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in
SALUTE DELL'UOMO

# Clinical significance of BAP1 in Malignant Pleural Mesothelioma

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# ABSTRACT

**Introduction:** Malignant pleural mesothelioma (MPM) is an aggressive disease, with few available treatment options. Identification of novel prognostic and predictive biomarkers is a priority. In MPM patients, BRCA-associated protein 1 (BAP1) alterations are detected in about 60% of cases and its prognostic role has been amply investigated in the last 10 years. However, the clinic relevance of BAP1 in MPM is still a matter of debate. In this study we aimed to clarify the prognostic role of BAP1 in MPM. Moreover, since miR-31 seems to be involved in BAP1 regulation at post-transcriptional level, we combined BAP1 status with the tissue expression levels of miR-31 in order to improve its prognostic performance.

**Methods:** A systematic literature search was conducted. The inclusion criteria were: immunohistochemical (nuclear positivity) investigation of BAP1 expression on tumor tissue; hazard ratio (HR) values for the overall survival (OS) obtained through multivariate analysis (or adjusted for histotype).

The expression of BAP1 and miR-31 was analyzed in tissues of 60 MPM patients treated with first-line chemotherapy. OS and progression-free Survival (PFS) were assessed by Kaplan-Meier method and Log-rank test was used to investigate differences among subgroups. Multivariate Cox regression analysis was used to evaluate independent predictors of survival.

**Results:** In our cohort, BAP1 was positive/retained (≥1%) in 23 samples (38%) and negative/loss in 37 samples (62%). BAP1 loss was significantly associated with epithelioid histotype (p=0.01). At univariate analysis, there were no significant difference in terms of OS between BAP1 retained group (median OS=18.1 months, 95% CI 11.2-

25.8) and BAP1 loss group (median OS=14.8 months, 95% CI: 10.7-29.3, p=0.17). Multivariate analysis showed that non-epithelioid histology was the only independent prognostic factor (HR 3.58, 95 % CI: 1.58–8.14, p=0.002). Even, from meta-analysis consisting of 698 patients, no differences were observed in term of OS according to BAP1 status (HR 1.11; 95% CI, 0·76-1·61; p=0.60).

Lower miR-31 levels were detected in epithelioid MPM (e-MPM) compared to the non-epithelioid subtypes, which was associated with BAP1 loss. By looking at the e-MPM subgroup, loss of BAP1 was not able to predict clinical outcome. Conversely, miR-31 levels were significantly associated with PFS (p=0.028), but not with OS (p=0.059). By combining the two biomarkers, e-MPM patients with BAP1 loss/low miR-31 levels showed a better prognosis compared to the ones with BAP1 retained/high miR-31 levels (median OS 22.6 months, 95% CI: 12.0-33.2 vs 17.0 months, 95% CI: 11.5-22.5, p=0.017 and median PFS 8.7 months, 95% CI: 3.3-14.1 vs 5.1 months, 95% CI: 2.5-7.6, p=0.020). The BAP1 and miR-31 combination was confirmed at multivariate analysis as an independent prognostic factor for e-MPM patients.

**Conclusion:** BAP1 alone was unable to stratify MPM patients based on its status when histotype was considered. However, in e-MPM patients, prognostic stratification may be improved by simultaneously assessing BAP1 status and miR-31 levels. The two-biomarker score is useful to identify a subgroup of e-MPM tumors characterized by BAP1 retained and high miR-31 levels with worse clinical outcome.

# ABBREVIATIONS

| | |
|---|---|
| AP-1 | activator protein 1 |
| AST | atypical Spitz tumor |
| ASXL | putative polycomb group protein |
| BAP1 | BRCA-associated protein 1 |
| BARD1 | BRCA1-associated RING domain protein 1 |
| Bcl-2 | B-cell lymphoma 2 |
| BRCA2 | breast cancer type 2 susceptibility protein |
| CD | cluster of differentiation |
| CDKN2 | cyclin-dependent kinase inhibitor 2A |
| CM | cutaneous melanoma |
| COX7C | cytochrome c oxidase subunit 7C |
| CT | computed tomography |
| CTD | C-terminal domain |
| CTLA-4 | cytotoxic T-lymphocyte–associated protein 4 |
| DAMP | damage associated molecular pattern |
| DUB | deubiquitinating enzyme |
| EPD | extended pleurectomy/decortication |
| EPP | extrapleural pneumonectomy |
| ER | endoplasmic reticulum |
| FFPE | Formalin-Fixed Paraffin-Embedded |
| FISH | fluorescence *in situ* hybridization |
| FoxK | Forkhead Box K |

| | |
|---|---|
| HBM | host cell factor 1 binding domain |
| HCF1 | host cell factor 1 |
| HMGB1 | high mobility group box 1 |
| HGF | hepatocyte growth factor |
| HR | hazard ratio |
| IGF | insulin-like growth factor |
| IHC | immunohistochemistry |
| IL | interleukin |
| IP3R3 | inositol 1,4,5-trisphosphate receptor type 3 |
| LATS2 | large tumor suppressor kinase 2 |
| LDH | lactate dehydrogenase |
| MAM | mitochondrial-associated membranes |
| MAPK | mitogen activated protein kinases |
| MBAIT | melanocytic BAP1-mutated Atypical Intradermal Tumor |
| MCP-1 | monocyte chemoattractant protein-1 |
| MCU | mitochondrial uniporter channel |
| miRNA | microRNA |
| MLH1 | mutL homolog 1 |
| MLH3 | mutL homolog 3 |
| MM | malignant mesothelioma |
| MPM | malignant pleural mesothelioma |
| MRI | magnetic resonance imaging |
| mTOR | mammalian target of rapamycin |
| NF2 | neurofibromin 2 |

| | |
|---|---|
| NF-kB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| NGS | next-generation sequencing |
| NLS | nuclear localization signal |
| OGT | O-linked N-acetylglucosamine transferase |
| OS | overall survival |
| PDGF | platelet-derived growth factor |
| PD-L1 | programmed death-ligand 1 |
| PET | positron emission tomography |
| PFS | progression-free survival |
| PMT | pemetrexed maintenance therapy |
| PRC | polycomb-repressive complexes |
| PR-DUB | polycomb group repressive deubiquitinase complex |
| qRT-PCR | real-time PCR |
| RAGE | receptor for advanced glycation endproducts |
| RCC | renal cell carcinoma |
| RNS | reactive nitrogen species |
| ROS | reactive oxygen species |
| SETD2 | SET domain containing 2 |
| SMRP | soluble mesothelin-related peptides |
| TGF-β | transforming growth factor β |
| TNF-α | tumor necrosis factor α |
| TP53 | tumor protein 53 |
| UCH | ubiquitin carboxyl hydrolase domain |
| ULK | Unc-51 like autophagy activating kinase |

| | |
|---|---|
| UM | uveal melanoma |
| VDAC | voltage-dependent anion channel |
| VEGF | vascular endothelial growth factor |
| WT1 | wilms' tumor protein |
| YY1 | Ying Yang 1 |

# 1. INTRODUCTION

## 1.1 Malignant Pleural Mesothelioma

Malignant pleural mesothelioma (MPM) is an aggressive treatment resistant tumor that arises from the neoplastic transformation of the pleural mesothelium, a thin membrane that covers and protects the lung (1). MPM represents up to 80% of all cases of malignant mesothelioma (MM). The other form of mesothelioma originates from other serous membranes coated with mesothelium and are peritoneal mesothelioma (other 20% of mesothelioma), pericardial mesothelioma and mesothelioma of vaginal tunic that are both very rare (2). Seventy per cent of MPM cases are associated with documented exposure to asbestos and develop after a latency period of 30-40 years (4). MPM develops insidiously in patients and is difficult to be diagnosed in the early stages because it does not show specific symptoms until advanced stages. Even diagnostic tools are not helpful for early detections of MPM and there is a lack of serum biomarkers that have not yet been determined. The body cavities where MPM initially develops, present anatomical location and characteristics that also causes malignant cells to easily spread and invade the adjacent cavities. Due to late diagnosis, only a few patients undergo surgery and current therapy is not very effective so life expectancy ranges from 9 to 18 months from diagnosis (5).

### *1.1.1 Epidemiology*

Until the second half of the 20th century MPM was extremely rare, but its incidence and mortality rates began to rise in the 1960s after the massive use of asbestos, a mineral fiber,

during World War II and thereafter (6). The first studies that confirmed the link between asbestos and MPM were two epidemiological studies conducted by Doll et al and Wagner et al in 1955 and 1960, respectively (7, 8).

There are approximately 400 different types of asbestos fibers present in nature, but those used commercially are six (amphiboles fibers [crocidolite, actinolite, tremolite, anthophyllite, and amosite] and serpentine fibers [chrysotile]) and were collectively called "asbestos" (**Figure 1**) (9).

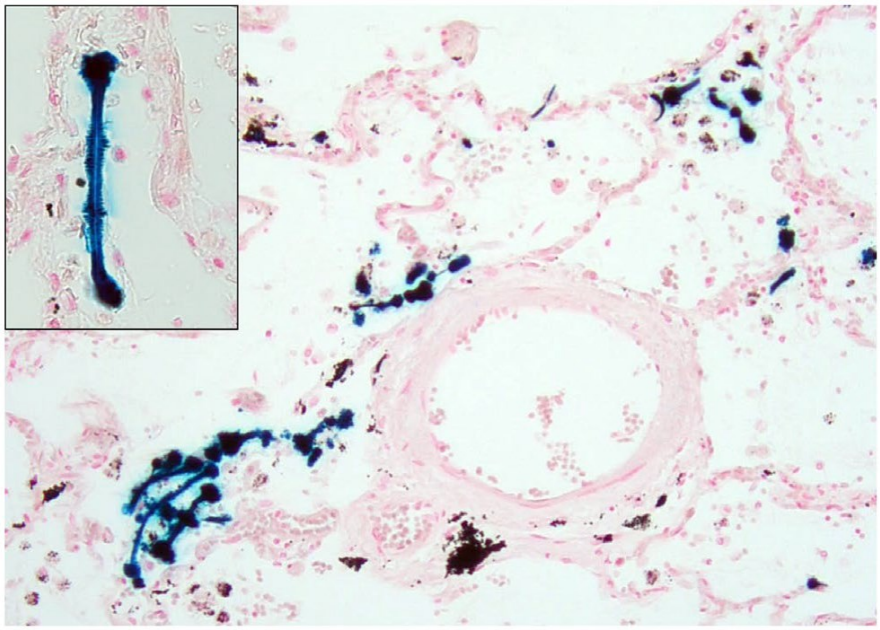

**Figure 1**. Presence of asbestos fiber in a lung alveoli's biopsy from a patient with mesothelioma who worked for "Eternit,"

The use of these six fibers were regulated in high-resource countries between 1970 and late 1980, but not the remaining approximately 400 mineral fibers, although many of them are carcinogenic and have been associated with mesothelioma (9, 10).

Asbestos is characterized by high resistance to heat, as well as chemical and biological agents, abrasion, and wear; therefore, it was widely used in the shipbuilding and construction industries, especially between the 1940s and 1979 in the United States and

Europe (11). MPM is a disease characterized by a long latency interval between the onset of exposure and the appearance of the disease (from 20 to 40 years and over) (12). Thus, despite controls on the use of asbestos introduced over the years, the incidence and the annual number of deaths from mesothelioma has continued to rise worldwide with a deaths peak expected between 2015-2025 and, according to some experts, even in 2040 (13–18).

According to WHO estimates, more than 100,000 deaths are due to the consequences of occupational exposure to asbestos (mesothelioma, lung cancer, and asbestosis) and it has been estimated that, between 1994 and 2008, age-adjusted mesothelioma mortality rates increased by 5.37% per year worldwide (19). In Western Europe, due to asbestos exposure, are expected 500,000 deaths in the first thirty years of 2000 (20-22) and the underlying level of the mortality rate from mesothelioma is about 1–2 per million/year (23-25). Currently there are about 2,000 to 3,000 cases per year in the United States, but the incidence rate varies between states with or without asbestos industry (from less than one case to 2-3 cases per 100.000 inhabitants). In the U.S. there are 3000 deaths per year and projections estimates 100.000 deaths over the next 40 years (9,10,19, 26-28). According to the Surveillance, Epidemiology, and End Results (SEER) database, the MPM in the USA reached the peak in the 1980s to 1990s with an incidence in men of 2.5 cases per 100.000 and now is stable to 1.8 cases per 100.000. In women the rate has been 0.4 cases per 100.000 and has not changed over time. Women have also threefold better survival than men (29). In the US the mean age of death for MPM was 72.8 years, with a male-to-female (M:F) mortality ratio of 4.2:1, since men were traditionally more likely to be employed in works involving asbestos exposure. But, with equivalent asbestos exposure, men and women have a similar incidence of MPM (30).

The highest age-standardized incidence rates in 2018 were observed in the US, Western Europe, Australia, Russia, Turkey, South Africa, and Argentina (31). The incidence varies between 7 per million in Japan and 40 per million in Australia (32, 33). In the last twenty years, the incidence has steadily increased in Europe in the industrialized countries, with an average incidence of 20 per million inhabitants/year and its peak is expected to be around 2020–2025 (15, 34). In addition, a temporal decline in mesothelioma mortality rates in males has been observed in Sweden and in the United Kingdom, probably thanks to the introduction of regulatory laws during 1970s (35). Instead, rates in those countries among women are still rising as of 2018 (19, 36, 37). Europe incidence data for the year 2000-2007 are available on RARECARE, which estimates the frequency of rare cancers in Europe. (38) (**Table 1**).

| Tumor | Crude Incidence Rate Per 100.000 | 95% Confidence Interval | | Number of Cases Collected in the RARECAREnet Database from 2000-2007 | Estimated New Cases EU 2013 |
|---|---|---|---|---|---|
| Malignant Mesothelioma | 2.14 | 2.12 | 2.16 | 33.552 | 12.526 |
| Mesothelioma of pleura and pericardium | 1.83 | 1.80 | 1.85 | 28.627 | 10.703 |
| Mesothelioma of peritoneum and tunica vaginalis | 0.13 | 0.12 | 0.13 | 2.065 | 746 |

**Table 1.** RARECARE incidence of malignant mesothelioma (MM) 2000-2007. Source: http://www.rarecarenet.eu/analysys.php, Updated 10 June 2019.

Russia, China, Thailand, Brazil, India, Kazakhstan, Iran, and Ukraine were the highest worldwide consumption of asbestos from 1995 to 2003, but the World Health Organization include mesothelioma incidence and mortality data only for Kazakhstan,

where incidence rates increased from approximately 0 to 0.26 cases per 100,000 persons in the past 10 years (31).

Unfortunately, each country banned and regulated the use of asbestos in different times, so, in the next decades, it is expected that mesothelioma rates will follow dissimilar patterns. By 1990, in the most industrialized countries, the use of asbestos had been reduced by at least 75% from the peak asbestos consumption. In 1999, Iran, Korea, Chile, and Egypt reached the same level of reduction, as did Nigeria, Zimbabwe, the United Arab Emirates, Ukraine, and Kazakhstan between 2000 and 2005. But, where the asbestos is still used, such as the Russian Federation, India, and China, an increase in age-adjusted mesothelioma incidence and mortality rates is expected in the coming years (39).

In the European Union, the directive 2003/18/CE of the European Parliament and of the Council of 27 March 2003 provides the obligation to the completely stop of the asbestos use by 15 April 2006. In Italy, the law n. 257 of 27 March 1992 declared the "cessation of the use of asbestos", and the ban on the extraction, import, export, marketing, and production of asbestos products and products containing asbestos, but the law did not prohibit the indirect use. Therefore, the Italian territory is disseminated of several million tons of compact materials containing asbestos and many tons of friable asbestos are still present in many contaminated sites, industrial and nonindustrial, public, and private. In Italy, the number of exposed worker is very high because the raw asbestos produced or imported has been used in a wide range of industrial activities such as sectors of industrial production of asbestos-cement manufactured articles, textile manufactures containing asbestos, shipbuilding, repair and demolition of railway rolling stock, construction, and in many other sectors of economic activity (2).

Moreover, given the presence of numerous asbestos mines, in Italy the production of raw asbestos was very high, with 3,748,550 tons of raw asbestos produced up to 1992, and with a peak of more than 160,000 tons/year between 1976 and 1980. For these reasons, Italy is one of the most involved and sensitive country in asbestos related diseases monitoring and control (2). In particular, Piedmont was one of the most affected Italian regions, since it hosted the main European chrysotile quarry (Balangero, operating from 1917 through 1990) and the largest facility for the manufacture of asbestos-cement products (Casale Monferrato, operating from 1907 through 1985) (40-43). The asbestos cement factory, owned by Eternit, in Casale Monferrato, produced plain and corrugated sheets, tubes, and high-pressure pipes. The asbestos cement factory had an average workforce of over 1000 workers and was located close to the town center of Casale Monferrato, causing also airborne asbestos contamination in the town. Therefore, Casale Monferrato is one of the Italian towns with the highest incidence and mortality of malignant mesothelioma [44, 45]. The Registry of Malignant Mesothelioma – ReNaM – of Piedmont reported that, between 2010-2014, the incidence rates in Casale Monferrato was of 90.2/100.000 person years in men and 45.4 in women [Registry of Malignant Mesothelioma – ReNaM – of Piedmont, www.cpo.it/it/articles/show/incidenza-e-sopravvivenza-dei-mesoteliomi-1990-2014/]. The case of Casale Monferrato is even an example that mesothelioma does not occur only in people who had work in direct contact with asbestos, but even to people that had an environmental exposure to asbestos. Between 2001-2006, an analysis of exposure histories of 847 cases of mesothelioma demonstrated that only 475 cases (56%) was considered occupationally exposed, whereas, 357 (42%) was classified as non-occupationally exposed, mostly due both to living in proximity to the asbestos-cement factory (“environmental” exposures: 200

cases) or to have a factory workers in family ('familial' exposure: 144 cases). These evidences make known the how persisting is asbestos contamination in the workplace and in the general environment from asbestos-cement production (46). Unlike MPM caused by occupational exposure, those caused by the environment tend to occur at a younger age (<55 years) with an M:F ratio close to 1:1.6, probably why environmental exposure often begins at birth and occurs randomly among sexes (19).

In Italy, the epidemiological surveillance of mesothelioma cases is assigned by the Decree of the President of the Council of Ministers n. 308/2002 to the ReNaM established at the National Institute for Insurance against Accidents at Work (INAIL) [47]. For the incidence period between 1993 and 2015, ReNaM has collected 27,356 MM and the modalities of exposure to asbestos have been investigated for 21,387 (78%) cases (**Table 2**).

| **Modality of exposure** | **Incidence period (1993-2015)** | | |
|---|---|---|---|
| | **Male (%)** | **Female (%)** | **Total (%)** |
| Occupational, define | 9.300 (59.3) | 987 (17.3) | 10.287 (48.1) |
| Occupational, probable | 1.358 (8.7) | 191 (3.3) | 1.549 (7.2) |
| Occupational, possible | 2.246 (14.3) | 736 (12.9) | 2.982 (13.9) |
| Familial | 152 (1.0) | 895 (15.7) | 1.047 (4.9) |
| Enviromental | 409 (2.6) | 530 (9.3) | 939 (4.4) |
| Other non-occupational | 128 (0.8) | 194 (3.4) | 322 (1.5) |
| Unlikely | 268 (1.7) | 308 (5.4) | 576 (2.7) |
| Uknown | 1.824 (11.6) | 1.861 (32.6) | 3.685 (17.2) |
| Total defined | 15.685 (100.0) | 5.702 (100.0) | 21.387 (100.0) |
| Total | 19.633 (100.0) | 7.723 (100.0) | 27.356 (100.0) |
| Total defined | 15.685 (79.9) | 5.702 (73.8) | 21.387 (78.2) |
| Total undefined | 3.948 (20.1) | 2.021 (26.2) | 5.969 (21.8) |

**Table 2** Italian National Mesothelioma Register (ReNaM) archives. Collected malignant mesothelioma cases by modality of asbestos exposure and gender. ReNaM archives updated at December 2016, diagnosis period 1993–2015*, Italy

Among them, an occupational exposure has been defined for 14,818 (around 70%) of defined cases. Non-occupational exposure has been defined for 9.3% of cases of which 4.9% due to family exposure and 5% due to environmental exposure. The exposure median period was 1959 [1951-1966]. Average age at diagnosis of malignant mesothelioma (MM) was 70 years, with no appreciable gender differences (70.8 years in woman and 69.5 in men). MM cases younger than 45 years at diagnosis are very rare (less than 2%). More than 90% of collected cases are pleural mesotheliomas (93%). Peritoneal MM cases are 6.5% (5.3% and 9.4% in men and women respectively) and pericardial and tunica vaginalis testis MM cases are very rare (58 and 79 collected cases respectively among the entire ReNaM archives). Epithelioid histology represented the 55% of cases. Gender ratio is, constantly in time, equal to 2.54 (M/F) and to 2.64, if restricted to pleural cases (48). From this analysis it was possible an examination of the geographic distribution of mesothelioma cases in Italy which enabled the identification of clusters in the municipalities with the highest incidence rates, such as Casale Monferrato, Broni, Genoa, La Spezia, Grugliasco-Collegno, Monfalcone, Trieste, Castellamare di Stabia, Bari, Taranto, Biancavilla, and Augusta (49-52) (**Figure 2**).

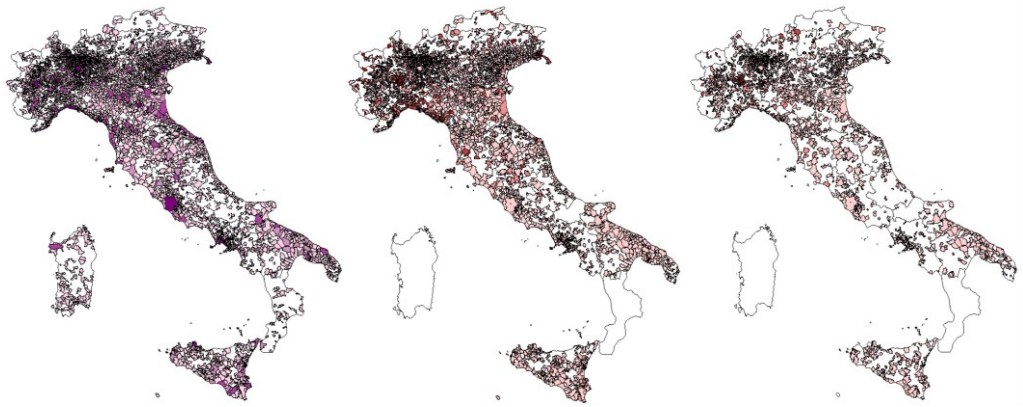

**Figure 2.** Italian National Mesothelioma Register (ReNaM) archives. Number of MPM cases in men and women (**a**) and incidence rates of MPM in men (**b**) and woman (**c**), by municipality of residence at the time of diagnosis, Italy. ReNaM archives updated to December 2016, diagnosis period 1993–2015. Sardinia, Molise and Calabria are excluded due to lack of data.

### *1.1.2 Etiopathogenesis*

MPM has been associated with asbestos exposure and over 80% of MPM patients have a history of asbestos exposure (53). Although asbestos, in particular crocidolite, is considered to be the carcinogenic agent mostly involved in MPM etiology, only the 5% of asbestos-exposed subjects develop the disease (54). As MPM is a relatively rare malignancy, it is still not clear how much exposure is needed to cause MPM, and what mechanisms are triggered during the long latency from the time of exposure to the time of tumor development. In vivo experiments have shown that there is a clear dose-response, thus, MPM does not develop below a certain dose of exposure (about 1 mg) (55). However, in human, there is no defined threshold limit for mesothelioma risk, which may arise even after short asbestos exposure (24, 56, 57).

When long and thin asbestos fibers are inhaled deeply into the lung and penetrate the pleural space, the interaction between asbestos fibers, mesothelial cells and inflammatory cells occur, causing chronic inflammation and atypical mesothelial hyperplasia. The single flat layer of mesothelial cells that form the pleura and peritoneum round up and forming multicellular layers from which over time mesothelioma may arise (58). Chronic inflammation is the primary mechanism of asbestos-related carcinogenesis. The continuous generation of highly reactive oxygen species (ROS) promotes DNA mutation, and trigger transformation (59). The presence of iron (II) ions (Fe2+) in asbestos fibers can also induce hemolysis by sequestering Fe(II) from hemoglobin (60); this lead to a particular reaction (Fenton reaction), where free Fe(II) disproportionate $H_2O_2$ into hydroxyl radicals that oxidize DNA, free nucleic acids, proteins, and lipids (61). Also, Macrophage are unable to complete digest asbestos fiber after phagocytosis, and, over time, this generates abundant ROS and even reactive nitrogen species (RNS). Asbestos

fibers are also engulfed by mesothelial cells where can physically interfere with the mitotic process of the cell cycle by disrupting mitotic spindles. This may result in chromosomal structural abnormalities and aneuploidy of mesothelial cells. Asbestos fibers absorb a variety of proteins and chemicals on their broad surface and may result in the accumulation of hazardous molecules including carcinogens. The fibers can also bind important cellular proteins necessary for the normal function of mesothelial cells (62, 63). All these processes lead to DNA damage in the forms of single-strands breaks, crosslinks, and double strand-breaks. Particularly, the oxidation of the $8^{th}$ carbon on the DNA base guanine (8-oxo-2'deoxyguanosine) changes normal 2'deoxyguanosine Watson-Crick base pairing preference from 2'deoxycitosine to 2'deoxyadenosine, resulting in G to T and C to A transversions (64).

Paradoxically, human mesothelial cells are very susceptible to this asbestos cytotoxicity, which raises an issue of how asbestos causes MPM if human mesothelial cells exposed to asbestos die (65). This paradox was addressed by recent findings which demonstrated a critical role for TNF-α and NF-kB signaling in mediating human mesothelial cell responses to asbestos. TNF-α signaling through NF-kB-dependent mechanisms increases the percent of human mesothelial cells that survive to asbestos exposure, and which can therefore undergo to malignant transformation. These findings suggest a pathogenic model where asbestos causes expression of several cytokines, including MCP-1, which in turn cause the accumulation of macrophages in the pleura and lung where they can encounter asbestos and release TNF-α. At the same time, in mesothelial cells, asbestos directly induces the expression of the TNF-α receptor and the secretion of TNF-α. When TNF-α, released by macrophages and by human mesothelial cells, binds its own receptor this activates NF-kB signaling which promote survival of human mesothelial cells

following asbestos exposure (66). This process allows human mesothelial cells with accumulated asbestos-induced DNA damage to survive, divide and propagate genetic aberrations in pre-malignant cells. Finally, if sufficient genetic damage accumulates occur; these cells could eventually develop into a MPM (**Figure 3**).

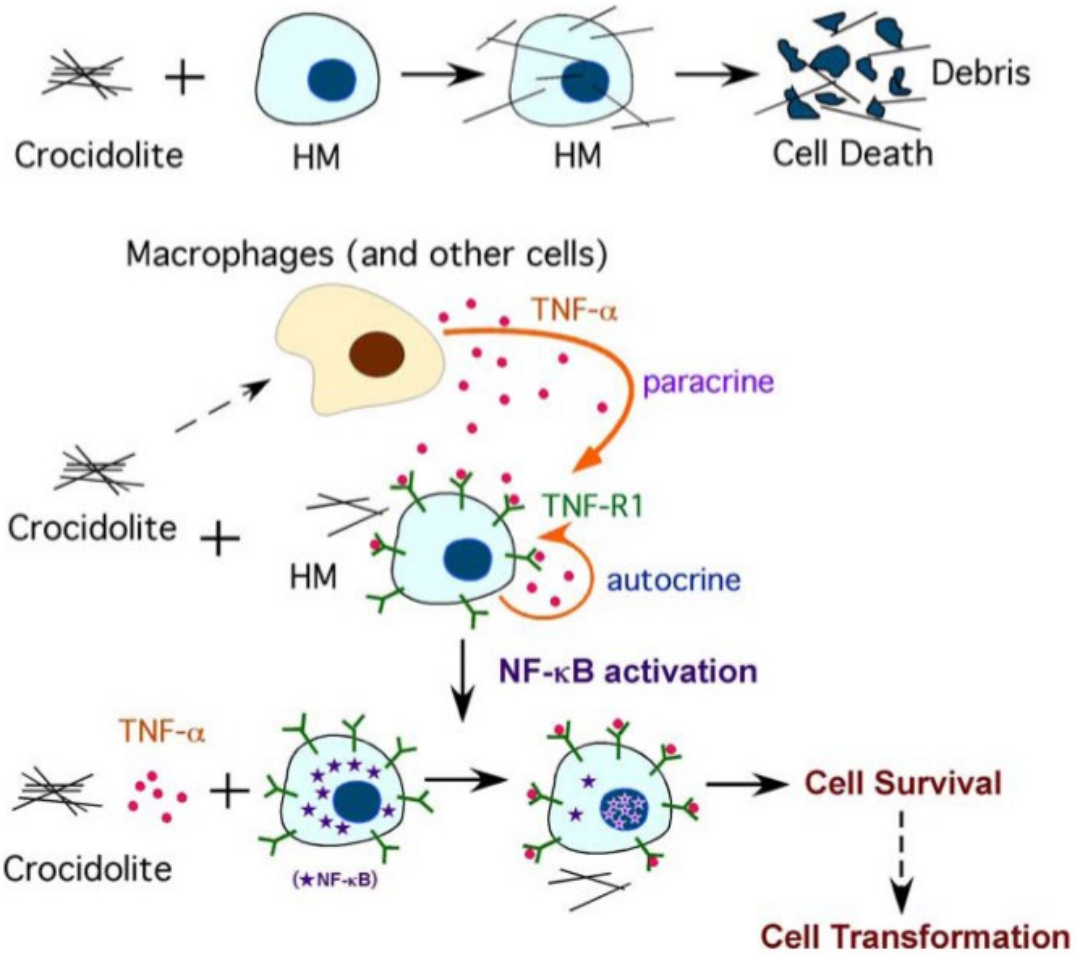


**Figure 3.** Mechanisms of asbestos-induced carcinogenesis

Even autophagy has a role in asbestos-induced carcinogenesis via the pro inflammatory protein high mobility group box 1 (HMGB1). There are two type of autophagy: constitutive (background) autophagy, that recycles cellular components from aged/damaged organelles and induced (reactive) autophagy, that occurs in response to environmental challenges and protect cells from apoptosis and necrosis (66-69). Yang et

al demonstrated that asbestos induced programmed cell necrosis in human mesothelial cells and induced necrotic cell to release HMGB1 into extracellular space (70). In addition to stimulate inflammation (71-73), HMGB1 triggers the inflammasome pathway (74) and sustain the chronic inflammatory process induced by asbestos; HMGB1 modulates even autophagy in cells under pro-autophagic stressed (75-77). Finally, in a recent study, Xue et al demonstrated the link between autophagy and asbestos-induced HMGB1 translocation from the nucleus to the cytoplasm and to the extracellular space. They found that both cytoplasmic and extracellular HMGB1 mediated asbestos-induced autophagy through the RAGE-mTOR-ULK and Beclin 1 pathways (78). Cytoplasmic HMGB1 can activate p-Beclin 1 displacing Bcl-2 (77) and extracellular HMGB1, thus contributing to p-Beclin 1 activation and autophagy via binding to the cell-surface receptor RAGE, which, in turn, initiated a downstream pathways that culminated in increased levels of p-Beclin 1 (**Figure 4**) (78). This mechanism allows asbestos-exposed mesothelial cells that have sustained DNA damaged to survive. Otherwise, in human mesothelial cells with HMGB1 silenced, autophagy is inhibited and cells undergoing to apoptosis or necrosis (78).

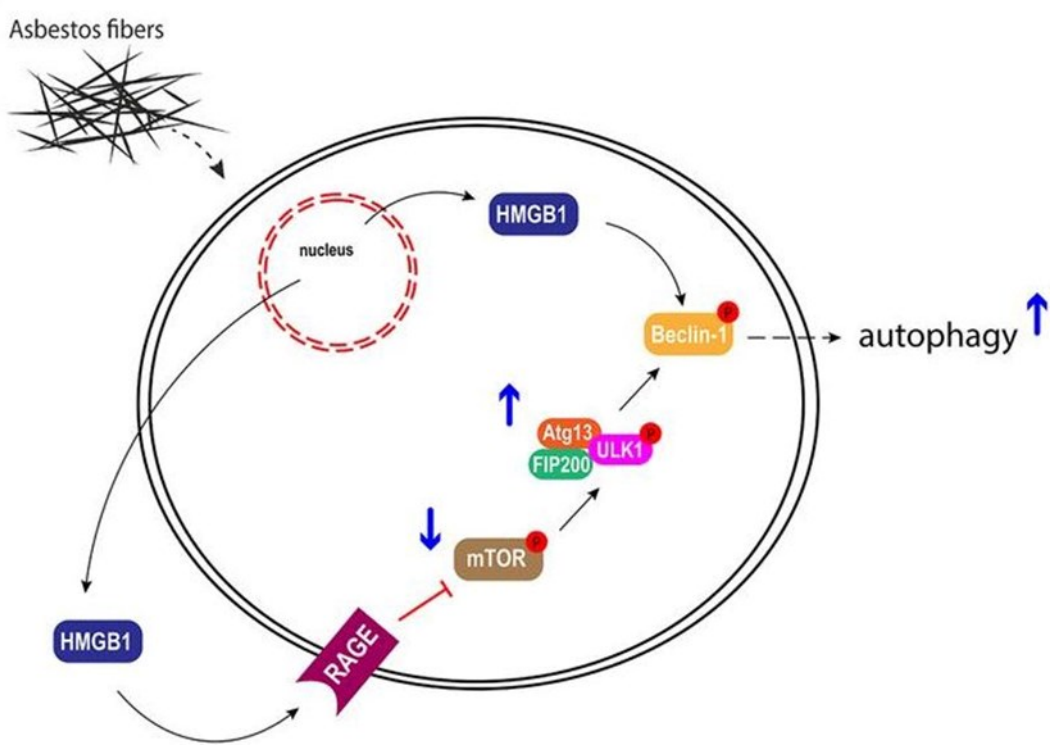


**Figure 4**. Schematic representations of the asbestos-HMGB1-autophagy pathway.

In addition to TNF-α, other growth factors and cytokines are involved in the carcinogenesis process caused by asbestos: transforming growth factor β (TGF-β), which may play a role in stimulating the growth of cancer cells; platelet-derived growth factor (PDGF), an important mitotic agent, which may act as a regulatory factor in the proliferation of MPM cells; the insulin-like growth factor (IGF), which it would promote tumor proliferation and cell migration (79). Furthermore, interleukins, such as IL-6 and IL-8, could promote tumor cell growth and neo-angiogenesis with the development of newly formed capillaries. In particular, IL-8 plays the role of an autocrine factor for cell growth (80). Other growth factors involved in the pathogenetic mechanism of mesothelioma are the vascular endothelial growth factor (VEGF), that promotes neo-angiogenesis (81) and the hepatocyte growth factor (HGF) which stimulates cell proliferation, as well migration and invasiveness in tissues by the tumor (82).

In addition to the production of growth factors, asbestos stimulates and interacts with several signaling pathways, such as the mitogen activated protein kinases (MAPK) signaling that lead to the activation of the transcription factor AP-1 that stimulates the mitosis of mesothelial cells (62).

Because of the carcinogenic "field effect" caused by asbestos, mesotheliomas are often polyclonal (83, 84). Recently, the Cancer Genome Atlas program, published a study aimed to investigate the genetic alterations of 74 mesotheliomas using next-generation sequencing (NGS). As in the previous comprehensive NGS study by Bueno et al (85), Hmeljak et al (86) reported frequent somatic mutations and/or copy-number alterations of CDKN2A, NF2, TP53, LATS2, and SETD2 (**Figure 5**) (85, 86). In addition, the authors (85) reported a 57% prevalence of BAP1 mutations, confirming the previous comprehensive analysis reporting a 60% prevalence of BAP1 mutations (87).

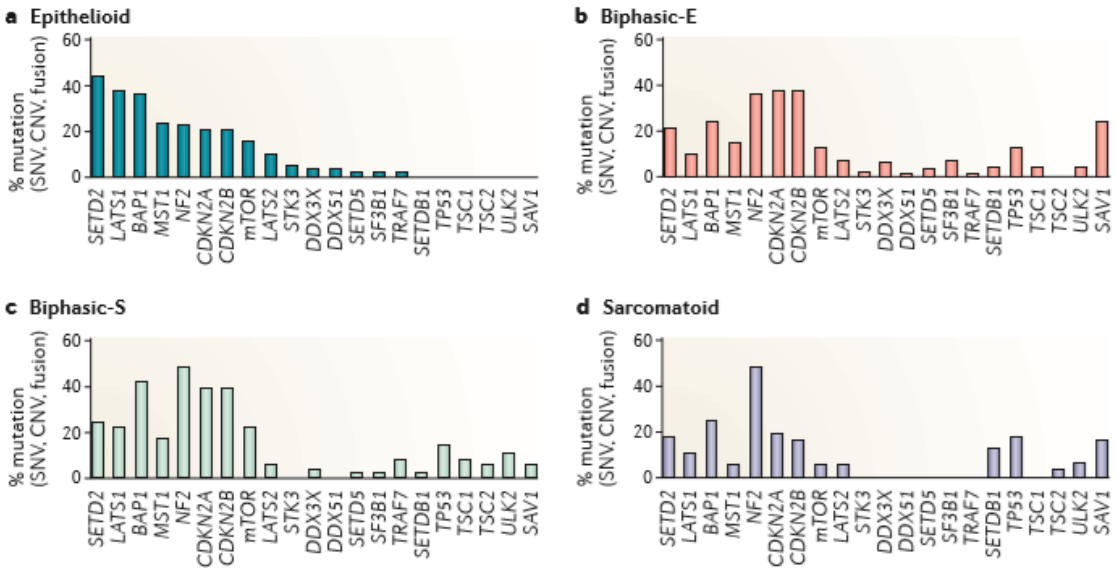


**Figure 5**. Mutational hierarchy in mesothelioma subtypes.

As previously reported only 5% of asbestos-exposed subjects developed MPM. It has been seen that, in addition to the mechanisms described above, gene predisposition is also involved in the development of mesothelioma (88). Inherited mutations of DNA repair genes and of other genes could accelerate the accumulation of DNA damage and/or the percentage of cells carrying DNA damage (89). In addition, inherited mutations may also increase susceptibility to environmental carcinogens (GxE interaction) (89). Several tumor suppressor genes have recently been found to cause a hereditary predisposition to mesothelioma and, overall, at least 12% of mesotheliomas occur in carriers of genetic mutations (90-92). Most of these heterozygous germline mutations occur in genes that regulate DNA repair, such as *MLH1*, *MLH3*, *TP53*, BRCA2, but the first and the most studied gene associated with mesothelioma predispositions is BAP1 (90-92).

### *1.1.3 Clinical presentation and diagnosis*

MPM symptoms are non-specific and may mimic other respiratory diseases (93,94). Most patients with MPM present dyspnea which is associated with breathlessness, chest pain, weight loss and fatigue (95, 96) and is predominantly related to the development of a pleural effusion. Thoracic pain is common and multifactorial in MPM and is due by tumor invasion of chest wall. When tumor invades neural intercostal, paravertebral or brachial plexus structures may cause bone pain and neuropathic pain. At advanced stages, MPM symptoms include weight loss, fatigue, fevers, cachexia and nights sweats and are often detected hypoalbuminemia, thrombocytosis, elevated erythrocyte sedimentation rate and anaemia (32, 97)

MPM develops initially unilaterally and local invasion of neighboring structures, including lymph node involvement, can occur, causing superior vena cava syndrome,

pericardial effusion and subsequent cardiac tamponade, spinal cord compression as well as a subcutaneous involvement. The affected site becomes fixed and cannot expand. Other MPM progression may involve invasion of contralateral pleural cavity and peritoneum. Unlike lung cancer, distant metastases rarely occur in MPM since patients die before metastases occur (98, 99).

Standard diagnostic work-up in patients with MPM starts with Chest X-ray and/or computed tomography (CT) scan of chest and upper abdomen in order to shows pleural effusion at disease site, pleural thickening and involvement of the interlobar fissure and invasion of the chest wall (**Figure 6**).

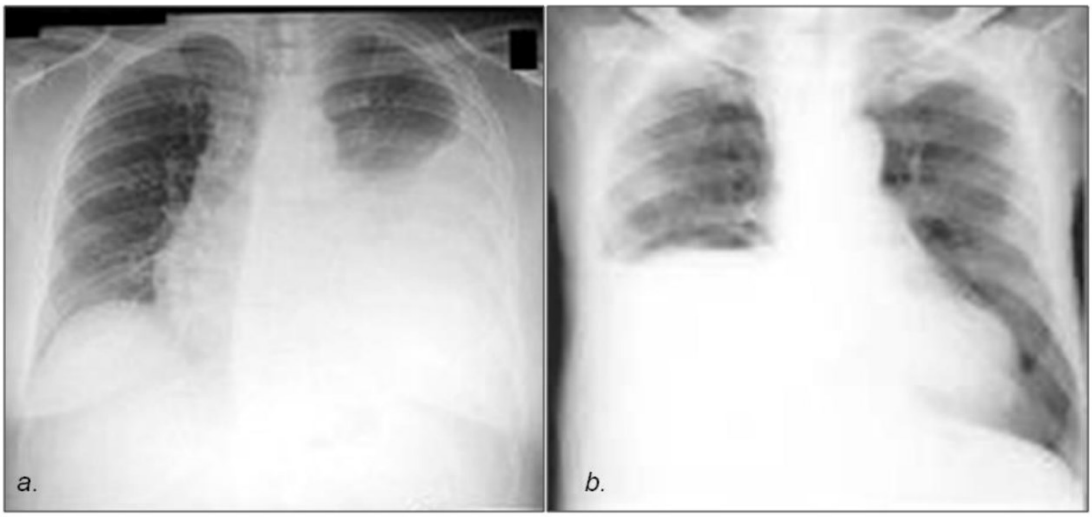


**Figure 6**. Chest radiographs in P-A projection of the chest with left (**a**) and right (**b**) pleural effusion, respectively.

Then, by thoracentesis or by fine-needle aspiration biopsy the pleural effusion is drained, and the fluid is examined cytologically. Pleural biopsy is often required for a definitive diagnosis histological and bio-molecular diagnosis, and pleurodesis with talc poudrage is often performed during the same surgical setting. Recognition and rapid investigations of the pleural or peritoneal effusion are key for early diagnosis. Delayed diagnosis will

inevitably lead to tumor progression, limiting the therapeutic options. Also, by PET and MRI, it is possible define the extension of the disease. Additional investigations include blood markers and pulmonary function tests (100, 101).

### *1.1.4 Histology and staging*

As previously said, a definitive diagnosis of mesothelioma requires histological evaluation. Biopsies are mostly obtained by thoracoscopy or laparoscopy.

According to the 2015 World Health Organization Classification of Tumors of the Pleura, malignant mesothelioma is broadly classified as epithelioid, sarcomatoid, or biphasic types (102) In the NCDB (*National Cancer Database, USA*) from 2003 to 2014, MPM subtypes were 38.4% epithelioid, 12.3% sarcomatoid, 11% biphasic, and 44.7% not otherwise specified (103)

Epithelioid MPM (**Figure 7a**) is the most common histotype (60%–70%) of MPM and is associated with less severe prognosis than non-epitheliod MPM. (104, 105). The median overall survival for the epithelioid mesothelioma is 24.9 month (106). Histologically, the tumor cells have an epithelioid morphology with an ample cytoplasm with well-defined cell borders. The nuclei are frequently bland and can look like reactive mesothelial cells with moderate cytologic atypia. Growth patterns include tubulo-papillary, microglandular (adenomatoid), acinar, and solid. Morphologic variants of epithelioid mesothelioma include clear cell, small cell, deciduoid, adenoid cystic, lymphangiomatoid, and signet ring cell morphology (106).

Sarcomatoid MPM (**Figure 7b**) is the most aggressive subtype with a median survival of 7 months (106) It is characterized by a proliferation of spindle cells infiltrating dense fibrous stroma and exhibit a disorganized growth pattern. The spindle cells may show

marked nuclear atypia and hyperchromasia. Areas of osseous and cartilaginous differentiation have been reported (107). This subtype is non resectable by surgical intervention.

Biphasic MPM is characterized by the simultaneous presence of both epitheliod and sarcomatoid cells. This suggest that survival may correlate with the amount of sarcomatous component present within the tumor (**Figure 7c**) (102). This diagnosis is of clinical importance, because selecting patients for surgical intervention is dependent on the absence of a sarcomatoid component and tumor volume and resectability.

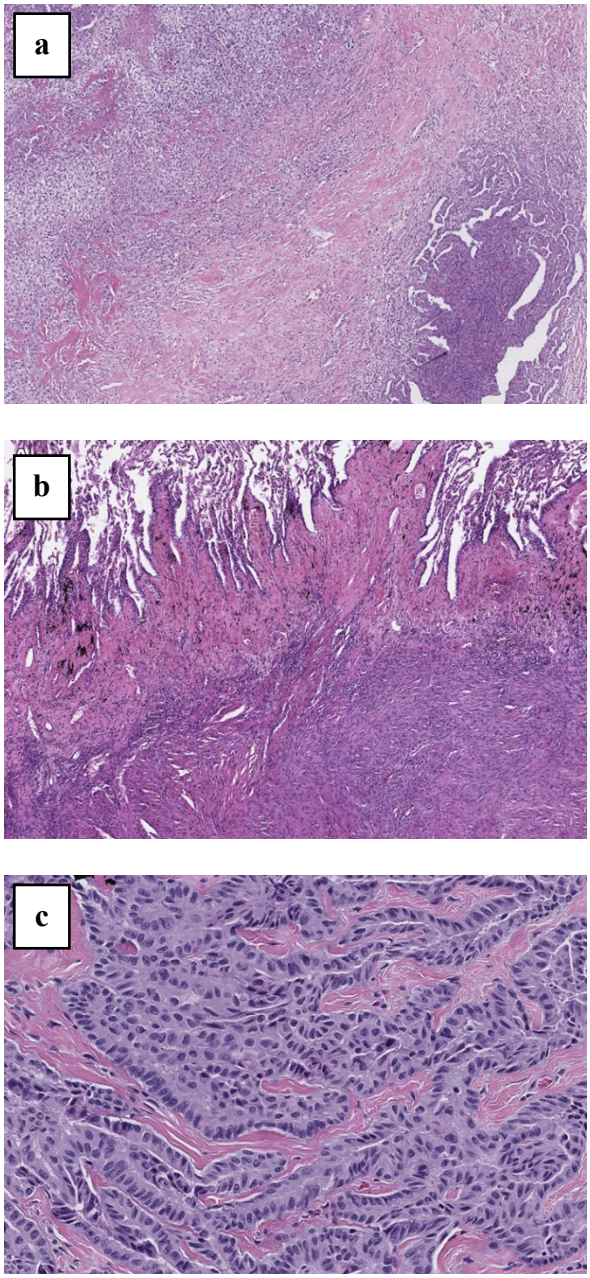


**Figure 7.** Immunohistochemistry imagines of mesothelioma; (**a**) epitheliod, (**b**) sarcomatoid and (**c**) biphasic subtype.

The staging classifications of MPM is based on the AJCC (The American Joint Committee on Cancer)/UICC (International Union Against Cancer) criteria (**Figure 8**) (108, 109). According to this, T classification is determined based on the extent of tumor invasion within the pleura and into adjacent thoracic structures so, T1 tumors, are those that remain confined to unilateral pleural surfaces. The T2 classification includes

extended tumors that involve ipsilateral parietal or visceral pleura with invasion of lung parenchyma or diaphragm muscle. T3 tumors are locally advanced and are extended to at least one of the following structures: endothoracic fascia, mediastinal adipose tissue, nontransmural invasion of the pericardium, or focal resectable soft tissue of chest wall. Finally, T4 are unresectable tumors with diffuse or multifocal chest wall soft tissue involvement, invasion of brachial plexus, bony components of chest wall or spine, mediastinal organs, contralateral pleura, or extension through diaphragm or pericardium. Unlike TNM staging of most solid tumors, due to the impracticality of measuring tumors with irregular and highly variable morphology, criteria for T classification of MPM do not include consideration of tumor size. N classification of MPM follows the lung cancer map (110). MPM are classified as N1 when invade pulmonary lymphatics that drain predictably and progressively through intraparenchymal and ipsilateral hilar lymph nodes; MPM N2 present metastasis in ipsilateral and midline mediastinal nodes; MPM N3 present metastasis in contralateral and extrathoracic stations. The lung map does not account for some nuances of MPM nodal invasion, however, MPM can even invade pulmonary parenchyma from visceral pleura following this metastatic pattern (111) TNM grouping criteria do not distinguish N1 from N2, although studies have demonstrated worse prognosis for N2 than N1 (112, 113). Nevertheless, evidence-based proposals have been made to refine N classification considering combined N1 and N2 involvement versus N1-only or N2-only disease (114), the number of involved nodes (112) or nodal stations (115), or the specific mediastinal stations involved (113). M classification of MPM indicates the absence (M0) or the presence (M1) of distant blood-borne metastasis. Usually, the presence in brain, bone, kidney and adrenal glands metastases is a rare condition, although it has been documented (116). Probably this is due to the

comparatively rapid and fatal progression of local T4 disease involving vital intrathoracic organs. (117).

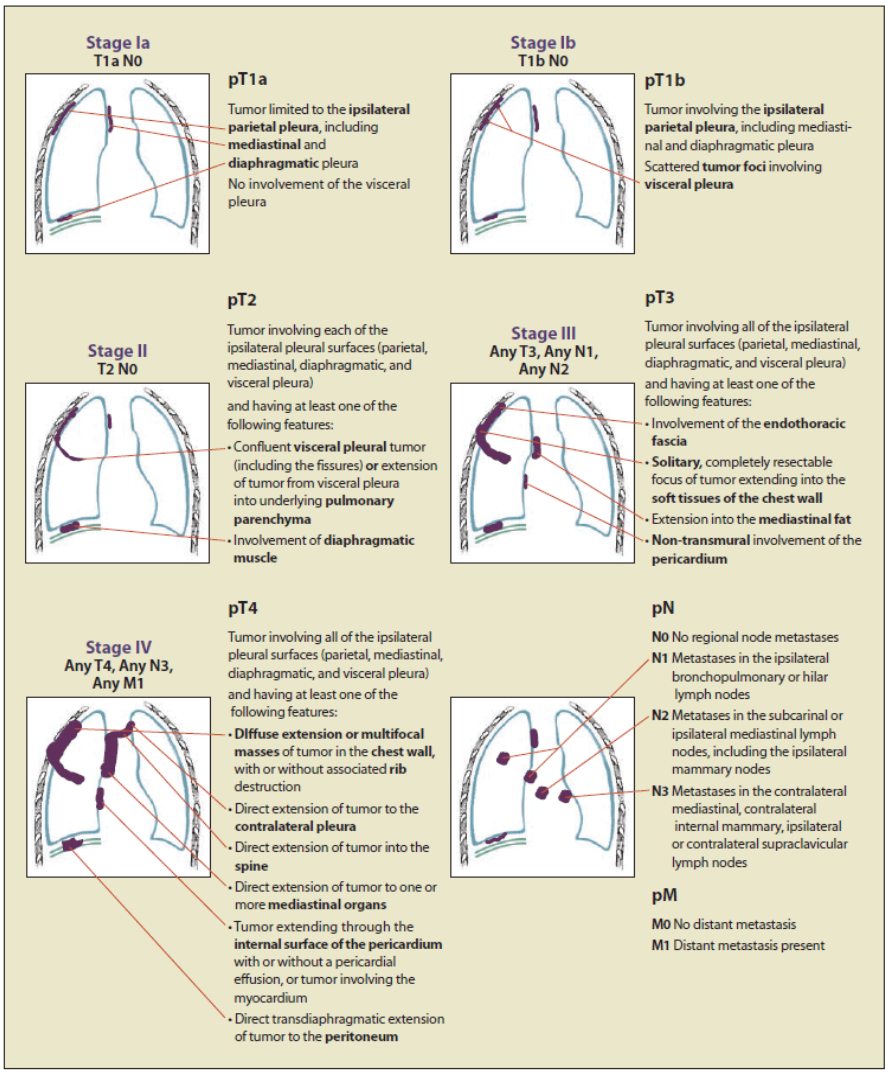


**Figure 8.** The proposed International TNM Staging System for MPM

## *1.1.5 Therapeutic approach*

Only a minority of MPM patients is fit enough to be a surgical candidate and the indication for surgery has become stricter in the last years. Complete microscopic surgical resection is highly unlikely and MPM almost always recurs after surgery alone (118). Therefore, surgery is usually always part of a multimodal treatment strategy and is

considered only in patients with stage I/II MPM and only with epithelioid histology. Non-epithelioid subtypes are “unresectable”.

The ideal surgical intervention to achieve maximal cytoreduction is still under debated (119). There are two types of surgical intervention for MPM, extrapleural pneumonectomy (EPP), and lung-sparing extended pleurectomy/decortication (EPD), but there is no difference in terms of survival between the two methods (120). Otherwise, many experts still prefer EPD, in part because of the preservation of lung parenchyma and theoretical postoperative functional improvement and capacity to tolerate further pulmonary insults (119 (**Figure 9).**

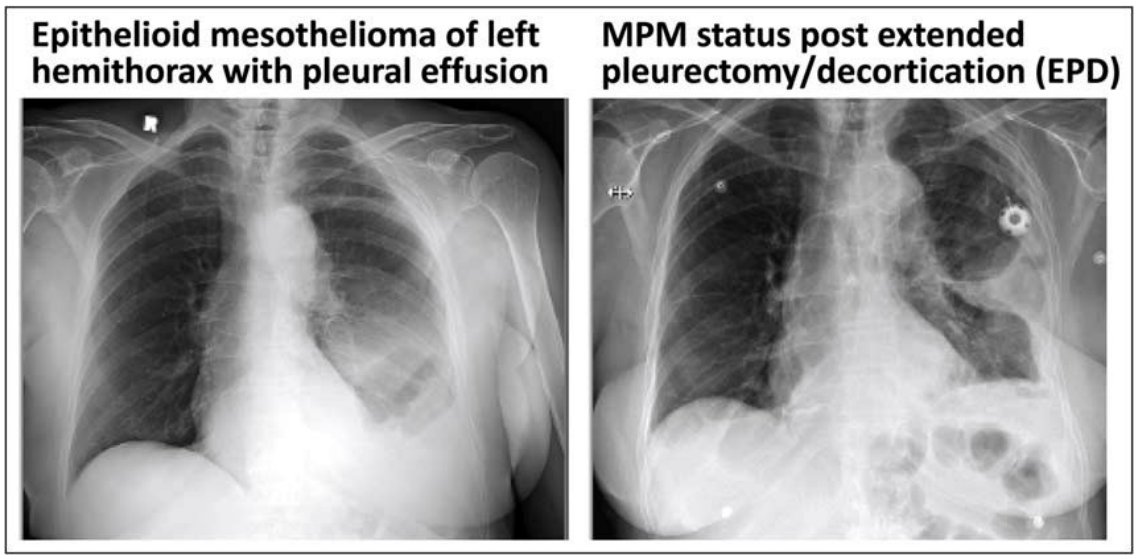


**Figure 9**. Demonstration of preservation of the lung parenchyma in epitheliod MPM before and after EPD

Most patients with mesothelioma are not offered surgery because of the extent of disease, advanced age, comorbidities, or poor performance status, and are considered for palliative chemotherapy instead. For more than fifteen years, the standard first line treatment, both for resectable and unresectable MPM, has been cisplatin-pemetrexed and it is currently the only regime approved by the Food and Drug Administration (FDA) for MPM. Cisplatin is a genotoxic chemoterapy that induces intra-strand DNA cross-linking,

causing DNA damage and interfering with DNA replication (121). Pemetrexed is a folate antimetabolite that inhibits three enzymes used in purine and pyrimidine synthesis. By inhibiting the formation of precursor purine and pyrimidine nucleotides, pemetrexed prevents the formation of DNA and RNA, both required for the growth and survival of normal and cancer cells. However, MPM is a highly heterogeneous cancer and chemotherapy treatment can cause additional selective pressure by eradicating only sensitive proliferative cells. Resistant cells remain and can still grow or regrow after treatment (122)

In a randomized study was observed that the median OS in the cisplatin-pemetrexed arm was 12.1 *vs.* 9.3 months in the control arm that received cisplatin mono therapy (P=0.020, two-sided log- rank test). The most common non-hematological toxicities, in both groups, were nausea, vomiting and fatigue within around 90% of patients experiencing grade-3 toxicity (123).

Although platinum-pemetrexed is an active agent in the first line treatment, chemotherapy may only alleviate symptoms but is not curative. Several studies have been conducted to improve the survival of MPM patients by combining the two classic drugs with drugs used for other tumors. VEGF signaling is an important concept in mesothelioma cell pathophysiology (124). The addition of anti-angiogenesis agents to chemotherapy has been tested in several clinical studies. In the phase III MAPS trial, addition of maintenance bevacizumab, an anti-VEGF antibody, to cisplatin-pemetrexed therapy in unresectable treatment naïve patients, prolonged median PFS from 7.3 to 9.2 months and median OS from 16.1 to 18.8 months. Unfortunately, in the bevacizumab combination arm more grade-3 toxicity occurred, like hypertension (23% *vs.* 0%) and thrombotic events (6% *vs.* 1%) (125).

In MPM there is no current evidence for maintenance chemotherapy and there is a lack of studies showing a better PFS or survival benefit. In a study with a cohort of 13 patients (out of 30 patients who started with platinum-pemetrexed), treatment with pemetrexed maintenance therapy (PMT) was associated to a better survival with a median OS of 8.5 *vs.* 3.4 months in the cohort without maintenance therapy. Grade-3 toxicity consisted of neutropenia, leucopenia and anemia. The only non- hematological grade-3 toxicity during PMT was fatigue (15%). The reason to stop PMT was disease progression (69%), toxicity (23%) and in patient's best interest (8%) (126).

Up today, there is no standard second line treatment in MPM, but if there is a good sustaining response at the time of initial chemotherapy interruption, the NCCN guidelines recommend the re-challenge of pemetrexed (if not administrated in the first- line). Other options like vinorelbine, gemcitabine, and immunotherapy (pembrolizumab and nivolumab-ipilimumab) could also be considered (1).

In MPM, prophylactic radiation therapy to prevent procedure-tract metastasis is not recommended on a routine basis (127). Radiotherapy could be considered in a trimodality treatment. In the NCDB, improved survival was associated to trimodality treatment including radiation therapy compared with surgical intervention and chemotherapy alone (103).

In conclusion, conventional treatments for MPM are ineffective and the prognosis is still very poor, with median survival for resectable pleural mesothelioma between 17 to 25 months and, for unresectable mesothelioma, between 9 to 12 months (128). Therefore, it is crucial to identify novel, well defined targets which can be used alone or in combinations with classic treatments.

In a study, which performed a semi-quantitative assessment of the inflammatory response in the tumor and in the stroma of 175 MPM patients, was found that patients who had a high-grade chronic inflammatory response in the stroma (n=59) presented improved survival compared with those who had a low-grade chronic inflammatory response (n=116; median OS, 19.4 months vs 15.0 months; p=0.01) (129).

Several studies even proposed the prognostic role of lymphocytes and macrophages and the presence of immuno-suppression in MPM through analysis of T-cell-inhibitory receptors and chemokines (130). In a study by Bueno et al (85), were identified, in 212 MPM patients, 4 different phenotypic clusters of molecular expression with divergent associated survival and mutational characteristics. Programmed death-ligand 1 (PD-L1) was expressed in 39% of patients and was associated with a worse survival. PD-L1 expression was higher in non-epithelial MPMs (85).

This has led to considering immunotherapy as a possible treatment strategy for MPM. Clinical trials using cytotoxic T-lymphocyte–associated protein 4 (CTLA-4) inhibitors failed to improve survival in MPM (131), while subsequent trials suggested that PD-L1 inhibitors may benefit some patients (132).

Systemic dendritic cell immunotherapy is another immunotherapy treatment that has shown promising result. This therapy lead to a significant antitumor immune responses in MPM as demonstrated in a pilot study of 10 advanced non-sarcomatoid MPM patients. These patients underwent to an 8-10-week course of adjuvant cyclophosphamide and autologous dendritic cell immunotherapy which resulted associated with impressively prolonged survival, with seven patients survived for at least 24 months and with two patients still alive after 50 and 66 months (133).

### *1.1.6 Prognosis*

As discussed above, for most patients, treatment for MPM is palliative. for this reason, every intervention must take into account improved life expectancy with quality of life. A CT scan, in patients who receive chemotherapy, is often performed mid-cycle to assess response. However, MPM is difficult to monitor using conventional CT scanning, so a biomarker that could measure response to chemotherapy or predict recurrence would offer considerable advantages. Currently, the clinical prognostic scoring systems used for MPM patients are established by European Organization for Research and Treatment of Cancer (EORTC) and Cancer and Leukemia Group B (CALGB) (134, 135). This scoring system define that non-epithelioid histology, male gender, anemia, thrombocytosis, leukocytosis, and elevated LDH are associated with poor prognosis. Otherwise, overall survival remains dismal and there is still a need for better prognostic biomarkers.

The research in this field has focused both on circulating blood-based markers and on genetic alterations identifiable on tumor tissue that could predict patient's outcome.

The soluble mesothelin-related peptides (SMRPs) are found in normal mesothelial cells and are overexpressed in various cancers. SMRP has been analyzed in serum and was studied as a diagnostic marker, with promising results (136), and even as a prognostic factor, but for the latter the results are inconclusive. Indeed, no correlation has been shown between serum SMRP level and progression-free or overall survival in several study (137-138). However, some study found that the SMRP level was inversely associated with overall survival, but, in multivariate analysis limited to epithelial MPM, the prognostic value of SMRP on overall survival was lost (139, 140). Thus, suggesting that histology remains a critical determinant of prognosis.

Osteopontin is a cell adhesion protein that mediates, via interaction with integrin and CD44 receptors, cell-matrix interaction and cell-signaling (141). The prognostic value of osteopontin has been analyzed in several studies which have shown that low baseline plasma levels of the protein were independently associated with favorable progression-free and overall survival (137).

HMGB1 is a damage associated molecular pattern (DAMP) and a key mediator of inflammation. Studies showed that HMGB1 serum levels were higher in MPM patients than in control group with asbestos-exposure (142). Furthermore, another study demonstrated a possible prognostic role of HMGB1 given that, at a cutoff value of 9 ng/mL, there was a significant negative correlation between serum HMGB1 level and survival (143).

In conclusion, SMRP, osteopontin and HMGB1 have been associated with poor prognosis and show potential as prognostic markers in MPM. However, none of them are currently used routinely in clinic for this purpose, as majority of them have not been validated prospectively and their superiority used alone has not been proven over the conventional prognostic EORTC or CALGB models (144).

MicroRNAs (miRNAs) are another group of markers being investigated as diagnostic and prognostic biomarkers due to their tissue specificity, to their ability to classify several types of tumors and to their stability in circulation (145). Even in the MPM, deregulated miRNAs have shown potential as diagnostic and prognostic factors, like miR-29 which if it is up regulated predicts longer survival (146). MiRNAs can be combinated each other in order to improve their prognostic value. For example, in a subsequent study involving MPM patients undergoing surgery, a six-miRNA signature including miR-21-5p, miR-23a-3p, miR-30e-5p, miR-221-3p, miR-222-3p, and miR-31-5p was found to predict an

overall survival of more than 20 months (147). However, the use of miRNAs as a biomarker is not yet applicable to clinical practice and larger studies are required to validate their diagnostic and prognostic values.

Taken together, there has been a strong interest in the research of circulating biomarkers due to their non-invasiveness, but many of them have varying levels making them unsuitable for diagnostic or prognostic use. For this reason, a strong interest has also been placed in the search for tumor markers on biopsy sections. Genetic aberrations, identifiable by immunoistochemestry (IHC) or FISH analysis on tumor tissue, were studied as prognostic factor.

One of the most common events found in MPM is the 9p21 deletion. The *CDKN2A* gene contained in this region, encodes for p16 protein, a well-established tumor suppressor in a variety of tumor types, including MPM. This genetic aberration has been confirmed in several studies and has been correlated with poor patient prognosis (148, 149).

Cedres *et al.* have been demonstrated that the expression of WT1 gene in tumor section of MPM is associated with a better survival, but they don't find a correlation between calretinin expression, another promising prognostic factor in MPM, and survival (150)

The expression of the Programmed death-ligand 1 (PD-L1) in MPM has been correlated with shorter survival, compared to MPMs without this condition (151-153). Tumor cells that express PD-L1 on their extracellular surface downregulate the anti-tumor activity of infiltrating lymphocytes. Positive PD-L1 expression, determined by IHC (>1% tumor cell staining), is reported in 11–72% of MPMs (131-138) and allows patients to be subjected to immunotherapy treatments such as Pembrolizumab (an anti-PD-1 monoclonal antibody). Instead, nivolumab (another anti-PD-1 agent) is accepted as salvage therapy regardless of PD-L1 IHC findings. (154).

Finally, interest was placed on the BAP1 gene. BRCA-1-associated protein 1 (BAP1) is a deubiquitinase enzyme, involved in the regulation of various cellular pathways (155). The prognostic role of BAP1 expression in MPM has been studied, but the results are still confusing.

## 1.2 BAP1

### *1.2.1 Structure and function*

BRCA1-associated protein 1 (BAP1) was identified in 1998 and, from the first studies, it was reported that it had a growth suppression activity in breast cancer cells. Moreover, it was also observed that this anti-tumor activity was carried out in cooperation with BRCA1 in cultured cells.

BAP1 is a deubiquinating enzyme, with the gene is located on the short arm of chromosome 3 (3p21.1) and consists of 17 exons (157). BAP1 protein is a 90 kDA, nuclear localized deubiquitinating enzyme (DUB) consisting of 729 amino acids (2). BAP1 protein has three main domain: an N-terminal ubiquitin carboxyl hydrolase domain (UCH); a host cell factor 1 (HCF1) binding domain (HBM) in the middle portion; a C-terminal domain (CTD) containing a coiled-coil motif for interaction with ASXL1/2 and a nuclear localization signal (NLS). There are also various binding regions to other protein interaction partners (**Figure 10**) (158-160).

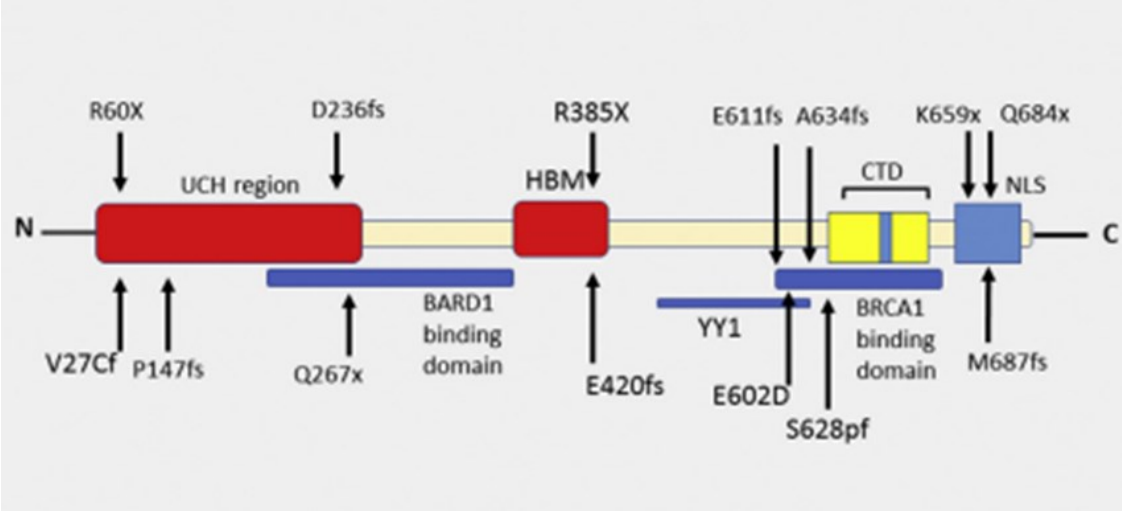


**Figure 10.** Schematic structure of BRCA1-associated protein-1 (BAP1) domains and locations of reported germline mutations.

Many cellular processes are regulated by protein ubiquitination and deubiquitination, a reversible post-translational modification. BAP1 works as a tumor suppressor by its deubiquitinase activity, regulating target genes involved in transcription, cell cycle control, DNA repair and cellular differentiation (**Figure 11**) (161).

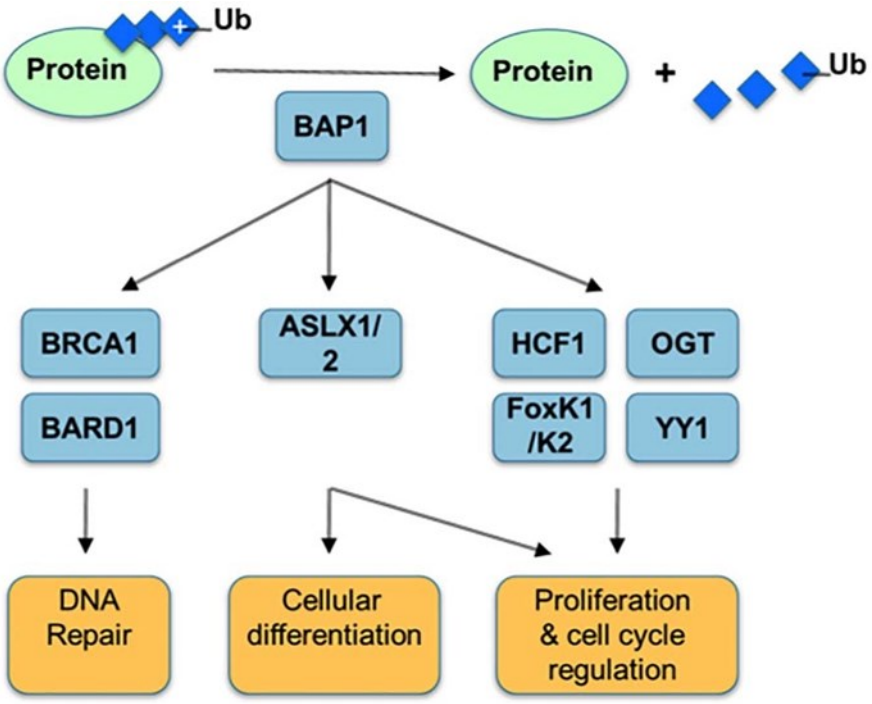


**Figure 11**. Schematic rapresentations of BRCA1-associated protein 1 activity.

BAP1 interacts with numerous proteins and carries out its anti-tumor activity in various ways. BAP1 can activate the transcriptional regulator HCF1 (162). Once activated, HCF1 modulates chromatin architecture by recruiting histone-modifying complexes and activating transcription factors such as the E2F family, which controls G1/S phase progression in the cell cycle (158). BAP1 also deubiquitinates O-linked N-acetylglucosamine transferase (OGT), which in turn modifies and activates HCF1 (163). BAP1 has been shown to form a ternary complex with HCF1 and transcription factor Ying Yang 1 (YY1), that controls cellular proliferation (164). This complex is recruited to the promoter of COX7C which encodes a component of the mitochondrial respiratory chain (164). Furthermore, BAP1 is involved in cell proliferation and cell cycle control even by forming a ternary complex with HCF1 and the forkhead transcription factors FoxK1/K2 (159)

BAP1 form the polycomb group repressive deubiquitinase complex (PR-DUB) by interacting with ASXL1/2 and regulate physiological processes such as stem cell pluripotency, embryonic development, self-renewal and differentiation. This group of protein contain even a polycomb-repressive complexes (PRCs) that ubiquitinate histones and lead to gene silencing (158). There is a transcriptional balance and control due by ubiquitination by PRCs and deubiquitination by PR-DUB (158).

BAP1 is involved even in the DNA damage repair process. BAP1 form a complex with several recombination proteins including Breast Cancer type 1 (BRCA1) and BRCA1-associated RING domain protein 1 (BARD1), which promotes E3 ubiquitin ligase activity to regulate DNA damage response (165).

Initially, it was thought that BAP1, given its NLS domain, was present only in the nucleus, where it is involved in several anti-tumor processes such as DNA repair.

However, in a recent study conducted by Bonomi et al, the presence of BAP1 was also found in the cytoplasm. Here, BAP1 is found in the endoplasmic reticulum (ER) fraction where it is involved in the apoptotic process (166, 167).

In detail, cytoplasmic BAP1 regulates $Ca^{2+}$ transfer from the ER, where $Ca^{2+}$ is normally stored in the cell, to the cytoplasm by deubiquitinating the IP3R3 receptor channel. $Ca^{2+}$ is released in areas of the ER, called MAMs (mitochondrial-associated membranes), that are in close contact with the mitochondrial outer membrane. Here, $Ca^{2+}$ flows inside the mitochondria first through the voltage-dependent anion channel (VDAC) channel localized on the outer mitochondrial membrane and then by the mitochondrial uniporter channel (MCU) located on the inner mitochondrial membrane.

This process is finely regulated, because $Ca^{2+}$ in the mitochondria is necessary for the Krebs cycle, but if DNA damage occurred and cannot be repaired, higher amounts of Ca2+ is released from the ER through the IP3R3 leading to high mitochondrial Ca2+ concentrations. Finally, mitochondria release cytochrome c into the cytosol, thus inducing the apoptotic process (167) (**Figure 12)**. In case of BAP1 inactivating mutation, cells cannot release enough amounts of $Ca^{2+}$ to induce the apoptotic process and with low level of $Ca^{2+}$ the Krebs cycle is impaired. This cause the cells to switch into anaerobic glycolysis (Warburg effect), a metabolic shift that favors malignant growth (166).

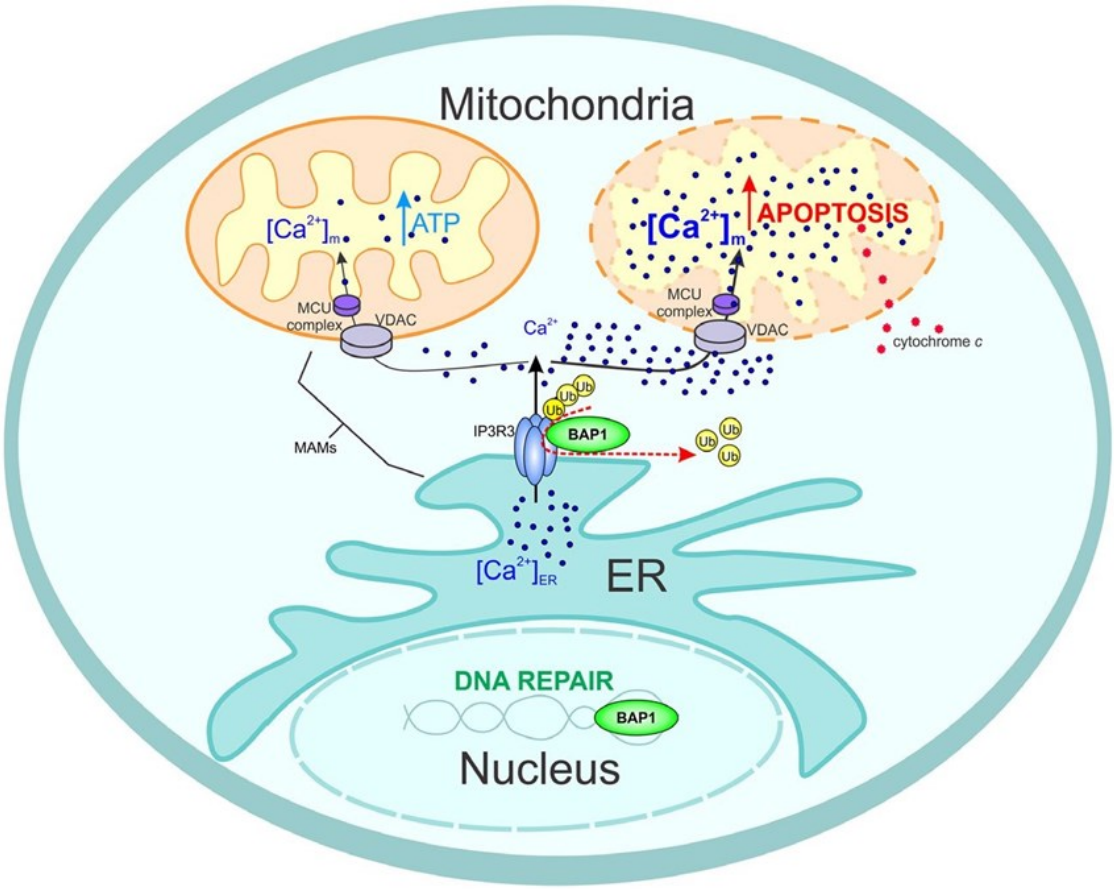


**Figure 12**. Representations of BAP1 activity in the cytoplasm.

Subsequently, Zhang et al showed that cells with reduced BAP1 activity also have impaired ferroptosis (168) providing an additional mechanism by which BAP1-mutated cells escape cell death (155). Thus, cells with reduced or absent BAP1 activity could accumulate more DNA damage (166) since they cannot properly repair the DNA (167, 169) and, at the same time, they cannot execute apoptosis, which normally eliminates cells that contain genetic mutations. This leads to a possible transformation in cancer cells

### *1.2.2 BAP1 and MPM*

In a study by Carbone et al it was shown that the high incidence of MPM in some Cappadocian and American families was due to the presence of germline mutations of

the BAP1 gene. The study demonstrated the existence of a predisposition to MPM which was transmitted in an autosomal dominant fashion (88, 170-172). Like other tumors suppressors, affected individuals inherited a non-functional BAP1 allele. The remaining functional allele was inactivated later in life.

Since 2011, over 600 articles have confirmed and expanded the pathogenic role of BAP1 mutations in MPM and in other cancers (10, 172-175).

This condition was named the "BAP1 cancer syndrome" (BAP1-TPDS) because affected family members present an high risk of developing tumors, predominantly MPMs, uveal melanomas (UM), cutaneous melanoma (CM) and renal cell carcinomas (RCC). While, basal cell carcinoma, breast carcinomas, cholangiocarcinomas, sarcomas, and various types of brain tumors were less frequently (10, 164, 173-175). In addition, Wiesner et al. recognized that a benign atypical skin melanocytic tumor was associated with germline BAP1 mutation (176). This melanocytic lesion was histologically between benign Spitz nevus and malignant melanoma, so was named Melanocytic BAP1-mutated Atypical Intradermal Tumor (MBAIT), or atypical Spitz tumor (AST) (176). The detection of MBAIT, which develop between 20-30 years, allows dermatologists to suspect the diagnosis of BAP1-TPDS, which is then verified and confirmed by DNA sequencing (173-175, 177).

The incidence of these tumors in people with BAP1 syndrome is very high and, in most cases, they have a positive family history of at least two of the main cancers (including UM, MPM, MBAIT, CM, and RCC) among their first- or second-degree relatives (178). Moreover, affected individuals may have more than one type of primary cancer (172, 173, 178).

Furthermore, somatically mutated (acquired mutations occurring during tumor cell growth) BAP1 has been found in approximately 60% of MPMs, underscoring the critical role that BAP1 has in preventing MPM growth (85, 87, 179-181).

Several different alterations in the BAP1 gene have been described, including large deletions of exons leading to loss of the N-terminal region, focal deletions, frameshift mutations due to insertions or deletions, splice site mutations, and base substitutions leading to non-sense and missense mutations (158, 167). In MPM, the BAP1 gene is commonly lost by chromosomal deletion, and more than 70% of reported germline BAP1 mutations are truncation (167, 178).

From these studies it was found that BAP1 plays a key role in MPM, therefore it was also analyzed as a possible diagnostic and prognosis factor.

BAP1 status determinations by IHC has improved the ability to diagnose MPM. BAP1 wild-type ($BAP1^{WT}$) is found in the nucleus and the cytoplasm, with strong nuclear staining and less intense cytoplasmic staining (87), while MPM BAP1 loss are determined by the complete absence of staining or by cytoplasmic staining without nuclear staining. BAP1 positivity nuclear staining is a specific and reliable marker to distinguish benign atypical mesothelial hyperplasia at its earliest stages of development from mesothelioma because benign cells always express BAP1 in the nucleus (87, 182-184). Overall, approximately 70% of epithelial and 50% of sarcomatoid MPMs contain somatic BAP1 gene mutations, resulting in an absence of BAP1 nuclear staining (87, 182-186).

BAP1 loss is highly specific for MPM and this is helpful in the differential diagnosis with carcinoma of the lung, breast, and stomach. Only one study showing BAP1 loss in just 1% of lung cancers (187-189). Furthermore, multiple studies have indicated that BAP1 loss is highly specific for MPM in the differential diagnosis with ovarian carcinoma (188,

190, 191). However, multiple other malignancies (including renal cell carcinoma and melanoma) also frequently show BAP1 loss (158, 187, 192). These tumors may occasionally metastasize to the serosal surfaces, thus careful consideration of the clinical and radiographic data, tumor morphology, and lineage-specific markers is critical when interpreting BAP1 IHC (154).

In addition to its diagnostic role, multiple studies indicate that BAP1 even show a prognostic value and the preponderance of them supports a better outcome in MPM with BAP1 loss (185, 193-199). Instead, one study found that BAP1 loss was correlated to a poorer prognosis and suggests that the improvement in prognosis identified in the other studies was likely due to confounding factors (200). Importantly, these studies have not generally discriminated between germline and sporadic BAP1 loss.

Specific studies showed that germline *BAP1* mutations were associated to better prognosis compared to non-germline-mutated MPM, irrespective of tumor site, with 5-year overall survival of 47%, and 7%, respectively (90, 201).

In addition to germline and somatic gene mutations (202), post-transcriptional mechanisms are involved in the regulation of BAP1 expression. For instance, 25% of MPM with negative nuclear IHC staining of BAP1 are also negative for BAP1 gene mutations (158). MiRNA are small noncoding RNAs that control gene expression at post-transcriptional level binding by 7-8 nucleotides the complementary ones in the 3'-untranslated regions of their targets. MiRNA may function as either oncogene or tumor suppressors depending on target genes and cancer type (203). It has been demonstrated in pre-clinical studies that miR-31 is a post-transcriptional regulator of BAP1 (204-206).

# 2. AIM OF THE THESIS

BAP1 loss has been detected in about 60% of MPM and its prognostic role has been amply investigated in order to find a potential link between BAP1 status and patient outcome. However, the clinic relevance of BAP1 loss-of-function in MPM still remain under debates, with conflicting reports that it may be associated with epithelioid subtype. In order to clarify the prognostic role of BAP1 expression in MPM, we conducted a systematic review and meta-analysis for summarize the available evidence in this setting. To improve the performance of BAP1 in distinguishing patients with a worse outcome, BAP1 status has been combined with the tissue expression of miR-31, a miRNA involved in the BAP1 regulation at post-transcriptional level.

# 3. MATERIAL AND METHODS

## 3.1. Cohort study

### *3.1.2. Study population*

Between 2003 and 2019, clinical records of 85 patients with a histological diagnosis of MPM were retrospectively collected at the Clinical Oncology, Polytechnic University of Marche, AOU Ospedali Riuniti.

Formalin-Fixed Paraffin-Embedded (FFPE) tissues with BAP1 IHC staining (n=60) were collected at the Clinic of Pathological Anatomy before any treatment including the first-line chemotherapy.

The collected patients characteristics and clinical-pathological features were: BAP1 expression (BAP1 retained vs BAP1 loss, as assessed by IHC), age, gender, smoking status (current/former smokers vs never smokers), asbestos exposure, histotype (epithelial, biphasic and sarcomatoid), side of disease, clinical TNM stage (stage IV versus others [VIII edition]) (207), treatment modality (chemotherapy plus surgery versus only chemotherapy), type of chemotherapy, response to chemotherapy according to modified Response Evaluation Criteria in Solid Tumors for assessment of response in malignant pleural mesothelioma (mRECIST criteria) (208).

### *3.1.3. Immunohistochemical analysis*

All selected MPM histological samples and relative diagnoses were reviewed by an experienced mesothelioma pathologist and divided into epithelioid (e-MPM), sarcomatoid (including desmoplastic, s-MPM) and biphasic (b-MPM) MPM according to the 2015 World Health Organization (WHO) classification (209). For the inclusion of MPM sample in the b-MPM subgroup was required at least the presence of both sarcomatoid and epithelioid components in 10% of the tumor.

All samples were FFPE and, for each patient, a single 4-mm-thick paraffin section was cut from the sample with the greatest amount of tumor tissue. All sections were deparaffinized and rehydrated in graded concentrations of xylene and ethanol. Sections were coated with 1:50 mouse monoclonal BAP1 antibody (clone C4:sc-28383; Santa Cruz Biotechnology, USA) and incubated at 4°C overnight. Then, automated IHC was performed on Omnis platform (Agilent, USA).

BAP1 IHC status was considered as “positive/retained” if there was an unambiguous positive nuclear staining in any number of tumor cells, and “negative/loss” if the nuclear staining was absent in neoplastic cells. Tumor cells with cytoplasmic reactivity without a clear nuclear staining were considered negative. Non-neoplastic cells, such as vascular endothelium, fibroblasts or inflammatory cells, were considered as internal positive control.

### *3.1.4. Mir-31 assay*

Total RNA was extracted from FFPE tissue samples (10–100 µg) using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Thermo Fisher Scientific) according to the manufacturer's protocol. The RNA concentration and purity were determined in the Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific). The retro-transcription reaction of miR-31 was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The reaction mix was prepared using 20 ng/$\mu$l of total RNA and incubated in the Biometra thermal cycler. The thermal protocol was the following: 16℃ for 30 minutes, 42℃ for 30 minutes and 5 minutes at 85℃.

Quantitative RT-PCR (qPCR) was performed using the TaqMan Fast Advanced Master Mix (Applied Biosystems) with U6 as the housekeeping gene. The qPCR assays were carried out using the Mastercycler EP Realplex instruments (Eppendorf) using the following thermal protocol: 95°C for 10 minutes for the polymerase activation, then 40 cycles at 95 °C for 15 seconds and at 60°C for 1 minute. MiR-31 levels were considered as percentiles and then divided into quartiles, in order to quantify the miR-31 expression for each sample. Because the $25^{th}$ and $50^{th}$ percentile showed similar miR-31 expression, the respective inter-quartiles were used as reference and results of remaining samples were expressed as relative level using the ΔCT method ($2^{-\Delta CT}$). MiR-31 expression was considered "high" if miR-31 levels were over the $75^{th}$ percentile and "low" if miR-31 levels were below the $75^{th}$ percentile.

### *3.1.5. Statistical analysis*

Discrete data were expressed either as mean, standard deviation, minimal and maximal values (if normally distributed), or as median, quartile and range (if not-normally distributed). Categorical variables were reported as either fractions or percentage. Differences between groups were analyzed using means of chi-squared method or Fisher's exact test for categorical variables and unpaired Student t test, the Mann-Whitney U and one-way analysis of variance (ANOVA) with Tukey post-hoc analysis test for continuous variables. OS was defined as the temporal interval between the date of the first cycle of first-line chemotherapy and the date of death or censoring at the date of last follow-up of alive patients. PFS was considered as the time from the first cycle of first-line chemotherapy until clinical or instrumental disease progression or last follow-up. Survival curves were constructed using the Kaplan–Meier method and log-rank method was used to assess difference between subgroups. Patients with missing survival data were excluded from the analysis.

Multivariate Cox regression analysis was used to evaluate independent predictors of survival. Nonsignificant prognostic factors were excluded from the model using backward elimination. A p value $< 0.05$ was considered statistically significant.

Statistical analysis was performed by using SPSS for Windows version 19.0 (SPSS Inc, Chicago, IL) and GraphPad PRISM (GraphPad Software, Inc., La Jolla, CA),

## 3.2. Systematic review and meta-analysis

### *3.2.1. Literature search*

We conducted a systematic review and meta-analysis following the procedures of the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement and the Meta-analysis of Observational Studies in Epidemiology (MOOSE) guidelines.

Relevant literature was identified by systematic search of PubMed (Medline) and Embase datasets until September 28, 2019, considering only English-written publications. Our search strategy included the following mesh terminology: (mesothelioma) AND (BAP1 OR bap1 OR UCHL2 OR hucep-6 OR HUCEP-13 OR "BRCA1 associated protein-1" OR "ubiquitin carboxy-terminal hydrolase") AND (mortality OR mor- talities OR fatality OR fatalities OR death OR survival OR prognosis OR "hazard ratio" OR HR OR "relative risk" OR RR OR prognosis OR progression OR recurrence OR PFS OR OS).

### *3.2.2. Study selection*

The inclusion criteria were as follows: (i) both prospective and retrospective full-text observational cohort studies regarding MPM; (ii) immunohistochemical (nuclear positivity) investigation of BAP1 expression on tumor tissue (iii) enough data to calculate the correlation of BAP1 status with OS.

Studies conducted on other cancer than MPM (e.g. peritoneal mesothelioma), with no report data on patient outcomes or that had investigated BAP1 status by other methods than IHC (e.g. DNA sequencing) were excluded. When more than one study from the

same institute/authors was reported, only the most recent or the highest quality study (reporting a higher number of patients) was included in the analysis.

Title and abstract of the primary studies identified in the electronic search were independently screened by two reviewers. Duplicate studies were excluded. Any disagreement or discrepancies was resolved by a third reviewer or contacting the study authors when necessary.

The reference list of all identified documents was scrutinized, with the aim of identifying additional potentially eligible studies.

### *3.2.3. Data extraction and meta-analysis outcomes*

For each study we extracted author name, year of publication, number of patients, mean age, gender, histotype, HR and 95 % CI of BAP1 immunohistochemical expression for OS.

Our primary objective of the meta-analysis was to determine the HR of death in patients whose tumor retains BAP1 expression (BAP1 retained) compared to patients whose tumor does not express BAP1 (BAP1 loss).

In the BAP1 retained MPM patients a HR > 1 indicates higher risk of death and a HR < 1 lower risk. We were considered only HR values obtained through multivariate analysis (adjusted for histotype). Finally, if the survival information was only available in Kaplan–Meier curves, we collected HR only when epithelioid and non-epithelioid subtypes were separately analyzed.

### *3.2.4. Data analysis*

DerSimonian-Laird random-effects model was used for calculating pooled HRs with correspondent 95% CIs of death between BAP1 retained versus BAP1 loss MPM patients. Because of the heterogeneity of the observational studies, this method was chosen a priori. To determine inconsistency across the results of the studies was used the Higgins $I^2$ index and chi-square statistics. Funnel plots were drawn to evaluate the publication bias of the included literature. All p values were two sided and $p < 0.05$ was considered significant. Data analyses and generation of forest plots were performed using R 3.6.0 (R Foundation for Statistical Computing) and RevMan 5.2 (Copenhagen, Denmark).

# 4. RESULTS

## 4.1. Demographic and clinic-pathological characteristics of MPM patients

Our cohort consisted of 85 patients (81% male). Fifty-one patients had progression of disease and 41 patients died at a median follow-up time of 41.28 months (interquartile range [IQR]: 14.64-not reached). Median age at diagnosis was 71 years (range 52-81 years). Median OS was 14.76 (95% CI: 7.92–26.52) months and median PFS was 4.56 (95% CI: 2.88–9.48) months. Asbestos exposure was documented in 55% of patients, and 57% of patients were current/former smokers. Epithelioid MPM (e-MPM) represented 66% of cases (n=56), while 15% was represented by sarcomatoid MPM (s-MPM) (n=13) and 10% was biphasic MPM (b-MPM) (n=10). Only 18 patients (21%) underwent surgical resection. 74% of patients (n=63) received first-line platinum-based chemotherapy in association with pemetrexed (83%) and 17% (n=20) pemetrexed in monotherapy (17%). Demographic and clinicopathological characteristics are summarized in **Table 3**.

| Characteristic | Total patients (n=85) | | Patients with BAP1 staining available (n = 60) | | | | |
|---|---|---|---|---|---|---|---|
| | | | BAP1 loss (n=37) | | BAP1 retained (n=23) | | *P valu* |
| | n | % | n | % | n | % | |
| **Age** | | | | | | | |
| ≤ 70 years | 41 | 48 | 13 | 35 | 10 | 43 | 0.11 |
| > 70 years | 44 | 52 | 24 | 65 | 13 | 57 | |
| **Gender** | | | | | | | |
| Male | 69 | 81 | 28 | 76 | 20 | 87 | 0.46 |
| Female | 16 | 19 | 9 | 24 | 3 | 13 | |
| **Smoking** | | | | | | | |
| Current/former | 48 | 57 | 21 | 57 | 13 | 57 | 1.00 |
| Never | 31 | 36 | 14 | 38 | 9 | 39 | |
| Unknown | 6 | 7 | 2 | 5 | 1 | 4 | |
| **Asbestos exposure** | | | | | | | 0.51 |
| Yes | 47 | 55 | 19 | 51 | 9 | 39 | |
| No | 38 | 45 | 18 | 49 | 14 | 61 | |
| **Histotype** | | | | | | | |
| Epithelioid | 56 | 66 | 32 | 87 | 11 | 48 | |
| Sarcomatoid | 13 | 15 | 2 | 5 | 5 | 22 | 0.01[a] |
| Biphasic | 10 | 12 | 3 | 8 | 5 | 22 | |
| Unknown | 6 | 7 | 0 | 0 | 2 | 8 | |
| **Clinical stage** | | | | | | | |
| I/II/III | 64 | 21 | 31 | 84 | 19 | 83 | 0.35 |
| IV | 11 | 13 | 1 | 3 | 3 | 13 | |
| Unknown | 10 | 12 | 5 | 13 | 1 | 4 | |
| **Surgery** | | | | | | | |
| Yes | 18 | 21 | 10 | 27 | 3 | 13 | 0.31 |
| No | 66 | 78 | 26 | 70 | 20 | 87 | |
| Unknown | 1 | 1 | 1 | 3 | 0 | 0 | |
| **First-line chemoterapy** | | | | | | | |
| Platinum-based chemoterapy | 63 | 74 | 29 | 78 | 18 | 78 | 0.22 |
| Pemetrexed | 17 | 20 | 6 | 16 | 4 | 18 | |
| Other | 1 | 1 | 1 | 3 | 0 | 0 | |
| Unknown | 4 | 5 | 1 | 3 | 1 | 4 | |
| **BOR** | | | | | | | |
| PR | 10 | 13 | 7 | 19 | 2 | 10 | 0.58 |
| SD/PD | 68 | 87 | 29 | 81 | 18 | 90 | |

**Table 3**. Patient demographics and baseline clinical characteristics.
[a] Comparison between epithelioid subtype and non-epithelioid subtype (sarcomatoid and biphasic).

## 4.2. Outcomes according to BAP1 status

Immunohistochemical staining for BAP1 expressions was conduct only for patients that had adequate tissue (n=60, 70%). Immunohistochemical BAP1 positive/retained (≥1%) was found in 38% of cases (n=23) and BAP1 negative/loss in 62% of cases (37%). BAP1 loss was mainly associated with the epithelioid subtype (p=0.01; 74% of e-MPM were BAP1 negative/loss and 33% of non-epithelioid MPM were BAP1 negative/loss; **Table 3**). Among patients with BAP1 loss and BAP1 retained there was no difference in age, gender, smoking status, asbestos exposure, clinical stage, and surgery. In **table 3** is summarized BAP1 expression status according to clinical data.

At univariate analysis, there were no difference in terms of OS between the two group (BAP1 retained vs BAP1 loss), with a median OS of 18.1 months (95% CI: 11.2-25.8) for positive BAP1 expression and 14.8 months (95% CI: 10.7-29.3 months) for negative BAP1 expression (p=0.17, **Figure 13a**). At multivariate analysis, after adjusting for age and histotype, no significant differences were observed among the two groups for OS (HR 1.09, 95% CI: 0.51-2.31, p=0.81). Conversely, non-epithelioid histology held its independent negative prognostic value for OS (HR 7.03, 95% CI: 2.48-19.86, p=0.0002)

Looking at PFS, both groups showed a similar trend, with median PFS of 5.04 months (95% CI: 3.00-8.64) for BAP1 retained tumors and 6.36 months (95% CI: 3.24-11.64) for BAP1 loss tumor (p=0.14, **Figure 13b**). Even for PFS, at multivariate analysis, non-epithelioid histology was the only independent prognostic factor (HR 3.58, 95% CI: 1.58-8.14, p=0.002).

Similarly, no differences in response to treatment (BOR) were noted (10% in BAP1 retained patients versus 19% in BAP1 loss, p 0.58).

In the platinum-based chemotherapy subpopulations (n=47) were no differences according to BAP1 status (18.1 months vs 14.8 months, p=0.3)

Finally, for the 12 patients who underwent surgery (before or after chemotherapy), we found an improved OS for patients with BAP1 loss (median OS of 25.9 months, 95% CI: 11.1-not reached, vs 5.5 months 95% CI: 5.0-not reached for BAP1 retained group, p=0.02). However, after adjusting for histotype, BAP1 status has lost its prognostic value (p 0.14).

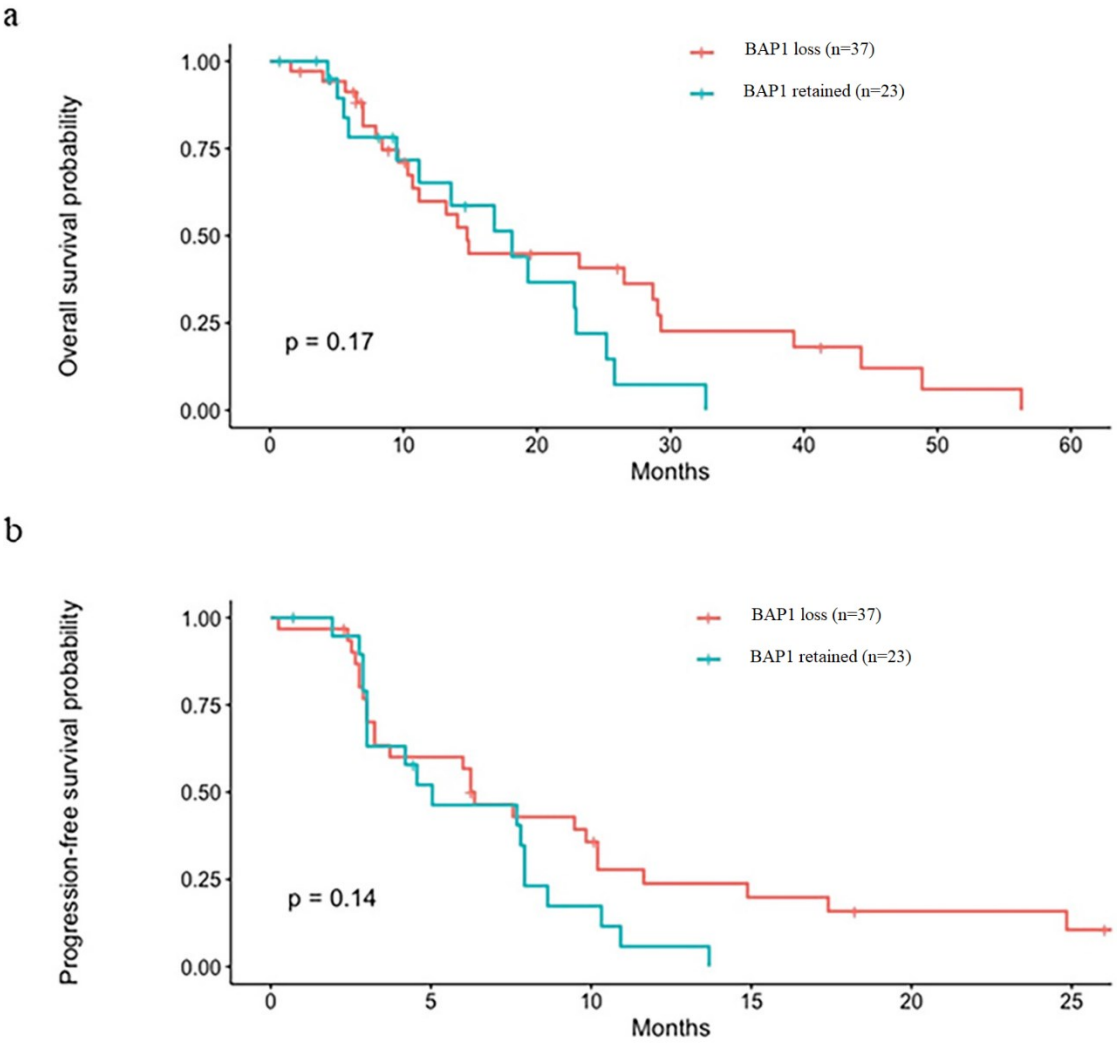


**Figure 13**. Kaplan-Meier curves of survival according to BRCA1 associated protein-1 (BAP1) expression. (**a**) Overall survival. (**b**) Progression-free survival.

## 4.3. Meta-analysis results

### *4.3.1. Search results and study population of included studies*

From systematic research was extracted 346 potentially relevant articles and, after the exclusion of duplicates, 243 were considered for meta-analysis. After screening of title and abstract, 19 articles were selected for full-text review. Among them, 14 articles were excluded, since they evaluated BAP1 status with techniques other than IHC (n=5) (85, 180, 210-212), took into account only germline BAP1 mutations (n=2) (90, 92), did not report any survival analysis (n=1) (30), confounding factors were not taken into account in a multivariate analysis (n=4) (194, 199, 213, 214) and two because they were redundant from the same institution. Finally, five studies for a total of six cohort, (87, 185, 196, 198, 200) qualified all the selection criteria and were included for the meta-analysis.

The PRISMA flow chart showed the detailed literature search steps (**Figure 14**).

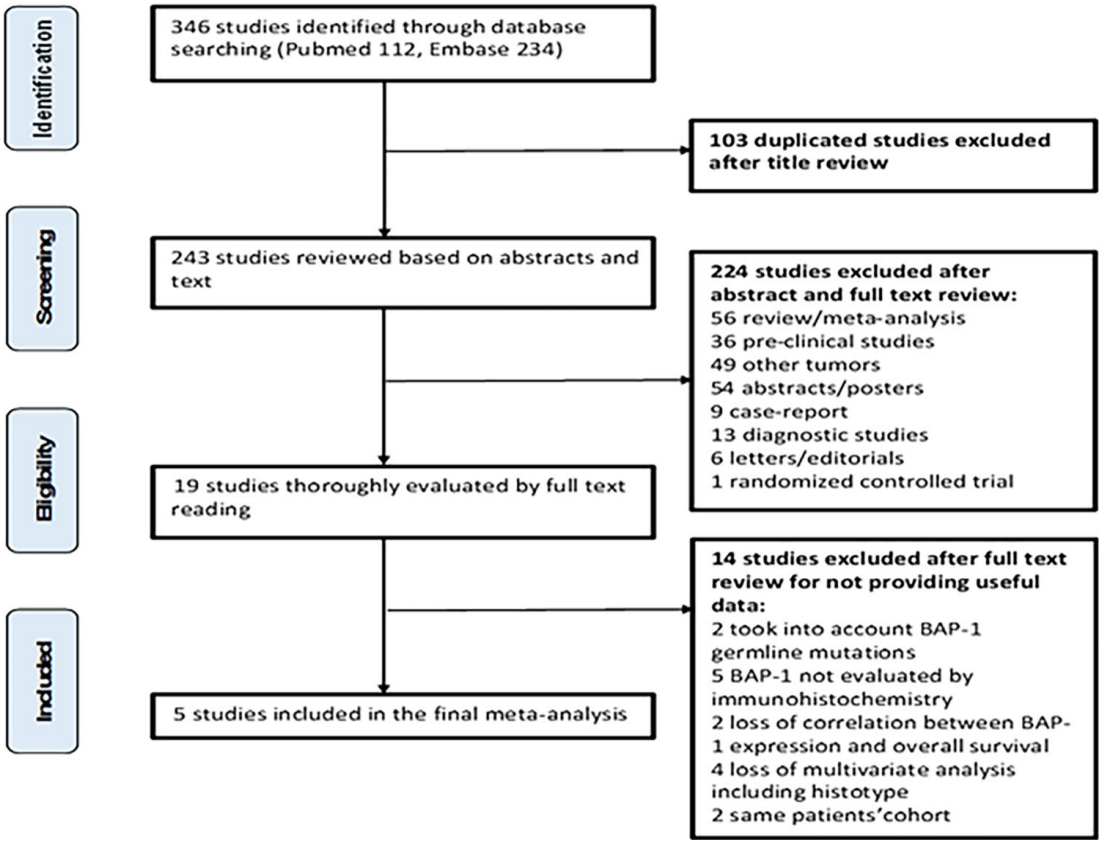


**Figure 14**. PRISMA flow chart summarizing the process for the identification of eligible studies for the meta-analysis.

All studies were published after 2015 and assessed for BAP1 status by IHC on whole-sections or tissue microarrays (185, 196). The number of adjustments in the multivariate analyses ranged from 1 to 3 and, in particular, histotype was always included.

We also added our cohort to the meta-analysis, thus, the overall populations consisted of 690 MPM patients. Seventy-eight per cent (n=543) were male and the proportion of epithelioid MPM was 62 % (n=428). Detailed descriptions of the 5 included studies (in addition to ours) are summarized in **Table 4**.

| | Forest et al. | Pulford et al. | Farzin et al. | McGregor et al. | Nasu et al. |
|---|---|---|---|---|---|
| **Year** | 2018 | 2017 | 2015 | 2015 | 2015 |
| **Country** | France | Australia | Australia | USA | USA |
| **Patients** | 117 | 81 | 229 | 111 | 92 |
| **Age**[a] | | | | | |
| Total cohort | 73 (43-92) | 74 (35-94) | 72 (35-93) | 70 (43-89) | 62 (20-85) |
| BAP1 loss | NA | 74 (53-92) | 70 (35-93) | 69 (43-89) | 62 (20-82) |
| BAP1 retained | NA | 74 (35-91) | 75 (44-93) | 69 (45-81) | 62 (23-85) |
| **Gender**[a] | | | | | |
| **Male** | | | | | |
| Totale cohort | 88 (79 %) | 63 (78 %) | 188 (82 %) | 87 (78 %) | 67 (73 %) |
| BAP1 loss | NA | 39 (62 %) | 83 (44 %) | 57 (65 %) | 44 (66 %) |
| BAP1 retained | NA | 24 (38 %) | 105 (56 %) | 30 (35 %) | 23 (34 %) |
| **Female** | | | | | |
| Total cohort | 29 (21 %) | 18 (22 %) | 41 (18 %) | 22 (25 %) | 25 (27 %) |
| BAP1 loss | NA | 8 (44 %) | 23 (56 %) | 11 (50 %) | 17(68 %) |
| BAP1 retained | NA | 10 (56 %) | 18 (44 %) | 11 (50 %) | 8(32 %) |
| **Histotype**[a] | | | | | |
| **e-MPM** | | | | | |
| Total cohort | 87 (74 %) | 57 (70 %) | 120 (53 %) | 58 (52 %) | 64(70 %) |
| BAP1 loss | 62 (71 %) | 35 (61 %) | 75 (63 %) | 47 (81 %) | 48(75 %) |
| BAP1 retained | 23 (26 %) | 22(39 %) | 45 (37 %) | 11(19 %) | 16(25 %) |
| **Non e-MPM** | | | | | |
| Total cohort | 30 (26 %) | 24 (30 %) | 109 (47 %) | 53 (48 %) | 28(30 %) |
| BAP1 loss | 6 (20%) | 12 (50 %) | 31 (28 %) | 21 (40 %) | 13(46 %) |
| BAP1 retained | 23 (77 %) | 12 (50 %) | 78 (72 %) | 32 (60 %) | 15(54 %) |
| **Variables included in multivariate analysis** | BAP1 loss, histotype, absence of necrosis, nuclear grading | BAP1 status, age, gender, histotype | BAP1 status, age, gender, histotype | BAP1 status, histotype | BAP1 status, histotype |
| **OS (months)** [a, b] | | | | | |
| BAP1 loss | 17.9 (e-MPM) p = 0.034; 5.3 (non e-MPM) p = 0.60 | 6 (surgical sections) p = 0.015; 8 (cytology sections) p = 0.205 | 16.1 (all patients) p < 0.01 | NA | NA |
| BAP1 retained | 12.4 (e-MPM) p = 0.034; 14.1 (non e-MPM) p = 0.60 | 11 surgical sections) p = 0.01; 9(cytology sections) p = 0.205 | 6.3 (all patients) p < 0.01 | NA | NA |

Table 4. Characteristics of studies included in the meta-analysis.

[a] Continuous variables are reported as mean, minimal and maximal values; categorical variables are reported as absolute numbers and percentage; OS is reported as median.

[b] OS was calculated from the date of diagnosis in two studies (Forest et al., McGregor et al.) and from the date of surgery in other two studies (Pulford et al., Farzin et al.). The numeric value of median OS referred to the whole cohort of patients was not reported in the studies by McGregor et al. and Nasu et al.

### *4.3.2. Association with clinical-pathological characteristics and prognostic role of BAP1*

Overall, BAP1 loss was the most represented status (388 out of 690, 56%) and was strongly associated with the e-MPM subtype (p for difference<0.0001). In the total populations, BAP1 loss was found in 70 % of epithelioid MPM and in 34 % of non-epithelioid MPM.

The median OS ranged between 6 months (200) and 17.9 months (198) and no association between BAP1 expression and gender was found (p for difference 0.94).

Pooling data from the 7 cohorts, BAP1 loss was not associated with a lower risk of all-cause mortality. The pooled HR was 1.11 (95 % CI, 0.76–1.61; p 0.60, $I^2$=78 %, p for heterogeneity=0.0001, random effect model) (**Figure 15a**) which indicate that BAP1 expression had no obvious impact on patient survival when histotype is taken into account. The funnel plot showed no evidence of publication bias (**Figure 15b**).

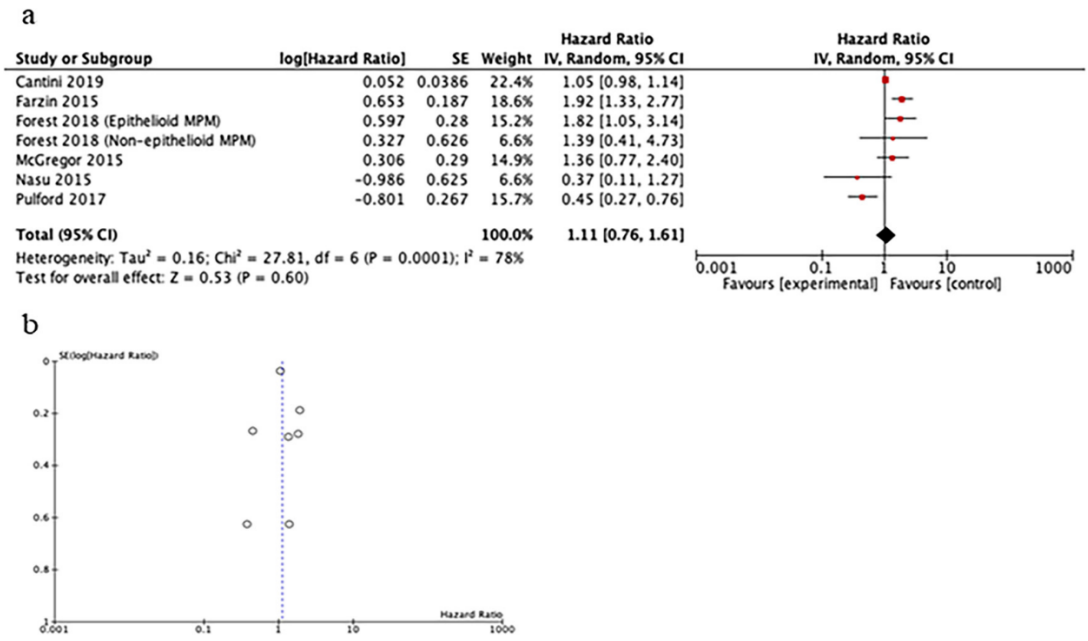


**Figure 15**. (**a**) Adjusted risk of death for MPM patients with retained BAP1 expression. (**b**) Funnel plot for the assessment of potential publication bias among included studies.

## 4.4. Prognostic value of BAP1 and miR-31 in epithelioid MPM patients

Considering the significant prognostic impact of epithelioid histotype on OS, as reported both in our cohort and from meta-analysis, we analyzed the role of BAP1 status within the e-MPM subgroup (n=40). In this specific subtype, BAP1 was not able to significantly predict OS (median OS of 19.8 months, 95% CI: 6.3-33.3 for BAP1 loss vs 18.4 months, 95% CI: 15.6-21.2 for BAP1 retained, p=0.271) and PFS (median PFS 6.3 months, 95% CI: 0.4-12.2 for BAP1 loss vs 8.1 months, 95% CI: 5.0-11.2 for BAP1 retained, p=0.453), (**Figure 16**).

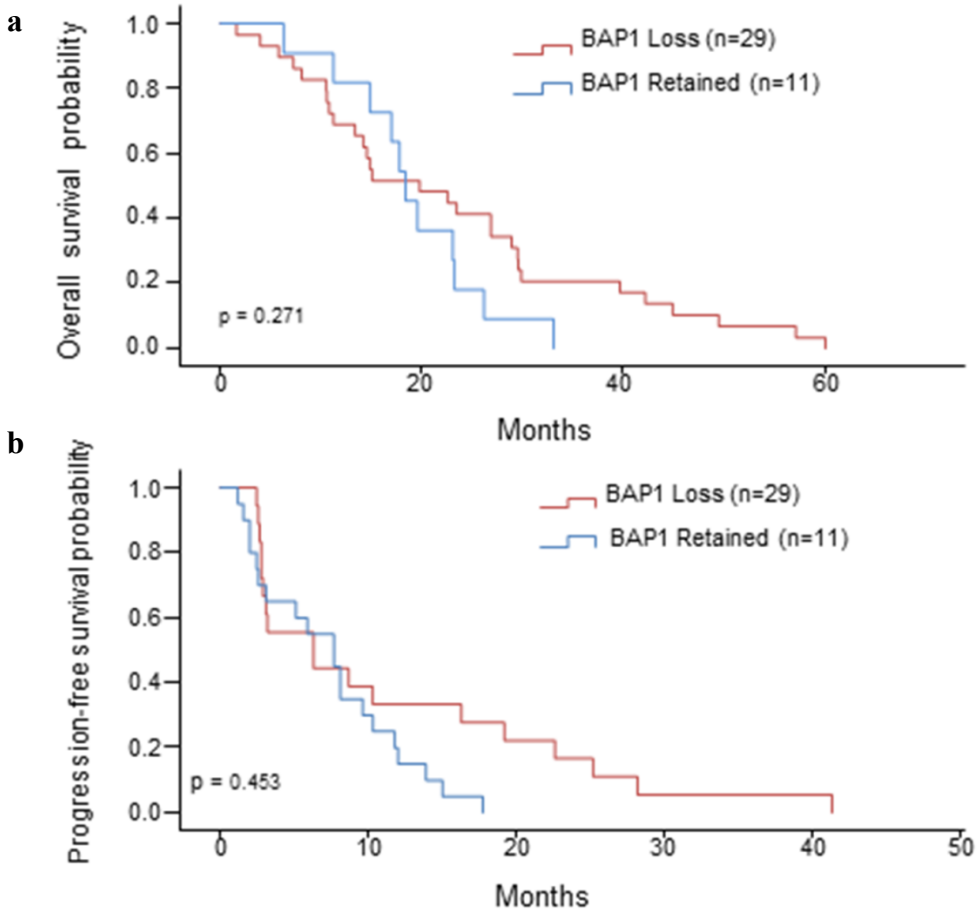


**Figure 16.** Kaplan-Meier survival curves for MPM epithelioid subtype subdivided by BAP1 expression. (**a**) Overall survival. (**b**) Progression-free survival. Comparisons between groups were made using log-rank test and two-sided p values lower than 0.05 were considered statistically significant.

In order to improve the performance of BAP1, we combined BAP1 status with the tissue expression of miR-31. We analyzed the expression level of miR-31 on the whole cohort (n=60). As shown in **Figure 17**, miR-31 was differently expressed between the different histotype of MPM as it was poorly expressed in the e-MPM and increased in the b-MPM until reaching higher levels in the s-MPM. Furthermore, low miR-31 levels were associated with the high percentage of MPM patients with BAP1 loss.

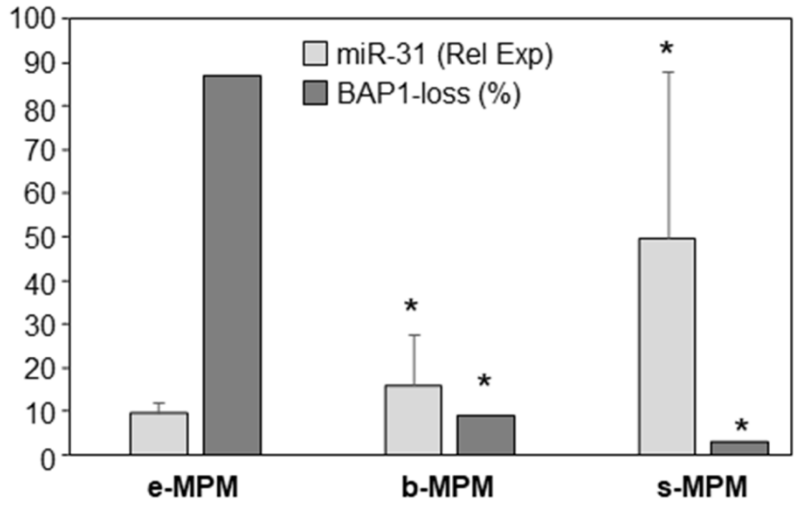


**Figure 17.** Distribution of miR-31 and BAP1 loss among the MPM histotypes. MiR-31 relative expression and the percentage of BAP1 loss in epithelioid MPM subtype (e-MPM), biphasic MPM subtype (b-MPM) and sarcomatoid MPM subtype (s-MPM). Comparisons among groups were determined by one-way ANOVA with Tukey post-hoc analysis. The symbol "*" indicates significant differences compared with the e-MPM with $p<0.05$.

We evaluated the prognostic role of miR-31 alone in e-MPM and we observed that Low miR-31 levels were significantly associated with better PFS (median PFS 7.7 months, 95% CI: 2.95-12.4 for low miR-31 levels vs 5.9 months, 95% CI: 0.0-15.5 for high miR-31 levels, p=0.028). A trend, but not significant, toward better OS was also detected (median OS 18.4 months, 95% CI: 8.3-28.5 for low miR-31 levels vs 17.0 months, 95% CI: 10.9-23.1 for high miR-31 levels, p=0.059), (**Figure 18**).

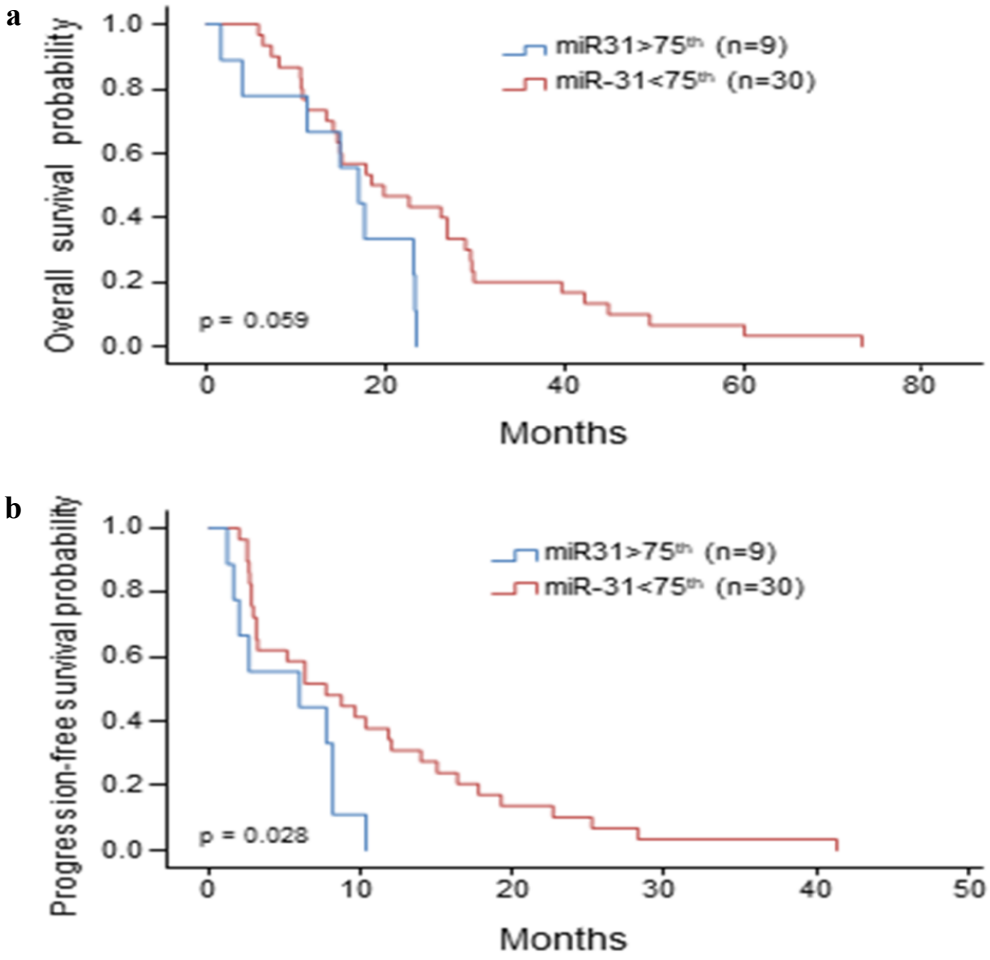


**Figure 18**. Kaplan-Meier survival curves for MPM epithelioid subtype subdivided by miR-31 level. (**a**) Overall survival. (**b**) Progression-free survival. Comparisons between groups were made using log-rank test and two-sided p values lower than 0.05 were considered statistically significant.

Finally, in order to further improve patient risk stratification, we stratified e-MPM patients according to BAP1 status and miR-31 levels taken together, obtaining a combined score (**Figure 19**).

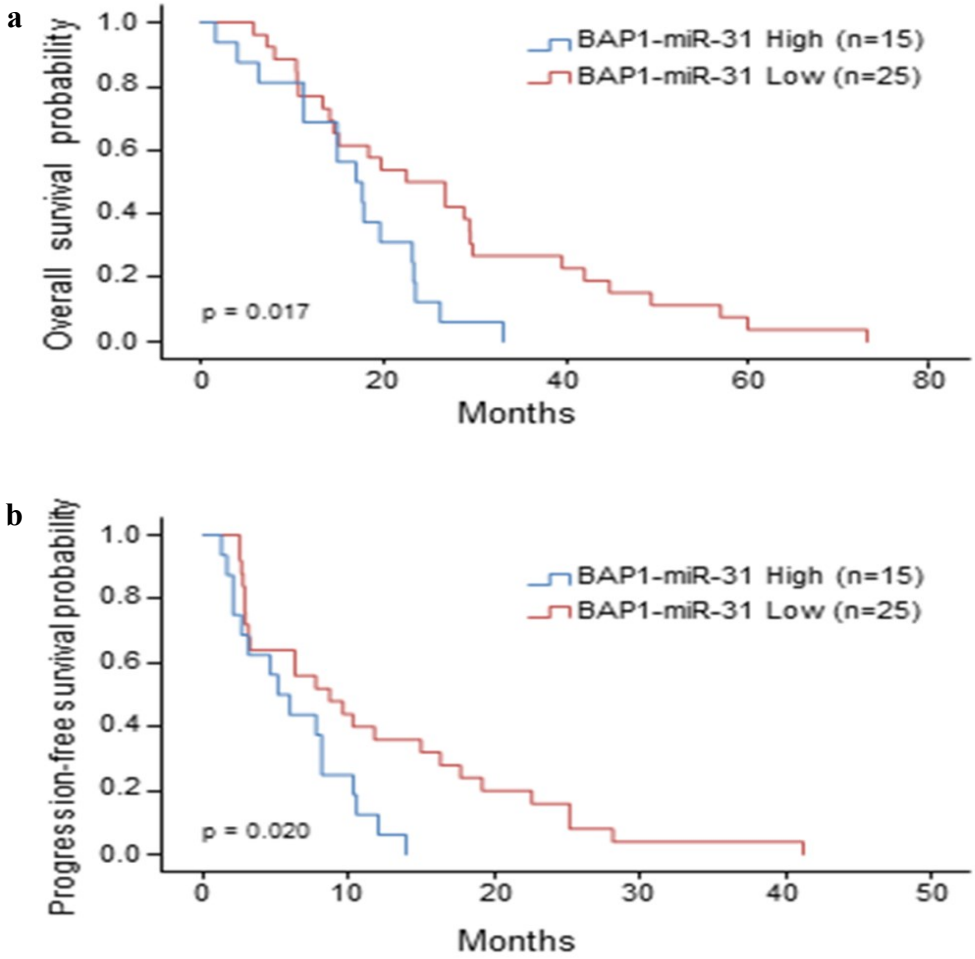


**Figure 19**. Kaplan-Meier survival curves for MPM epithelioid subtype subdivided by miR-31-BAP1 combination (**a**) Overall survival. (**b**) Progression-free survival. Comparisons between groups were made using log-rank test and two-sided p values lower than 0.05 were considered statistically significant.

Patients with BAP1 loss/low miR-31 levels (BAP1-miR-31 Low) had significantly better OS (median OS 22.6 months, 95% CI: 12.0-33.2 vs 17.0 months, 95% CI 11.5-22.5, p=0.017) and PFS (median PFS 8.7 months, 95% CI: 3.3-14.1 vs 5.1, 95% CI: 2.5-7.6, p=0.020), compared to the BAP1 retained/high miR-31 (BAP1-miR-31 High) subgroup. The multivariate analysis, showed in **Table 5**, confirmed BAP1 status/miR-31 level combination as an independent prognostic factor for e-MPM patients (HR of the BAP1 retained and high miR-31 level group 2.207, 95% CI: 1.062-4.587, p=0.034 for OS and HR 2.146, 95% CI: 1.050-4.388, p=0.036 for PFS).

| B) MPM epithelioid (n=40) | | | | | | | |
|---|---|---|---|---|---|---|---|
| **OS** | | | | **PFS** | | | |
| **Variable** | HR | 95% CI (OR) | p-value | **Variable** | HR | 95% CI (OR) | *P value* |
| Age | 1.017 | 0.949-1.089 | 0.641 | Age | 0.999 | 0.931-1.073 | 0.980 |
| Gender | 0.731 | 0.198-2.695 | 0.638 | Gender | 1.377 | 0.319-5.954 | 0.668 |
| Smoking | 0.605 | 0.246-1.483 | 0.272 | Smoking | 0.753 | 0.311-1.823 | 0.529 |
| **Asb-exp** | **2.082** | **1.082-4.004** | **0.028** | Asb-exp | 1.513 | 0.745-3.072 | 0.252 |
| Surgery | 0.420 | 0.173-1.021 | 0.056 | Surgery | 0.424 | 0.156-1.152 | 0.093 |
| **miR-31-BAP1** | **2.207** | **1.062-4.587** | **0.034** | **miR-31-BAP1** | **2.146** | **1.050-4.388** | **0.036** |

**Table 5**. Multivariate Cox regression analysis associated with OS and PFS.
Regression model with stepwise Wald-backward adjusted for age, gender, smoking, surgery, histotype, and miR-31-BAP1. OS, Overall Survival; PFS, Progression-Free Survival; HR, hazard ratio; CI, confidence interval.

# 5. DISCUSSION

MPM is an aggressive tumor, mostly unresectable, that responds poorly to current platinum-based chemotherapy. In daily clinical practice, heterogeneity of treatment response, also within the same MPM histotype, remains a hard challenge for clinicians. Therefore, identification of novel prognostic biomarkers may be helpful for patient risk stratification (215). BAP1 is a deubiquinating enzyme with tumor suppressor and apoptotic activity. BAP1 gene alterations, both inherited and somatic, have been detected in about 60% of MPM and, in the last 10 years, its prognostic role has been investigated. At today, this argument is still under debate and the prognostic role of BAP1 remains unclear. The past analyses were conducted in heterogeneous cohorts of patients, in which histology, treatment and other patient characteristics were not deeply analyzed (216-218). Furthermore, germline mutations could have a different prognostic significance than somatic ones, as suggested by Hassan et al. (92), but the analysis of BAP1 through IHC does not allow distinguishing between them.

In our study, conducted on MPM patients who homogenously received first-line chemotherapy, we revealed that there no differences in terms of OS according to BAP1 expression assessed by IHC (BAP1 retained vs BAP1 loss). We also showed that, at multivariate analysis, histological subtype mainly affected the patient survival and we confirmed that BAP1 loss is more frequent in epithelioid histotype, as previously observed (85, 90, 92, 180, 209, 211, 212). We only found a link between BAP1 loss and better outcome in patients who underwent surgery, but, after adjusting for histology at multivariate analysis, BAP1 status lost its prognostic value.

In order to definitely clarify the prognostic role of BAP1, we pooled our data with past studies in a comprehensive meta-analysis of 690 MPM patients. From this analysis, we demonstrated that, when tumor histology is taken into account, BAP1 immunohistochemical expression does not represent an independent prognostic factor.

Several reasons might explain the discrepancy of our data with previously meta-analyses that had investigated the relationship between BAP1 expression and prognosis (216-218).

In the meta-analysis conduct by Luchini et al (216), only two studies were included, and only one reported the adjusted HR for all-cause mortality (196). This study was also included in our meta-analysis (HR for BAP1 loss versus BAP1 retained 0.52, 95 % CI: 0.36–0.75).

Wang et al (217), from their meta-analysis, associated worse prognosis with BAP1 retained tumor (HR for BAP1 retained versus BAP1 loss 2.03, 95 % CI: 1.67–2.47). However, not all of the seven studies included in the analysis have conducted a multivariate analysis and some potential confounding factors (such as the tumor histotype) might have influenced the data.

The last meta-analysis presents data in agreement with ours but in this case, there was a focus on BAP1 gene mutation instead of protein expression (218).

We demonstrated that BAP1 loss was strongly associated with epithelioid histotype both in our cohort ($p=0.01$) and in meta-analysis ($p$ for difference $< 0.0001$). E-MPM is strongly associated with better prognosis than non-e-MPM tumors.

This association between BAP1 loss and epithelioid histotype might have affected the previous meta-analysis, where the prognostic value of BAP1 is instead due to the e-MPM subtype.

By looking only at epithelioid cases in our cohort, no association between BAP1 expression and survival was observed. Despite BAP1 loss has been associated with mutations or chromosomal deletions, BAP1 loss assessed by IHC has been found also in tumors without BAP1 genetic alterations (158).

In this context, a post-transcriptional regulation mediated by miRNAs might be involved. MiR-31 showed altered levels of expression in different tumors and it has been investigated as a potential upstream regulator of BAP1 (204, 219-221). In MPM the role of miR-31 is still under investigation and data are conflicting. Ivanov et al. (222) reported that miR-31 loss in MPM cell lines was associated with cell cycle progression and its restoration inhibited cell proliferation and migration. Conversely, miR-31 over-expression in miR-31-null NCI-H2452 cells significantly increased resistance to cisplatin and carboplatin (223). In the present study, we found that miR-31 was differentially expressed accordingly to the histotype: miR-31 was highly expressed in s-MPM compared to e-MPM. High miR-31 levels were also associated with BAP1 retained, worse PFS and a trend toward a shorter OS. In the cancerous tissue the direct relationship between miR-31 levels and BAP1 expression (high miR-31 corresponded to low BAP1 expression) previously described was lost (204, 205). Most probably, the genetic and epigenetic alterations in the malignant tissues contributed to a genetic rearrangement, thus affecting gene expression.

By focusing on the e-MPM subgroup, high miR-31 levels correlated with worse PFS, and a trend to worse OS was also detected. Our results are consistent with data described by Matsumoto et al. showing a miR-31 upregulation in patients affected by s-MPM, which correlated with worse prognosis (224). Notably, the BAP1-miR-31 combination was strongly associated with OS and PFS, which was further confirmed in the multivariate

model. Accordingly, retained BAP1 and high miR-31 expression was associated with a non-epithelioid and a more aggressive phenotype.

The MPM is a highly heterogeneous cancer characterized by multiple molecular profiles. Molecular diversity has been shown to occur between different histotypes, as well as within specific histological subtypes. Therefore, we can postulate that the combination of BAP1 status with miR-31 levels may help to detect within the e-MPM an aggressive subtype with BAP1 retained and high miR-31 associated with a worse outcome.

Recently the EURACAN/IASLC (pathology) group published a study on "Updating the Histologic Classification of Pleural Mesothelioma", underlining the importance of a multidisciplinary approach based on the integration of both histological and molecular parameters (225). More detailed diagnosis may lead to improved patients risk stratification, which is essential for guiding treatment. Alongside, a better knowledge of miRNAs role among different MPM histotypes may lead to a better understanding of the complex MPM biology, as well as to the development of new miRNA-based targeted therapies. Despite being limited by the small sample size and the retrospective nature, we can postulate from our study that the combination of BAP1 status and miR-31 levels is helpful in the context of e-MPM subtype for identifying a tumor subgroup (BAP1 retained-miR-31 high) with a worse outcome. Prospective studies are needed to better analyze the role of this combined score in predicting outcome in MPM and explore the emerging idea of a molecular model classification complementary to the histological one, where miRNAs might play a key role.

# 6. CONCLUSION

In this study we demonstrated that BAP1 alone was unable to stratify MPM patients based on its expression when histotype was considered. We also confirmed that the epithelioid histotype was associated with better survival in terms of OS and PFS and that BAP1 loss was more represented in this histotype. However, we observed that within the epithelioid histotype, the prognostic value of BAP1 improved, but did not reach statistical significance. Therefore, in order to improve its prognostic performance, the BAP1 status was combined with miR-31 expression levels. We showed that the combination of BAP1 retained and miR-31-high was significantly associated with a worse prognosis in MPM patients.

In conclusion, BAP1 and miR-31 can be routinely detected in diagnostic biopsies and can help to identify epithelioid MPMs with worse prognosis. The combinations of IHC method (BAP1) and molecular assay (miR-31) allow to detection prognostic/predictive factor in cancer.

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