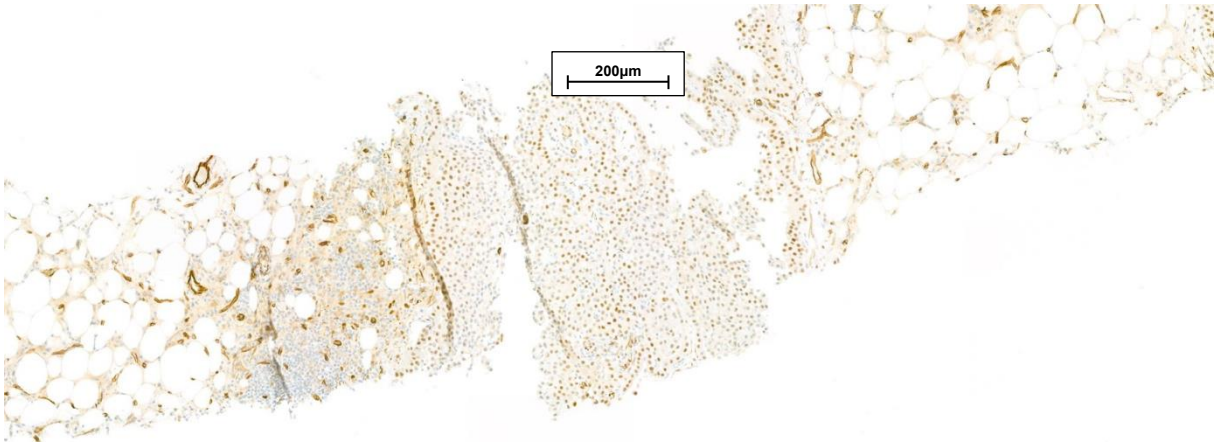

Figure 4: Positive staining of WT-1 in 70% of tumor cells

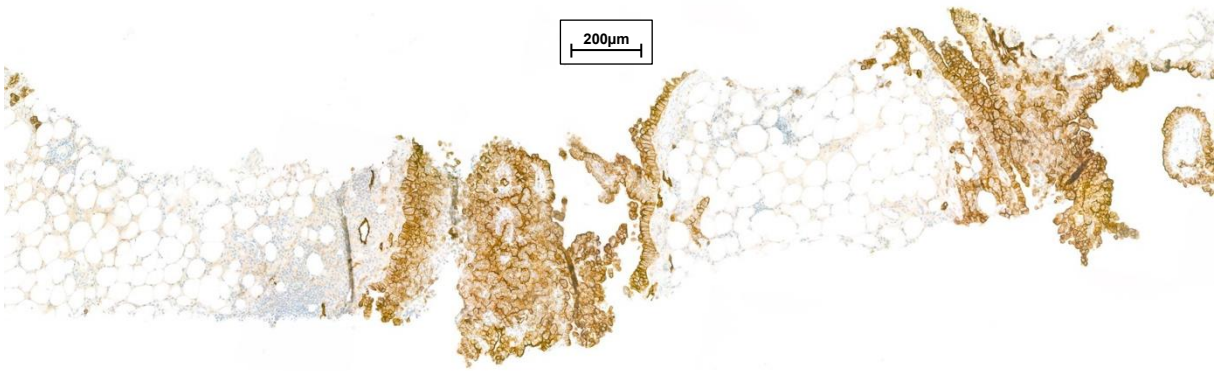

Figure 5: Positive staining of D2-40 (podoplanin) in all tumor cells

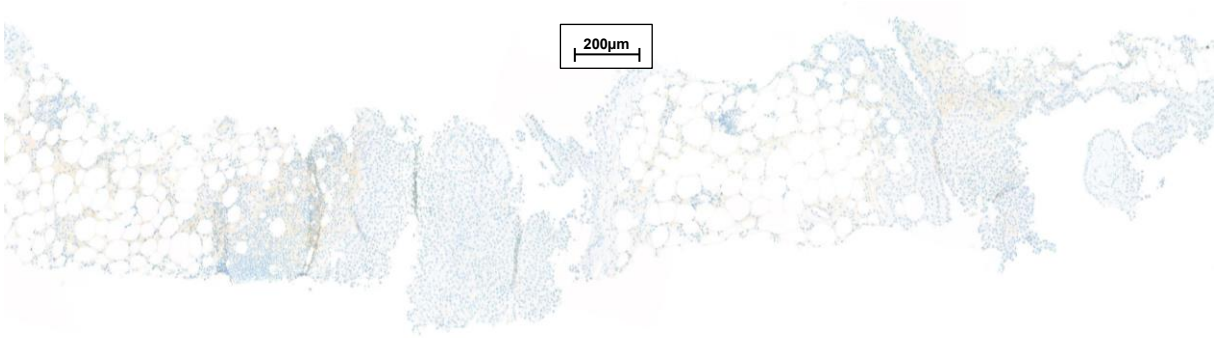

Figure 6: Negative staining of CEA in all tumor cells

Some further immunohistochemical markers, like pancytokeratins, can be useful in excluding some non-epithelial tumors, which can mimic epithelioid mesothelioma. Especially melanoma, large-cell lymphoma, epithelioid hemangioendothelioma, and angiosarcoma have been reported to have similar histological features. EPM stains positive for pancytokeratins, while these tumors (with only little and focal exemptions) do not. A negative immunohistochemical reaction wit (pan)keratins speaks also against the diagnosis of epithelioid mesothelioma (88-91). A more detailed composition of possible immunohistochemical markers for different malignant tumors is shown in Table 1, as previously presented by Monaco *et al*. (92)

Type of malignancy	*Immunohistochemical markers*
Breast carcinoma	GATA3, Mammaglobin, GCDFP15, ER, PR, SOX10 (in triple-negative carcinomas)
Squamous cell carcinoma	p40, p63+
Melanoma	S100, Melan A, SOX10, HMB45
Undifferentiated thoracic tumors (+SMARCA4 deficiency)	Loss of SMARCA4, SOX2, Cytokeratin, CD34, SALL4, Claudin4,
Vascular neoplasms	CD34, CD31, ERG
Prostate carcinoma	NKX3.1, PSA, AR
Renal cell carcinoma	PAX8

Table 1: Useful immunohistochemical markers in malignant neoplasms [modified from ref. (92)]

1.4.2 Differentiation of benign and malignant

After a suspicious proliferation is proven to be of mesothelial origin, IHC further is of great use, to confirm potential malignancy. Especially so, if a suspected invasion of the tumor into the underlying tissue is unclear (for instance in small biopsies). Staining for calretinin and/or cytokeratin, can highlight the tumor cells and make them easily visible in fat tissue (see Figure 3) (83). A marker indicating malignancy in mesothelioma is BAP1, known to be lost in about 15% of sarcomatoid pleural mesotheliomas and up to 65% of epithelial pleural mesotheliomas (see Figure 7 and Figure 8). Especially when looking at the differential diagnosis of reactive benign mesothelial proliferation, loss of BAP1 is highly specific for malignant tumors. It is expressed in virtually all cases of mesothelial hyperplasia and multiple studies have reported BAP1 to be present in 100% of their benign samples. Nuclear staining loss is therefore a certain sign of malignancy (29). However, loss of BAP1, is not purely specific to pleural mesotheliomas and can occur in some carcinomas as well as in malignant melanoma (79, 93, 94). For this reason, it is commonly combined with the examination of CDKN2A. The isolated detection of homozygous deletion of CDKN2A through FISH though, lacks specificity to pleural mesothelioma, in the same way as BAP1 (95). Recently the cytoplasmatic absence of MTAP, proven by IHC, has been reported to be a good surrogate marker for the loss of CDKN2A (see Figure 9) (96). Yoshimura *et al.* enhanced the panel combination of BAP1 and MTAP in IHC, by adding an enhancer of zeste homolog 2 (EZH2). Overexpression of EZH2 in IHC adds further accuracy to

the distinction of pleural mesothelioma from benign reactive mesothelial hyperplasia (97). Another recently presented marker is nuclear loss of 5-hmc. Extensive loss of this by-product of gene methylation is reported to be highly specific for pleural mesothelioma, while 5-hmc stains positive in virtually all benign proliferations. Chapel *et al.* found a panel combination of BAP1 and 5-hmc to be sensitive for pleural mesothelioma in 100% of cases, paired with a 98% specificity in comparison to benign tumors (98).

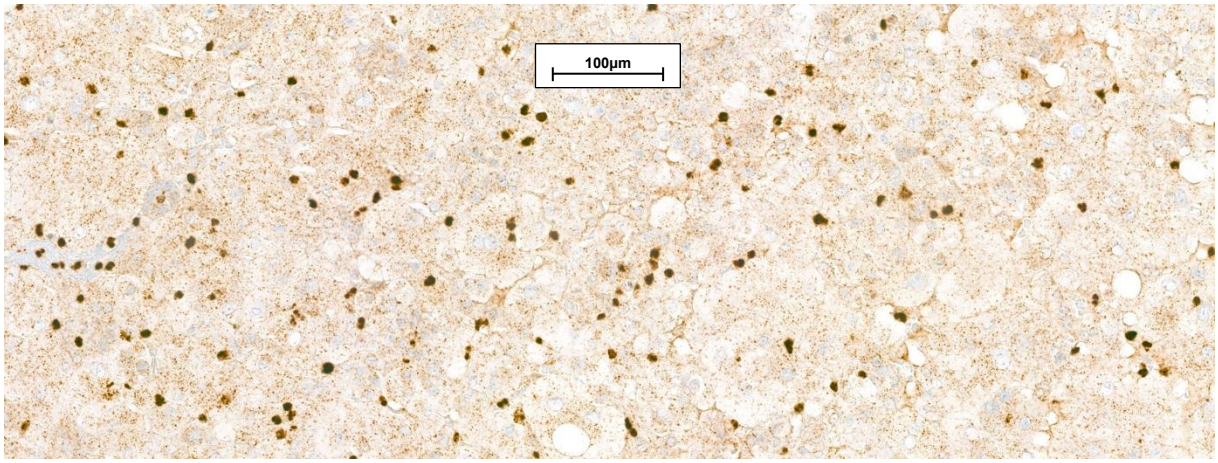

Figure 7: BAP1 staining, cells of epithelioid mesothelioma without nuclear reaction, and inflammatory cells with clear positive nuclear staining (internal control for the antibody reaction)

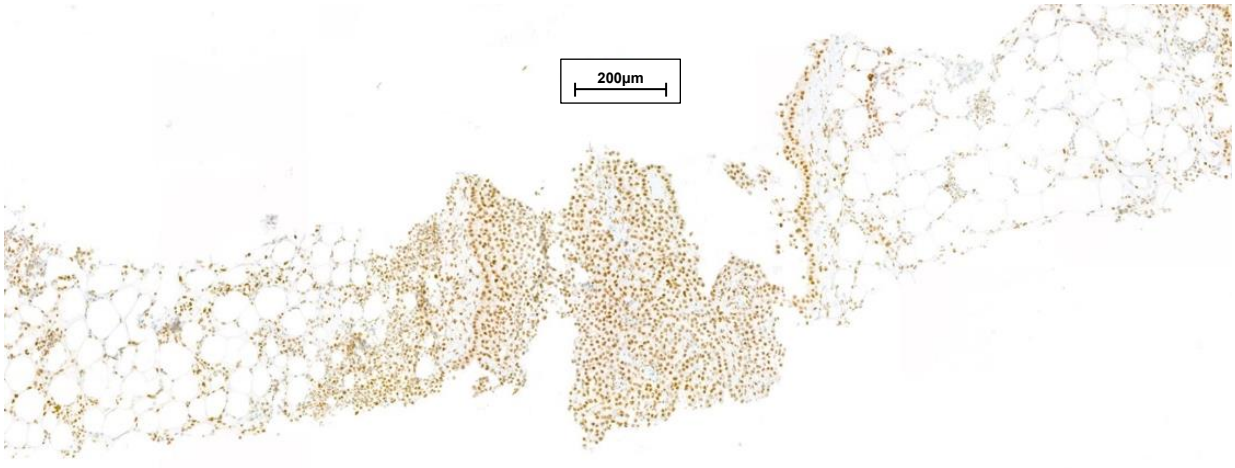

Figure 8: Nuclear BAP1 staining of tumor cells with preserved reaction oft BAP1

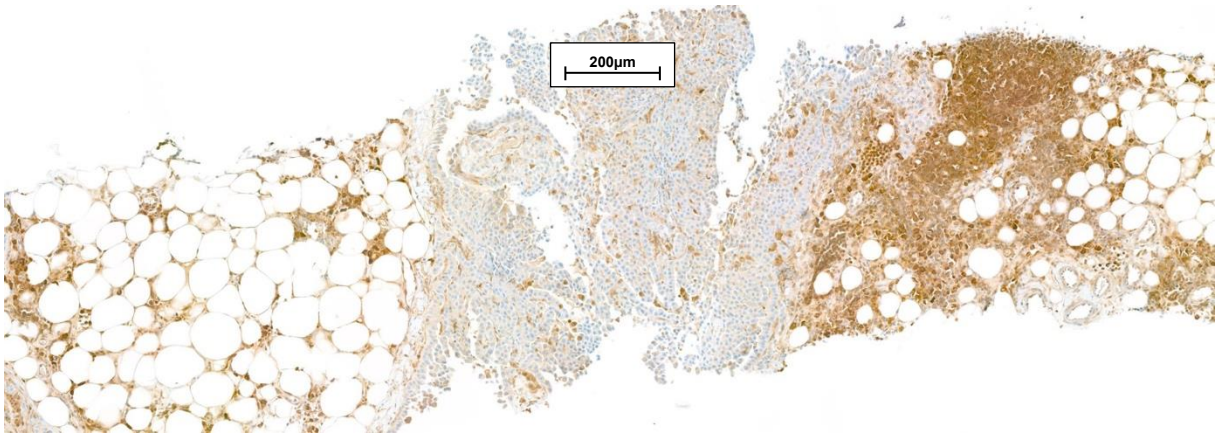

Figure 9: Lost cytoplasmic staining of MTAP in tumor cells

1.4.3 Application of IHC in various mesothelial neoplasms

Sarcomatoid/desmoplastic pleural mesothelioma

In the assessment of sarcomatoid mesothelioma, traditional mesothelial markers are of limited usefulness. Especially the proven mesothelial markers WT1 and CK5/6 have been reported to be of low sensitivity when applied to SPM (52). Reliable diagnosis through immunohistochemistry can be challenging, especially as the neoplasm can appear similar in molecular expressions to sarcomatoid carcinomas. Antibodies that are targeted against pancytokeratins (most commonly AE1/AE3, KL1, and OSCAR) usually stain well in sarcomatoid pleural mesothelioma, at least in parts of the tumor (53, 99). The same goes for CAM5.2, which was found by Chirieac *et al.* to be positive in 96% of sarcomatoid mesotheliomas (100). However, the possibility of the tumor being negative in the assessment of cytokeratin is not excluded (52). The role of calretinin in SPM has been diminishing recently, as it is expressed in only one-third of sarcomatoid mesotheliomas (99). The marker D2-40 on the other side has been reported to stain positively more often than calretinin and enable better differentiation from pulmonary adenocarcinoma (no reported positive staining) and sarcomatoid carcinoma (7% positive staining). Most sarcomatoid/desmoplastic pleural mesotheliomas show strong staining for GATA3, whereas sarcomatoid carcinoma shows virtually no or just focal or patchy staining, making it an excellent marker in differentiating these neoplasms (101). A helpful marker in distinguishing sarcomatoid from epithelioid pleural mesothelioma can be vimentin, which will be negative in EPM and positive in SPM (100).

Adenomatoid tumor of the pleura

This benign tumor is more common along the genital tract than in the pleura. However, since their common origin is mesothelial-type cells, studies that focus on the immunohistochemical markers of genital tract adenomatoid tumors, to a major extent, apply to their pleural counterpart (38, 102). They express typical mesothelial markers like calretinin, D2-40, WT1, and stain positive for cytokeratin. BAP1 is intact, the same goes for CDKN2A, which is why they serve as a distinction marker from pleural mesothelioma (38, 103).

Mesothelioma in situ

Immunohistochemistry is the method of choice to distinguish mesothelioma in situ from benign reactive pleural proliferation. This can be achieved by focusing on nuclear BAP1 and/or cytoplasmatic MTAP, which both are positive (retained nuclear, and cytoplasmic staining, respectively) in benign proliferations, but show no staining in mesothelioma in situ, due to their loss of function (see Figure 7 and Figure 9). Homozygous deletion of CDKN2A, which can be found by FISH, is also a possible combination with BAP1 (32, 33, 104). MTAP has been reported to often be co-deleted with CDKN2A, hence making it a suitable surrogate marker (105). It is important to stress, that negative results for these markers do not exclude (invasive) malignancy.

Localized pleural mesothelioma

The immunohistochemistry of localized pleural mesothelioma is the same as in diffuse pleural mesothelioma. This also accounts for its possible subtypes, since, as the name suggests, the only difference is that localized pleural mesothelioma grows in a circumscribed mass, rather than diffuse spreading (62-64).

Well-differentiated papillary mesothelial tumor of the pleura

Immunohistochemistry in this tumor shows the same mesothelial markers as in diffuse pleural mesothelioma (84). Only a few studies have been performed on BAP1, NF2, and CDKN2A regarding well-differentiated papillary mesothelial tumor of the pleura with only limited significance due to the low number of cases. Hee *et al.* found BAP1 to be deleted only in cases of WDPMT, where simultaneous pleural mesothelioma was present (106), while Stevens *et al.* did not find any alterations of BAP1 at all (the latter study focused on peritoneal WDPMT only and therefore has limited implications

concerning its pleural equivalent) (107). None of the examined cases in either of those two studies showed deletion of NF2 in IHC or CDKN2A in FISH.

1.5 Molecular changes

1.5.1 CDKN2A

One of the most relevant eukaryotic tumor suppressor genes, second only to p53 in terms of commonness, is CDKN2A. It is located on 9p21.3. To understand its clinical importance, it is necessary to have some understanding of its function. CDKN2A, or p16 as the protein is also often referred to, has one of its main purposes in slowing down the progress of cell proliferation and therefore avoiding excessive growth. More specifically, it does so by decelerating the progress from the cell cycle G_1-phase to the S-phase. In G_1, a mitotic cell will execute copious amounts of mRNA and protein production, which further will be relevant for the synthetization of DNA (108). It will then proceed to the S-phase in which the DNA will then be synthesized (hence the “S”). After a cell enters the S-phase, it requires no further extracellular stimulation to continue mitosis. There is no turning back, it is a full commitment to proliferation, regardless of whether the organism can provide the nutrition and energy resources that are necessary to guarantee flawless progression of the remaining cell cycle. To point out the gravity of this process: If unlimited amounts of cells would enter the S-phase without proper regulation, genetic alterations would be replicated, and excessive proliferation would lead to an uncontrolled build-up of cancerous cells. For that reason, in between G_1 and S, there is the so-called G_1/S-Restriction-Point or just R-Point (109, 110). Ideally, at this point, a cell is found to be large enough and have sufficient capacities for producing DNA in the S-phase. If so, cyclin-dependent kinases 4 and 6 (CDK4/6) bind cyclin D. The resulting complex is active and capable of phosphorylating retinoblastoma protein (RB), a protein which usually is bound to the transcription factor E2F. Due to the phosphorylation by the Cyclin D-CDK4/6 complex, RB dissociates from E2F. The so-freed E2F will now find the entrance to the nucleus. There it activates its target genes, induces the continuation of the G_1-Phase into the S-Phase, and allows the cell to complete mitosis (110, 111). With this underlying mechanism in mind, it is easier to explain the role CDKN2A takes in this context. CDKN2A binds to CDK4 and CDK6, resulting in less availability of those kinases to form complexes with cyclin D. Because those are necessary for the phosphorylation of RB (as explained above) and its following dissociation from E2F, CDKN2A indirectly prevents the cell from entering

the S-phase (112-114). Concerning mesothelial proliferations, CDKN2A has proven to be a very useful marker in differentiating pleural mesothelioma from reactive mesothelial hyperplasia (RMH). While in MM, it has been reported that around 47-80% show loss of CDKN2A, there is no reported case of RMH that does so (115, 116). Even though this sets the specificity of CDKN2A deletion at 100% when distinguishing between those two, the fraction of PM cases without the loss of CDKN2A is still large enough to prevent CDKN2A from being the sole and ideal marker for diagnosing mesothelioma (117).

1.5.2 BAP1

BRCA Associated Protein 1, or BAP1, has recently been reported to be one of the main protagonists when it comes to genetic alterations in the development of MM. Encoded on 3p21.2, the complex is situated in the cell nucleus, where its main purpose is deubiquitination. It can be seen as the antagonist of ubiquitination, a post-translational process that adds ubiquitin to proteins, changing their interactions, location, and activity level or marking them as targets for certain processes like degradation via proteasomes. BAP1 can reverse those effects by removing the added ubiquitin molecules (118). Among the many pathways where this function shows its potential influence, two especially stand out when talking about the types of cancer typically associated with BAP1 aberrations. One is the Polycomb repressive deubiquitinase complex (PR-DUB). It is formed by BAP1 together with ASXL1, a protein that interacts with chromatin and enhances or represses the transcription of certain genes at the targeted loci (119). By deubiquitination, PR-DUB has effects on the H2A-Histone, which arranges DNA into nucleosomes and further into chromatin. Originally this packaging gives shape to the otherwise loose DNA, it consequently plays a role in the expression of genes, depending on their position in the so-generated structure. Considering this underlying mechanism, those expressions are also influenced by the deubiquitination of H2A by the PR-DUB complex (120-122). Aberrations, like a loss of function of BAP1, can cause malignancies by derailing the expression of tumor suppressor genes along those DNA-arrangements (123). Another pathway where BAP1 plays a key role is the deubiquitination of the host cell factor C1 (HCF1). HCF1 has responsibilities in controlling the cell cycle. For example, among other functions, it promotes the transition from G_1 to the S phase and is involved in the sequence of steps that ensure a normal progression of cell division during the M phase (124-126). HCF1

has also been reported to be a coregulator of the E2F transcription factor, which also is involved in the regulation of the cell cycle and the modification of chromatin structure. Loss of BAP1 and its consequences for the deubiquitination of HCR-1, therefore, results in disturbances of the cell cycle regulation. This causes the excessive proliferation of cells, potentially leading to the growth of cancerous neoplasms (127). In the diagnosis of MM, BAP1 has become an important maker to distinguish malignancies from RMH. Like CDKN2A, there have been no cases of RMH with a reported loss, while between 60-70% of MM show deletion of BAP1. But even with a specificity of 100% BAP1 is not feasible as a standalone marker due to the existing fraction of MM without its deletion. For this reason, when used for diagnostic purposes, BAP1 is paired with a second marker like CDKN2A to increase the chances of correctly telling apart MM from RMH (29, 128).

1.5.3 NF2

The gene NF2 is well known for its role in the genesis of neurofibromatosis type 2. It encodes for a protein called Schwannomin, or MERLIN. This protein is part of the cytoskeleton and especially present in nervous cells. Therefore, malfunctions of MERLIN can lead to various nervous cell tumors, like schwannomas, meningiomas, or ependymomas, commonly found in patients with neurofibromatosis type 2 (129). It has further been determined that mutations of NF2 are not only present in the same name disease but are also found in many cases of mesothelioma. This is attributed to the fact, that MERLIN, next to it being involved in the structure of the nervous cell cytoskeleton, also is a tumor suppressor gene. It comes into play along the Salvador-Warts-Hippo pathway, a signaling cascade responsible for the control of cell proliferation, apoptosis, and organ size (130). In pleural mesothelioma, NF2 loss of function is especially cancerogenic when combined with the loss of BAP1 or CDKN2A or both. In 2019 Kukuyan *et al.* demonstrated, that 85% of triple conditional knockout mice developed pleural mesothelioma. In double conditional knockout individuals, with BAP1 either combined with CDKN2A or NF2, this number was still at 20%, while single conditional knockout mice showed no rise in pleural mesothelioma (131). The combined loss of NF2 and CDKN2A or BAP1 has also been shown, in 2016, by Singhi *et al.* to be a negative prognostic factor not only for the overall survival rate, but also for progressive free intervals, with both of them being significantly shorter (132).

1.6 Etiology

1.6.1 Asbestos

The term “asbestos” is used for a bunch of naturally occurring fibrous silicate minerals. They can be structurally divided into two groups, serpentines (chrysotile) and amphiboles (amosite, crocidolite, tremolite, actinolite, and anthophyllite). Around the first half of the 19th century, asbestos was discovered to be a very versatile material, and since then found its main use in the isolation of buildings and ships. Due to its durability and resistance against acidic conditions and extreme temperatures (up to 1000 °C for a short time and 300-400 °C without limitations) it was also used in many everyday consumer products like hair dryers, fridges, or car tires (133). Technical fibers have a diameter from 0,75 to 5,0 µm, depending on the different types of asbestos. Due to the critical geometry, the small fibers that make up the material can not only be inhaled into the alveoli but can puncture their path through the tissue of the lung. They then reach the pleura, where phagocytosis usually fails because of the fibers' size. Asbestos causes inflammation and irritation to the macrophages. To break down the foreign material, they produce high amounts of reactive oxygen species as a by-product, which can interact with the DNA and promote the genesis of pleural mesothelioma. The exact mechanism of its development remains subject to future research (134-136). The various subtypes of asbestos fibers are associated with different relative risks of developing pleural mesothelioma. Crocidolite is reported to be the most harmful one, around five times more potent than amosite and 50 times more than the most common fiber chrysotile (137). More than 80% of the patients have been exposed to asbestos. Most of them frequently had occupational contact with the silicate, but also more sporadic exposure increases the risk for mesothelioma. Because of the reported damages to health, the use of asbestos has been widely prohibited, especially in most “western” and south american countries including the whole European Union, Canada, and Brazil (although Brazil implemented a ban in 2018, many asbestos mines are still active). The United States of America has strict limitations with few exemptions, but no definite ban of the material (138-140). Restrictions aside, asbestos is still commonly used today in large parts of the world, especially in emerging or economically weak countries, like China, India, and most african countries. This mainly is because the material is a lot cheaper than less harmful alternatives. The biggest producer of asbestos is the Russian Federation (around 60%

of the worlds annual production of 1,2 Million tons), followed by Kazakhstan, the People´s Republic of China, and Brazil (141). While asbestos consumption per capita in Europe has practically reached zero, even in most countries, that still favor its use, asbestos has been on the fall. As reported by United States Geological Surveys from 2007 and 2016, this is especially clear in former member states of the Soviet Union (see Table 2) (141, 142).

	2007	2016
Ukraine	1,8 kg	0,4 kg
Russian Federation	2,0 kg	1,6 kg
Uzbekistan	3,2 kg	2,2 kg
Belarus	3,5 kg	0,6 kg
Kyrgyzstan	4,0 kg	1,1 kg
Kazakhstan	7,0 kg	1,4 kg

Table 2: Asbestos consumption per capita in former Soviet Union states [modified from ref. (142)]

1.6.2 BAP1 tumor predisposition syndrome

Germline mutations have been reported to be a significant cause of neoplasms in family accumulations of pleural mesothelioma. The rate at which such inherent mutations are responsible for the development of MM over all patients, not considering their history or clinical presentation, is found by Hassan *et al.* (143) to be at 12%. Out of this fraction, more than 60% show alterations to 3p21.2 or close loci which encode for BAP1, making it the most affected enzyme. Due to the autosomal dominant heredity, germline BAP1 mutations show very high penetrance (143, 144).

In 2011 Testa *et al.* published a study that, for the first time, linked BAP1 to increased family rates of PM. Underlying their research were reported family clusters of PM in turkish and U.S. families with up to 50% of the members being affected. These numbers surpass the rates of even high-risk occupational asbestos exposure (4,6 % of affected workers develop PM) by far, indicating a hereditary cause (145, 146). In their 14-year-long prospective study, they focused on two U.S. families, one originating from Wisconsin (W-family), and the other one from Louisiana (L-family). In both families manifold types of cancer were diagnosed, the most common one being PM (11 out of 21 cases). In both families combined, 17 of 21 neoplasms were successfully linked to

BAP1 mutations, with only three of the examined members of the W-family and none of the L-family being cancer free. The families that they focused on were not exposed to the known high-risk environmental factors like asbestos at their workplace. They did, however, live in houses that contained asbestos, which is linked only to a moderate increase in PM, hence not being an explanation for the extraordinarily high rates of neoplasms that occurred in those families (147). Since then, hundreds of families with germline BAP1 mutations have been subject to extensive research, with some of them having PM rates up to over 50%, compared to the 4,6% of patients with excessive asbestos exposure, that develop the same type of cancer.

BAP1 mutations not only are connected to PM but also to other types of cancer, most notably uveal melanomas, cutaneous melanomas, or atypical Spitz-tumors. Even though the linked neoplasms are relatively rare, they are reported to occur in the same patient and accumulate in families. This reportedly led to the conclusion of them being caused by the same autosomal dominant BAP1 germline mutations, being consecutively labeled as “BAP1 tumor predisposition syndrome.” Even though, there are many pathological findings, in which this hereditary syndrome should be suspected, diagnostic criteria have yet to be established (94, 148). When set in relation to sporadic PM, mesothelioma caused by germline BAP1 mutation sets itself apart in multiple ways. To conclude epidemiological differences: Generally speaking, patients with BAP1-related PM caused by familial germline mutations presented at an earlier age, showed multiple neoplasms at once and associated tumors more often, and had a higher peritoneal fraction of mesotheliomas. A larger percentage of them was female and the histological subtype was exclusively epithelioid (144). The supposedly most significant distinction was shown in the patient's prognosis. The carriers' 5-year survival rate was reported to be 47%, much higher than wild-type PM patients, which only showed one of 6,7% (149).

1.7 Radiological examination

Because of the diffuse, circumferential rind-like growth pattern of most cases of pleural mesothelioma, its radiographic evaluation is a complex and challenging task. Here, radiographic imaging does not necessarily have the equivalent diagnostic value as in other types of malignant neoplasms. Imaging focusses on the assessment of the tumor’s extension along the pleura, the potential involvement of nearby structures, that might show signs of invasion, and distant metastasis. The main goal of these

examinations is to make reliable statements about potential resectability, clinical staging, and response to performed therapeutical measurements (150, 151).

1.7.1 X-Ray

Since most patients with symptomatic MM present with chest pain and/or dyspnea, chest X-rays are performed in most cases. They usually happen without the concrete though of a possible mesothelioma, but rather as the most common first imaging of a patient. In the early stages of PM, chest radiography can show unspecific signs of pleural thickening or plaques in some cases but normally is without any pathological findings. In later stages, some signs that can be seen are extensive opacity of the lumen, lung encasement, ipsilateral shift of the mediastinum (due to contraction, the tumor “pulls” towards the affected side of the thorax), calcification or deconstruction of the chest wall. Sometimes, pleural effusion can be visible. Another possible finding is pathological changes in the mediastinal lymph nodes. A diagnosis of MM cannot be made based solely on chest radiography. Most of the possible results are too limited in their utility and can also occur in many other diseases that are far more common, like lung cancer or pneumonia. However, especially with a matching history of asbestos exposure, it can be the first step towards more detailed imaging like a CT scan, which is of higher diagnostic value (152-154).

1.7.2 CT-Scan

Computer tomography is the ruling imaging modality when it comes to the evaluation of pleural mesothelioma. It not only plays an important part in diagnosis but also in the planning of therapeutical measurements and post-treatment evaluation. A wide variety of radiological signs can be present. Yoon *et al.* found pleural thickening to be the most frequent one at 96,1%, followed by pleural effusion (91,3%). Other common findings of the study were pleural plaque (66,0%), nodular pleural thickening (59,2%), and pericardial involvement (56,3%) (155). Present manifestations, among others, might also include the ipsilateral shift of the mediastinum and diaphragmatic elevation, chest wall destruction, or circumferential lung encasement by the tumor. The diagnostic value of computed tomography, concerning pleural mesothelioma, is limited by the fact, that none of its possible findings are pathognomonic to the disease. Pleural irregularities can be of many other origins. The most common reason, for misdiagnosing pleural mesothelioma, is a metastatic pleural disease, caused by other primaries (156, 157). Despite these restrictions, CT remains the most reliable

radiological tool for the initial assessment of patients with suspected pleural mesothelioma. However, to secure the diagnosis, further verification is needed (154). If a patient is considered for surgical treatment, CT offers a good modality for preoperative assessment. If distant metastasis, peritoneal participation, or extensive and diffuse chest wall invasion is found, most of the time surgery should be precluded (158).

1.7.3 MRI

While initial radiographic assessment of pleural mesothelioma virtually exclusively relies on CT, MR imaging is a great tool for further evaluation. Especially patients, that are considered for elective surgical resection of the tumor benefit from additional staging information through MRI. In T1-weighted imaging, pleural mesothelioma appears iso- or slightly hyperdense compared to adjacent muscle tissue and moderately hyperdense on T2-weighted MR images. Enhancement of the tumor, through contrast medium based on gadolinium, allows excellent distinction between the neoplasm and its surrounding tissue. This is also an alternative for patients, where iodinated contrast material in CT is contraindicated. Enhanced contrast by gadolinium not only accounts for mesothelioma but also other kinds of malignancies, so gadolinium-based MRI is not suitable to make a statement about what type of tumor is present (159). The information, gained through MRI, is used to assess for the exact expansion and possible invasion of other structures. MRI has been found by Truong *et al.* to be especially superior to CT in cases of suspected diaphragmatic (69% versus 46% accuracy) and/or endothoracofascial or single focus chest wall invasion (82% to 55%) (160). After performed surgery, the increased precision of MRI in comparison to CT is helpful to evaluate if the targeted definitive resection seems to have been successful (161).

1.7.4 PET/CT

Positron emission tomography combined with CT shows great value in the diagnosis and preoperative staging of pleural mesothelioma. Its biggest strength lies in the graphic integration of radiopharmaceutical [18F]-Fluorodeoxyglucose (FDG) resorption into the depiction of anatomical structures by computed tomography. This means, that high metabolic turnover (like in pleural mesothelioma and its metastatic offspring) of the marked FDG appears as enhanced areas on the underlying CT scan. Although PET/CT has strong limitations, when it comes to evaluating the local

expansion of a primary tumor, it is the superior imaging modality for staging and the detection of distant metastasis, which can easily be missed in other radiological examinations. Additionally, PET/CT only shows little interobserver variability (162). This is especially important in the preoperative staging of a patient and assessing tumor resectability. Ambrosini *et al.* showed in a controlled clinical trial, that a third of patients´ treatment plans had to be modified after additional PET/CT was performed on them, compared to just CT alone. In those cases, PET/CT did not unveil new information about the primary neoplasm but was significantly more precise in detecting spreading to mediastinal lymph nodes and distant metastasis (163). With applying additional PET/CT in mesothelioma, another study by Wilcox *et al.* found 40% of its cases were not suitable for surgical intervention and led to the upstaging of 70% of the examined patients (164). Staging and preoperative assessment are not the only applications of PET/CT concerning pleural mesothelioma. It can also be used to distinguish between benign and malignant tumors in the first place. Terada *et al.* reported 18F-FDG PET/CT to have a high specificity for malignancy in mesothelial tumors; meaning that benign mesothelial proliferations show no enhancement and can be excluded by positive uptake of the radiopharmaceutical marker. Sensitivity, however, only lies at around 60%; meaning that negative results do not eliminate potential malignancy (165).

1.8 TNM - Grading and Staging

1.8.1 TNM categories

The eighth edition of TNM contains the most recent staging method of pleural mesothelioma. After a retrospective analysis of three large groups (2414, 2432, and 3519 cases), some adjustments to previous TNM versions have been made, as proposed by the research team in 2016. The analysis was carried out by the International Association for the Study of Lung Cancer (IASLC) and is applicable for pathological as well as clinical staging. In earlier stages, CT and MRI are not particularly precise and therefore clinical staging is not very accurate (166). 18F-FDG PET/CT can enhance the hit rate, but nevertheless, clinical staging is mostly used in advanced cases (75% are staged III or higher), where imaging becomes more reliable (162). Pathological staging should be applied in definitive resection specimens (extrapleural pneumonectomy/EPP or extended pleurectomy decortication/EPD). If small intraoperative findings occur, a multidisciplinary approach is feasible (167).

T – Tumor

Because of its mostly circumferential and unusual growth pattern, which differs from the concentric expansion of most malignant tumors, defining prognostic criteria for the T-section has shown to be challenging. In the 2016 IASLC Proposal, different methods of assessing T (pre-/postsurgical and others) have been merged and statistically weighted, to evaluate its prognostically most reliable stages. This led to the abandonment of discriminating between T1a (involving parietal pleura) and T1b (involving visceral pleura) since it didn't show any significant benefit.

Table 3 shows the current qualitative criteria for T in pleural mesothelioma, as adapted by the eighth TNM edition (168).

Category	*T-Description*
TX	Primary tumor cannot be assessed
T0	No evidence of a primary tumor
T1	Tumor involving the ipsilateral parietal and/or visceral and/or diaphragmatic and/or mediastinal pleura
T2	Tumor involving each of the ipsilateral pleural surfaces and at least one of the following: • diaphragmatic muscle • pulmonary parenchyma • confluent visceral pleural tumor, with the inclusion of the fissures
T3	Potentially resectable, but locally advanced tumor, that involves all ipsilateral pleural surfaces and at least one of the following: • definitively resectable solitary focus of tumor extending into the chest walls soft tissue • non-transmural invasion of the pericardium • mediastinal fat • endothoracic fascia

T4	Unresectable tumor, that involves all ipsilateral pleural surfaces and at least one of the following: • transdiaphragmatic extension to the peritoneum • extension to mediastinal organs • multifocal and/or diffuse extension to the chest wall +/- associated rib destruction • extension to the spine • extension through the internal pericardium +/- pericardial effusion • involvement of the myocardium

Table 3: Criteria for T in pleural mesothelioma [modified from ref. (168)]

N – Nodes

Assessment comprises the same lymph nodes as in lung cancer plus nodes in the anterior peridiaphragmatic and internal mamillary artery region. In the previous edition of TNM, N1 accounted for intrapleural and N2 for extrapleural lymph node metastasis. In the 2016 proposal of the IASLC, no significant difference in survival between these two categories has been evident. The same has been reported by multiple studies before the proposal (51, 169). Therefore, they have collapsed into a single category. This can be explained because the pleural lymphatic drainage does not necessarily have intrapleural nodes as an initial station, so no universal assumption about the prognosis of intra- versus extrapleural lymph node metastasis can be made (170). For the study, that was underlying the IASLC proposal, only cases with complete information about lymph node involvement have been considered. The extent of N sometimes can be unclear, especially in unresectable tumors. Survival rates in patients with lymph node invasion drop by 50% compared to cases without nodal involvement. Therefore, an accurate staging system with empiric validation is important, as it has major effects on treatment and prognosis. The IASLC has located future potential for N classification. This accounts especially for a more detailed clinical assessment of N, before invasive measurements. Further improvements can be made in more precise reports of the number and extent of nodular involvement, as this shows a more significant prognostic impact than the location of the affected lymph nodes. However, due to inaccuracies and differences in clinical and surgical documentation, the exact implementation has yet to be empirically concluded.

Table 4 shows the updated criteria for N in the eight TNM edition (171, 172).

Category	*N-Description*
NX	Regional lymph nodes cannot be assessed
N0	No metastasis of regional lymph nodes
N1	Metastasis present in ipsilateral lymph nodes (bronchopulmonary, hilar, peridiaphragmatic, intercostal, pericardial fat pad, internal mamillary, or mediastinal)
N2	Metastasis present in contralateral lymph nodes (bronchopulmonary, hilar, or mediastinal) or ipsi-/contralateral supraclavicular lymph nodes

Table 4: Criteria for N in pleural mesothelioma [modified from ref. (172)]

M – Metastasis

In pleural mesothelial tumors, differentiation between M0 (if no distant metastasis is present) and M1 (if there is evidence for distant metastasis) has been sufficient. The IASLC proposal confirms the so far used staging for M, that was put in place in the seventh TNM edition, in 1994.

Consequently, the recommended staging of M in the updated TNM edition remains as shown in Table 5 (173).

Category	*Description*
MX	The presence of distant metastasis cannot be assessed
M0	No evidence of distant metastasis
M1	Evidence of distant metastasis

Table 5: Criteria for M in pleural mesothelioma [modified from ref. (173)]

1.8.2 Combined TNM Staging

After assessing T, N, and M individually, the present combination of each of the three factors results in a definitive staging of the tumor. There are other staging systems of mesothelioma, like the Butchart system or the Brigham system. Both are not or hardly used today, as the TNM system has proven to be the most accurate and become widespread and understood. In combination T, N, and M result in a grouped stage from I to IV. As a short overview, I, II, and III are considered potentially resectable, with the

(unsharp) border of resectability lying in between stages III and IV. I and II are mostly contained within the pleura, stage III shows extension beyond pleural borders. Stage IV is characterized solely by distant metastasis and therefore is attached to the worst prognosis.

Table 6 shows the combined and definitive TNM staging for patients with pleural mesothelioma (174).

		Individual category		
Stage		*Tumor*	*Nodes*	*Metastasis*
I	IA	T1	N0	M0
	IB	T2/3	N0	M0
II		T1/2	N1	M0
III	IIIA	T3	N1	M0
	IIIB	T1-3	N2	M0
IV		T4	N0-2	M0
		Any T	Any N	M1

Table 6: Combined TNM staging of pleural mesothelioma [modified from ref. (174)]

1.9 Treatment Options

Over the past years, the trend in the management of malignant tumors has been developing rapidly towards an interdisciplinary approach. Modern treatment options like immunotherapy, 3D-modular radiation, and improved best supportive care have changed the handling of pretty much all kinds of neoplasms. Pleural mesothelioma is no exception. Since the overall survival rate in PM is still low when compared to other tumors, true curative settings remain rare. Quality of live, however, has improved massively, especially due to tailored multimodule measurements for the best individual care of each patient. These are composed of surgical interventions, chemotherapy and supportive medicaments, radiation, psycho-oncological therapy, and best palliative support in incurable and terminal cases. The focus on different aspects of these components is subject to the decision of interdisciplinary tumor boards and depends on many varying factors in each case. The most common approaches are described in the following paragraphs (22, 175).

1.9.1 Curative surgical interventions

The pleural mesothelioma growth pattern, is different from other tumors, since it does not expand from a solitary lesion, but rather shows circumferential and areal expansion along pleural surfaces. This makes it impossible to achieve true R0-resection. The surgical intervention’s primary goal is macroscopic radical resection and must be embedded in a multimodal interdisciplinary treatment plan. Expedient objective comparison of the different techniques, described below, is somewhat challenging. This is because of the strong dependency on the performing surgeon's experience, as well, as the incomparability of older studies, due to divergent documentation and terminology. Decisions, regarding the best possible procedure, should therefore be made individually, and in a multidisciplinary setting. They should be based on the clinical presentation and the experience of the surgeon in the respective intervention. Comparative studies are running, most prominently the MARS-2 trial in the UK (176), to provide a clear path for finding the right surgery for each patient. Studies, examining the benefits of surgical cytoreduction concern the epithelioid subtype. To this day, no studies have shown any prognostic improvement of surgical interventions, in the sarcomatoid or biphasic subtype. The same goes for patients with N2 and/or stage IV disease, according to the eighth TNM edition (177).

Extrapleural pneumectomy (EPP)

This intervention is the oldest curative technique for radical macroscopic resection of pleural mesothelioma. It consists of the total removal of the visceral and parietal pleura, the pericardium, the diaphragm, and the lung of the affected side of the thorax. The pericardium and diaphragm must be reconstructed with a patch afterward. It is performed through posterolateral access in the sixth intercostal space, including the removal of a rib, if more space is required. Resection should be en-bloc and with caution to avoid tumor seeding into the surrounding tissue. EPP is a long and complex surgery, which should be performed in specialized centers. More recent studies have favored EPD in comparison to EPP, because of EPP´s prolonged morbidity and its lesser quality of live, due to the compromised respiratory capacity (175, 178).

Extended pleurectomy and decortication (EPD)

As the name suggests, EPD can be considered as a magnified version of the below described P/D. In addition to the en-bloc removal of the visceral and parietal pleura, mediastinal lymph nodes and the ipsilateral diaphragm and pericardium are resected

as well. It is the most extensive resection possible while leaving the affected lung inside the thorax. EPD is a complex and long surgery. It should be performed in specialized centers and only if the patients' physical conditions allow the laborious intervention and the required pre- and postsurgical measurements. Surgeons will usually enter through the antero- or posterolateral sixth intercostal space. Sometimes the removal of an adjacent rib is helpful if the thorax has shrunken, and conditions are less spacious. After the intended removal, the diaphragm and pericardium must be reconstructed. This is done with synthetic patches. Since the visceral pleura plays an important role in preventing pneumothoraxes, the lung must be sealed thoroughly, after the removal. This can be achieved with fibrin glue. To further prevent air from exiting the decorticated lungs into the thorax, drains must remain without suction. Intensive respiratory therapy should be performed soon after surgery (178, 179).

Pleurectomy and decortication (P/D)

The surgical performance P/D is similar to EPD but describes the en-bloc resection of only the visceral and the parietal pleura. It is performed in patients, that are not fit enough to undergo EPP/EPD or if radical resection is expected to be achievable without the removal of the diaphragm and pericardium. Like in EPD, the quality of life in patients that undergo P/D is improved, when compared to EPP. Because the lung is not resected, patients show better respiratory reserves as well as perfusion and ventilation capacities. This enhances their physical resilience towards further treatment, which positively affects survival rates. However, due to the dependency on the surgeon's experience and the heterogeneity of protocols and documentation in different hospitals, data on P/D remains rather inconclusive and should be seen critically (178, 179).

1.9.2 Oncological therapy

Systemic treatment in pleural mesothelioma is performed in three different settings. If performed as a neoadjuvant treatment, it aims at the presurgical reduction of tumor mass. The same can be done after surgery, to reduce tumor cells, which were not yet resected or discovered, to the furthest possible extent. Patients in advanced stages of the disease receive medication with the target to expand life expectancy and reduce symptoms. As of today, options regarding oncological therapy are limited, although there are many ongoing studies to widen the spectrum of possible medication. Reliable data, concerning effective medication, is rare and must be seen critically. Implementing

study-based combinations is challenging in clinical practice. The few existing underlying studies were performed on collectives, which did not represent clinical everyday life, resulting in strong recommendations but weak evidence. The latest ERS/ESTS guidelines for the treatment of pleural mesothelioma do not recommend different first-line medication, dependent on the various histological subtypes. Therefore, platin-based chemotherapy can still be considered the driving force behind the oncological treatment of PM. Extensive research is performed on other therapeutical modalities, such as immunotherapy. Especially for sarcomatoid and biphasic mesothelioma, the standard procedure is expected to change, in the nearer future, toward the use of two combined checkpoint inhibitors (175).

First-line therapy - Platin / Pemetrexed

Today combined Platin (mostly Cisplatin) and Pemetrexed chemotherapy is the most prominent systemic oncological medication in pleural mesothelioma. It is used in inoperable cases, as well, as in patients who receive neoadjuvant treatment, and is recommended without dependence on the histological subtype. Cisplatin/Pemetrexed was first proven to be an effective combination in 2003 when Vogelzang *et al.* reported it to increase median overall survival from 9,3 to 12,1 months when compared to Cisplatin alone (180). Because the study excluded patients with critical comorbidities and/or an ECOG score of >1, it is not universally applicable in a clinical setting. A patient's general condition, comorbidities, or other contraindications have a profound impact on the practicability of chemotherapy. According to the ERS/ESTS guidelines, patients who are fit for Cisplatin/Pemetrexed chemotherapy should also receive supplementary folic acid and vitamin B12. In case of toxic cumulation, progression of the disease, or grade 3-4 side effects (peripheral neuropathy, gastrointestinal and hematologic toxicity, and fatigue), treatment with Cisplatin/Pemetrexed should be terminated. Patients who respond to the medication can receive up to six cycles of chemotherapy (179). In all histologic subtypes, if a patient´s general condition after those cycles is suitable, he or she can be re-exposed to Pemetrexed-based medication (181). Certain contraindications can prevent the use of Cisplatin, the most common ones being nephrogenic or cardiac comorbidities. In that case, Carboplatin, which has been reported to positively affect survival rates, could be a viable substitute (182). However, no randomized phase-III studies, which directly compare the effectiveness of Cisplatin and Carboplatin, have been performed to this day. The use of Carboplatin,

therefore, remains limited to individual cases, and Cisplatin should be preferred in all histological subtypes if no contraindications are present (175). In cases where platin-based medication can be used, but Pemetrexed is contraindicated, Byrne *et al.* have reported Gemcitabine to be a viable alternative. Again, this is based on a phase-II study and should be considered only in individual cases (183). In patients, where no platin-based combination is administrable, options are mostly reduced to off-label monotherapies. This can for example be the use of just Pemetrexed, without additional platin-based medication (184). Various other second- and third-line therapies have been performed, but (except for Platin/Pemetrexed re-exposure as second-line medication) are based only on small sample retrospective analysis and individual experience (185-187).

Bevacizumab

This monoclonal antibody binds VEGF and inhibits angiogenesis. The National Comprehensive Cancer Network (NCCN) guidelines recommend its additional administration to patients, receiving first-line Cisplatin/Pemetrexed therapy (188). This applies to patients, whose physical condition allows these medications, but does not impede macroscopic resection. In a MAPS-study, Zalcman *et al.* reported the addition of Bevacizumab, to conventional first-line chemotherapy, to increase the median survival rate in pleural mesothelioma by 2,7 months. Along with this, they also showed increased side effects, namely grade III/IV hypertonia (23% versus 0%), grade III proteinuria (3,1% versus 0%), and grade III/IV thrombotic events (6% versus 1%) (189). However, although Bevacizumab shows a positive overall impact, its approval has neither been requested in the US nor in Europe (175, 179).

Immune checkpoint inhibitors

Immunotherapy and immune checkpoint inhibitors are commonly used in the treatment of many different neoplasms, especially in a multimodal approach and in addition to chemotherapy. In pleural mesothelioma, these options are less established than in other malignancies. In PM, immunotherapeutic substances like Interferon gamma, Interleukin-2, or tumor necrosis factor alpha have been examined in clinical studies for more than two decades without notable progress or effects on its treatment (190). Immune checkpoint inhibitors, a relatively new group of monoclonal antibodies, represent a promising new weapon against pleural mesothelioma. Multiple phase II and III studies (namely, among others, MAPS-2, DREAM3R, PRE0505) are currently

ongoing and already offer promising results, but so far do not have a major impact on guidelines or recommendations (191-193). Only a few phase III studies, concerning the effects of immune checkpoint inhibitors on neoplasms, and their further development and implementation into clinical practice have been concluded. The most notable ones are described below.

- *Checkmate-743-Study*

  This is the first concluded randomized phase-III study, concerning first-line ICI-use in patients with pleural mesothelioma. It compares the immunotherapeutic effects of two combined monoclonal antibodies (Nivolumab, targeted at PD-1 and Ipilimumab, targeted at CTLA-4) to established chemotherapy (Platin/Pemetrexed). Six hundred patients, all diagnosed with inoperable pleural mesothelioma, were included. They were stratified, based on their sex (male/female) and the histological subtype of their tumor (epithelioid/non-epithelioid). ICI-combination therapy showed a significant increase in median overall survival in all histological subtypes, especially in non-epithelioid mesothelioma (see Table 7). Further, median overall survival was significantly increased in ICI-recipients, whose tumor cells showed ≥1% PD-L1 expression (see Table 8). Patients who received combined immunotherapy had a better 2-year survival rate than those with chemotherapy (41% versus 27%) but also expressed more adverse grade III/IV side effects (15% versus 6%). Concluding, the combined therapy with Nivolumab and Ipilimumab did not only show significant benefits in the first-line therapy of inoperable pleural mesothelioma. It can also be considered a new standard procedure in patients with non-epithelial subtypes, where surgical resection is not an option (194).

	Platin/Pemetrexed	Nivolumab/Ipilimumab
All subtypes	14,1 months	18,1 months
Epithelioid PM	16,5 months	18,7 months
Non-epithelioid PM	8,8 months	18,8 months

Table 7: Median overall survival by histological subtype [modified from ref. (194)]

	Platin/Pemetrexed	Nivolumab/Ipilimumab
PD-L1 negative (<1%)	16,5 months	17,3 months
PD-L1 positive (≥1%)	13,3 months	18,0 months

Table 8: Median overall survival, dependent on PD-L1 expression [modified from ref. (194)]

- *PROMISE-MESO-Study*

  This multicentered randomized phase-III trial, by the European Thoracic Oncology Platform (ETOP), compared single-agent chemotherapy (Vinorelbine or Gemcitabine) to Pembrolizumab in 142 patients, who already received first-line platin-based chemotherapy. Pembrolizumab is a monoclonal antibody, targeted at the PD-1 receptor, to prevent PD-L1 from docking it. Progression-free survival (PFS) was determined as the primary endpoint. No significant difference could be shown, neither in PFS nor in median overall survival rates (see Table 9). Also, grade III/IV side effects were of similar prevalence in the two randomized groups (24% in chemotherapy, 19% in Pembrolizumab). However, patients who received Pembrolizumab had a much higher response rate to their medication, with 22% showing an initial response, compared to just 6% in single-agent chemotherapy (195).

	Vinorelbine *or* Gemcitabine	Pembrolizumab
PFS	2,5 months	3,4 months
mOS	10,7 months	11,7 months

Table 9: Single-agent chemotherapy compared to Pembrolizumab [modified from ref. (195)]

- *CONFIRM-Study*

  In this study, 332 patients with pleural or peritoneal mesothelioma, and evident progression of the tumor after first-line platinum-based chemotherapy were included. After a randomized allocation, two-thirds of the participants received Nivolumab (anti-PD-1 monoclonal antibody), and the other third obtained a placebo. The primary endpoint was the overall survival (OS) of the two groups, which was significantly increased in patients who were treated with Nivolumab. Additionally, median PFS was reported to be higher, while no correlation between PD-L1 expression and overall survival could be shown (see Table 10).

With manifestation in 45% of the Nivolumab and 42% of the placebo group, the prevalence of severe adverse side effects (grade III/IV) showed no significant difference (196).

	Nivolumab	Placebo
Overall survival	9,2 months	6,6 months
Median PFS	3,0 months	1,8 months

Table 10: Second line Nivolumab compared to placebo [modified from ref. (196)]

1.9.3 Radiotherapy

For the longest time, field experts considered radiation therapy to be of very limited use in curative settings of pleural mesothelioma, and to be mainly applicable as a palliative therapy. This can in large part be attributed to the rather experimental initial approach to the technique, and the limited knowledge of the exact short- and long-term effects, radiation would have on the tumor and the patient. Recent developments, especially during the last 15 years, have caused radiotherapy to be considered a potentially curative treatment, ideally embedded in a multimodal therapeutical approach (179). Important steps, which led to this change in perspective are:

- Personalized computer-based planning of radiation enabled focusing on target-volumes and precisely defined areas, and made it possible to define structures, which should be spared from radiation as much as possible. This replaced the rather inaccurate method, where radiation was applied onto a field on a patient´s body, supposedly equivalent to an imagined tumor projection. Further, computer-based planning made individual dose calculation easier and sent precalculated tables into redundancy (179).
- PET-CT made it possible to accurately display areas of enhanced tumor activity, making it superior to conventional CT in the planning of radiation treatment. To this point, CT could already show detailed images of internal structures, but the assessment of exact tumor expansion, and the consequential borders of radiation therapy represented the challenging task of the responsible radiologist. By highlighting areas of high proliferation activity, PET-CT enables

a three-dimensional demarcation of the tumor, where radiation then can be focused (197).

- Today radiation intensity can be modulated in specifically targeted areas. Rotating accelerators allow focusing on complex three-dimensional figures within the patient's body. This enables concentrated radiation in a location with high proliferative activity. The price for the higher intensity in the respective malignancy is a greater amount of healthy tissue exposed to low-dose radiation. This dose however, is nowhere near as aggressive, as in the center of a targeted location (198).
- The most recent major improvement was the implementation of a "fourth dimension," namely the adaption to temporal changes in a patient, during radiation exposure. Until this point, positional shifts of a tumor or organs were compensated by widening the targeted volume. With 4D-CT, these changes are automatically calculated, all the way down to small, breath-related movements. Due to the resulting reduction of targeted volume, the applied radiation dose is kept to the lowest necessary amount (179).

Today, radiation therapy has secured a place within the interdisciplinary approach to pleural mesothelioma. Precision and effectiveness of radio-therapeutical measurements depend heavily on the radiological and technical expertise and experience of the responsible therapeutic team and are not to be considered trivial. Radiotherapy usually takes place in either a neoadjuvant/adjuvant or palliative setting. The lung is extremely sensitive to radiation. More than 20Gy generate irreparable damage to the lung's parenchyma, if large areas are affected, doses as low as 5Gy can cause partial or complete loss of function. Therefore, the administered dose in a pre- and postsurgical application depends on the performed intervention. In EPP, the used amount of radiation can be as high as 50Gy. Damage to the ipsilateral lung should not be considered because it will be resected anyway. Today EPP has widely been replaced by the preferred P/D or EPD. Combining these surgical interventions with neoadjuvant/adjuvant radiotherapy has not been possible for a long time, due to the lack of selective radiation methods (199). With the developments, described above, it is now possible to focus on small malignant areas and prevent healthy residual lung parenchyma from being damaged in the process (200). In 2019, Minatel *et al*. published a randomized phase-III study,

including 108 patients that underwent neoadjuvant Platin/Pemetrexed chemotherapy and lung-preserving surgery. They then compared adjuvant palliative (20-30Gy onto thoracal access routes and macroscopic tumor) to radical experimental (up to 60Gy with the exclusion of healthy lung parenchyma) adjuvant radiotherapy. The study reported significantly higher two-year-survival-rates in patients, who received radical radiation (58%) than in those who underwent palliative doses (28%) (201). Although this study has several limitations, it can serve as a guiding point for further implementation of radiotherapy as an integral part of multimodule therapy concepts for mesothelioma. (179)

1.9.4 Palliative and psycho-oncological aspects

Consequential to the mesothelioma's abysmal long-term survival rates, palliative treatment, and best supportive care constitute an inherent part of pleural mesothelioma therapy. The focus lies on relief of physical and psychological symptoms and, in most cases, is required for a brief period, because of the disease's short average survival. Most patients experience strong physical, mostly pain-related symptoms. A wide variety of medications or interventions, like trigger point injections, can provide a diminution of pain. These will not be described in detail here. As in other palliative or terminal cancer patients, the importance of psychological aspects of disease management cannot be overstated. This holds true, even if a patient has just weeks to live. Depression, resulting from symptoms like pain or dyspnea, or the confrontation with the disease and death, heavily impacts patients' quality of live. In a large retrospective study by Zaorsky *et al.*, the included 8,6 million people cancer patients were 4,4 times more likely to commit suicide than the general population (202). Zaorsky *et al.* further reported in a different study, that among all cancer patients, those with malignancies related to the lung had the highest suicide ratio within the first year after diagnosis (203). This might be attributed to the high discomfort, which breathing-associated symptoms cause when compared to other malignancies. Within the first four weeks after diagnosis, about one-third of cancer patients develop a mental disorder (among others including anxiety, adjustment, or somatoform disorders), which further emphasizes the need for early psycho-oncological interventions (204).

1.9.4.1 Palliative surgery – Pleurodesis

One of the biggest subjective restrictions for patients´ quality of life, is increased dyspnea, associated with advanced stages of mesothelioma. The symptom is often

caused by malignant pleural effusion, preventing the lung from unfolding to its residual potential. The goal of pleurodesis is to counteract this mechanism, by merging the parietal and visceral pleura and closing the interpleural gap. This intervention prevents the leakage of malignant pleural effusion into the thoracic cavity, allowing patients to breathe more freely. In most cases, pleurodesis is performed by introducing talcum, a silicate, into the interpleural gap. The foreign material causes inflammation, which further results in the mutual adhesion of the parietal and visceral layers. Because this indirectly connects the lung to the thorax wall, ventilation is less laborious, which reduces patients' dyspnea. Pleurodesis is a cheap and easy intervention. Complications are rare and it shows high success rates (205). In a prospective study by Walter *et al.* 87,8% of patients that received pleurodesis reported a significant improvement in their subjective well-being, making it an important treatment option in palliative cases (206).

2 Material and Methods

2.1 Study cohort

Representative paraffin blocks of PM diagnosed between 2012 and 2019 were retrieved from the archives. Each case was reassessed using the WHO 2021 criteria (207) to confirm the diagnosis and identify paraffin blocks containing sufficient tumor tissue for constructing a tissue microarray (TMA). Suitable tissue regions were chosen, and three cores of tumor tissue were transferred to the TMA. The research adhered to the guidelines of the Helsinki Declaration and received approval from the Ethics Committee of the Medical University of Graz (30-475 ex17/18).

2.2 Immunohistochemistry

TMAs were constructed using the manual tissue microarrayer Quick-Ray (Unitma Co., LTD., Korea), employing 5x6 matrixes of 3 mm cores, with three samples taken per patient. These samples were stained with hematoxylin and eosin (HE) and subjected to a thorough review before proceeding to immunohistochemistry analysis.

For IHC analysis, 4 µm sections of the TMAs were utilized and processed using the BenchMark XT (Ventana) automated staining system. Various antibodies were employed, including BAP1/2433 from Novus Biologicals, BAP1 (C-4) Antibody from Santa Cruz Biotechnology, Inc., and BAP1 Monoclonal Antibody (1G8) from LifeSpan BioSciences. Despite employing different protocols and making variable adjustments, only the C-4 clone antibody proved effective in producing adequate nuclear staining. This assessment was conducted by comparing the staining intensity of tumor cells with internal positive controls such as lymphocytes and fibroblasts.

To ensure accuracy and consistency, each core was independently evaluated by two assessors, namely LB and AW. They assessed the presence or loss of nuclear staining in tumor cells and determined the percentage of stained tumor cells. The evaluation results for each core were recorded in an Excel table, and the mean value was calculated for each patient, providing a comprehensive overview of the staining characteristics.

3 Results

3.1 Study group

The examined study group consisted of 58 patients, 43 of which were male, and 15 were female (see Figure 10). Among all cases, the median age at the time of first biopsy was 68 years. The youngest patient was 43, while the oldest was 85 years of age.

In separate analyses of gender groups, the median age among female patients was lower by ten years. The male median age was 71 years, while among female patients it was 61 years. In all patients the second and third quartile, making up for 50% of the whole group, lied between 60 and 74 years. This was similar in the male subgroup, where the according quartiles were spread from 63 to 75 years. Again, the female subgroup was comparatively younger, with 50% of the group lying between 55 to 69 years (see Figure 11).

When looking at the overall age distribution in the examined group the peak was at the age of 71–75 years, followed by a drop off (see Figure 12).

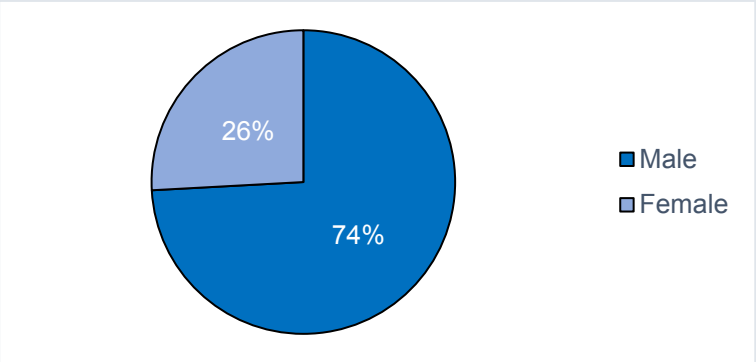

Figure 10: Gender ratio of examined patients

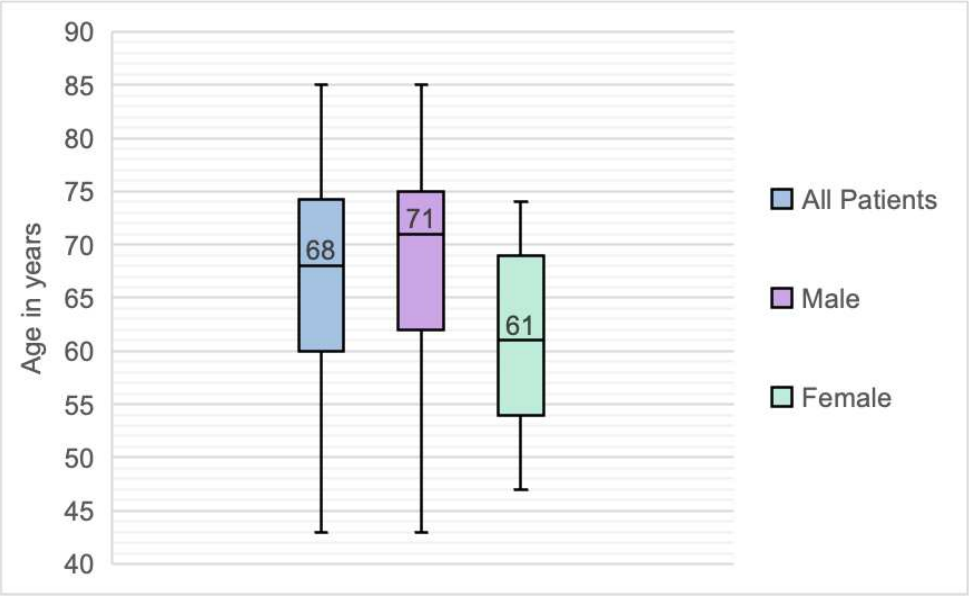

Figure 11: Median age of examined cases

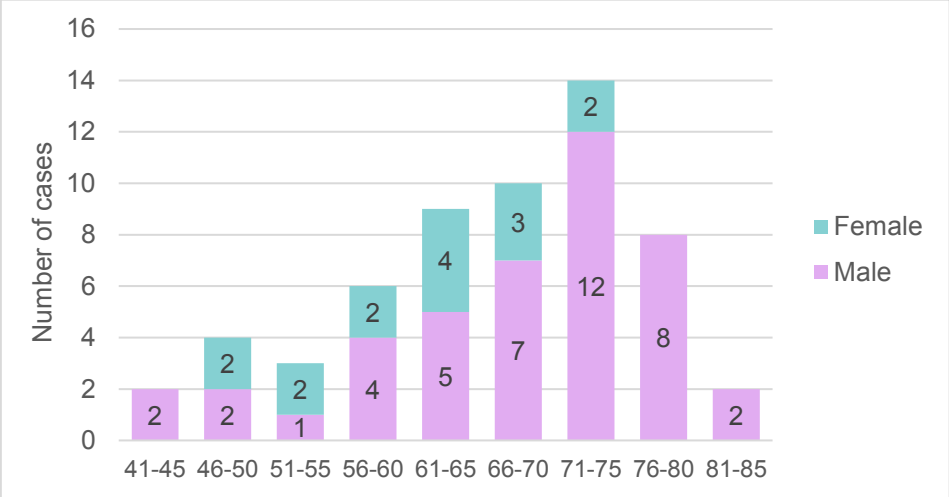

Figure 12: Age distribution among all cases

3.2 Histological subtypes and their distribution

Histologic subtyping of the examined mesotheliomas was performed before the application of the BAP1 antibody. Out of the 58 cases, 41 were diagnosed as epithelioid pleural mesothelioma. Nine were proven to be sarcomatoid and eight had both histological components in them and therefore were classified as biphasic pleural mesotheliomas (see Figure 13).

When looking separately at the study groups of male and female patients, the ratios of the individual histological subtypes differ from the overall collective. While the male subcategory showed very similar percentages in comparison to the overall patient

collective, the female group did not include any cases of sarcomatoid pleural mesothelioma (see Figure 14).

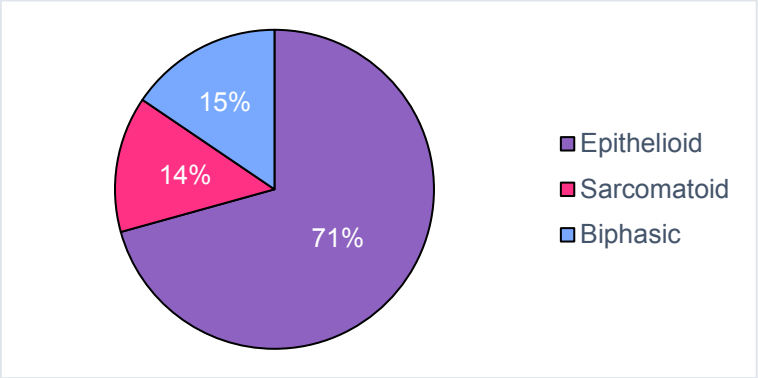

Figure 13: Histological subtypes of examined probes (all patients)

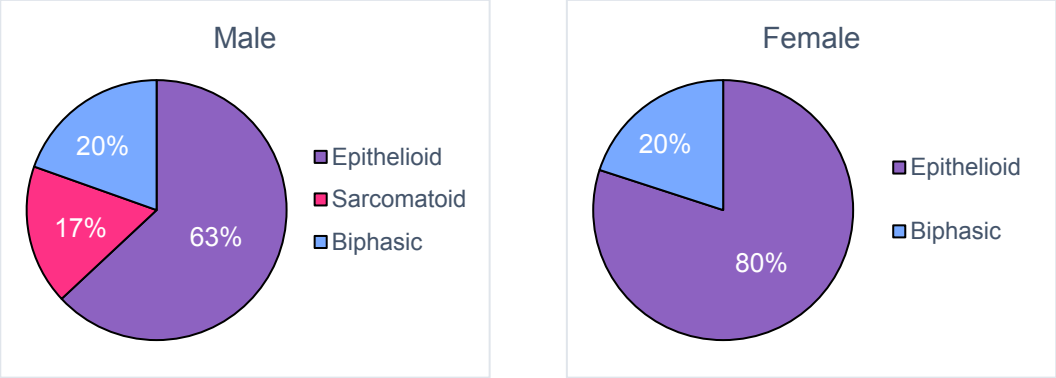

Figure 14: Histological subtypes of examined probes (by gender)

The gender ratio within the present study collective has been described above. Another aspect to show is the fraction of each gender within the different subtypes. Out of 41 patients with the epithelioid type of pleural mesothelioma, 29 were male, and 12 were female. Comparable relative distribution was present in the examined cases with biphasic subtype mesothelioma, with nine male and three female cases (Figure 15). The eight examined cases of SPM did not include any female patients (as portrayed in Figure 14).

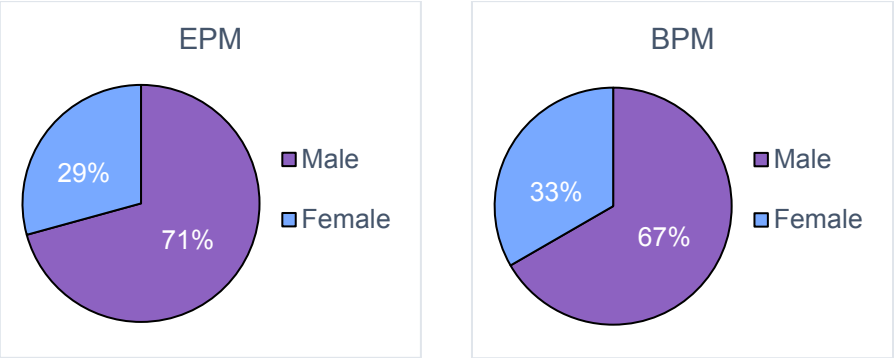

Figure 15: Study group gender ratios in EPM and BPM

3.3 BAP1 Immunohistochemistry

Out of the 58 patients, 41 had all three potential cores available in the TMA slides. Eleven cases had two useable cores and five cases had only one core. For one patient all three cores of the array were not evaluable (see Figure 16).

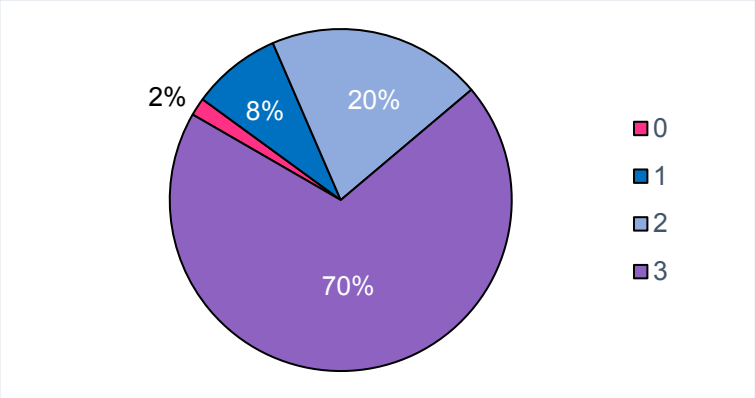

Figure 16: Usable microarray cuts per probe (all subtypes)

When looking at the individual histologic subtypes, a similar distribution to the overall collective was seen. All three subtypes had two or three successfully stained slides in at least 88% of the cases (see Figure 17). In all cases where multiple cores were available, the result of the BAP1 staining, positive or negative, showed consistent results. This means, that the immunohistochemical staining did not show any divergence within each sample in our TMAs. Accordingly, a singular viable slide might be considered representative of the whole sample.

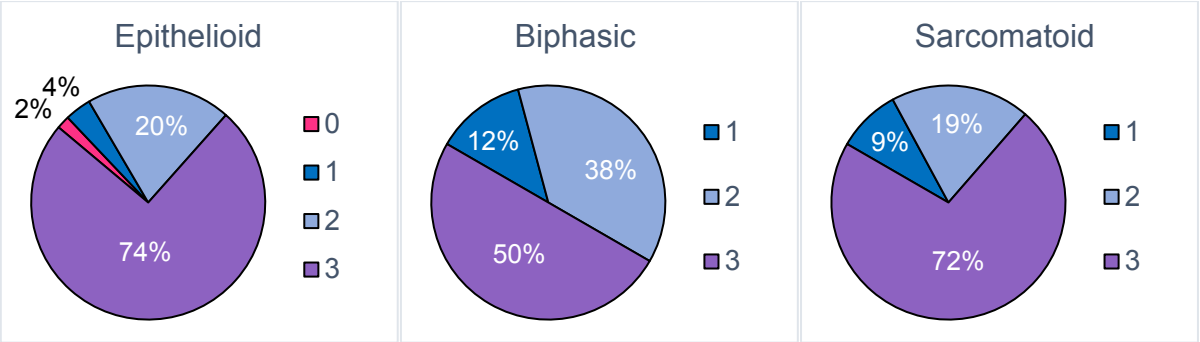

Figure 17: Usable microarray cuts per probe (separated by subtype)

The largest portion of our cohort consisted of epithelioid pleural mesotheliomas. 40 out of the 41 cases were successfully prepared, meaning that at least one of the three immunohistological staining was found to be adequate for examination. Out of these 40 cases, nuclear BAP1 staining was present in the probes of 13 individuals (see Figure 18). The other 27 samples showed a loss of BAP1 (see Figure 19). When separated by gender, the relation of positive and negative staining remained the same as in the whole cohort (see Figure 20). In all three groups (male, female, and overall patients), about two-thirds of the viable probes showed BAP1 loss compared to one-third that was proven to have retained nuclear BAP1 staining (see Table 11).

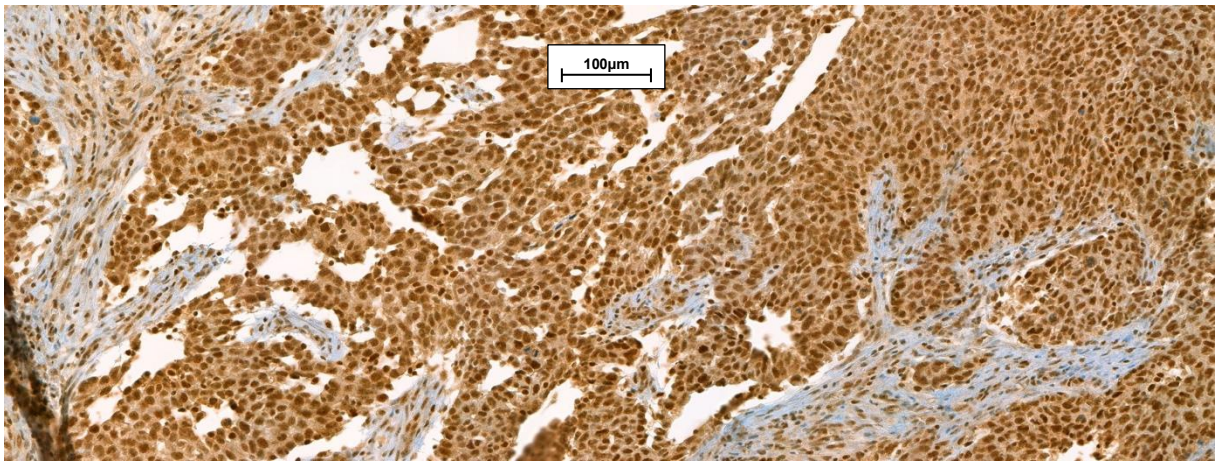

Figure 18: BAP1 staining presentation in epithelioid mesothelioma, without loss of nuclear staining in tumor cells

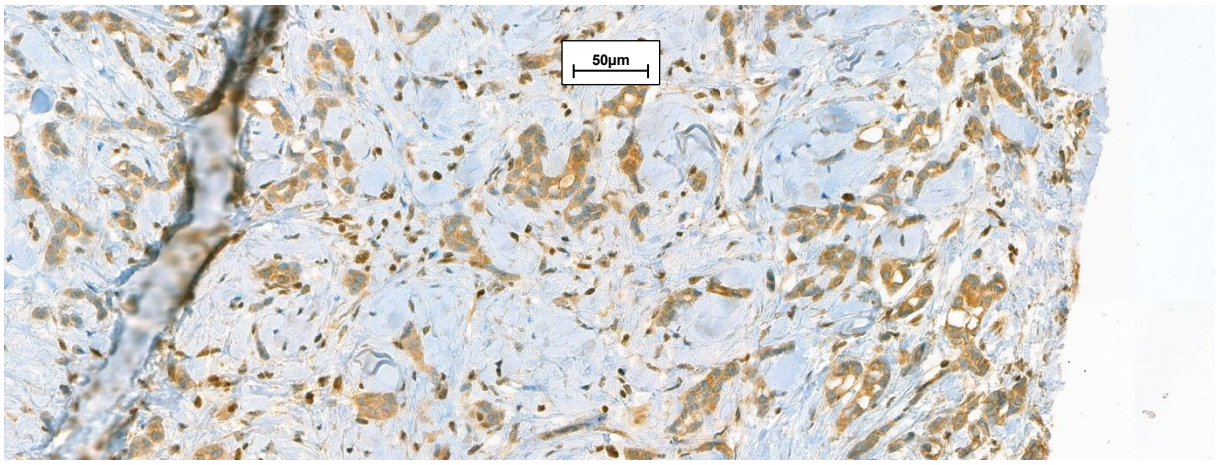

Figure 19: BAP1 staining in EPM, with loss of nuclear staining in tumor cells

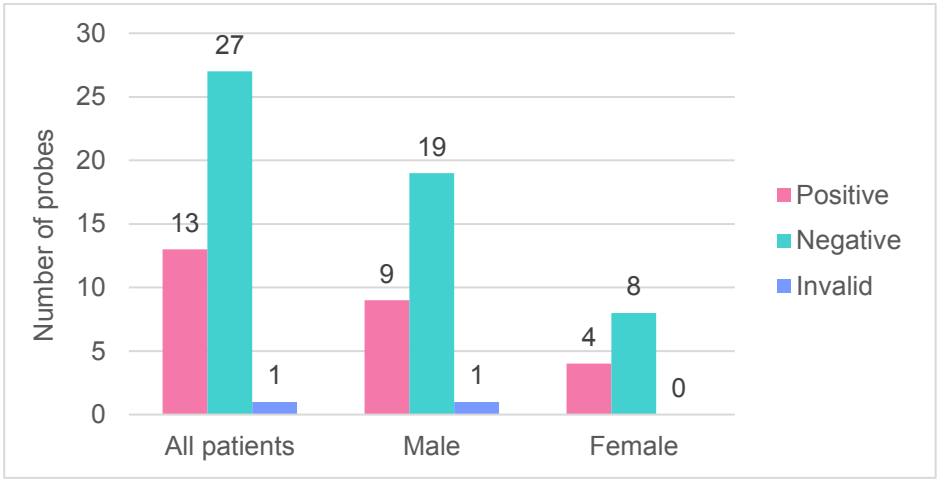

Figure 20: BAP1 staining of epithelioid type mesothelioma

	Positive		*Negative*	
All patients	13	32.50%	27	67.50%
Male	9	32.14%	19	67.86%
Female	4	33.33%	8	66.67%

Table 11: BAP1 staining in epithelioid type mesothelioma (valid cuts)

When looking at the staining results of biphasic pleural mesothelioma samples, an interesting difference to purely epithelioid samples became evident. Even though the same staining process was performed, only two out of nine BPM samples showed retained nuclear BAP1 staining of epithelioid components. At the same time, eight of them showed retained nuclear BAP1 reaction in the sarcomatoid part (see Figure 23).

Six of those showed loss of nuclear BAP1 in their epithelioid compartment (see Figure 21). Given the reliability of the immunohistochemical staining procedure, this means that in the examined BPM probes, the loss of nuclear BAP1 was more common in the epithelioid component, than in its sarcomatoid counterpart. Only one of the nine samples did not show staining in either of the two which represents a complete loss of nuclear BAP1 staining in the examined probe. No BPM sample showed positive results solely in its epithelioid fraction. Two probes showed fully intact nuclear BAP1, proven by positive staining in the epithelioid as well as the sarcomatoid compartment (see Figure 22). A quantitative comparison of the different component staining combinations is shown in Figure 24.

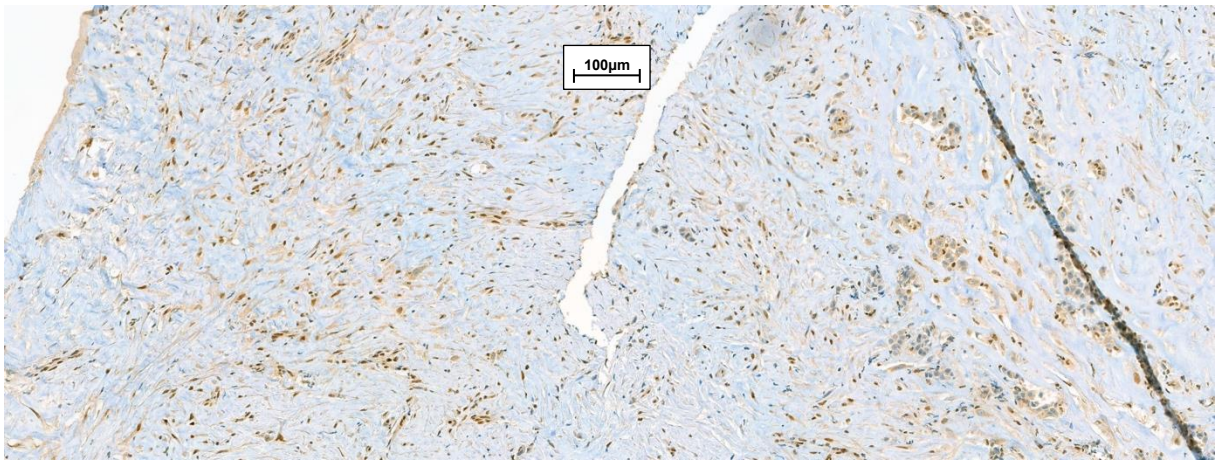

Figure 21: BAP1 staining presentation in biphasic mesothelioma, without loss of nuclear staining sarcomatoid part, and lost staining in epithelioid part

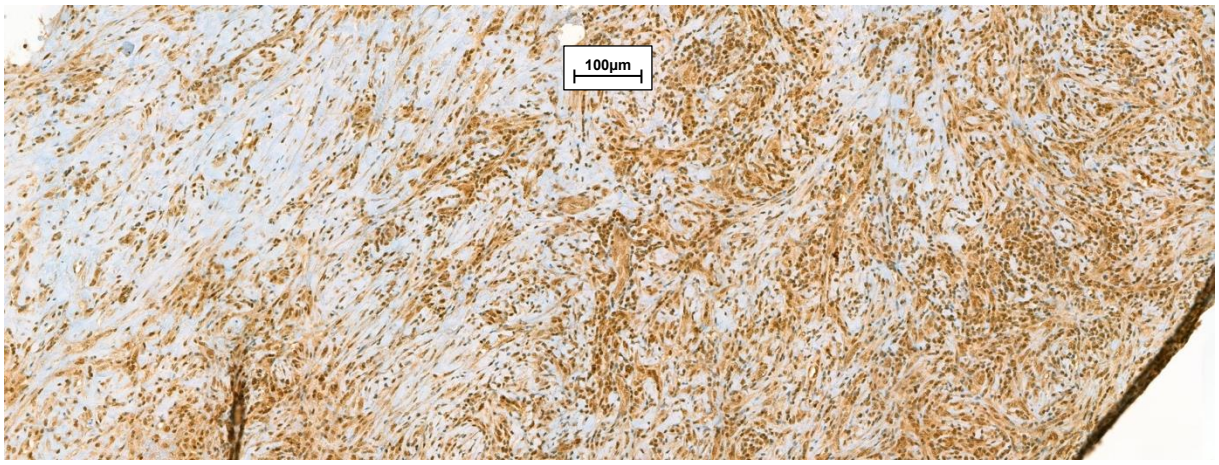

Figure 22: BAP1 presentation in BPM with positive nuclear staining in both compartments

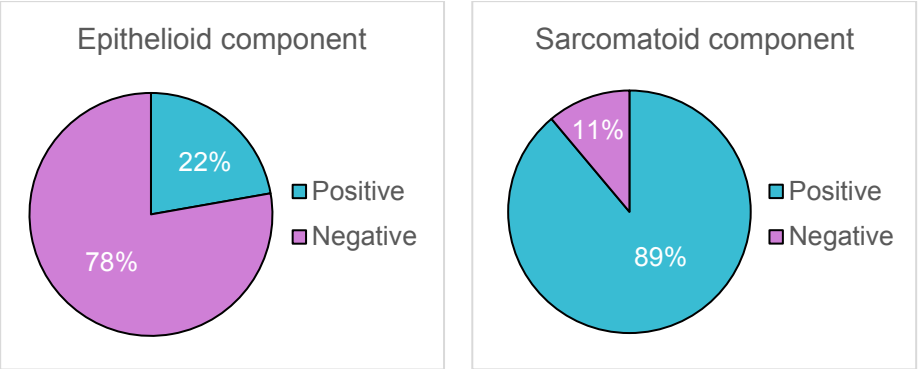

Figure 23: BAP1 staining results in BPM, separated by components

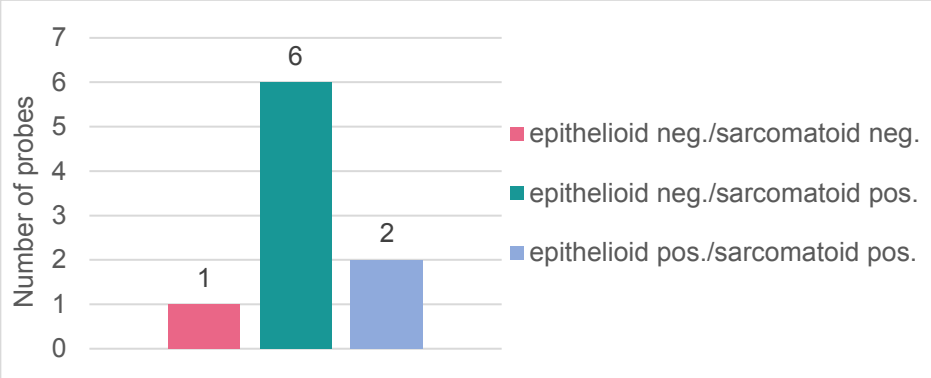

Figure 24: BAP1 staining results in BPM, component combinations

This study included eight examined cases of sarcomatoid mesothelioma. Five of them stained positive, showing the presence of nuclear BAP1 in the samples (see Figure 25), while three showed loss of BAP1 nuclear staining (see Figure 26). This is consistent with the BAP1 staining results in the sarcomatoid compartment in examined biphasic pleural mesotheliomas (see Figure 24 and Table 11).

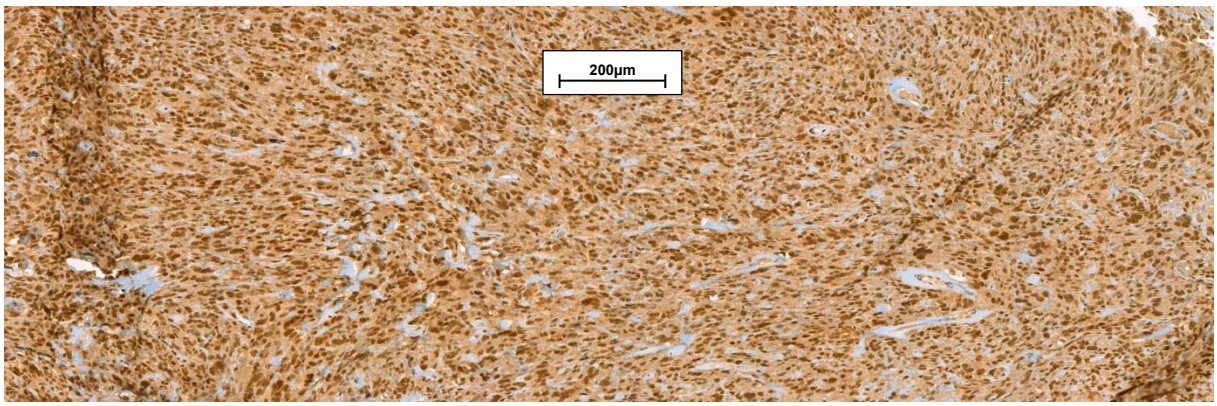

Figure 25: BAP1 staining presentation in SPM, without loss of nuclear staining

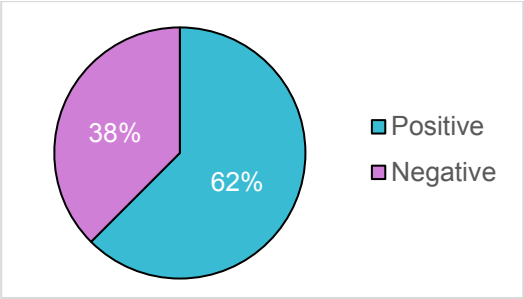

Figure 26: BAP1 staining results in SPM

4 Discussion

The role of BAP1 in pleural mesothelioma has become increasingly important over the past decades, especially in the last few years, with more precise, reliable, and available methods to examine its nuclear expression. It functions as a tumor suppressor gene (123) and accordingly, the loss of BAP1 expression is considered a major criterion for malignancy in pleural mesothelioma. This is especially important since it is known that the loss has not been described in any non-malignant lesion so far. In practice, this means if we have tumor cells/tissue, with proven mesothelial origin and loss of BAP1 immunohistochemical expression, it can certainly be defined as a malignant lesion (29). This is very useful, especially in cytological samples, where, before the use of BAP1 immunohistochemistry, it was very difficult to reliably diagnose mesothelioma, since also reactive mesothelial cells can show atypical features. However, retained BAP1 expression does not exclude malignancy, which is important to keep in mind in a diagnostic process (79, 97).

This study aimed to compare different clones of BAP1 antibody, and to analyze possible differences in staining pattern, percentage of positively or negatively stained cells, and heterogeneity of staining intensity and positivity. Unfortunately, we were not able to achieve adequate staining with two out of three tested BAP1 antibodies. Therefore, our results were produced only based on the staining with the most used BAP1 clone (C-4). Although we could not compare different clones, we have analyzed clinical characteristics as well as BAP1 positivity in our cohort and compared these with available published data. Major characteristics of our patient collective, including age and gender distribution, as well as histologic types, are comparable with the published data (16, 17). Our cohort was composed both of males and females with a ratio of 4:1. As described previously, this can be attributed to the higher exposure of the male population to identified risks, mainly asbestos (11, 13). The age distribution among the patient cohort also corresponds to previously presented data in different publications. The occurrence in older age groups is explained by the heavy use of asbestos in the mid 80´s and the long latency of mesothelioma development after exposure (8, 9). The fraction of each histologic subtype examined in our study group is also representative of the respective subtype distribution in the overall mesothelioma patient population (208, 209).

We have demonstrated loss of BAP1 nuclear staining in 67,5% of EPM, and 38% of SPM. Biphasic mesotheliomas demonstrated loss in 78% of samples in the epithelioid part, and in 11% of samples in the sarcomatoid part. However complete loss (in both parts) was present only in 1 case. Our data is comparable with published data when looking at EPM and SPM. A rather wide range of BAP1 loss has been shown in other studies, reaching 61-77% when looking at epithelioid mesothelioma (29, 34, 210, 211). Variation is much bigger looking at the sarcomatoid type. In a study by McGregor *et al*. (211), the authors did not find any losses of BAP1 in SPM, while other studies found losses in 15-36% (29, 34, 210). It is not easy to compare existing data for biphasic mesothelioma to our study since we have evaluated both components separately. In previous studies, loss of BAP1 expression in BPM was described in 49-75% of cases (29, 34, 210, 211)

One possible reason to explain such a wide range of results might be the usage of different antibodies. However, all four publications we compared our data with, used the most commonly used BAP1 antibody Clone C-4. Three of them used C-4 BAP1 mouse antibody, one used C-4 BAP1 rabbit antibody. Another reason for the rather large outcome spectrum can be found in different protocols and the type of used material. Different pre-analytical processes, as well as different staining protocols for C-4 antibodies, might be additional factors. Out of the cited studies we looked at, only McGregor *et al*. (211) used TMA (four cores per tumor sample) to examine BAP1 staining. This applies to all subtypes, except for BPM where they used whole paraffin slides for evaluation. Serial whole paraffin sections of tumor samples were used by Righi *et al.* (210) and Pulford *et al.* (34) in their respective studies. Cigognetti *et al.* (29) additionally used cytological samples embedded in paraffin. Cut thickness in these studies varied from 0,3µm to 0,4µm. Another possibility for varying results is tumor heterogeneity. It is known for practically all tumors to be composed of tumor cells driven by different mechanisms and expressing different molecular and sometimes IHC markers. Tumor heterogeneity is one of the main explanations for tumor resistance to therapy and the different potential of different tumors to invade and metastasize. The heterogeneity of BAP1 immunohistochemical expression is not fully understood so far. In our study, where we used three cores of tumor tissue, we did not observe any heterogeneity in its expression. However, due to the small proportion of tumor tissue, which is analyzed with this method, heterogeneity of expression cannot be excluded.

Although this is very important for future approaches, heterogeneity of BAP1 expression was not the aim of this study.

Our study has several limitations. It is a single-institution, retrospective study. A stronger, and more valid conclusion regarding the BAP1 expression throughout the mesothelioma samples can be made with a much larger cohort, from different institutions, with the prospective design of the study. In this way, the effect of the age of the paraffin blocks can also be avoided. Furthermore, as already mentioned, we did our analysis on TMAs. Although for each tumor sample, there were 3 cores, this is still representing only a small part of the whole tumor, therefore not really addressing the tumor heterogeneity issue.

To conclude, our goal was to perform a comparison of different BAP1 antibodies in a single institution cohort of mesothelioma. However, only one of the three tested antibodies (clone C-4) showed adequate and interpretable staining results, therefore a comparison of different clones was not possible. This BAP1 clone, which is the most commonly used one, showed reliable staining, with a distribution of BAP1 expression in our cohort in concordance with published data.

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