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Secondary prevention of malignant pleural mesothelioma in a population exposed to asbestiform fibers: identification of molecular and protein biomarkers

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ABSTRACT

Biancavilla town (Catania, Sicily) has been recognized as a Site of National Interest (SIN) by the Ministerial Decree 468/2001 whose perimeter was approved by the Ministerial Decree 231/2002. The Decree refers to the presence of a quarry of stone material contaminated by an asbestiform fiber called fluoro-edenite (FE). An environmental exposure is therefore configured for the general population since from 1950 to 1998 the houses and streets of Biancavilla were built with materials from the aforementioned quarry. In relation to this exposure, a cluster of malignant pleural mesothelioma (MPM) was found in the Biancavilla town, which led the scientific population to perform *in vivo* and *in vitro* studies on FE. These studies have demonstrated the carcinogenicity of FE, classified by the International Agency Research on Cancer (IARC) as definitely carcinogenic to humans (Group 1). MPM is an insidious and lethal cancer, characterized by long latency and nonspecific symptoms, which cause a late diagnosis, and it is very invasive for the patient. MPM is causally related to exposure to asbestos. Cases of MPM around the world are on the rise. In Italy, a peak of incidence is estimated between 2015-2025 due to the ubiquity and exceptional persistence of the material in the environment. Currently, 25% of MPMs derive from occupational exposure, 25% from indirect exposure of the family member and 50% from exposure to fibers present in the environment. The inhalation of asbestiform fibers and their accumulation in the lung triggers a series of biomolecular organ effects including the production of reactive oxygen species (ROS), chromosomal damage, alteration of the mitotic process, growth factor signals and the apoptotic cascade, genetic mutations, deregulation of the methylation state, chronic inflammation, aberrant microRNA (miRNA) expression. There is currently no cure for MPM, and early diagnosis could improve both prognosis and monitoring of therapeutic response. The clinical and imaging signs of MPM are non-specific; and a definitive diagnosis, which relies on histology, can sometimes be very difficult to achieve, even with the use of immunohistochemistry. To date, no single marker or panel of soluble biomarkers is available for a clear diagnosis of MPM, so the identification of specific, sensitive and minimally invasive biomarkers for MPM has been ongoing for years with encouraging but not definitive results for the prevention of

MPM. In recent years, several studies have investigated a large number of miRNAs, biomarkers with a diagnostic value already recognized for different types of carcinoma, looking for a correlation with MPM, in particular these researches were aimed at demonstrating a possible correlation between the miRNA expression, cancer type and degree of cancer differentiation. MiRNAs are highly conserved non-coding RNA molecules, about 20-25 nucleotides long, which play an important regulatory role at the post-transcriptional level. By virtue of their post-transcriptional activity, they have been identified as new potentials biomarkers, related to the degree of cell differentiation, and therefore could be correlated with the type and stage of MPM. The circulating miRNAs are considered diagnostic biomarkers for various pathophysiological conditions such as cancers, neurodegenerative diseases, diabetes and other pathologies. MiRNAs possess all the characteristics of the ideal marker and provide reliable indications before the onset of clinical symptoms (early diagnosis), are sensitive to changes in the pathology (evolution of the pathology or therapeutic response), can be easily detected from fluids (blood, urine, saliva) as liquid biopsy and are easily transferable from laboratory models to humans. MiRNAs are particularly attractive biomarkers as their short nucleotide sequence remains stable, making these molecules excellent candidates for extraction and quantification starting from Formalin-Fixed Paraffin-Embedded (FFPE) tissue and whole blood.

On these bases, the aim of the study was to identify and validate a panel of molecular biomarkers (miRNAs) and proteins in relation to the changes induced on the pleura, in subjects exposed to FE fibers in order to provide a minimally invasive screening tool for secondary prevention to MPM in a population at high risk of incidence and mortality. For these purposes, *in silico* analyses were first performed on healthy/exposed to asbestos fibers subjects *vs.* patients with MPM using the GEO2R tool available on GEO DataSets. These analyses revealed a set of miRNAs strictly involved in MPM by merging the lists of miRNAs found differentially expressed in the miRNA expression datasets contained in GEO DataSets database. The three miRNAs selected as statistically significant were hsa-miR-323a-3p, hsa-miR-101–3p, and hsa-miR-20b-5p. Secondly, functional *in vitro* experiments on normal pleural mesothelial cell line (MeT-5A) and MPM cell

line (JU77) have been performed to test the carcinogenetic effects and epigenetic modulation induced by FE exposure. The result of the computational evaluations allowed the analysis of the expression levels of the miRNAs previously identified *in silico* both *in vitro* and in MPM *vs.* nonmalignant pleura FFPE tissues to evaluate differences in the expression levels of the selected miRNAs and their MPM diagnostic and prognostic potential. A customized droplet digital PCR (ddPCR) assay was used to amplify the miRNAs previously identified *in silico* both *in vitro* and in FFPE samples. Subsequently, by consulting the Catalogue of Somatic Mutation In Cancer (COSMIC) it was possible to identify the 20 most mutated genes that are known to be involved in MPM development and therefore have a dysregulated expression. Furthermore, the clinical implication of the analyzed miRNAs was assessed through the clinic-pathological data and the miRNA expression profiles analysis contained in The Cancer Genome Atlas Mesothelioma (TCGA-MESO) database and downloaded using the online exploration tool UCSC Xena Browser. Additionally, using the bioinformatics prediction tool microRNA Data Integration Portal (mirDIP) the interaction levels between the miRNAs previously identified via computational analysis and the main genes mutated and altered in MPM were evaluated.

The *in vitro* data showed consistency between expression levels of hsa-miR-323a 3p and hsa-miR-101–3p and the *in silico* results (up-regulation and down-regulation respectively in MPM cases compared to controls). Instead, hsa-miR-20b-5p showed a no significant dose-dependent increase in MeT-5A cell line, but a significant up-regulation in JU77 cells *vs.* control and treated MeT-5A, in contrast with the *in silico* analysis that showed a downregulation in MPM cases compared to controls. The *ex vivo* data showed a different expression of the three miRNAs analyzed in MPM and healthy controls. There was a statistically significant trend of down-regulation observed for hsa-miR-323a-3p, hsa-miR-101-3p, and hsa-miR-20b-5p in MPM cases *vs.* controls. Furthermore, hsa-miR-101-3p showed a prognostic value for MPM because the analysis of miRNAs expression levels considering the median Overall Survival (OS), the disease-specific survival (DSS), and the progression-free interval (PFI) contained in the TCGA-MESO database revealed a significant association between hsa-miR-101-

3p high expression levels and increased OS. According to the mirDIP gene target analysis these three miRNAs can target and modulate both cancer suppressor and oncogene genes playing a potentially key role in cancer cell development.

With this research work, we would contribute to basic biomarkers research, and we hope to transfer these results to clinical practice for the identification of cancer cellular alterations at an early stage.

1. INTRODUCTION

Malignant pleural mesothelioma (MPM) is a malignant cancer originating from the mesothelial layer of the pleura and traditionally related to the exposure to asbestos fibers (Tomasson et al., 2016). There is evidence that the inhalation of asbestos fibers can provoke two types of inter-connected pathogenetic processes: chronic inflammation and carcinogenesis, involving the lung after inhalation and deposition of asbestos fibers (Travaglione et al., 2003). Therefore, it has been established that cancer frequently arises in areas of chronic inflammation (Pikarsky et al., 2004). Many lines of evidence have highlighted the ability of asbestos fibers to: interfere with the mitotic apparatus; stimulate host cell proliferation; induce genetic and epigenetic alterations; induce cytotoxicity and fibrosis; produce reactive oxygen species (ROS) release from inflammatory and other target cells such as lung epithelial cells and mesothelial cells (Liu et al., 2013). The ROS production results in DNA damage, release of inflammatory cytokines and growth factors that collectively contribute to fiber pathogenicity (Travaglione et al., 2003; Tokokuni, 2009), and H_2O_2 production in mediating asbestos pulmonary toxicity ((Liu et al., 2013). Several studies have reported a high incidence of MPM due to asbestos exposure in Finland (Koskinen et al., 1996), California (USA) (Pan et al., 2005), China (Luo et al., 2003), New Caledonia (Baumann et al., 2011), Corsica (France) (Rey et al., 1993), Cyprus (Greece) (McConnochie et al., 1987), and Greece (Constantopoulos, 2008). Yet, in many cases, these MPM cases were not associated to asbestos but asbestiform fibers including fluoro-edenite (FE). FE is a silicate mineral belonging to the amphibole family (Soffritti et al., 2004). This mineral has been identified in the lavic products of Monte Calvario from stone quarries located in the southeast of Biancavilla (Gianfagna and Oberti, 2001), a small town of the Etnean volcanic complex (Sicily, Italy) (Figure 1).

Figure 1. Coordinates of Biancavilla, a small town of the Etnean volcanic complex (highlighted in red on the map).

This silicate presents some characteristics similar to the asbestos group (Biggeri et al., 2004; Comba et al., 2003); in particular it presents the same morphological and compositional aspect of the two fibrous phases tremolite and actinolite. The mineralization process led to the development of large prismatic crystals embedded in the matrix, small acicular crystals that line cavities or also fibrous and asbestiform (Gianfagna and Oberti, 2001). The salient feature which distinguishes the FE of Biancavilla is the very anomalous composition characterized by high sodium, aluminum and fluorine contents, in comparison to other known oncogenic minerals (Gianfagna et al., 2003). Epidemiological studies have indeed confirmed that FE fibers have shown similar effects to those already reported after exposure to asbestos fibers (Ledda et al., 2016, 2017; Martinez et al., 2006), including: cellular multinucleation, increase in cell size, cytokine production by epithelial cells that activate neutrophils and macrophages which accumulate in the injured area, chronic inflammation, ROS production leading to DNA mutations and enhanced signal transduction that may lead to activation of oncogenes, and increased risk of cancer development (Filetti et al., 2020a) (Figure 2).

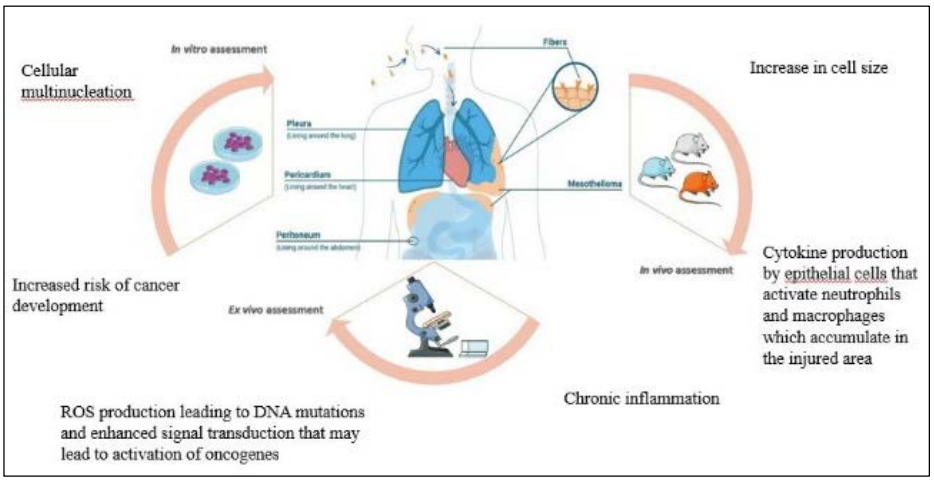


Figure 2. The main effects reported in the literature caused by exposure to FE fibers.

Several studies have reported a high incidence of MPM in Italy due to FE exposure in Biancavilla (Paoletti et al., 2000; Di Paola et al., 1996; Fazzo et al., 2012) concerning the time window 1980-2009. All of the data suggest that a mode of exposure to FE fibers is related to environmental contamination, rather than specific occupational activities (Paoletti et al., 2000). In fact, the stone material from the quarry of Monte Calvario has been used locally for about 50 years for building purposes (Rapisarda et al., 2015) and none of the residents diagnosed with MPM have been significantly exposed to asbestos during their professional lives (Travaglione et al., 2003). Several lines of evidence have led to the classification of FE as Group 1 human carcinogens (Grosse et al., 2014; IARC, 1987).

MPM is characterized by long latency and non-specific symptoms (Tomasson et al., 2016), only 5% of mesothelioma cases are diagnosed at an early stage (Ying et al., 2017) and despite advances in chemotherapy, radiation therapy and surgical management of MPM, the median survival remains less than 12 months (Tomasetti et al., 2017). The current standard for the diagnosis of pleural biopsies is difficult and requires histopathological and immunohistochemistry techniques when invasion is not clearly demonstrated based on the histology (Galateau-Salle et al., 2016). Several studies have tried to identify novel diagnostic and prognostic biomarkers for the management of MPM patients. However, the currently available diagnostic strategies, mainly based on the evaluation of

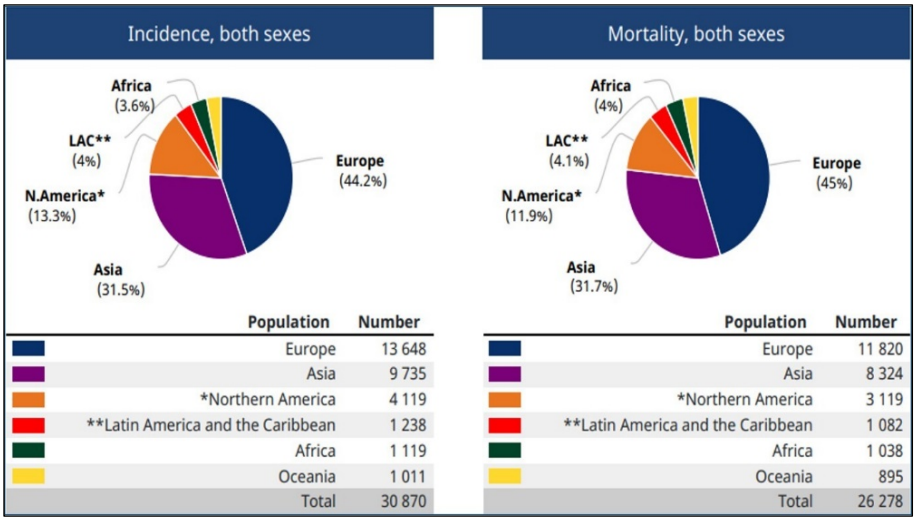
cancer biomarkers such as calretinin, cytokeratin 5, podoplanin, mesothelin, osteopontin, hyaluronic acid, fibulin-3 (Caltabiano et al., 2018), vascular endothelium growth factor (Arnold and Maskell, 2017), aquaporin-1 (Angelico et al., 2018), high mobility group box 1 (Wu et al., 2016), and macroH2A.1 (Loreto et al., 2020a), often fail to correctly diagnose MPM due to the low rates of sensitivity and specificity of these biomarkers. Even the prognostic indicators for MPM evaluated to date need further studies and insights. Among these, there are Serine and Arginine-Rich Splicing Factor 1 (SRSF1) (Broggi et al., 2021), ATG7 (Rapisarda et al., 2021), and ATG13 (Follo et al., 2016) that gave interesting preliminary data to provide the physician with important predictive data on the therapeutic response. To date, liquid biopsy is emerging as a helpful tool for non-invasive diagnosis, screening, prognosis, and stratification of cancer patients (Cavallari et al., 2019; Sato et al., 2019; Tuaeva et al., 2019) and to characterize cancer heterogeneity (Tomasetti et al., 2017). The literature already proposes an early diagnosis of MPM through the expression levels analysis of several “mesomiRs” (Micolucci et al., 2016). Circulating miRNA-126-3p, miRNA-625-3p, and miRNA-103a-3p in blood paired with mesothelin and fibulin-3 have been suggested as potential diagnostic biomarkers of MPM (Micolucci et al., 2016). This approach could avoid the histopathological and immunohistochemistry techniques used as the standard for the late diagnosis of pleural biopsies (Salle et al., 2016). It could be particularly helpful to study and subsequently use a combination of several proteins and molecular markers to improve diagnostic accuracy.

For this purpose, droplet digital PCR (ddPCR) investigations as well as *in silico* analyses were performed to assess the functional role of the selected miRNAs and their predictive value for MPM patients’ diagnosis and prognosis for a better management of this neoplasm.

1.1 Malignant pleural mesothelioma

MPM is an aggressive cancer of the pleural surface. It is associated with previous asbestos and asbestiform fibers exposure, with a latency period of ∼40 years between oncogenic fiber exposure and disease presentation (Sekido, 2013, Filetti et al., 2020b).

According to the Globocan cancer observatory of the International Agency for Research on Cancer (IARC), in 2020 the number of new malignant mesothelioma diagnoses for both sexes and all ages was about 30,870 with over 26,278 deaths (http://gco.iarc.fr/today). The incidence and mortality of this neoplasm in Europe was about 45% (Figure 3). Particularly, in Italy, the number of new malignant mesothelioma diagnoses for both sexes and all ages was about 1,973 and the number of deaths was about 1,774 (http://gco.iarc.fr/today).



Population	Number
Europe	13 648
Asia	9 735
*Northern America	4 119
**Latin America and the Caribbean	1 238
Africa	1 119
Oceania	1 011
Total	30 870

Population	Number
Europe	11 820
Asia	8 324
*Northern America	3 119
**Latin America and the Caribbean	1 082
Africa	1 038
Oceania	895
Total	26 278

Figure 3. Pie charts show the overall incidence (left) and mortality (right) rates of malignant mesothelioma for both sexes and all ages.

Prognosis with MPM is poor and median survival remains less than 12 months from diagnosis (Tomasetti et al., 2017). There are four main histological sub-types of malignant mesothelioma: epithelioid, sarcomatoid, biphasic, and desmoplastic. The sarcomatoid variant is associated with the worst outcomes, with a median survival of just 4 months. In contrast, epithelioid has the most favourable prognosis with a median survival of 13.1 months (Scherpereel et al., 2010). The incidence of the various sub-types of malignant mesothelioma may vary according to the cases, but 70-85% of mesotheliomas is the epithelioid type, 10-25% is biphasic, and 10% is sarcomatoid, while the desmoplastic type is the rarest form (<2%) (Bueno et al., 2004).

1.1.1 Epidemiology and risk factors

Malignant pleural mesothelioma is subjected to a specific epidemiological survey activity by the National Mesothelioma Registry (ReNaM), established pursuant to Legislative Decree 257/1992, the rule that in Italy prohibited the use of asbestos. The great attention to the asbestos-related diseases in Italy, 30 years after the prohibition of all forms of asbestos extraction, processing, import, and business, derives from the fact that in recent years there was the highest incidence of mesotheliomas due to the frequent use of this silicate from the second post-war period up to the 1980s, and the long latency of the pathology. The frequency of malignant pleural mesothelioma is higher among men (2/3 of cases), probably due to the more frequent exposure to asbestos (AIOM-AIRTUM, 2018). The incidence in Italy is higher in the northern areas (AIOM-AIRTUM, 2018), and in particular in the areas where the use of asbestos has been greater (Marinaccio et al., 2018). The median survival for MPM observed in two studies based on ReNaM data was 9.8 months, with 5% of patients who survived 5 years (Montanaro et al., 2009).

From the etiopathogenetic point of view, all types of asbestos caused the malignant mesothelioma (IARC, 2012), although others fibrous minerals present in nature as erionite, winchite, magnesio-riebeckite, richterite, Libby asbestos, antigorite, and FE share the siliceous architecture and the fibrous morphology of asbestos. There are no industrial uses of these substances, but environmental exposure has been associated with cases of malignant mesothelioma in humans (Grosse et al., 2014). Several studies have reported a high incidence of MPM due to erionite exposure in rural regions of Turkey, Central Anatolia (Yazicioglu et al., 1980; Dumortier et al., 1998; Senyigit et al., 2004; Metintas et al., 2005; Döngel et al., 2013). Clark and Nye counties, in southern Nevada (USA) have shown a significantly high incidence of MPM due to carcinogenic fibers including erionite, winchite, magnesio-riebeckite, and richterite (Baumann et al., 2015). These are the same fibrous minerals present in Libby, Montana (USA) where they have been related to MPM and other asbestos-related diseases (Konen et al., 2019). Antigorite is found in the Western Alps (Piemonte, Italy) (Cardile et al., 2007; Groppo and Compagnoni, 2007), in North America,

Australia-Oceania, and Rowland Flat in South Australia (FitzGerald et al., 2010; Fitzgerald et al., 2014). FE, as mentioned above, is the amphibole of Biancavilla (Sicily, Italy), where a high incidence of MPM related to its environmental contamination has been reported (Di Paola et al., 1996; Paoletti et al., 2000; Fazzo et al., 2012; Rapisarda et al., 2015).

The proportion of cases of familial MPM in Italy is between 1.3 and 2.5% of the total cases (Ascoli et al., 2014). The role of germline BAP-1 mutations is limited to cases that are part of the "BAP-1 cancer predisposition syndrome" (the increased frequency in families of cases of uveal melanoma, MPM, skin melanoma, renal carcinoma, but also in other types of neoplasia) while such alterations are very rare (1/300 cases) in sporadic malignant mesothelioma cases (Betti et al., 2015). Recently in literature have been shown that patients with familiarity, i.e. carriers of germline mutations in BAP1 or in cancer predisposing genes, to have better survival than patients with sporadic mesothelioma (Pastorino et al., 2018). Furthermore, younger patients (age < 50 years old) have better survival than older patients, regardless of BAP1 mutational status. On the basis of epidemiological observations it has been stated that the incidence of malignant mesothelioma increases proportionally to the exposure multiplied by a power equal to 3 or 4 of the time of the exposure (commonly named latency) (Health Effects Institute, 1991). An analysis of several studies shows that after about 45 years from the first exposure to asbestos, the trend of increase in the incidence and mortality of MPM tends to slow down. It is also possible that there will be a stabilization or a reduction in risk when exposure ceases (Magnani et al., 2008; Harding et al., 2010).

1.1.2 Health surveillance and early diagnosis

There are health surveillance programs for workers exposed to asbestos and for those potentially exposed for occupational reasons (Legislative Decrees 257/2006; 81/2008). The implementation of these programs is not uniform among the different regions despite the fact that guidelines have been issued by the Ministry of Health.

To date, there are no investigations with sensitivity and specificity useful to make an early diagnosis of malignant mesothelioma in asymptomatic people.

Health surveillance programs aimed at workers exposed to asbestos must:

1. inform individuals of the risk due to present or past exposure to asbestos;
2. inform family members of exposed individuals about possible health risks caused by passive exposure;
3. fully reconstruct work history, particularly on exposure to asbestos;
4. provide information on diagnosis, therapies, and medico-legal perspectives;
5. promote the recognition and compensation of cases due to asbestos exposure;
6. provide advice on quitting smoking and other health-threatening lifestyles (AIOM, 2018).

The diagnosis of malignant mesothelioma must always be reported by the doctor who makes it to the mesothelioma registers. In addition, malignant mesotheliomas should be reported to the ASL (prevention services or workplace prevention services). Patients and their families must be informed of their right to request recognition and compensation from INAIL, whether of professional or non-working origin. In fact, compensation is also provided for the latter (Novello et al., 2016).

1.2 Clinical and molecular features of malignant mesothelioma

The pathological classification of malignant mesothelioma is the most recent proposed by the World Health Organization (WHO) in 2015, periodically updated by a panel of international experts (Galateau-Salle et al., 2016).
Considering the histological and clinical features, malignant mesothelioma can be divided into four main sub-types: epithelioid, sarcomatoid, biphasic, and desmoplastic (AIOM, 2018).

1. The epithelioid variant represents about 70-85% of all mesothelioma diagnoses (Bueno et al., 2004). It consists of globose / polygonal cells with large eosinophilic cytoplasm and uniform nuclei with prominent

nucleolus, arranged in solid aggregates that tend to form 3D structures. The epithelioid variant with the greatest number of cyto-architectural patterns (papillary, tubulo-papillary, micro-papillary, trabecular, solid, deciduous, adenomatoid / micro-cystic, clear cell, transitional, ring with bezel or small cells). The presence of multinucleated anaplastic and / or giant cells is typical of the pleomorphic form, while the lympho-histiocytoid variant shows a dense mixed inflammatory infiltrate (including lymphocytes, plasma cells, histiocytes, and granulocytes) which can obscure mesothelial cells mimicking a lymphoma or a lymphoepithelial-like carcinoma. The stroma of epithelioid mesothelioma is often fibrous, dense and hypocellular with "wax casting" appearances, but hypercellular features with numerous fibro / myofibroblastic elements or Alcian-Blue positive myxoid areas may also be observed (Galateau-Salle et al., 2016).

2. The sarcomatoid variant represents about 10% of all mesothelioma diagnoses (Bueno et al., 2004). It is characterized by the presence of a proliferation of spindle cells arranged in short bundles with a historiform or disordered pattern that infiltrates the soft tissues of the parietal pleura or the lung parenchyma. Cells can be thin and long or swollen and short, while nuclear atypia and mitoses can be absent to prominent. The presence of foci of cancer necrosis is of diagnostic significance. Heterologous components of osteosarcoma, rhabdomyosarcoma or chondrosarcoma may also be observed alongside the conventional fusata component. The pleomorphism can be very marked (Galateau-Salle et al., 2016).
3. The biphasic variant represents about 10-25% of all mesothelioma diagnoses (Bueno et al., 2004). It involves the combination of the epithelioid and sarcomatoid pattern with the presence of at least 10% of one of the two components. It is recommended to report the percentage of sarcomatoid component globally represented in the whole mesothelioma (Galateau-Salle et al., 2016; Novello et al., 2016; Husain et al., 2017).

4. The desmoplastic variant is the rarest form (<2%) (Bueno et al., 2004). It is usually the most difficult form to diagnose. It is a mild proliferation of spindle elements arranged in a disordered manner in an ianilized collagen stroma. Invasion of soft tissue or lung parenchyma is difficult to demonstrate and immunohistochemical investigations for the mesothelium are often negative. The presence of hypercellulated nodulations alongside entirely fibrotic areas, mild cancer necrosis, and the search for conventional epithelioid or sarcomatoid focal areas are key aspects to support the diagnosis (Churg and Galateau-Salle, 2012; Galateau-Salle et al., 2016; Husain et al., 2017). The desmoplastic form has a similar prognosis to that of the sarcomatoid variant.

Other uncommon forms of mesothelioma are represented by localized mesothelioma, well-differentiated papillary mesothelioma, and adenomatoid mesothelioma. The localized mesothelioma is a rare presentation of malignant mesothelioma as a localized solitary mass with a pleural base. It can manifest all histological variants and it is in differential diagnosis with solitary fibrous cancer and synovial sarcoma (Henderson et al., 2013a). The well-differentiated papillary mesothelioma is a rare form of low-grade mesothelioma that often involves the peritoneum. It is characterized by exophytic papillary growth with a monolayer coating of mesothelial cells with mild atypia and myxoid stroma, in the absence of clear invasion aspects. Cases of well-differentiated papillary mesothelioma with initial soft tissue infiltration have been reported (Churg et al., 2011). The adenomatoid mesothelioma is a rare benign form of mesothelial proliferation characterized by a solitary nodular growth with gland-like, irregular spaces with flat or cuboidal mesothelial cells devoid of atypia. The differential diagnosis is with an adenomatoid-like form of epithelioid mesothelioma (with diffuse growth pattern) and with epithelioid hemangioendothelioma (negative for mesothelial markers and positive for vascular markers) (Galateau-Salle et al., 2016).

1.2.1 Staging of malignant pleural mesothelioma

At present, the American Joint Committee on Cancer (AJCC) staging system is the most used classification method worldwide. This system is constantly updated adding novel molecular and clinical findings obtained in recent years. The AJCC system classifies malignant pleural mesothelioma according to the TNM scoring parameters that are cancer size (T), lymph node involvement (N) and presence of metastases (M) (Brierley et al., 2017).

According to this staging system, there are four staging levels of malignant pleural mesothelioma where the higher levels correlate with a worse prognosis:

I. The cancer is confined to the parietal pleura and is not present in the lymph nodes;
II. The cancer involves, in addition to the parietal pleura, the visceral pleura (the membrane in direct contact with the lung), and the lung or the diaphragm;
III. The cancer has invaded the first layer of the chest wall, part of the mediastinum or a point on the chest wall; it can affect the outer surface of the pericardium and the lymph nodes on either side of the chest;
IV. The cancer has spread to other organs (metastases) such as the liver, brain, bones or lymph nodes on both sides of the chest (Stahel et al., 2010).

1.2.2 Molecular alterations in malignant mesothelioma

For the correct classification of malignant mesothelioma, it is also important to evaluate the molecular alterations harbored by neoplastic cells that could be useful to clinicians to establish the prognosis of patients and to define the therapeutic schedule.

Of note, numerous cancer suppressor genes are altered in malignant mesothelioma, in particular NF2, CDKN2A (p16INK4a), CDKN2B (p15INK4b), e BAP1. NF2 results inactive through mutations and deletions.

The NF2 product, neurofibromin 2 protein, is a membrane protein associated with the cytoskeleton and it regulates several signal transmission pathways including the mTOR and Hippo pathways. In fact, the inactivation of NF2 is

associated with the activation of mTOR and Hippo (Galateau-Salle et al., 2016; Novello et al., 2016; Husain et al., 2017).

The deletion of the 9p21 locus is one of the most common alterations and involves the loss of p16, p14, and p15, cancer suppressor genes that code for inhibitory proteins of the cyclin-dependent kinase, fundamental in the regulation of the cell cycle. The deletion in homozygosity is the alteration that most frequently leads to the inactivation of p16 and it is present in 70-85% of malignant mesotheliomas (higher in the sarcomatoid form).

On average, 60% of malignant mesotheliomas are characterized by loss of BAP1 following deletions and gene mutations. Germline mutations of BAP1 are present in <5% of patients with MPM. To date, no association between BAP1 mutation and p16 deletion or NF2 deletion / mutation has been demonstrated. BAP1 is a nuclear protein with deubiquitinase function, also critical in the regulation of the repair mechanisms of the DNA double helix (Hida et al., 2017, Yap et al., 2017).

The commonest genetic variations in malignant pleural mesothelioma were clustered in two main pathways: the p53/DNA repair (TP53, SMACB1, and BAP1) and phosphatidylinositol 3-kinase–AKT pathways (PDGFRA, KIT, KDR, HRAS, PIK3CA, STK11, and NF2), some of them with prognostic value (Lo Iacono et al., 2015). PIK3CA, STK11, and TP53 mutations have been associated with significantly shorter disease progression and OS (Lo Iacono et al., 2015).

The activation of CTLA4, and of PD-1/PD-L1 checkpoint, the prominent role of angiogenesis with VEGFR overexpression, have proved to be promising biological mechanisms in MPM pathogenesis and the future therapeutics choices (Yap et al., 2017).

Recently, higher levels of osteopontin and mesothelin have been associated with a worsening of prognosis. Inserting osteopontin and mesothelin levels as predictors could play an important role in improving the prognostic capacity of MPM (Pass et al., 2016).

Finally, other molecular alterations associated with malignant mesothelioma are related to the alteration of the expression levels of several factors, and in

particular of autophagy-related (ATG) proteins, primarily implicated in different functions of the autophagic process. Among ATG proteins, the most involved in MPM progression are ATG7 and ATG13 whose high expression levels correlate with higher increased survival, with late recurrences and death for the neoplasm (Follo et al., 2016; Rapisarda et al., 2021). In particular, the autophagy process plays a dual role, both pro-survival and cancer-suppressive (Follo et al., 2016, 2018). This cancer-suppressive role for autophagy is stated by evidence that allelic loss of Beclin 1 in mice is associated with the progression of hepatocellular carcinoma and other cancers (Galluzzi et al., 2015; Maes et al., 2013; White, 2015). Hepatocyte-specific deletion of ATG7 promotes liver size, fibrosis, progenitor cell expansion, and hepatocarcinogenesis, which is rescued by concurrent deletion of Yap (Lee et al. 2018), an important transcription activator in MPM (Zhang et al.,2017). Literature data demonstrated that autophagy represents a mechanism of cell death that can be established even without detectable apoptosis (via autophagic death) or at the same time as apoptosis (Yonekawa and Thorburn, 2013). Besides, autophagy regulatory and executor ATG genes can also interact with other processes, such as apoptosis. It was reported that ATG7 protein, involved in the maturation of the autophagosome, regulates p53 function (Lee et al., 2012). Several common oncogenes (such as those encoding PI3K class I, Pkb, Tor, AKT) inhibit autophagy, while cancer suppressor genes (such as those encoding p53, Pten, Tsc1, Tsc2) induce the autophagic process (Botti et al., 2006). Then, autophagy constitutes a stress adaptation that escapes cells from apoptosis, whereas it represents an alternative cell-death pathway in other cellular settings. In severe cell damage, both these cell death processes may be activated by common upstream signals and cooperate to escape cell transformation. In this setting, massive autophagy activation leads to cell death through apoptosis (Maiuri et al., 2007). Previous studies conducted by our research group demonstrated the activation of the apoptosis cascade in MPM tissues. This is a critical mechanism that leads tissue to irreparable FE-induced damage and, if not activated enough, predisposes it to a neoplastic evolution (Loreto et al., 2020b).

Some studies have been conducted to understand the link between common genetic variations in the molecular pathways and cancer risk with the final goal to develop novel therapeutic targets. Lim et al. (2013) reported mutations in SMO and SUFU and a novel multi-exonic deletion in PTCH1 in MM cell lines and cancers. These data suggest that aberrant activation of the Hedgehog (HH) signaling in MM is unlikely to be driven by mutations in the majority of cancers but instead activated through autocrine signaling (Shi et al., 2012; Lim et al., 2013). This pathway may represent a novel therapeutic target in MPM for recently developed HH pathway inhibitors.

Currently, none of these biomarkers is used as gold-standard in the malignant mesothelioma clinical practice.

1.2.3 Diagnosis of malignant mesothelioma

The diagnosis of MPM can sometimes be very complicated and generally requires a multidisciplinary approach by correlating the cyto-histological and immunomolecular aspects with clinical and radiological data (Galateau-Salle et al., 2016; Novello et al., 2016; Husain et al., 2017). The definitive diagnosis requires biopsy confirmation, although in selected cases also cytological or cell-block samples of pleural effusions can be used for diagnostic purposes by observing the criteria recently approved by a panel of international experts of the International Mesothelioma Interest Group (IMIG) (Chen et al., 2014; Hjerpe et al., 2015).

In most cases, however, the diagnosis of malignant mesothelioma can be suspected or identified directly on hematoxylin-eosin morphology reports, particularly in the epithelioid sub-type. Despite this, several groups of pathologists recommend the confirmation of the morphological diagnosis with appropriate immunohistochemical investigations, which must always take into account the context of the differential diagnosis in each individual case (lung adenocarcinoma *vs.* MPM, sarcoma *vs.* MPM, mesothelial hyperplasia *vs.* MPM) (AIOM, 2018).

In case of surgical resection, the International Collaboration on Cancer Reporting (ICCR) group of mesothelioma pathologists identified eight required points in the MPM reporting, as follows:

1. operational procedure performed to obtain samples;
2. sample type;
3. macroscopic site of sampling;
4. histological type;
5. state of the margins;
6. extent of cancer invasion;
7. lymph node stage;
8. pTNM stage (AIOM, 2018).

In addition, seven points recommended in the pathology report were identified:

1. clinical history;
2. neoadjuvant therapy performed;
3. size of major cancer nodules and maximum thickness of neoplastic invasion;
4. number of mitoses per mm^2 of neoplastic tissue;
5. response to neoadjuvant therapy;
6. associated neoplastic or non-neoplastic pathologies;
7. ancillary studies performed (immunohistochemistry, molecular investigations, etc.) (Churg et al., 2016).

It should be emphasized that there is no specific primary antibody for mesothelial origin, so the sensitivity and specificity of the various antibodies used in the diagnosis of MPM vary considering the type of differential diagnosis. The most sensitive and specific markers in confirming mesothelial differentiation are calretinin, WT-1 (clone C19), cytokeratin 5 or the combination CK5 / 6, D2-40 (podoplanin), mesothelin, and HEG1. Unfortunately, as we said, there are no specific markers for malignant mesothelioma, in fact calretinin and mesothelin can be positive in breast cancer and pancreatic adenocarcinoma, respectively.

Immunohistochemical investigations are frequently used in confirming the mesothelial derivation of the neoplasm with respect to the possibility of a

metastasis from carcinomas in other sites, in particular carcinomas of the lung, breast, kidney, female genital tract, intestine (Galateau-Salle et al., 2016; Husain et al., 2017). Negative markers for mesothelium that favor carcinoma metastasis are CEA (clone DAKO A0115), B72.3, Ber-EP4, Bg8, MOC-31, CD15, MUC4, claudin 4 (Galateau-Salle et al., 2016; Husain et al., 2017; Henderson et al., 2013b; Facchetti et al., 2007; Tsuji et al., 2017; Comin et al., 2014; Klebe et al., 2016; Amatya et al., 2017).

In the differential diagnosis between epithelioid mesothelioma and carcinoma metastases the recommendations of some International Societies suggest the use of two positive and two negative markers (Galateau-Salle et al., 2016; Husain et al., 2017). In consideration of the type of differential diagnosis, there are immunohistochemical markers not expressed in mesothelioma and specific for some types of carcinoma (Galateau-Salle et al., 2016; Husain et al., 2017).

Furthermore, the choice of the most specific clone of primary antibody is very important, because markers believed to be highly specific for pulmonary and extra-pulmonary adenocarcinoma can be expressed in a significant subset of mesotheliomas (Klebe et al., 2016). However, recent literature shows that antibodies initially considered specific for non-mesothelial neoplasms are also expressed in mesotheliomas (Berg et al., 2017).

Mesothelial markers are insensitive or negative in sarcomatoid or desmoplastic forms of mesothelioma. This aspect is particularly important in the legal field because it is not possible to state that in the absence of expression of mesothelial markers, a diagnosis of malignant mesothelioma cannot be performed, with certainty (AIOM, 2018). Recently, some criteria have been proposed to try to improve the objectivity of the diagnosis of sarcomatoid mesothelioma compared to sarcomatoid forms of lung cancer, based on clinical-radiological and immunomorphological reports (Marchevsky et al., 2017). To date, for sarcomatoid mesothelioma it is necessary to make a differential diagnosis with some forms of sarcoma such as synovial sarcoma, solitary fibrous cancer, and desmoid cancer (Galateau-Salle et al., 2016; Husain et al., 2017; Henderson et al., 2013a). In these cases, the immunomolecular investigations can be fundamental. Synovial sarcoma can present in a monophasic spindle, biphasic

form with spindle, pseudo-glandular and pleomorphic aspects, can express cytokeratins, EMA and calretinin, but is characterized by the expression of TLE1 and the presence of the rearrangement t (X; 18) with involvement of SYT gene (Galateau-Salle et al., 2016; Husain et al., 2017; Henderson et al., 2013a). Solitary fibrous cancer is usually characterized by spindle elements interspersed with collagen or myxoid stroma with areas of hypo- and hyper-cellularity, expression of CD34 and STAT6 with rearrangement between NAB2-STAT6 (Henderson et al., 2013a; Robinson et al., 2013; Galateau-Salle et al., 2016; Husain et al., 2017). Desmoid cancer is a spindle cell neoplasm with expression of smooth muscle actin, nuclear beta-catenin and mutation of CTNNB1 in the absence of cytokeratins (Colombo et al., 2013; Henderson et al., 2013a; Galateau-Salle et al., 2016; Husain et al., 2017).

Epithelioid hemangioendothelioma and angiosarcoma can also be differentially diagnosed with MPM. Indeed, these can express cytokeratins but are usually positive for the vascular markers CD31 and CD34 (Henderson et al., 2013a; Anderson et al., 2015; Galateau-Salle et al., 2016; Husain et al., 2017). Epithelioid hemangioendothelioma then presents the t (1; 3) translocation with formation of the WWTR1-CAMTA1 fusion gene and immunohistochemical expression of CAMTA1 (Anderson et al., 2015).

The differential diagnosis between reactive mesothelial process and malignant mesothelioma is one of the diagnostic problems of the pathologist. Identification of soft tissue invasion of the parietal pleura or lung parenchyma is the most important criterion for determining malignancy. Other morphological aspects present in the malignant nature are the absence of zoning (cellularity distant from the surface of the effusion), historiform growth patterns, evident cytological and nuclear atypia, capillaries distributed in a casually and inconspicuous way, even mild cancer necrosis (Churg and Galateau-Salle 2012; Churg et al., 2015; Galateau-Salle et al., 2016; Husain et al., 2017).

However, especially in small surface biopsies or in cytological specimens, neoplastic invasiveness may not be evident. In these cases, the presence of strong and circumferential membrane expression patterns of EMA (clone E29) favors the diagnosis of MPM (Churg and Galateau-Salle 2012; Henderson et al., 2013a;

Galateau-Salle et al., 2016; Husain et al., 2017). The deletion of p16 in superficial mesothelial cells is often associated with the deletion of p16 in malignant mesothelial cells. Therefore, superficial biopsies with evident deletion of p16 can support the diagnosis of malignant mesothelioma in the presence of clinical-radiological evidence of diffuse pleural thickening. However, the absence of p16 deletion in superficial mesothelial cells does not rule out the diagnosis of MPM (Chung et al., 2010; Monaco et al. 2011; Wu et al., 2013). The expression of GLUT-1 and IMP3 can be associated with the diagnosis of malignant mesothelioma, but the absence of their immunoreactivity does not exclude MPM (Lee et al., 2013). The combined use of these markers significantly increases the diagnostic value (Churg et al., 2015; Galateau-Salle et al., 2016; Husain et al., 2017).

Recently, the absence of BAP1 expression has been associated with malignant mesothelial proliferations. BAP1 represents the most reliable and specific diagnostic marker in dubious mesothelial proliferations even on cytological samples because all reactive mesothelial processes are positive for the expression of BAP1 (Shinozaki-Ushiku et al., 2017).

The cytological diagnosis of mesothelioma can be performed in most cases in the epithelioid and biphasic variant, but almost never in the sarcomatoid variant (Chen et al., 2014; Hjerpe et al., 2015; Churg et al., 2016; Galateau-Salle et al., 2016; Novello et al., 2016; Husain et al., 2017). Diagnosis requires the demonstration of malignant cells on morphological findings and mesothelial differentiation with immunocytochemical investigations. In equivocal cases, immunomolecular investigations can support the malignant nature of the mesothelial elements. In the absence of a biopsy or a cell-block, as in the case of an elderly patient with important comorbidities, the cytological diagnosis of mesothelioma is acceptable in the presence of neoplastic cells with mesothelial differentiation, taking into consideration the clinical-radiological context (AIOM, 2018).

Finally, the correct diagnosis of MPM is performed by the pathologist who evaluates the histological features of mesothelial cells thus establishing their malignant potential. However, no effective tumoral biomarkers are available for

the effective diagnosis of this tumor or to define the prognosis of the patients. Some studies have tried to identify biomarkers for this neoplasm, however, inconclusive results were obtained. Among the currently used biomarkers there is the mesothelin, the only US Food and Drug Administration (FDA)-approved biomarker for malignant mesothelioma (Creaney et al., 2013, 2014; Chen et al., 2017), but with limitations (Boudville et al., 2011). In fact, the poor sensitivity of mesothelin clearly limits the added value to the diagnosis of MPM (Cui et al., 2014).

Radiological imaging plays an important role in the surveillance, differential diagnosis (Aluja Jaramillo et al., 2018), staging, and treatment response of MPM (Armato et al., 2016; Cardinale et al., 2017). In fact, despite active research in the field, the point of view is that MPM screening is not possible to date (Falaschi et al., 2018).

Recent studies have tried to evaluate the diagnostic and prognostic significance of miRNAs for MPM, however, these studies need further validation (De Santi et al., 2017; Martínez-Rivera et al., 2018). In several studies, *in silico* analysis to identify selected miRNAs highly deregulated in cancer samples when compared with normal control have been developed (Falzone et al., 2016, 2018; Hafsi et al., 2016). This *in silico* approach could represent an effort in the field of biomarker discovery for MPM because it is possible to have a large series of samples useful to obtain truthful expression data concerning miRNAs with a potential diagnostic and prognostic role in cancer.

1.2.4 Management of malignant mesothelioma

There is no curative treatment for MPM. Systemic treatment options include chemotherapy, targeted therapy and radiotherapy, delivered separately or as part of multimodality treatment. Surgery is controversial and limited to patients with early stage disease and good functional status. Palliative care and symptom management are essential and the control of pleural effusions is an important factor (Bibby et al., 2016). A number of novel therapeutic agents are under investigation, and may provide further treatment options for MPM in the future. Chemotherapy is the only treatment modality that has been shown to improve survival in MPM. However, prior to 2003 the evidence was poor and based on

underpowered, early phase trials (Bibby et al., 2015). Response rates were low and survival was universally <10 months (Fennell et al., 2008). One large randomised trial demonstrated that the addition of chemotherapy to active symptom control offered no survival benefit and no improvement in quality of life compared with active symptom control alone (Muers et al., 2008).

However, in 2003 two pivotal phase III trials were published that changed the landscape of chemotherapy in MPM (Vogelzang et al., 2003; Van Meerbeeck et al., 2005). The trials used third-generation anti-folate agents aimed at inhibiting DNA synthesis and preventing cancer proliferation. The first trial randomised 448 treatment-naïve participants to receive either pemetrexed and cisplatin or cisplatin alone (Vogelzang et al., 2003). Median survival in the pemetrexed arm was 12.1 months, compared with 9.3 months with cisplatin alone (p=0.02). Toxicity rates were high initially, but fell after the addition of vitamin B12 and folic acid supplementation. On the basis of this trial, pemetrexed was approved by global marketing authorities for use in combination with cisplatin for MPM. Over 10 years later, it remains the standard first-line chemotherapy for patients with MPM. The second trial compared raltitrexed and cisplatin with cisplatin alone in 250 participants (Van Meerbeeck et al., 2005). Survival benefit was similar to that seen in the pemetrexed trial (11.4 months *vs.* 8.8 months, p=0.048) although objective response rates were lower (Vogelzang et al., 2003; Van Meerbeeck et al., 2005). The study appeared underpowered, and consequently had less impact on clinical care. At present raltitrexed is not licenced by the US FDA or the European Medicines Agency for use in MPM (Bibby et al., 2016).

Carboplatin can be substituted for cisplatin in older patients, patients with comorbidities or patients who experience toxicity with cisplatin, as it is generally better tolerated. In a meta-analysis, carboplatin demonstrated similar efficacy to cisplatin, and phase II trials have shown enhanced OS and longer progression-free survival (PFS) in patients with MPM treated with carboplatin and pemetrexed (Berghmans et al., 2002; Ceresoli et al., 2006, 2008; Castagneto et al., 2008).

An important issue in MPM chemotherapy is predicting which patients will respond to treatment. An evaluation of over 1700 patients who received pemetrexed with either cisplatin or carboplatin as part of an expanded access programme demonstrated response rates of 26.3% and 21.7%, respectively (Santoro et al., 2008). These low response rates, combined with a lack of reliable biomarker, are likely to be responsible for the low uptake of chemotherapy.

There are many unanswered questions regarding chemotherapy and MPM. It is not known whether immediate chemotherapy is more effective than chemotherapy delayed until the appearance of symptoms. A small pilot study suggested a trend for slower progression and longer survival with early chemotherapy, but larger trials are needed (O'Brien et al., 2006). Similarly, the optimum number of cycles of chemotherapy and the role of maintenance pemetrexed are unclear.

Vascular Endothelial Growth Factor (VEGF) plays a key role in MPM by promoting angiogenesis and stimulating cancer growth (Ohta et al., 1999; Sekido, 2013). Recently, bevacizumab, an anti-VEGF monoclonal antibody, has been shown to be effective in MPM (Zalcman et al., 2016). The multicentre, phase III MAPS trial randomised 448 participants with MPM to receive cisplatin and pemetrexed chemotherapy with or without bevacizumab. Patients who received bevacizumab had significantly longer median (95% CI) overall survival at 18.8 (15.9–22.6) months compared with 16.1 (14.0–17.9) months in the chemotherapy alone arm ($p=0.017$). Patients given bevacizumab alongside chemotherapy also showed longer PFS of 9.2 (8.5–10.5) months versus 7.3 (6.7–8.0) months in those receiving standard care ($p<0.0001$) (Zalcman et al., 2016), although thromboembolic complications and kidney impairment were more common with bevacizumab. To date, the use of bevacizumab is warranted alongside first-line standard chemotherapy in patients with unresectable MPM (Zalcman et al., 2016; Zauderer et al., 2016).

The benefit of surgery in MPM is much debated, and there is a need for robust randomised trial data to elucidate its efficacy and clarify its role in management (Treasure and Sedrakyan, 2004; Clive et al., 2016a). There are two approaches

to surgery in MPM: radical removal of all visible disease or a more conservative, tissue-sparing, debulking procedure.

The more radical option is extrapleural pneumonectomy (EPP), an operation which aims to eradicate all macroscopic cancer via the removal of lung, pleura, pericardium and diaphragm (Butchart et al., 1976). The original descriptions of EPP reported enhanced survival, but mortality and complication rates were high (Butchart et al., 1976; Sugarbaker et al., 2004; Opitz et al., 2006). Additionally, these reports were based on retrospective data from highly selected patients, with no control arm and no information about the population from which they were drawn. Consequently, a systematic review concluded that it was impossible to determine whether EPP extended survival in people with MPM (Maziak et al., 2005). The only randomised trial to assess EPP in MPM was the Mesothelioma and Radical Surgery trial (MARS) (Treasure et al., 2011). Analysis of clinical outcome data revealed potential harm associated with EPP with an adjusted hazard ratio for death of 2.75 (95% CI 1.21–6.26; p=0.016). As a result of this, and observational studies suggesting similarly poor outcomes, EPP has been largely abandoned in favour of less radical procedures (Batirel et al., 2016; Flores, 2016).

Extended pleurectomy with decortication (also known as pleurectomy/decortication) is a lung-sparing procedure in which the visceral and parietal pleura are removed. It is associated with fewer surgical complications than EPP, and potentially better survival (Cao et al., 2014; Batirel et al., 2016). Pleurectomy/Decortication (PD) is a “de-bulking” procedure, and unlike EPP does not aim for macroscopic complete resection. Consequently it is often employed alongside multi-modality treatment (Richards et al., 2006; Lang-Lazdunski et al., 2012). Another non-radical approach is partial pleurectomy via VATS. Non-randomised studies suggested that VATS-partial pleurectomy controlled symptoms in MPM, and possibly improved survival (Halstead et al., 2005; Nakas et al., 2008). However, the only suitably powered randomised trial to examine this demonstrated no survival difference compared with talc pleurodesis via a chest drain (Rintoul et al., 2014). VATS-partial pleurectomy had higher rates of pleurodesis in the first 12 months, but at the expense of a

greater number of surgical complications and a longer hospital stay. Consequently VATS-partial pleurectomy cannot be recommended for MPM, and non-surgical pleurodesis methods should be employed to control pleural fluid (Bibby et al., 2016).

Trimodality treatment for MPM consists of induction chemotherapy followed by EPP with subsequent hemithoracic radiotherapy. Non-randomised studies in carefully selected patients reported median survival times of up to 29 months (Krug et al., 2009; Van Schil et al., 2010; Cao et al., 2012). However, a subsequent randomised trial reported no difference in PFS or overall survival in patients treated with neoadjuvant chemotherapy and EPP with or without radiotherapy (Stahel et al., 2015). The study concluded that the addition of hemithoracic radiotherapy to EPP and chemotherapy added an unnecessary burden without offering benefit.

An alternative approach involves delivering high-dose radiotherapy prior to EPP, followed by adjuvant chemotherapy if there is lymph node involvement at surgery. This approach, nick-named SMART (Surgery for Mesothelioma After Radiation Therapy) appeared to be particularly efficacious in patients with epithelioid sub-type cancers (Cho et al., 2014), showing a median survival of 36 months (De Perrot et al., 2016). However, at present trimodality treatment is not recommended in the standard care pathway for MPM.

Radiotherapy is used in two main settings in MPM: as a palliative measure to treat symptoms or an adjuvant to surgery and chemotherapy in the context of trimodality treatment. Evidence for the latter technique is limited to anecdotal reports in highly selected patients, and as a result trimodality treatment is not considered standard care for MPM. Radiation is administered using the highly precise intensity-modulated radiotherapy technique as the alternative, high-dose external-beam hemithoracic radiotherapy, was associated with significant toxicity. Intensity-modulated radiotherapy technique allows accurate 3D mapping of the cancer, thus reducing the likelihood of radiation injury to surrounding organs (Rimner et al., 2016). Nonetheless, toxicity remains a risk, with eight (30%) out of 27 patients experiencing radiation pneumonitis (Rimner et al., 2016). In the palliative setting, radiotherapy can reduce cancer bulk and

relieve symptoms, particularly in the context of chest wall invasion, nerve root involvement or painful cutaneous metastases (MacLeod et al., 2015). Radiotherapy has been used prophylactically to prevent sub-cutaneous metastases developing due to cancer seeding along procedure tracts. The only suitably powered, randomised trial to date reported no difference in the frequency of tract metastases in patients receiving immediate radiotherapy after large-bore chest interventions compared with those receiving it when required. Consequently, the use of prophylactic radiotherapy is likely to diminish (Bibby et al. 2016).

The majority of patients with MPM experience a pleural effusion at some point. Drainage of pleural fluid improves breathlessness and prevention of fluid re-accumulation can improve quality of life long term (Davies et al., 2012). There are a number of methods for achieving pleural fluid control, each with benefits and disadvantages.

Therapeutic pleural aspiration is the simplest approach, and most patients will undergo this intervention at least once in their disease pathway. However, fluid inevitably re-accumulates and further interventions are required (Roberts et al., 2010; Bibby et al., 2015). Repeated aspirations may be appropriate for patients with a very short life expectancy, but a definitive procedure is generally preferable (Scherpereel et al., 2010; Roberts et al. 2010; Bibby et al., 2015).

Definitive fluid control can be achieved with chemical pleurodesis, a procedure aiming to obliterate the pleural space and render fluid re-accumulation impossible. For successful pleurodesis to occur there must be direct apposition of enough healthy pleural tissue to allow pleural inflammation and adhesion to occur when a chemical irritant is instilled into the pleural space. Consequently patients with trapped lung or extensive cancer bulk should be considered for alternative methods of pleural fluid control. Many chemical agents have been used for pleurodesis, but sterile, medical-grade talc appears to be the safest and most effective (Ong et al., 2000; Keeratichananont et al., 2015; Clive et al., 2016b), although other agents such as bleomycin and tetracycline are used (Antony et al., 2001; Roberts et al., 2010). Talc pleurodesis is successful in 60–80% of people provided the underlying lung is not trapped (Roberts et al., 2010;

Davies et al., 2012). An alternative to pleurodesis is the placement of an indwelling pleural catheter. This allows regular home drainage, and provides long-term relief of breathlessness (Davies et al., 2012). Indwelling pleural catheters are as effective at relieving symptoms and have the additional benefit of being appropriate for patients with trapped lung (Davies et al., 2012). In situations where both indwelling pleural catheters and talc pleurodesis are available, management should be led by patient choice (Maskell et al., 2012).

Numerous novel agents have been investigated in MPM. Amongst those that have demonstrated efficacy are targeted therapies to epidermal growth factor receptor antagonists and platelet-derived growth factor receptor inhibitors (Govindan et al., 2005; Mathy et al., 2005; Garland et al., 2007; Nowak et al., 2012).

Immunotherapy has shown promise in MPM. Checkpoint inhibitors such as tremelimumab and pembrolizumab have shown impressive disease control rates and prolonged disease stability when used as first-, second- or third-line treatment (Calabrò et al., 2013, 2015a; Cornelissen et al., 2014; Alley et al., 2015; Kindler et al., 2015). The combination of chemotherapy with immunotherapy, or multiple immunotherapy agents, appears synergistic in other cancers and warrants further exploration in MPM (Emens et al., 2015; Calabrò et al., 2015b; Larkin et al., 2015).

Mesothelin-targeted treatments are another area of interest in MPM. Agents that have undergone early phase clinical trials include mesothelin-specific monoclonal antibodies (e.g. amatuximab), anti-mesothelin immunotoxins (e.g. SS1P), mesothelin cancer vaccine (CRS-207) and chimeric antigen receptor T-cells targeted to mesothelin (Hassan et al., 2010; 2014a, 2014b, 2015).

Finally intra-pleural gene therapy using an adenovirus vector has been shown to be safe and feasible in MPM, with promising median survival (Sterman et al. 2016).

Overall, the future of MPM looks likely to contain many more therapeutic options than are currently available, but given the lack of curative treatment and the limited life expectancy, to date the overriding aim with MPM is to maintain quality of life and allow patients to live a meaningful and dignified life.

1.3 Epigenetic Alterations in Oncology

Epigenetics (from the Greek ἐπί, epì, "above" and γεννητικός, gennetikòs, "relating to family inheritance") is a branch of genetics that investigate the mechanisms responsible for the modification of the phenotype without any changes in the DNA sequence or the genotype of individuals. There are several mechanisms of epigenetic modification including modifications of DNA (methylation) and histone proteins that can be subjected to methylation, phosphorylation, acetylation and ubiquitination phenomena. These alterations are also called “epimutations” and originate during the lifetime as a consequence of exposure to several environmental factors. In addition, such epigenetic modifications can be transmitted to the other cells through cell division (Bird, 2002). If epigenetic alterations affect germinal cells (ova or spermatozoa), such modifications can be inherited by the next generation (Chandler, 2007).

Several studies have demonstrated that epigenetic alterations are involved in both physiological and pathological processes (Esteller, 2007). From a functional point of view, epigenetic modifications related to the alterations of histone proteins contribute to condensation or decondensation of chromatin that in turn alter the transcription of those genes with histone modifications. Another important epigenetic modification is mediated by the modification of the methylation status of DNA. In particular, different enzymes are able to induce hyper-methylation or hypo-methylation in specific DNA sites adding a methyl group to the cytosine nucleotide of a 5'-cytosine-guanine-3'dinucleotide (CpG) forming 5’-methylcytosine (5-MeC) (Levenson, 2010). The first association between DNA methylation and gene silencing in cancer was observed in 1989 where it was observed that p16INK4a, a cancer suppressor gene, was down-regulated in retinoblastoma as a consequence of methylation phenomena occurring at the promoter level. At present, it was widely demonstrated that methylation occurring in specific genomic portions is associated with the dysregulation of gene expression and consequently to human pathologies (centromeric instability, immunodeficiency and dimorphisms) (Maunakea et al., 2010). Therefore, it is evident how gene expression is strongly influenced by the covalent modifications of histone proteins, by DNA methylation, and the

alteration of the expression levels of non-coding RNAs, including miRNAs, by the insertion of histone variants and by the remodeling of nucleosomes able to modify the structural organization of chromatin (Jaenisch and Bird, 2003).

1.3.1 non-coding RNAs (ncRNAs) and microRNAs (miRNAs)

Gene expression is profoundly modulated by non-coding RNAs (ncRNAs), i.e. RNA molecules that are transcribed but not translated into proteins. Of note, ncRNA molecules include long non-coding RNAs (lncRNA from 200 nucleotides to 100 kb), short interfering RNAs (siRNA less than 25 bp), circular RNA (circRNA) in length and microRNAs (miRNA) of 20-25 nucleotides in length (Costa, 2006; Bartel, 2009; Wapinski and Chang, 2011).

Among ncRNAs, miRNAs are the most studied and their dysregulation was associated with different diseases including cancers. In particular, miRNAs genes are transcribed by an RNA polymerase II in a primary transcript called pri-miRNA which has a 5' CAP and a 3' polyadenylated tail. The length of a pri-miRNA is not well defined but is thought to be hundreds of nucleotides. The pri-miRNAs are processed in the nucleus by Drosha, a type III RNase aided by a ds-RNA binding protein (DGCR8), the resulting cleavage product is a miRNA precursor named pre-miRNA (Han et al., 2004). The pre-miRNA is about 70-90 nucleotides long and with a secondary “hairpin” and stem-loop structure, in which the two arms of the stem may not be fully complementary. The pre-miRNAs are characterized by a protrusion of two nucleotides at 3' and are transported into the cytoplasm with the aid of an exportin, esportin 5 and the Ran-GTP cofactor. In the cytoplasm they are further processed at the base of the stem loop by Dicer, a ribonuclease of the RNase III family together with the ds-RNA binding partner (TRBP), and are then released as short double-stranded RNA fragments of about 22 nucleotides (miRNA duplex). Each duplex contains the mature miRNA strand (5'-3') and a miRNA * fragment (3'-5') complementary to the first. The two nucleotides at 3' of the miRNA duplex: miRNA* are recognized by the Ago2 protein, recruited by TRBP, which guides the duplex in incorporating it into a RISC complex (RNA Induced Silencing Complex) (Hammond et al., 2000) which is activated consequentially to the development of the duplex. Subsequently, the miRNA* strand is degraded in the cytoplasm

while the mature miRNA strand drives the RISC complex towards the target mRNA. The recognition between a miRNA and its target mRNA occurs between the 5' end of the miRNA named "seed region" and, the 3' region of the target, for about 2-8 nucleotides. In case of a perfect match between miRNA and the targeted mRNA the RISC complex binding the miRNA is able to induce the complete degradation of the mRNA. On the contrary, if a partial match between miRNA and mRNA exists, as in the case of most mammals, the RISC complex operates a transient block of targeted mRNA translation (Figure 4) (He and Hannon, 2004; Lynam-Lennon et al., 2009).

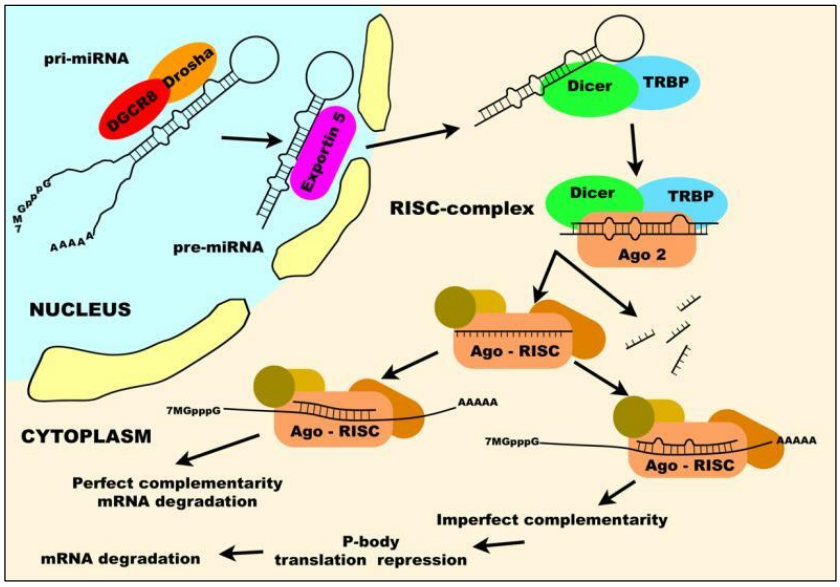


Figure 4. Mechanism of miRNA biogenesis.

The position of the miRNA genes within the genome is particularly interestingly, in fact most of them are found in the intergenic sequences, at least 1 Kb from known genes, indicating that they are transcribed regardless of these. Many miRNAs also form clusters suggesting that they could be transcribed as a single strand polycistronic, a theory supported by the coordination of gene expression in the cluster. It is because of the imperfect complementarity that a single miRNA can probably regulate several mRNAs (Dalmay, 2008). This multiple binding activities of miRNAs exerted towards different genes (Ramírez-Salazar et al., 2014) makes it particularly difficult the search for specific targets with the consequence that the functions of many miRNAs are still unknown.

1.3.2 miRNAs in cancer

In cancer, several alterations in the expression levels of specific miRNAs have been observed; in particular, miRNAs are differentially expressed in cancers compared to normal tissues thus representing good diagnostic and prognostic biomarkers (Falzone et al., 2019; Falzone et al., 2020; Giambò et al., 2021). MiRNAs can be also distinguished into cancer suppressor miRNAs directed against oncogenic factors and oncogenic miRNAs directed against mRNAs with cancer suppressor functions. The recent literature revealed that cancer suppressor miRNAs, such as miR-7a, are decreased in melanoma cells compared to healthy melanocytes; this leads to an increase in its targets, such as the RAS oncogene and the β3 integrin, causing an increase in the invasive capacity of neoplastic cells (Varamo et al., 2017). On the contrary, miRNAs with an oncogenic function, have an opposite trend; indeed these miRNAs, like miR-221 and miR-222 able to target the p27 protein, appear to be increased in different types of cancer (Galardi et al., 2007; Mercatelli et al., 2008), inducing abnormal cell proliferation (Varamo et al., 2017).

Of note, several miRNAs have already been associated with the development of different cancers, including breast cancer, glioblastoma multiform, lymphomas, pancreatic cancer, hepatocellular carcinoma, lung adenocarcinoma, thyroid carcinoma, and melanoma (Jin et al., 2011; Liu et al., 2012; Satzger et al., 2012; Hafsi et al., 2016; Ahmadinejad et al., 2017; Kanno et al., 2017; Lu et al., 2017; Peng et al., 2017; Rosignolo et al., 2017; Candido et al., 2019; Falzone et al., 2020).

1.4 miRNAs in malignant mesothelioma

A recent review of the literature (Ledda et al., 2018) indicates a list of miRNAs potentially involved in MPM. Potential miRNA biomarkers for this malignant neoplasm include the following: miR-126-3p, miR-625-3p, miR-103a-3p, miR-16-5p, miR-143-3p, miR-145-5p, miR-192-5p, miR-193a-3p, miR-200b-3p, miR-203a-3p, and miR-652-3p. Some miRNAs with potential diagnostic value for MPM have been validated by the scientific community using RT-qPCR. Among these downregulated miRNAs there are miR-126, miR-145, miR-16, miR-200c, miR-103 (Benjeamin et al., 2010; Gee et al., 2010;

Santarelli et al., 2011, 2015; Kirschner et al., 2012; Weber et al., 2012, 2014; Reid et al., 2013; Andersen et al., 2014; Cioce et al., 2014; Ramírez-Salazar et al., 2014; Cappellesso et al., 2016). The scientific community has revealed that several miRNAs are involved in deregulation and in all molecular mechanisms associated with MPM development (De Santi et al., 2017; Martínez-Rivera et al., 2018) and constantly updates the miRNAs which can be associated with MPM early diagnosis and prognosis (Filetti et al., 2020a). Therefore, it is essential to know the molecular mechanisms and specific targets of the different miRNAs in order to develop personalized anticancer therapies or evaluate the expression levels of miRNAs for both diagnostic and prognostic purposes.

2. AIM OF THE STUDY

The epidemiological data and clinical characteristics of MPM highlight how the management of this cancer is particularly complex from a diagnostic, prognostic and therapeutic point of view. Indeed, the diagnosis of MPM is still often formulated when the cancer has already at an advanced stage thus limiting the survival rates of the patients. An explanation of the late diagnosis of MPM is related to the lack of effective diagnostic and prognostic biomarkers. Therefore, the identification of novel factors capable of predicting the risk of the onset of MPM and its aggressiveness is essential to better manage this malignant neoplasm.

Based on these premises, the aim of the present project was to identify and validate a panel of molecular biomarkers (miRNAs) and proteins in relation to the changes induced on the pleura, in subjects exposed to FE fibers in order to provide a minimally invasive screening tool for secondary prevention to MPM in a population at high risk of incidence and mortality.

For these purposes, *in silico* analyses were first performed on healthy/exposed to asbestos fibers subjects *vs.* patients with MPM using the GEO2R tool available on GEO DataSets. These analyses revealed a set of miRNAs strictly involved in MPM by merging the lists of miRNAs found differentially expressed in the miRNA expression datasets contained in GEO DataSets database. The three miRNAs selected as statistically significant were hsa-miR-323a-3p, hsa-miR-

101–3p, and hsa-miR-20b-5p. Secondly, functional *in vitro* experiments on normal pleural mesothelial cell line (MeT-5A) and MPM cell line (JU77) have been performed to test the carcinogenetic effects and epigenetic modulation induced by FE exposure. The result of the computational evaluations allowed the analysis of the expression levels of the miRNAs previously identified *in silico* both *in vitro* and in MPM *vs.* nonmalignant pleura FFPE tissues to evaluate differences in the expression levels of the selected miRNAs and their MPM diagnostic and prognostic potential. A customized droplet digital PCR (ddPCR) assay was used to amplify the miRNAs previously identified *in silico* both *in vitro* and in FFPE samples. Subsequently, by consulting the Catalogue of Somatic Mutation In Cancer (COSMIC) it was possible to identify the 20 most mutated genes that are known to be involved in MPM development and therefore have a dysregulated expression. Furthermore, the clinical implication of the analyzed miRNAs was assessed through the clinic-pathological data and the miRNA expression profiles analysis contained in The Cancer Genome Atlas Mesothelioma (TCGA-MESO) database and downloaded using the online exploration tool UCSC Xena Browser. Additionally, using the bioinformatics prediction tool microRNA Data Integration Portal (mirDIP) the interaction levels between the miRNAs previously identified via computational analysis and the main genes mutated and altered in MPM were evaluated. The identification and validation of the miRNAs identified in this study through the computational and experimental approaches described in the following chapter will represent a promising strategy for the identification of new diagnostic and prognostic biomarkers for a better management of malignant mesothelioma patients.

3. MATERIALS AND METHODS

3.1 *In silico analyses*

The selection of miRNA expression profiling datasets was performed using the publicly available GEO DataSets database, as previously reported (Falzone et al., 2016; Hafsi et al., 2016). For the selection of miRNA expression profiling datasets the following search terms were used: "((“non coding rna profiling by array" [DataSet Type]) AND Malignant Mesothelioma) AND “Homo sapiens” [porgn:__txid9606]. Datasets including the expression data of malignant mesothelioma cell culture or animal models were not considered for the analysis. Following dataset selection, the data matrices were downloaded and differential analyses were performed between normal/asbestos-exposed and pathological samples using the GEO2R tool available on GEO DataSets, as previously described (Falzone et al., 2018, 2019, 2020). Since different miRNA microarray platforms were adopted, the ID value of the differentially expressed miRNAs of each dataset was annotated using the last version of miRBase (miRBase v.22) (Kozomara et al., 2019). The miRNA expression fold change (FC) was expressed as base-2 logarithm of FC (log2FC) to normalize the miRNA expression values obtained from different microarray platforms.

The lists of differentially expressed miRNAs obtained from the malignant mesothelioma datasets were merged through a Venn diagram calculating tool, to obtain miRNAs shared in at least two out of the three datasets analyzed (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Through the dataset analysis of miRNAs expression profiling, we analyzed the data coming from multiple studies of miRNA expression profiling and thus had a large series of samples from which to obtain truthful expression data concerning miRNAs with a potential diagnostic role in malignant mesothelioma. To clarify the role of hsa-miR-323a-3p, hsa-miR-101-3p, and hsa-miR-20b-5p in MPM, different computational tools were used.

By consulting COSMIC (http://cancer.sanger.ac.uk/cosmic) it was possible to identify the 20 most mutated genes that are known to be involved inMMdevelopment and therefore have a dysregulated expression. The selection

was performed using the search term “Malignant Mesothelioma” including the terms “Pleura” in tissue selection and “Mesothelioma” in histology selection.
Subsequently, using the bioinformatics prediction tool mirDIP (version 4.1.11.2, Database version 4.1.0.3) (http://ophid.utoronto.ca/mirDIP) (Shirdel et al., 2011; Tokar et al., 2018), the interaction levels between the miRNA previously identified via computational analysis and the main genes mutated and altered in MPM were evaluated.
Furthermore, the clinical implication of the three analyzed miRNAs was assessed through the clinic-pathological data and the miRNA expression profiles analysis contained in The Cancer Genome Atlas Mesothelioma (TCGA-MESO) database and downloaded using the online exploration tool UCSC Xena Browser (https://xenabrowser.net/) (Goldman et al., 2020). In particular, the TCGA database was used to verify if the three miRNAs here analyzed were dysregulated in MM according to asbestos exposure, cancer stage, and patient survival. A total of 17 MPM patient-related datasets were found containing a total of 87 MPM samples (35 exposed to asbestos, 49 not exposed to asbestos, 3 excluded due to lack of useful information). The datasets contained the expression levels of 1,964 different miRNAs, but we focused on hsa-miR-323a-3p, hsa-miR-101-3p, and hsa-miR-20b-5p for further investigation.

3.2 Cell cultures and treatments

Normal MeT-5A cells and malignant mesothelioma JU77 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Both cell lines have been cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum, 1% L-glutamine (Lonza; Walkersville, MD, USA), 1% non-essential amino acids solution (Gibco by Thermo Fisher Waltham; Massachusetts, USA), 1% penicillin/streptomycin (Lonza; Walkersville, MD, USA). The culture conditions were 37 °C in a humidified atmosphere with 5% CO_2. The MeT-5A and JU77 cells were split 1:3 and 1:6, respectively, twice a week. Mycoplasma testing has been done for the cell lines used before the experiment. The supernatant of antibiotic-free cell cultures (> 7 days) and of Mycoplasma culture (positive control) have been used for PCR reaction. PCR water has been used as negative control. The absence of

Mycoplasma has been detected by verifying the bands of amplified DNA fragments in electrophoresis (amplicon size 270 bp).

Cells from confluent cultures were separated from the culture flask (SPL Life Sciences; Korea) using 0.25% trypsin in 2.21 mM EDTA solution (Corning; Manassas, VA, USA) and counted using Bürker chamber by Trypan Blue Stain 0.4% (Gibco by Life Technologies; NY, USA).

The cells have been used for the experiments between the V and VI passages. FE fibers collected around the farms of Biancavilla (Sicily, Italy) are been provided by Prof. A. Gianfagna, Department of Earth Sciences, University “La Sapienza” of Roma (Italy). FE fibers are presented as small needles of transparent and intense yellow color. These were sterilized under UV light for 10 min, suspended in RPMI-1640 medium used for cell cultures and sonicated through Omni-Ruptor 4000 Ultrasonic Homogenizer (OMNI International Inc.; Kennesaw, GA, USA) for 10 min. The stock solution was then diluted appropriately to obtain the different concentrations for *in vitro* treatments.

Growth curves and dose-response curves for both cell lines were determined before performing *in vitro* functional experiments.

First, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay has been used to determine the cell metabolic viability of both cell lines. MeT-5A and JU77 were plated onto 96-well plates (Thermo Fisher Scientific; Roskilde, Denmark) for growth curve at a range density of $2x10^3$ - $8x10^3$ cells/100 µl. At each time point (0, 24, 48, 72, 96 h after seeding), 10% MTT in Dulbecco's Phosphate-Buffered Saline (DPBS) (Corning; Manassas, VA, USA) has been added to each well. After 4 h of incubation, 100 µl of the lysis solution (0.1% $HCl_{conc.}$ in absolute isopropyl alcohol) have been added to each well. The optical density was measured with an absorbance microplate reader (TECAN Trading AG; Switzerland) at $\lambda = 620$ nm. The experiment was performed in triplicate.

Duplication time of both cell lines obtained by MTT colorimetric assay have been compared with Trypan Blue cell counting. Trypan Blue is a stain used to selectively stain dead cells blue. To minimize operator bias, trypsinized cells

were stained with Trypan Blue, and live and dead cells enumerated by two different operators.

Cell viability by MTT assay was calculated as the percentage of viable cells as follows:

$$\text{Cell viability (\%)} = [\text{OD (Control)} - \text{OD(Blank)}] \times 100$$

Duplication times have been calculated through the following formulas (t = Time; OD=Optical Density; n = Cells Number):

$$[(\text{t2} - \text{t1})/\log 2\ (\log OD_2 - \log OD_1)] \text{ by MTT assay,}$$

$$[(\text{t2} - \text{t1})/\log 2\ (\log n_2 - \log n_1)] \text{ by Trypan Blue results.}$$

Secondly, MeT-5A were plated onto 96-well plates for the dose-response curve at the density of $3x10^3$ and $6x10^3$ cells/50 µl while JU77 were plated at the density of $2x10^3$ and $4x10^3$ cells/50 µl. After 24 h of incubation, 50 µl of FE fibers solutions were added to the cell cultures in amounts corresponding to final concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 µg/ml. Both cell lines grown in FE-free medium were used as controls. At each time point (6, 24, 48, 72 h of FE exposure) in cell culture, 10% MTT in DPBS has been added to each well. After 4 h of incubation, the lysis solution has been added to each well. The optical density was measured with an absorbance microplate reader at λ = 620 nm. For each sample, three replicates were performed.

Cell viability by MTT assay was calculated as the percentage of viable cells treated with FE fibers *vs.* untreated control cells as follows:

$$\text{Cell viability (\%)} = [\text{OD (Treatment)} - \text{OD(Blank)}]/[\text{OD (Control)} - \text{OD (Blank)}] \times 100$$

IC_{50} values have been calculated through the following equation:

$$[\text{Inhibitor}]\ vs.\ \text{normalized response} - \text{Variable slope}$$

(https://www.graphpad.com/guides/prism/7/curve-fitting/reg_dr_inhibit_normalized_variable_2.htm)

Finally, for the functional *in vitro* experiments MeT-5A and JU77 were plated onto 100 × 20 mm Petri Dishes (Eppendorf; Hamburg, Germany) at the density of $1x10^6$ cells and $8.5x10^5$ cells, respectively. After 24 h of incubation, the medium of MeT-5A cell line has been removed and replaced with FE fibers

solutions to final concentrations of 100, 50, 10, 5 μg/ml. MeT-5A and JU77 cells grown in normal medium were used as controls. After 48 h from FE exposure, supernatant and pellet have been collected in duplicate for the subsequent analysis. The supernatant has been collected after centrifugation at 0.3×g for 5 min at 4 °C. Cells were harvested on ice by scraping in cold DPBS. Cells are then centrifuged at 0.2×g for 5 min at 4 °C and suspended in 1 ml cold DPBS. The cell solution was transferred to eppendorf tubes. Cells were then centrifuged at 0.8×g for 5 min to 4 °C and supernatant has been removed. The samples have been stored to −80 °C until RNA extraction.

3.3 Collection of malignant mesothelioma FFPE samples and controls

Tissue specimens of ten cases of malignant mesothelioma and eight cases of healthy pleural mesothelium were retrospectively analyzed. Formalin-fixed and paraffin-embedded (FFPE) tissue specimens were obtained from the biobank of the Section of Anatomic Pathology, Department Gian Filippo Ingrassia, University of Catania. The exclusion criteria adopted in the choice of the cases were the following: (i) it was not possible to obtain additional slides from FFPE blocks for the analysis; (ii) no representative neoplastic tissue was contained in FFPE blocks. No written informed consent was necessary because of the retrospective nature of the study; the study protocols conformed to the ethical regulations of the Helsinki Declaration.

The cohort of patients of Biancavilla with FE-mediated MM was composed of six men and four women (mean age: 68.4 ± 13.9 years; age range: 50–93 years). Agreeing to the World Health Organization (WHO) criteria, six cases were histologically classified as epithelioid, three were classified as biphasic subtypes, and one was classified as sarcomatoid (Salle et al., 2016).

The cohort of control cases was composed of eight men (mean age: 44 ± 25.5 years; age range: 15–76 years). These patients did not live in Biancavilla, and they did not show oncological pathologies but pulmonary emphysema (n = 3) and pleurisy (n = 5). Data including MPM cases and controls are summarized in Table 1.

Table 1. Features of the FE-related MPM cases and controls.

	Age range (Years)	Mean age (Years)	Gender	Pathologies	Pathological Subtype	Survival Time range (Days)	Mean Survival Time (Days)
Cases (n=10)	50 - 93	68.4 ± 13.9	60% men, 40% women	100% MPM	60% epithelioid, 10% sarcomatoid, 30% biphasic	45 - 1800	579 ± 525
Controls (n=8)	15 - 76	44 ± 25.5	100% men	37.5% pulmonary emphysema, 62.5% pleurisy			

Freshly cut sections of FFPE tissue, each with a thickness of 20 μm, were obtained using a rotary microtome. Two sections for each sample were collected and stored at room temperature.

3.4 RNA isolation and Reverse Transcription

Total RNA, containing small non-coding RNA, was extracted from MeT-5A and JU77 using miRNeasy Mini Kit (QIAGEN; Venlo, Netherlands) while from supernatant miRNeasy Serum/Plasma Advanced Kit (QIAGEN; Venlo, Netherlands) has been used, according to the manufacturer's recommended protocols (miRNeasy Mini Handbook 07/2012, miRNeasy Serum/Plasma Handbook 02/2012). A synthetic miRNA, cel-miR-39, corresponding to Caenorhabditis elegans was introduced in the extraction phase as exogenous control.

Total RNA containing small non-coding RNA was extracted from FFPE tissue using miRNeasy FFPE Kit (QIAGEN; Hilden, Germany) according to the manufacturer’s recommended protocol (miRNeasy FFPE Handbook 01/2020).

The extracted RNA from all samples was directly quantified by the absorbance ratio at λ = 260/280 nm through NanoDrop (ND-1000) UV/Vis spectrophotometer. All samples were stored at −80 °C. The purified RNA was reverse transcribed into cDNA and pre-amplified, using TaqMan Advanced miRNA cDNA Synthesis Kit (cat. n. A28007 - Thermo Fisher Scientific) in C1000 Touch Thermal Cycler (Bio-Rad Laboratories; Hercules, California,

USA) according to the manufacturer's recommended protocol [TaqMan Advanced miRNA Assays User Guide (Single-tube Assays) - Catalog Number A25576 - Publication Number 100027897 - Revision C].

3.5 Analysis of miRNA expression levels by ddPCR

A customized droplet digital PCR (ddPCR) assay was used to amplify hsa-miR-323a-3p, hsa-miR-101–3p, hsa-miR-20b-5p. In the case of *in vitro* samples also cel-miR-39–3p as exogenous control has been add (miRNeasy Serum/Plasma Spike-In Control, cat. n. 219,610 – Qiagen). Briefly, 22 µL of reaction mixture was prepared by adding 11 µL of ddPCR Supermix for probes (no dUTP) (cat. n. 1,863,010 – Bio-Rad Laboratories), 1 µL of TaqMan Advanced miRNA Assays specific for each miRNA (cat. n. 477,863, 477,804, 477,853, 478,293 – Thermo Fisher Scientific), 5 µl of miR-Amp cDNA sample and 5 µl of PCR water. A Non-Template Control (NTC) has been considered for each probe. Twenty microliters of PCR reaction were loaded on the cartridge containing 70 µL of Droplet Generation Oil (cat. n. 1,863,005 - Bio-Rad Laboratories) in appropriate wells, and then Droplet Generator QX200 was used to generate droplets. Subsequently, the generated droplets were transferred to a 96-well PCR plate (Eppendorf, Hamburg, Germany) and were amplified by using C 1000 Touch Thermal Cycler (Bio-Rad Laboratories) as previously described (Salemi et al., 2018; Battaglia et al., 2019). PCR amplification was carried at the following cycling conditions: 10 min at 95 °C, 40 cycles of 94 °C for 30 s, 60 °C for 1 min, followed by 98 °C for 10 min (ramp rate 2 °C/s).

After the amplification, the plate has been incubated at 4 °C for 2 h to stabilize the fluorescence signal. After balancing at room temperature, the plate was loaded on QX200 Droplet Reader (Bio-Rad Laboratories) and both positive and negative droplets were read.

Finally, the absolute quantification of each miRNA was calculated automatically by using the QuantaSoft software, version 1.7.4 (QuantaSoft, Prague, Czech Republic) as previously described (Salemi et al., 2018). The quantification of the miRNAs was reported as relative expression within the ddPCR mixture.

3.6 Statistical analyses

To further strengthen the significance of the *in silico* results obtained, the miRNAs with a value of $P \leq 0.01$ were selected in this analysis. These were considered for the following identification of miRNAs involved in malignant mesothelioma.

As regards the experimental results obtained from the cell lines and the FFPE samples, the unpaired Student's t-test was used to compare data between the two groups. The Shapiro-Wilk normality test was applied for the calculation of the distribution of hsa-miR-323a-3p, hsa-miR-101-3p, and hsa-miR-20b-5p expression levels observed with ddPCR and deposited on the TCGA-MESO database. The Mann-Whitney test was utilized for the comparison between miRNAs expression vs. controls by ddPCR.

Receiver operating characteristic (ROC) curves were obtained to evaluate the specificity and sensitivity of the analyzed miRNAs in FFPE samples. An unpaired Student t-test and one-way ANOVA test were used for assessing the statistical differences existing between the expression levels of hsa-miR-323a-3p, hsa-miR-101-3p, and hsa-miR-20b-5p reported in the TCGA-MESO database according to the asbestos exposure and the MPM tumor stages, respectively. Cancer-specific survival analysis was performed using the Kaplan-Meier method, and for comparison of the survival curves, the Mantel-Cox log-rank test was used.

A value of $p < 0.05$ was considered statistically significant. The graphs were plotted using Prism for Windows version 7.00 (Graphpad Software; San Diego, CA, USA), and data were represented as the mean ± SD.

4. RESULTS

4.1 *In silico* miRNAs selection

The research of miRNA expression profiling datasets performed with GEO DataSets allowed the identification of three miRNA profiling datasets for malignant mesothelioma. The information regarding the selected datasets for malignant mesothelioma is reported in Table 2. In particular, two datasets were developed by Illumina (Illumina Human MicroRNA expression beadchip) and one developed by IPA (Center of Molecular Medicine Human Mouse Rat Ambion mirVana miRNA v 2.0 array).

Table 2. The information regarding the selected datasets for malignant mesothelioma considered for computational analysis.

Series Accession	n. normal	n. cancer	Samples	Platform	Ref.	Total Samples
GSE40345	6	13	Normal parietal pleura and malignant pleural mesothelioma	GPL8179 Illumina Human v2 MicroRNA expression beadchip	Xu et al., Chest 2013	27
GSE99362	5	51	Normal pleura and fresh frozen diffuse malignant peritoneal mesothelioma	GPL8179 Illumina Human v2 MicroRNA expression beadchip	Cimino-Reale et al., J Hematol Oncol 2017	58
GSE29707	17	18	PBMC Asbestos-exposed control and PBMC malignant mesothelioma	GPL13618 IPA/Center of Molecular Medicine Human_Mouse_Rat Ambion mirVana miRNA v2.0 array	Weber et al., PLoS One 2012	35

Merging the lists of differentially expressed miRNAs obtained from the three malignant mesothelioma datasets a panel of miRNAs strictly involved in malignant mesothelioma was identified. Seven miRNAs were found to be deregulated with concordant expression levels (downregulated or upregulated in all datasets) in at least two of the three malignant mesothelioma miRNA expression datasets (Table 3). In particular, two miRNAs (hsa-miR-1248 and hsa-miR-323a-3p) were upregulated and five miRNAs (hsa-miR-101–3p, hsa-

miR-129, hsa-miR-20b-5p, hsa-miR-520g-3p and hsa-miR-597–5p) were downregulated.

Table 3. Differentially expressed miRNAs between normal/asbestos-exposed pleura and malignant mesothelioma samples.

	Datasets ID		
miRNA ID	GSE40345	GSE99362	GSE29707
Upregulated miRNAs			
hsa-miR-1248	1.7183916	1.072063	
hsa-miR-323a-3p	1.7815888		2.27108
Downregulated miRNAs			
hsa-miR-101-3p	-0.824242		-2.12356
hsa-miR-129	-0.469861	-1.354274	
hsa-miR-20b-5p	-1.094756		-2.51629
hsa-miR-520g-3p	-1.615968	-0.524565	
hsa-miR-597-5p	-0.665968	-0.576755	

The miRNAs selected for further experimental analyses were indicated in bold in Table 3. In particular, hsa-miR-323a-3p was upregulated, hsamiR-101–3p and hsa-miR-20b-5p were downregulated with concordant expression levels in two MPM miRNA expression datasets (GSE40345, GSE29707). The hsa-miR-323a-3p, hsa-miR-101–3p, hsa-miR-20b-5p miRNAs were selected as targets primarily for performing *in vitro* functional experiments on MeT-5A exposed to FE fibers and untreated MeT-5A and JU77, and secondly in FFPE samples.

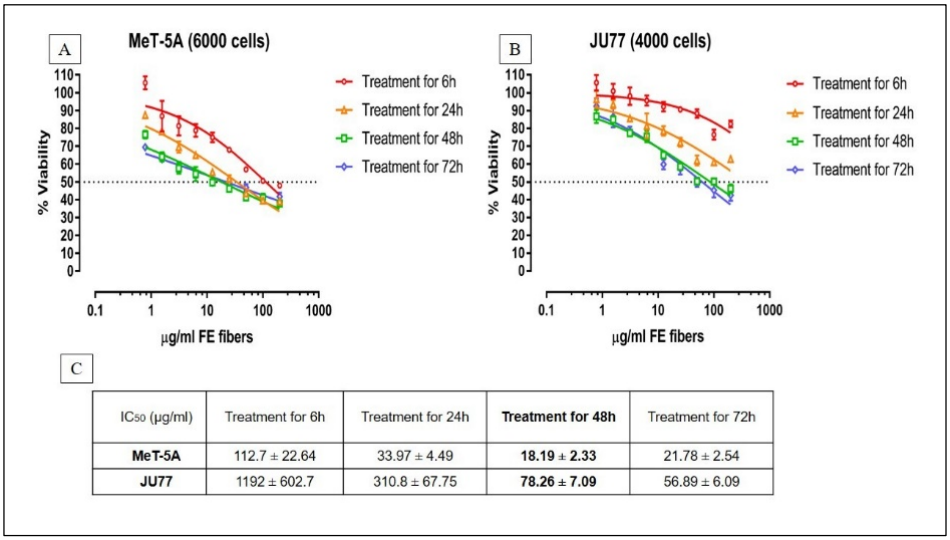
4.2 Choice of fluoro-edenite concentrations and exposure time for *in vitro* experiments

To evaluate the optimal cell density to use for the experiments, growth curves for both MeT-5A and JU77 cell lines at a range density of $2x10^3 – 8x10^3$ cells/100 µl onto 96-well plates have been performed. The curves that showed a linear growth of cells have been chosen as ideal densities for the experiments. Furthermore, cells at these densities were confluent at the end time point of 72 h. The data showed that optimal MeT-5A density is $6x10^3$ cells, while for JU77 the ideal density is $4x10^3$ cells for the end time point. Subsequently, duplication times obtained by MTT colorimetric assay have been compared with Trypan Blue cell counting for both cell lines (Table 4).

Table 4. Duplication times obtained by MTT colorimetric assay and Trypan Blue cell counting for MeT-5A and JU77 cell lines.

	Duplication time (h)	
	MTT assay	Trypan Blue counting
MeT-5A	55.07 ± 2.92	54.95
JU77	35.71 ± 0.21	32.00

In order to examine the effects of FE fibers upon mesothelium and MPM, the *in vitro* viability of MeT-5A and JU77 from 6 to 72 h exposure to FE (from 200 to 0.78 μg/ml) by MTT assay has been evaluated. Results were determined by constructing dose–response curves (Figures 5A and B) and calculating the IC_{50} values by non–linear regression analysis of the data for MeT-5A and JU77 cell lines (Figure 5 C). The results showed that MeT-5A cells were more sensitive to FE fibers compared to JU77 tumor cells.



IC50 (μg/ml)	Treatment for 6h	Treatment for 24h	**Treatment for 48h**	Treatment for 72h
MeT-5A	112.7 ± 22.64	33.97 ± 4.49	**18.19 ± 2.33**	21.78 ± 2.54
JU77	1192 ± 602.7	310.8 ± 67.75	**78.26 ± 7.09**	56.89 ± 6.09

Figure 5. A) Dose – response curves of MeT-5A with FE fibers from 200 to 0.78 μg/ml until 72h of treatments; B) Dose – response curves of JU77 with FE fibers from 200 to 0.78 μg/ml until 72h of treatments; C) IC50 values (μg/ml) for MeT-5A and JU77 cell lines.

The results of MeT-5A dose-response curves (Fig. 1A) made it possible to identify the FE concentrations and exposure time useful for in *vitro* experiments. This experimental phase has been very important to establish the two fundamental variables, FE concentrations and exposure time, for the following analysis steps with MeT-5A cell line. The results of the dose-response curves of

MeT-5A showed a significant difference between 6 h treatments and all other time points at all tested concentrations exposure to FE fibers. Incubation of 48 h after FE treatments was chosen as the final time of the experiment. Four different FE concentrations have been tested to MeT-5A cells (100, 50, 10, 5 μg/ml); two lower and two higher values than the IC50 value obtained after 48 h of treatments (IC_{50} = 18.19 μg/ml ± 2.33), in accordance with previous studies (Cardile et al., 2004a,b; Pugnaloni et al., 2007; Rapisarda et al., 2017). It was also decided to test higher FE concentrations than that obtained for the IC_{50} value to evaluate whether these doses can produce an adverse effect at the molecular level that does not occur at lower concentrations. Then, after 48 h of FE treatments at the different concentrations of 100, 50, 10, 5 μg/ml, supernatant and pellet have been collected for RNA isolation and ddPCR assay.

4.3 *In vitro* validation of miRNA expression levels

The expression levels of hsa-miR-323a-3p, hsa-miR-101–3p, hsamiR- 20b-5p were examined in MeT-5A exposed to different concentrations of FE fibers for 48 h compared to untreated MeT-5A and JU77. The analysis was performed both in collected cells and supernatants.

Shapiro-Wilk Normality test showed that the expression levels of secreted hsa-miR-323a-3p detected in cell supernatants did not show a normal distribution in all the samples analyzed. On the contrary, a normal distribution was observed by analyzing hsa-miR-323a-3p extracted from cell pellets. The same trend was observed for the distribution of hsa-miR-101–3p expression values. miRNA expression levels distribution in supernatants of JU77 pathological control, MeT-5A control and MeT-5A exposed to FE fibers did not show a normal distribution, while in cells a normal distribution of expression levels has been observed. For hsa-miR-20b-5p the expression levels of both supernatants and cells did not show a normal distribution.

The data showed that the expression levels of hsa-miR-323a-3p vary significantly when the supernatant- or cell-derived miRNAs were taken into account. In particular, no significant dose-dependent modulation of hsa-miR-323a-3p was shown in the supernatant after treatment with an increasing dose of FE fibers until the concentration of 50 μg/ml. However, at high-dose of FE fibers

(100 μg/ml), hsa-miR-323a-3p supernatant expression increased significantly. The expression levels of hsa-miR-323a-3p in the supernatant of JU77 cells was significantly higher compared to control MeT-5A and exposed to all FE concentrations. On the contrary, a significant difference in cellular hsa-miR-323a-3p levels was observed between MeT-5A exposed to all tested FE concentrations compared to JU77 cells. Indeed, cellular hsa-miR-323a-3p expression levels increased significantly in a dose-dependent manner in the treated MeT-5A as predicted with the in silico analyses while in JU77 cell line the hsa-miR-323a-3p expression was comparable with control MeT-5A (Figure 6).

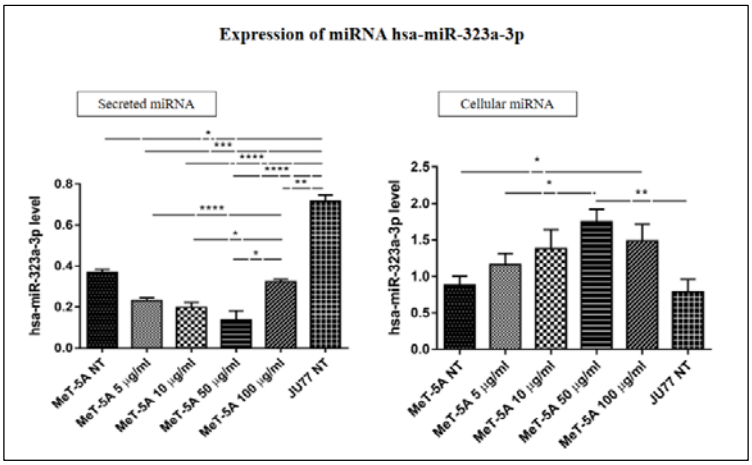


Figure 6. Normalized hsa-miR-323a-3p expression, reported as relative expression, in supernatants and cells: untreated MeT-5A, treated with 5, 10, 50, and 100 μg/ml of FE fibers for 48 h, and untreated JU77.

Secreted and cellular hsa-miR-101–3p in MeT-5A treated with FE fibers and JU77 cells showed different trends of expression. The expression levels of this miRNA in the tumor cell line would seem to be significantly opposite to the control MeT-5A in both biological matrices. No significant dose-dependent de-regulation of hsa-miR-101–3p has been observed in the supernatant of control MeT-5A compared to MeT-5A FE treated. However, significant differences have been observed between MeT-5A cells exposed to all tested FE concentrations and JU77 cells where hsa-miR-323a-3p was significantly higher. In opposite with this trend, cellular hsa-miR-101–3p has been shown a dose-dependent up-regulation in MeT-5A exposed to FE fibers. JU77 showed a down-regulation of

cellular hsa-miR-101–3p vs. control MeT-5A and exposed to all concentrations of FE fibers, in concordance with *in silico* analysis (Figure 7).

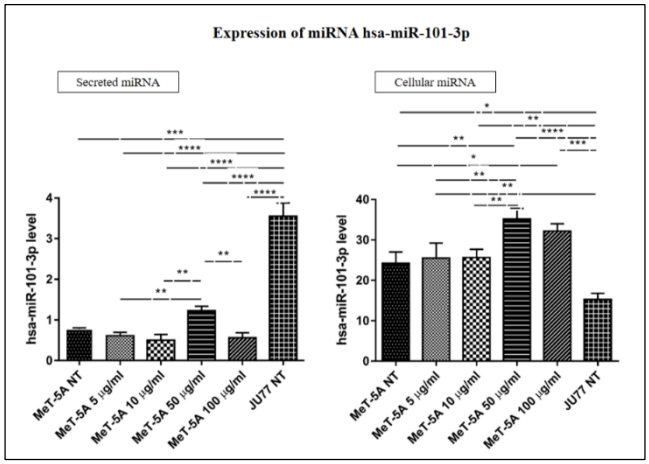


Figure 7. Normalized hsa-miR-101–3p expression, reported as relative expression, in supernatants and cells: untreated MeT-5A, treated with 5, 10, 50, and 100 µg/ml of FE fibers for 48 h, and untreated JU77.

As regard hsa-miR-20b-5p, there was no differential expression between secreted and cellular hsa-miR-20b-5p. This miRNA in both biological matrices has been shown a no significant dose-dependent increase in MeT-5A cell line, but a significant up-regulation in JU77 cells *vs.* control and treated MeT-5A. This result was in contrast with the *in silico* analysis that showed the down-regulation of hsa-miR-20b-5p down-regulated in MM cases compared to controls (Figure 8).

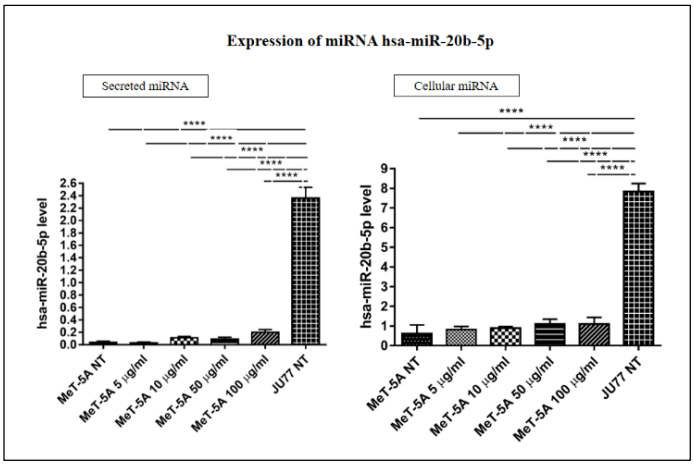


Figure 8. Normalized hsa-miR-20b-5p expression, reported as relative expression, in supernatants and cells: untreated MeT-5A, treated with 5, 10, 50, and 100 µg/ml of FE fibers for 48 h, and untreated JU77.

The data showed consistency between expression levels of hsa-miR-323a-3p and hsa-miR-101–3p and the *in silico* results (up-regulation and down-regulation respectively in malignant mesothelioma cases compared to controls). The hsa-miR-323a-3p expression in MeT-5A cells increased until the dose of 50 μg/ml of FE fibers and after it suffered a decrease. It is reasonable to think that this miRNA is secreted and its expression starts to increase in the supernatant at a concentration higher of 50 μg/ml of FE fibers (100 μg/ml). The expression trend of hsa-miR-101–3p was uneven due to the effect of the treatments with FE fibers, but also in this case the dose of 50 μg/ml of FE fibers caused a reversal of the expression of the miRNA. In both biological matrices, hsa-miR-20b-5p showed a no significant dose-dependent increase in MeT-5A cell line, but a significant up-regulation in JU77 cells vs. control and treated MeT-5A, in contrast with the *in silico* analysis that showed a downregulation in malignant mesothelioma cases compared to controls.

4.4 Validation of the diagnostic and prognostic values of miRNAs on FFPE samples

To evaluate the translational impact of the *in silico* and *in vitro* findings obtained through the ddPCR analysis performed on normal mesothelium cells and malignant mesothelioma cells, the expression levels of hsa-miR-323a-3p, hsa-miR-101-3p, and hsa-miR-20b-5p were evaluated in FFPE tissues obtained from ten MPM patients and eight individuals with healthy pleural mesothelium.

The Shapiro-Wilk normality test showed that the expression levels of the three miRNAs analyzed in MPM cases and healthy controls differed significantly from a normal distribution.

The comparison between tumor and normal tissues showed a different expression of the three miRNAs analyzed in MPM and healthy controls. The expression levels of hsa-miR-323a-3p, hsa-miR-101-3p, and hsa-miR-20b-5p in MM cases were significantly lower compared to controls (Figure 9). There was a statistically significant trend of down-regulation observed for the three selected miRNAs analyzed in MPM cases *vs.* controls.

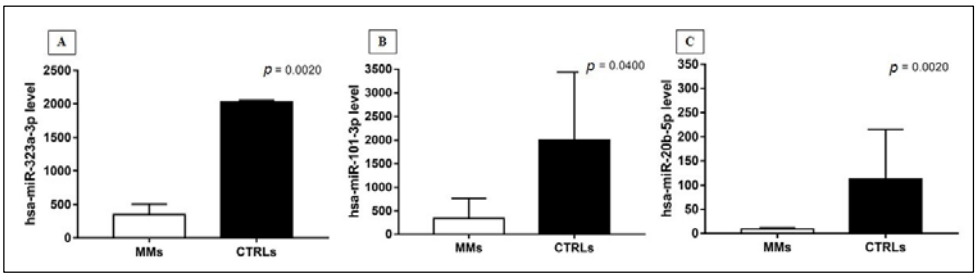


Figure 9. MiRNAs expression, reported as number of copies/µl of reaction in MPM cases and healthy controls according to: A) hsa-miR-323a-3p, B) hsa-miR-101-3p, C) hsa-miR-20b-5p.

To evaluate the sensitivity and specificity of these miRNAs and their role as novel, promising diagnostic biomarkers for MPM, ROC (receiver operating characteristic) curves were calculated (Figure 10). ROC analysis revealed high specificity and sensitivity rates for both hsa-miR-323a-3p and hsa-miR-20b-5p. In particular, the sensitivity and specificity for hsa-miR-323a-3p and hsa-miR-20b-5p were 100% and 100% (AUC (area under the curve) = 1). For hsa-miR-101-3p the sensitivity was 100% and the specificity was 40% (AUC = 0.8625). However, the AUCs of all analyzed miRNAs were statistically significant ($p < 0.05$).

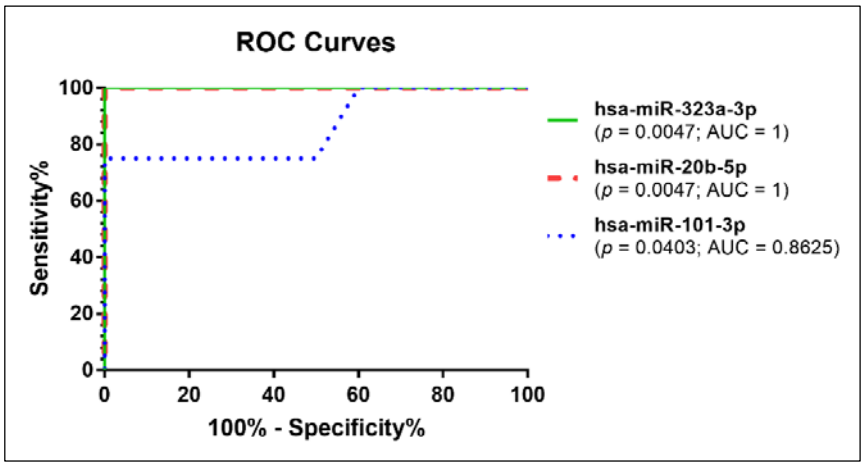


Figure 10. ROC curves demonstrated the diagnostic value of hsa-miR-323a-3p (100% sensitivity and 100% specificity), hsa-miR-20b-5p (100% sensitivity and 100% specificity), and hsa-miR-101-3p (100% sensitivity and 40% specificity).

Overall, these further results obtained in clinical samples revealed high specificity and sensitivity rates for both hsa-miR-323a-3p and hsa-miR-20b-5p, which thus acquire a diagnostic value for malignant mesothelioma.

4.5 *In silico* interaction between miRNAs and asbestos exposure, tumor stage, and patient survival

The Shapiro-Wilk normality test showed that the expression levels of the three miRNAs contained in the TCGA - MESO database have a normal distribution.

The analysis of miRNAs expression levels according to the asbestos exposure data contained in the TCGA - MESO database revealed that the expression levels of hsa-miR-323a-3p, hsa-miR-101-3p and hsa-miR-20b-5p did not change significantly in malignant mesothelioma patients exposed and not exposed to asbestos fibers (Figure 11).

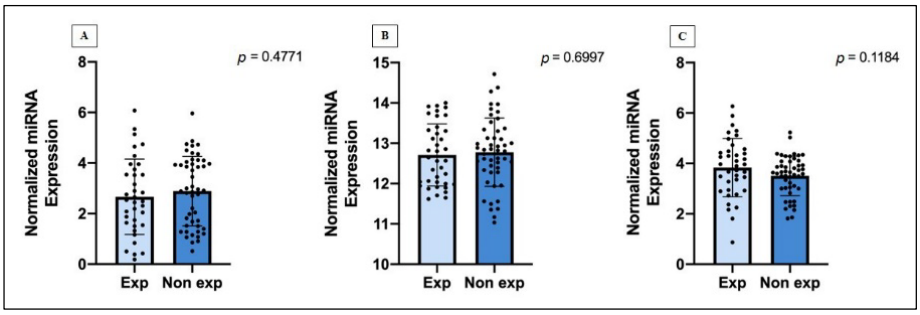


Figure 11. Normalized miRNAs expression in MM cases exposed and not exposed to asbestos according to: A) hsa-miR-323a-3p; B) hsa-miR-101-3p; C) hsa-miR-20b-5p.

The analysis of miRNAs expression levels according to the clinic-pathological data contained in the TCGA-MESO database showed that the expression levels of hsa-miR-323a-3p, hsa-miR-101-3p, and hsa-miR-20b-5p did not change significantly in malignant mesothelioma patients with different tumor stages (Figure 12).

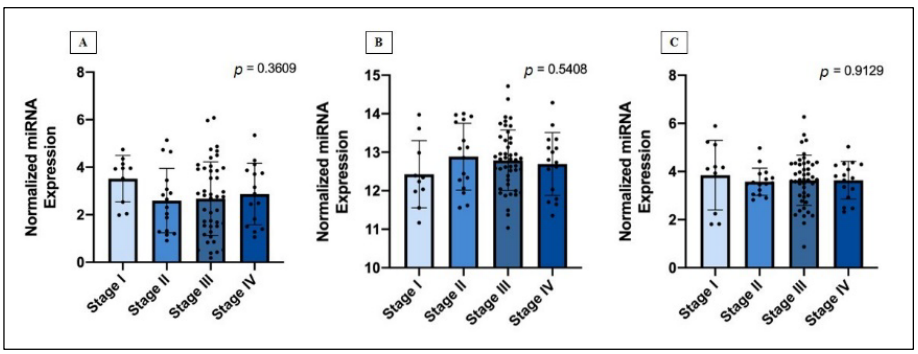


Figure 12. Normalized miRNAs expression in different MM stages according to: A) hsa-miR-323a-3p; B) hsa-miR-101-3p; C) hsa-miR-20b-5p.

Finally, considering the median overall survival (OS), the disease-specific survival (DSS), and the progression-free interval (PFI) between high and low miRNAs expression, significance for hsa-miR-101-3p ($p < 0.0001$) was shown. In particular, there was an association of high hsa-miR-101-3p expression and increased OS time (Figure 13A), DSS time (Figure 13B), and PFI time (Figure 13C). Thus, i*n silico* results showed a potential prognostic role of hsa-miR-101-3p due to a significant association of its higher expression and increased OS of malignant mesothelioma patients. On the contrary, hsa-miR-323a-3p and hsa-miR-20b-5p did not show significant results in malignant mesothelioma patients' survival.

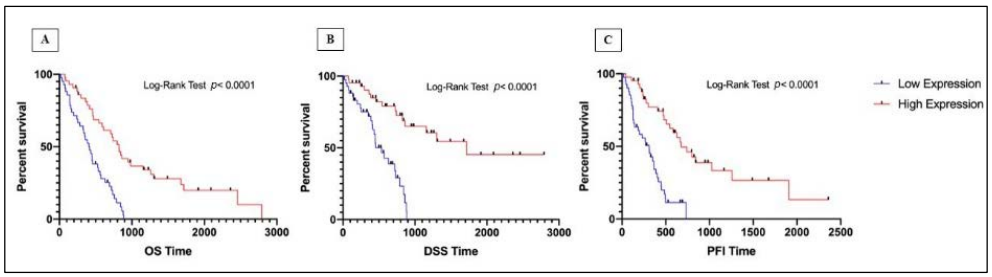


Figure 13. Kaplan-Meier survival curve of hsa-miR-101-3p expression in MM patients according to: A) OS Time; B) DSS Time; C) PFI Time.

4.6 *In silico* interaction between miRNAs and main genes involved in malignant mesothelioma

Gene target analysis performed using the bioinformatics tool mirDIP showed the level of interaction of the three computationally identified miRNAs with the main altered or mutated gene in malignant mesothelioma.

We took into account 20 different genes obtained from COSMIC and the mirDIP analysis revealed that the three evaluated miRNAs were able to interact with all genes involved in MPM with high levels of intensity. These genes were: BAP1 (24%), NF2 (18%), TP53 (12%), SETD2 (7%), LATS2 (5%), FBXW7 (2%), DDX3X (3%), EGFR (1%), SF3B1 (2%), PBRM1 (2%), KRAS (1%), PIK3CA (1%), CTNNB1 (1%), CREBBP (2%), NSD1 (2%), ZFHX3 (2%), APC (1%), LATS1 (2%), LRP1B (2%), BRAF (1%). In particular, for each miRNA the intensity of interaction was reported to be from medium to very high specificity. All miRNAs showed very high and high interaction levels with at least 50% of the genes analyzed, suggesting a potential role of these miRNAs in the

development of MPM. According to this analysis, hsa-miR-20b-5p showed very high and high interaction levels with 85% of the genes mutated in MPM. The genes linked with higher levels of intensity by miRNAs were found to be PBRM1 and ZFHX3, which showed in all cases very high levels of interaction. In addition, the KRAS gene showed very high levels of interaction with hsa-miR-323a-3p and hsa-miR-20b-5p and high levels of interaction with hsa-miR-101-3p. In opposition, the genes associated with medium levels of intensity for all miRNAs were found to be BAP1 and BRAF. None of the genes analyzed showed low levels of interaction intensity with the miRNAs evaluated (Figure 14).

Top 20 genes	miRNAs	Intensity of interaction
BAP1	hsa-miR-323a-3p	Medium
BAP1	hsa-miR-101-3p	Medium
BAP1	hsa-miR-20b-5p	Medium
NF2	hsa-miR-323a-3p	Medium
NF2	hsa-miR-101-3p	Medium
NF2	hsa-miR-20b-5p	High
TP53	hsa-miR-323a-3p	Medium
TP53	hsa-miR-101-3p	Medium
TP53	hsa-miR-20b-5p	High
SETD2	hsa-miR-323a-3p	High
SETD2	hsa-miR-101-3p	High
SETD2	hsa-miR-20b-5p	Very High
LATS2	hsa-miR-323a-3p	High
LATS2	hsa-miR-101-3p	Medium
LATS2	hsa-miR-20b-5p	High
FBXW7	hsa-miR-323a-3p	Medium
FBXW7	hsa-miR-101-3p	Very High
FBXW7	hsa-miR-20b-5p	Very High
DDX3X	hsa-miR-323a-3p	Very High
DDX3X	hsa-miR-101-3p	Very High
DDX3X	hsa-miR-20b-5p	High
EGFR	hsa-miR-323a-3p	Medium
EGFR	hsa-miR-101-3p	High
EGFR	hsa-miR-20b-5p	Very High
SF3B1	hsa-miR-323a-3p	High
SF3B1	hsa-miR-101-3p	High
SF3B1	hsa-miR-20b-5p	Medium
PBRM1	hsa-miR-323a-3p	Very High
PBRM1	hsa-miR-101-3p	Very High
PBRM1	hsa-miR-20b-5p	Very High
KRAS	hsa-miR-323a-3p	Very High
KRAS	hsa-miR-101-3p	High
KRAS	hsa-miR-20b-5p	Very High
PIK3CA	hsa-miR-323a-3p	High
PIK3CA	hsa-miR-101-3p	Medium
PIK3CA	hsa-miR-20b-5p	High
CTNNB1	hsa-miR-323a-3p	Medium
CTNNB1	hsa-miR-101-3p	High
CTNNB1	hsa-miR-20b-5p	High
CREBBP	hsa-miR-323a-3p	High
CREBBP	hsa-miR-101-3p	Medium
CREBBP	hsa-miR-20b-5p	High
NSD1	hsa-miR-323a-3p	Medium
NSD1	hsa-miR-101-3p	Very High
NSD1	hsa-miR-20b-5p	High
ZFHX3	hsa-miR-323a-3p	Very High
ZFHX3	hsa-miR-101-3p	Very High
ZFHX3	hsa-miR-20b-5p	Very High
APC	hsa-miR-323a-3p	Medium
APC	hsa-miR-101-3p	Very High
APC	hsa-miR-20b-5p	Very High
LATS1	hsa-miR-323a-3p	Medium
LATS1	hsa-miR-101-3p	Medium
LATS1	hsa-miR-20b-5p	High
LRP1B	hsa-miR-323a-3p	High
LRP1B	hsa-miR-101-3p	Medium
LRP1B	hsa-miR-20b-5p	Very High
BRAF	hsa-miR-323a-3p	Medium
BRAF	hsa-miR-101-3p	Medium
BRAF	hsa-miR-20b-5p	Medium

	Medium	High	Very High
hsa-miR-323a-3p	50%	30%	20%
hsa-miR-101-3p	45%	25%	30%
hsa-miR-20b-5p	15%	45%	40%

Figure 14. Interaction between selected miRNAs and main altered genes in MPM by mirDIP gene target analysis. For each miRNA the level of interaction with the 20 genes involved in MPM is reported. The intensity of interaction is highlighted with a color scale ranging from yellow (medium interaction) to red (very high interaction).

According to this analysis, these three miRNAs can target and modulate both tumor suppressor and oncogene genes playing a potentially key role in tumor cell development.

5. DISCUSSION

MPM is still today a highly aggressive neoplasm characterized by high mortality rates and poor therapeutic options. Despite the advancement of cancer pharmacological treatments ameliorate the therapeutic strategies for MPM, still today a significant fraction of MPM patients do not benefit from treatments mainly because of the lack of effective diagnostic biomarkers and the consecutive late diagnosis resulting in a 5-years survival rate of less than 5% (Lagniau et al., 2017). To date, there are no diagnostic tools with high sensitivity and specificity that can be used to perform an early diagnosis of MPM in asymptomatic people. Several studies reported a high incidence of malignant mesothelioma due to environmental exposure of different asbestiform fibers including FE, an amphibole site in Biancavilla (Sicily, Italy), classified by the IARC as definitely carcinogenic to humans (Group 1) (Grosse et al., 2014; IARC, 1987). Many biomarkers have been proposed for the screening and diagnosis of MPM in exposed subjects, but without results traslate to the clinical practice.

It is necessary to have valid biomarkers for this pathology because to date the overriding aim with MPM is to maintain quality of life and allow patients to live a meaningful and dignified life.

In this context, different studies have demonstrated a strong impact of epigenetic alterations on the development of malignant mesothelioma, in particular the miRNAs expression profiling is one of the most studied (Benjeamin et al., 2010; Gee et al., 2010; Santarelli et al., 2011, 2015; Kirschner et al., 2012; Weber et al., 2012, 2014; Reid et al., 2013; Andersen et al., 2014; Cioce et al., 2014; Ramírez-Salazar et al., 2014; Cappellesso et al., 2016; Micolucci et al. 2016). The scientific community has revealed that several miRNAs are involved in deregulation and in all molecular mechanisms associated with MPM development (De Santi et al., 2017; Martínez-Rivera et al., 2018). Therefore, it is essential to know the molecular mechanisms and specific targets of the

different miRNAs in order to develop personalized anticancer therapies or evaluate the expression levels of miRNAs for both diagnostic and prognostic purposes.

Recently, different bioinformatics and computational tools have been developed to fasten the analysis of omics data collected on public databases. Through the use of these tools, it is possible to predict the miRNAs expression in specific pathologies and to identify potential biomarkers or altered genes responsible for the development and progression of cancers.

Starting from these observations, through the adoption of both *in silico* and *in vitro* evaluations the pathogenetic role of miRNAs expression profiling was investigated in malignant mesothelioma induced by FE fibers (Filetti et al., 2020b). The data showed consistency between the *in silico* results and the expression levels of hsa-miR-323a-3p and hsa-miR-101-3p (up-regulation and down-regulation respectively in MPM cases compared to controls). In particular, the hsa-miR-323a-3p expression in MeT-5A cells increased until the dose of 50 μg/ml of FE fibers and after it suffered a decrease. It is reasonable to think that this miRNA is secreted and its expression starts to increase in the supernatant at a concentration higher of 50 μg/ml of FE fibers (100 μg/ml). The expression trend of hsa-miR-101–3p was uneven due to the effect of the treatments with FE fibers, but also in this case the dose of 50 μg/ml of FE fibers caused a reversal of the expression of the miRNA. In both biological matrices, hsa-miR-20b-5p showed a no significant dose-dependent increase in MeT-5A cell line, but a significant up-regulation in JU77 cells vs. control and treated MeT-5A, in contrast with the *in silico* analysis that showed a downregulation in MPM cases compared to controls.

Finally, the *in silico* and *in vitro* results were further partially confirmed on FFPE MPM samples *vs.* nonmalignant pleura tissue (Filetti et al., 2020c). In particular, hsa-miR-323a-3p, hsa-miR-101-3p, and hsa-miR-20b-5p tested in MPM tissue showed a down-regulation compared to controls. The translational data obtained for hsa-miR-101-3p were totally in accordance with our previous computational study which showed a significant downregulation in MPM samples compared to controls. Furthermore, this miRNA showed a prognostic value for MPM because

the *in silico* analyses performed here demonstrated a significant association between hsa-miR-101-3p high expression levels and increased OS. In line with our research, a study by Ramírez-Salazar et al. (2014) demonstrated that the targets of the down-regulated hsa-miR-101-3p in MPM were significantly enriched in pathways in cancer, including the signaling molecule mitogen-activated protein kinase 1 (MAPK1), the transcription factor v-ets erythroblastosis virus E26 oncogene homolog 1 (ETS1), and the mesenchymal transition-associated molecule frizzled class receptor 4 (FZD4). Multiple down-regulated miRNAs targeted multiple common oncogenic genes, as their reduced expression could increase the expression of these genes and consequently promote tumorigenesis (Ramírez-Salazar et al., 2014). This explains why an increase in hsa-miR-101-3p levels increases the survival of MPM patients, in which the high expression of hsa-miR-101-3p decreases the expression of these oncogenic genes and consequently counteracts tumorigenesis. Interestingly, although hsa-miR-20b-5p showed higher interaction levels with the most altered genes in MPM, the expression levels of this miRNA were not associated with the OS or PFS of patients. This phenomenon may be related to the multiple binding activities of miRNAs exerted towards different genes. Our results may support the hypothesis that miRNAs reach their biological impact by targeting multiple genes with similar biological effects. Therefore, although hsa-miR-20b-5p showed high levels of interaction with the genes analyzed, hsamiR-101-3p can activate a more complex network of genes involved in the progression of cancers and the survival of patients, as demonstrated in other studies (Ramírez-Salazar et al., 2014; Lu et al., 2018; Yao et al., 2018; Hu et al., 2018). It is noteworthy that the results obtained for hsa-miR-323a-3p and hsa-miR-20b-5p were opposite to those obtained in our previous *in silico* and *in vitro* analyses, however, for these miRNAs ROC analysis revealed high sensitivity and specificity in correctly distinguishing MPM and normal samples.

In addition, the computational analyses to establish the functional role of these three miRNAs in MPM pathogenesis has shown that these miRNAs can targete and modulate both tumor suppressor and oncogene genes playing a potentially key role in cancer cell development. In particular, the genes targeted with higher

levels of interaction by the selected miRNAs were PBRM1, ZFHX3, and KRAS. The alteration of the expression levels of PBRM1 was associated with the development of both renal cell carcinoma and malignant mesothelioma (Ross et al., 2018). Recent evidence suggests that PBRM1 genomic alterations are strongly associated with neoantigen production and responsiveness to immune checkpoint inhibitors (ICIs), therefore, the analysis of the expression levels and gene mutation affecting this gene may be predictive for the therapeutic choice in MM. ZFHX3, named also ATBF1, has been widely associated with the development of lung cancer when dysregulated. In addition, the analysis of ZFHX3 is also a positive prognostic biomarker for patients treated with ICIs (Zhang et al., 2021). Similarly, other studies have demonstrated the predictive role of KRAS alteration for the treatment of lung cancer (Kim et al., 2017). In addition, a recent study also established an important role of KRAS mutation in the development of malignant mesothelioma (Marazioti et al., 2020).

On these bases, the results here obtained encourage the adoption of ddPCR investigations as well as *in silico* analysis to assess the functional role of the selected miRNAs and their predictive value for MPM patients' diagnosis and prognosis. However, further validation experiments performed on a wide cohort of MPM patients and normal individuals are mandatory to confirm these preliminary results.

6. CONCLUSIONS

Asbestos and asbestiform fibers like FE continue to cause a high health concern due to the long latency period of related diseases including malignant mesothelioma.

Early detection of circulating tumor biomarkers represents one of the most promising strategies to enhance the survival of MPM patients by increasing treatment efficiency. Our goal is the validation of these results in a subset of patients chronically exposed to FE using liquid biopsy, to provide a minimally invasive screening tool for the secondary prevention of MPM. Besides these preliminary data, further studies will be designed for the validation of "mesomiRs" with diagnostic potential, alone or in combination with other protein biomarkers, to test their clinical role in high-risk individuals.

The computational and molecular results obtained in the present study confirm the high predictive value of the *in silico* analysis performed and the sensitivity of ddPCR assay which can also be used for low concentrations of circulating microRNAs potentially useful in malignant mesothelioma management.

In conclusion, the results here obtained pave the way for the development of new strategies for the identification of diagnostic and prognostic epigenetic biomarkers for malignant mesothelioma.

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