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Dysregulated circulating microRNAs as potential new biomarkers of asbestos exposure and malignant pleural mesothelioma

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1.INTRODUCTION

1.1. MALIGNANT PLEURAL MESOTHELIOMA

1.1.1 Malignant Pleura Mesothelioma (MPM): an overview

Malignant mesothelioma (MM) is an aggressive tumor, that originates from the inner lining of the body's serous cavities, such as pleura (pleural mesothelioma), peritoneum (peritoneal mesothelioma), pericardium (pericardial mesothelioma), and vaginal tunic of the testicle (tunica vaginalis mesothelioma) [1].

Malignant pleural mesothelioma (MPM) is the most common type among different mesotheliomas, accounting for 80 –90% of this cancer. MPM, mostly associated to asbestos exposure, remains one of the most lethal cancers [2]. The median overall survival rates (OS) range from 4 to 13 months for untreated patients and from 6 to 18 months for treated patients, whereas only 7% of patients are still alive at five years from the diagnosis [3], [4].

The majority of patients affected by MPM are 60 years old at manifestation, showing peaks of the age-specific incidence at 80–84 years old for males and 75–79 years old for females. Considering the occupational asbestos exposure, the prevalence is higher among males compared to females, with a male–female ratio of approximately 4:1–8:1 [5].

MPM is a tumor characterized by a long latency period between the asbestos exposure and the appearance of the malignancy, up to 50 years and over. The per capita consumption of asbestos in different countries of the world was analyzed demonstrating how the mortality curve for mesothelioma significantly reflects the consumption trend after a long latency period [4], [6].

Published data estimate an average of 14,200 new cases of MPM worldwide each year [4], [7] and the global MPM incidence is increasing, with an estimated peak in 2025-2030 [8], based on high exposure to asbestos in past years. In some Countries, such as India, Brazil and Russia, the use of the asbestos remains unregulated [5]. As a consequence, MPM will continue to represent a significant health concern even after the peak incidence mentioned above.

MPM is classified as an occupational disease since asbestos exposure occurs mainly in the workplace.

However, para-occupational exposure can occur: exposure to asbestos workers clothes, asbestos-containing commercial products, asbestos-containing buildings [9], [10]. Several epidemiological studies demonstrated an increased incidence of MPM cases among subjects, including women, with low levels or no history of occupational asbestos exposure [11], [12]. In addition, asbestos as a natural mineral [13] is present in the soils and/or rocks of specific geographical regions, while asbestos composed manufactures are becoming wreaks, often abandoned in the environment, overall contributing to the asbestos pollution. These data indicate that asbestos is an environmental contaminant, too, and consequently highlight the seriousness of asbestos and MPM issues for public health [4], [14], [15].

1.1.2 MPM risk factors

1.1.2.1 Asbestos

The most of MPM cases (>80%) is due to the asbestos exposure [16]. Asbestos refers to a group of natural mineral silicate fibers with physical properties causing diseases [15], [17], [18]. Among asbestos fibers identified in nature there are actinolite, amosite, anthophyllite, crocidolite, tremolite, and chrysotile. The International Agency for Research on Cancer (IARC) classified all types of asbestos fibers as certain carcinogens for humans (IARC 1973. *Some Inorganic and Organometallic Compounds*; IARC *Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man*; IARC: Lyon, France, 1973; Volume 2) [4]. The carcinogenicity, and in general the ability of asbestos fibers to interact with biological systems, are dependent on several fiber parameters including fiber dimensions. Specifically, the fibers considered the most damaging are those with an average diameter of 3 μm and a length of 5-100 μm [19]. Considering that asbestos is a powerful carcinogenic agent, there is no safety "threshold" below which there is no risk [20]. The analysis of data provided by epidemiological studies has shown that the risk of MPM correlates with the increase of the asbestos

exposure, indicating a proportional relationship between cumulative dose and MPM frequency [4], [6].

Asbestos has been used all over the world, whereas its use dates back to the time of the ancient Persians, Greeks, and Romans [4]. In particular the increase in asbestos global production is constant from the post-world war II period to the mid-seventies, a period in which asbestos reached its peak with more than 4.5 million tons/year produced [21].

The two historical epidemiological studies that established with certainty the causal role of asbestos in the onset of pulmonary carcinoma and MPM were, respectively, Doll's in 1955 and Wagner's in the 1960s [22], [23]. The degree of exposure associated with the development of MPM is relatively lower compared to that which causes lung fibrosis and lung cancer [24]. In a 2012 monograph of IARC, asbestos was confirmed as the only certain risk factor for mesothelioma in a series of target organs including the pleura, the peritoneum, the pericardium, and the vaginal tunic of the testicle [25]. However, nowadays asbestos is still used in several countries [5]. Italy was both an importer and a producer. Until the end of the 1980s, Italy was the second largest European fiber asbestos producer after the Soviet Union and the largest in the European community [17], [26]. The use of asbestos in Italy in the last century has grown to around 220,000–240,000 tons/year, reached in the second half of the 1970s. In the 1980s, use gradually decreased, although later and more slowly than in other European countries. Finally, asbestos was banned in 1992 [4]. The Italian law n. 257 of 27 March 1992 declared the "cessation of the use of asbestos", and in particular the ban on the extraction, import, export, marketing, and asbestos containing products. However, the law did not prohibit indirect use, and, therefore, several million tons of compact materials containing asbestos and many tons of friable asbestos are still present in many contaminated sites throughout the Italian territory [26]. Data provided by the Italian National Research Council (32 million tons of asbestos cement still to be reclaimed) and ISPRA (Higher Institute for Environmental Protection and Research), in the 2011 Yearbook, reports that waste containing asbestos produced in Italy in 2009 amounted to about 380,000 tons (Stato dell'arte e prospettive in materia di contrasto alle patologie asbesto-correlate.

Quad. Del Minist. Della Salute. 2012, 15, 2038–5293. Available online: http://www.salute.gov.it/imgs/C_17_pubblicazioni_2570_allegato.pdf). If the estimates demonstrate to be exact based on the asbestos still present in the national territory (32 million tons) and the annual amount removed (380,000 tons), the disposal process could last another 85 years [4]. The directive 2003/18/CE of the European Parliament and of the Council of 27 March 2003 provides for the obligation to completely stop the use of asbestos by 15 April 2006. However, despite this directive and the scientific evidences providing a clear and strong association between asbestos and MPM, many western countries, and newly industrializing economies, are still using asbestos [5], [17], [27], [28]. Recent data, from the Asbestos Ban Secretariat (2019) (http://www.ibasecretariat.org/ Updated 16 May 2019), related to 2015, reported that among the largest asbestos producers there were Russia (650,000 tons), Brazil (270,000 tons) and China (210,000 tons); while among the top five users there were India (370,000 tons) , China (287,000 tons), Brazil (163,000 tons), Russia (124,000 tons) and Indonesia (120,000 tons). The World Health Organization estimates that 125 million people annually around the world are exposed to asbestos [27]. Moreover, even in countries where asbestos was banned early, the incidence of MPM and other asbestos-related diseases is still increasing [7], [18], [29]. After the cessation of asbestos use at work, the public health risk is caused by the presence of a large amount of materials containing asbestos in a friable matrix, in civil and industrial buildings, as well as in transport sector facilities. In addition, there is still a considerable quantity of material containing asbestos in a compact matrix whose progressive deterioration can cause the release of fibers, representing a risk to public health. Overall, asbestos is extremely widespread and can be hidden in common living areas and in everyday objects, such as ironing board covers, curtains, cardboard and toys [4], [9].

1.1.2.2 Additional risk factors

In addition to asbestos exposure, other environmental interactions may increase the risk of developing MPM [7]. In Italy there are asbestosimic fibers, such as fluoro-edenite, naturally present in the area

of Etna, and balangeroite, found in Balangero mine (Turin), which are able of inducing mesothelioma [15]. Moreover, some studies have reported cases of MPM in individuals exposed to erionite, one of the most potent carcinogenic mineral fiber, present naturally in Cappadocia, Turkey, and employed for building. Although the high level of risk, erionite is not regulated, because it is not defined as asbestos [5], [7], [30].

Potential cofactors for MPM development are also exposure to other elongated mineral particles such as synthetic materials (ceramics, nanoparticles), ionizing radiation, and Simian Virus 40 (SV40) infections [7], [13], [31], [32]. Actually, the link between SV40 and malignant mesothelioma is controversial. On one hand, studies *in vitro* and *in vivo*, along with the detection of viral sequences in human samples, have shown an association between MPM and the oncogenic SV40. SV40 large T antigen has been shown to inactivate the tumor suppressor gene products Rb and p53, suggesting the possibility that asbestos and SV40 could act as co-carcinogens in MPM [33]–[35]. In addition, recent immunological investigations detected a higher prevalence of SV40 antibodies in sera of MPM patients in comparison with healthy blood donors, strengthening the association between MPM and SV40 [36], [37]. On the other hand, no association between SV40 and MM was found in some studies, e.g., no SV40 viral sequences were detected in paraffin block samples from both Iranian [38] and Korean [39] MM patients. Moreover, a recent high throughput study by Alchami et colleagues [40], revealed the complete absence of SV40 Tag mRNA in a large series of mesothelioma cases evaluated in the United Kingdom (UK) population. The negative findings of this investigation do not support a role of SV40 in the progression of human MPM in the UK, even if they do not exclude its role in the initiation of this tumor. Overall, the association between SV40 and MPM remains to be elucidated.

It is worth recalling that just a small percentage of asbestos exposed subjects develop MPM, suggesting the role of individual genetic susceptibility in the disease onset [16], [41], [42]. Family clusters in blood relatives have been described [43], [44] and a very high mesothelioma risk was discovered in family members who are heterozygous for inherited/germline BAP1 mutations [45]. Moreover, many studies have shown that polymorphisms in the genes involved in xenobiotic and

oxidative metabolism or in DNA repair processes may play an important role in the etiology and pathogenesis of MPM [46].

1.1.3 Molecular mechanisms of the MPM onset

The Cancer Genome Atlas (TCGA), and specifically the TCGA-MESO which includes 87 MPM cases with comprehensive multi-omic analyses, that are publicly available [47], has been a valuable resource for MPM research. This and other high-throughput approaches allowed to gain insights in the complex MPM biology. However, despite these global efforts, the mechanisms of MPM development after asbestos exposure have not been fully elucidated, yet. Several hypotheses have been formulated based on experimental data and investigations of clinical samples [48]–[54]. It was demonstrated that mesothelial cells are very sensitive to asbestos fibers, which, after being endocitated by the cell, interact directly with chromatin, destabilizing the structure and causing mutations on tumor suppressor genes and oncogenes [55], [56]. The accumulation of numerous chromosomal deletions in most MPMs suggests a multistep process of tumorigenesis, characterized by the loss and/or inactivation of multiple tumor suppressor genes [46]. Cytogenetic and loss of heterozygosity analyses of MPMs have demonstrated frequent deletions of specific sites within chromosome arms 1p, 3p, 6q, 9p, 13q, 15q, and 22q. Tumor suppressor genes within some of these regions, i.e. BRCA1-associated protein 1 (BAP1) at 3p21, p16/CDKN2A-p14ARF at 9p21, and NF2 at 22q12, are frequently altered in MPMs [50], [57]–[59]. Furthermore, a comprehensive integrative analysis of 74 cases of MPM confirmed the high frequency of BAP1 inactivation, as well as recurrent inactivating alterations in CDKN2A, NF2, TP53, LATS2 and SETD2, so suggesting that MPM is driven primarily by inactivation of tumor suppressor genes [60]. In particular, BAP1 germline and acquired mutations are the most common alterations observed in mesothelioma [57]. Among the investigated functions of BAP1, it promotes the maintenance of genomic stability and modulates calcium (Ca2+) release through binding and deubiquitination of the type 3 inositol-1,4,5-

trisphosphate receptor (IP3R3) [61]. Consequently, low levels of BAP1 promote both genomic instability and reduced cell death, thus favoring malignant transformation [57].

Numerous evidences indicated that the chronic inflammatory processes, related to asbestos or other fibers, are mainly responsible for carcinogenesis. Indeed, because of their physical characteristics, asbestos fibers remain in the lung, regional lymph nodes, and pleural cavity for months or years. Asbestos fibers trapped between the pleural layers and the wall of the chest cavity induce chronic inflammation, driven by the production of reactive oxygen/nitrogen species [62], as well as the release of High Mobility Group Box Protein-1 (HMGB1), [63] and related inflammasome activation. Consequently, nuclear factor kappalight-chain–enhancer of activated B cells (NF-κB) and the phosphatidylinositol 3-kinase (PI3K) pathways are activated in mesothelial cells [64]–[66]. These events overall promote the growth of aberrant mesothelial cells [7], [30]. In addition, asbestos fibers are strongly immunosuppressive [24]. Indeed, asbestos is a mineral silicate and it was reported that exposure to silica (SiO_2) causes dysregulation of immune system. Specifically, cellular and molecular features of immunocompetent cells can be altered by chronic and recurrent encounters with asbestos fibers, all of which eventually lead to tumor immunity decrease. These cellular events limit and / or inhibit the immune system in controlling and eliminating transformed cells, thus promoting the tumor development [24], [49] (Figure 1).

Moreover, some studies suggested a potential pro-tumorigenic role of hedgehog (Hh) signaling in a subgroup of MPMs [67]–[69]. Indeed, a high through-put analysis of MPM patients showed that the Hh pathway is active in a subset of patients and both Hh ligand-dependent and ligand-independent effects promote MPM cell growth in experimental models [70]. In this context, it was reported a cross talk between tumor cells and stroma, specifically Hh ligands produced by tumor cells lead to Gli-1 mediated “M2-polarization” of macrophages [71], which is associated with immunosuppression and pro-tumorigenic activity [70].

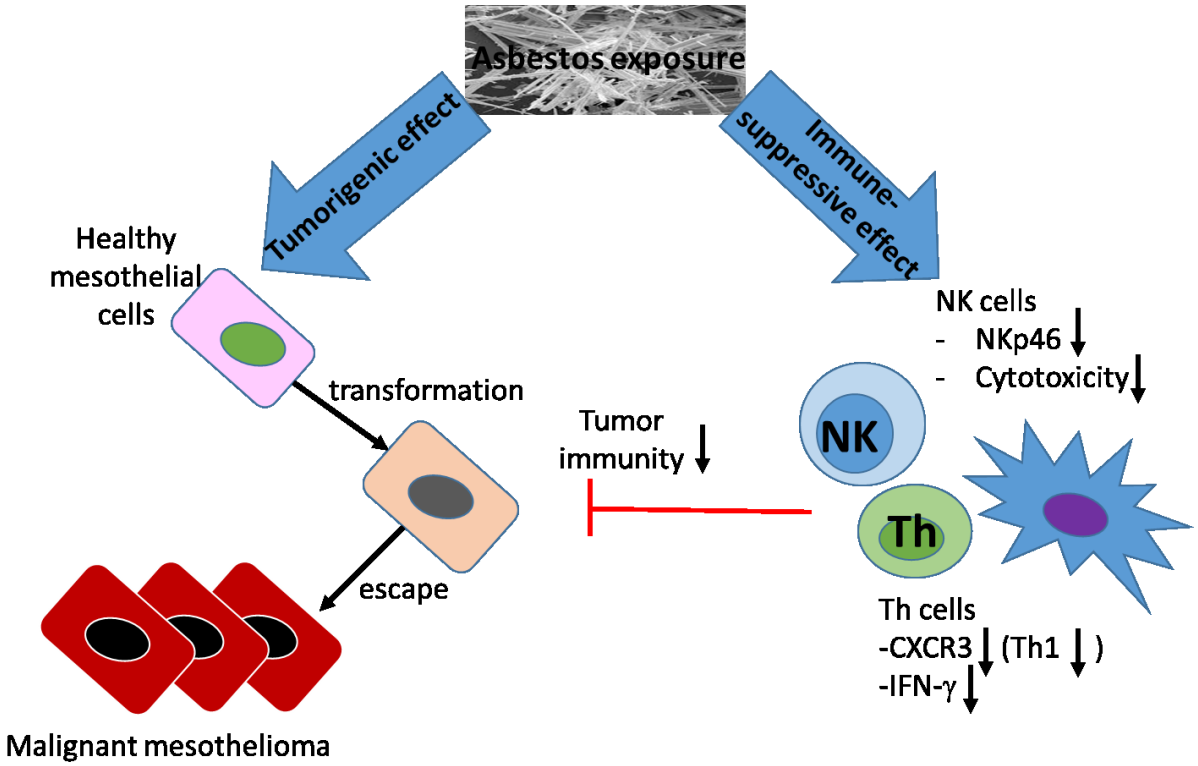

***Figure 1**: Tumorigenic and immune-suppressive effects of asbestos exposure. Adapted from* [49]

1.1.4 MPM: histology, diagnosis and prognosis

MPM is histologically heterogeneous [72]. MPM tumors are divided into three main histological subtypes: epithelioid, sarcomatoid and biphasic (or mixed) [73]. It depends both on predominant cellular component and different biological behavior. However, a recent large-scale study, characterizing most genetic alterations, identified four distinct molecular profiles in MPM: sarcomatoid, epithelioid (which actually include mostly pure epithelioid histotype), biphasic-epithelioid and biphasic-sarcomatoid [58]. Epithelioid mesothelioma, the most common form (50–70% of cases), is characterized by polygonal, oval, or cuboidal cells similar to carcinomas; the sarcomatoid type (10–20%), with a spindle cell morphology is similar to sarcomas; while the biphasic (30%) is composed of both epithelioid and sarcomatoid forms, in different proportion, within the same tumor [72].

The median survival varies significantly among the different histological subtypes. Patients affected by sarcomatoid MPM have particularly poor outcomes compared to patients with epithelioid histology. Indeed, sarcomatoid subtype is more aggressive, whereas the epithelioid subtype is high sensitive and responds better to chemotherapy, resulting in a longer survival than the sarcomatoid or biphasic subtypes [74]. The correct identification of the MPM histological subtype facilitates the differential diagnosis, influencing subsequent prognosis and therapeutic decisions. However, classification of histological subtypes is often challenging [5].

In the early phase of MPM, clinical signs are absent or not specific. For this reason, MPM is diagnosed in the advanced and incurable stages. The histopathological diagnosis is very complex [75]. Cytological examination of cells detectable in pleural fluids can be performed. However, the reported sensitivity of the cytological diagnosis of mesothelioma is highly variable and only 60% of true positive cases can be identified using this technique [76]–[79]. The cytopathological diagnosis should be confirmed by thoracoscopy-biopsy whenever possible [7]. The confirmatory test is based on qualitative analysis of markers in pleural tissue by immunohistochemical (IHC) staining. BAP1 IHC and fluorescence in situ hybridization or other analyses to detect homozygous deletion of the

CDKN2A (p16) are very useful to discriminate between benign mesothelial hyperplasia and mesothelioma [80], [81]. However, this approach requires pleural tissue samples obtained by invasive techniques [75], [82].

To evaluate the clinical staging and prognosis there are several methods, but to date there is no universal standard. The main imaging modality is computed tomography (CT) of the chest and upper abdomen. Further methods, including positron emission tomography (PET), magnetic resonance imaging (MRI) and endobronchial ultrasound (EBUS) with lymph node biopsies, can provide additional important diagnostic and prognostic information, helping to assess the extent of disease [83], [84]. Each method has its advantages and limitations, consequently their combined use is fundamental for determining the most appropriate treatment options for MPM patients [7], [85], [86]. Overall, although there have been improvements in diagnostic and prognostic tools for MPM, to date there is still a lack of adequately specific and sensitive biomarkers.

1.1.5 Biomarkers in asbestos exposure and MPM

A highly referenced definition of biological markers (biomarkers) was expressed by Hulka and colleagues [87]: "*cellular*, *biochemical or molecular alterations that are measurable in biological media such as human tissues*, *cells*, *or fluids*". Biomarkers potentially track all steps from the exposure to etiological factors to the complete evolution of a disease. Nowadays biomarkers are used in fields of clinical investigation (biomarkers of early diagnosis, prognosis, treatment response, etc.), as well as of prevention (biomarkers of exposure, risk prediction, etc.) [88]. Considering the prevention purpose, it is fundamental that people at high risk for cancer can be targeted through screening programs and health medical surveillance. Due to the highly carcinogenic potential of asbestos fibers, asbestos exposed individuals are considered high risk subjects and follow-up programs for asbestos exposed population should aim at the early diagnosis of asbestos-related lung malignancies, in particular MPM, which is diagnosed at an advanced and incurable stage.

According to the ATS guidelines, people with a minimal 10-year long history of asbestos exposure even with no apparent disease, should undergo a preventive follow-up with chest films and pulmonary function respiratory tests every 3 to 5 years [89]. Many asbestos-exposed workers around the world have been periodically in the past and are currently followed-up by chest X-ray and computed tomography (CT) examinations, however these methods resulted inadequate for an effective thoracic cancer diagnosis. Guglielmi et colleagues [90], reported an explicative example of health surveillance applied to a high risk for asbestos exposure population. This health surveillance protocol is based on a detailed asbestos-related work history data collection, aimed at a risk stratification, respiratory function tests, thoracic X-ray and/or low-dose CT. The frequency of radiological exams should be set on a risk/benefit balance, considering both intrinsic risk for health caused by imaging. Along with these exams a research program for evaluating the role of some blood-based biomarkers is performed. Among these biomarkers, aiming at MPM early detection, prediction of the risk or of the treatment effectiveness, mesothelin is one of the most investigated [91]–[94]. Mesothelin is a cell surface protein expressed by pleural, peritoneal, pericardial normal mesothelial cells. It is also highly expressed in many cancers, including malignant mesothelioma [95], [96]. Mesothelin can be shed from the cell surface and consequently it is found in blood, urine, and tumor-associated fluids. The term "soluble mesothelin-related peptides" (SMRP) refers to a number of similar proteins deriving from subsequent cleavages of cell-surface precursor. The first study of mesothelin as a potential diagnostic biomarker for mesothelioma, reported that the 87% of mesothelioma patients had increased levels in the serum compared with healthy asbestos-exposed and non-asbestos-exposed controls and individuals with other malignant or inflammatory lung and pleural diseases [97]. Moreover, SMRP has been found to be able to discriminate between epithelioid MPM histotype and controls, with a high specificity (80–85%), though with less consistent sensitivity (60–90%) [88], [98]–[100]. Some evidence was reported that mesothelin might promote tumor invasion [101], as well as resistance to certain chemotherapy drugs such as TNF-α, paclitaxel, and a combination of platinum and cyclophosphamide [88], [102]. Finally, serum mesothelin level may be useful for monitoring response

to treatment [103] and its cancer-specific expression makes mesothelin a potential target for monoclonal antibody therapy [96]. On the other hand, prospective studies have shown that monitoring serum mesothelin levels in asbestos exposed subjects, shows increased levels of this marker in only around 14% of individuals before diagnosis [104]. Moreover diagnostic value of mesothelin and SMRP may be influenced by the prevalence of specific genetic polymorphisms within the *MSLN* gene, which were found to be associated with high levels of SMRP in healthy subjects [105], [106].In addition, it is necessary to take in account that some clinical conditions such as renal failure or hypertension, body mass index, may significantly affect diagnostic ability of SMRPs in differentiating MPM from healthy people [107], [108].

Other potential biomarkers for asbestos-exposure and MPM have been investigated. Among these, 8-hydroxy-2'-deoxyguanosine (8OHdG), one of the main forms of free radical-induced oxidative lesions [109], has been widely used as biomarker for DNA damage in humans after exposure to cancer-causing agents, such as tobacco smoke, asbestos fibers, and heavy metals [110], and as factor of initiation and promotion of carcinogenesis [111]. Some investigations reported that 8OHdG content is related to grade of asbestosis and asbestos cumulative exposure [112]. Other authors showed that the high levels of 8OHdG in the circulating leukocyte DNA of asbestos workers was not correlated with possible confounding factors, so indicating that previous inhalation of asbestos fibers is the main factor responsible for the difference observed in oxidative DNA damage between asbestos workers and controls [113]. However, considering the value of 8OHdG levels as a biomarker of exposure and for predicting cancer, it should be taken into account that there is a range of variables that affect the interpretation of the data and 8OHdG measurements cannot be used to discriminate between asbestos exposed subjects with and without MPM [42].

Levels of SMRPs, DNA adduct 8OHdG, factors involved in tumor growth, including Platelet derived growth factor (PDGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and cancer progression markers such as metalloproteinases (MMP2 and MMP9) and tissue inhibitor metalloproteinases (TIMP1 and TIMP2),

were assessed in malignant mesothelioma patients, high-risk asbestos-exposed subjects, and healthy individuals. It was reported that 8OHdG, VEGF, SMRPs or a panel of the three biomarkers might be used to discriminate among the three cohorts under investigation [114].

Potential biomarkers for MPM were investigated also in tumor infiltrating lymphocytes (TILs): programmed cell-death-ligand-1 (PDL-1) and vascular endothelial growth factor receptor 2 (VEGFR-2) in TILs were reported to correlate with MPM histological subtype. Furthermore, the overexpression of the Insulin-Like Growth Factor 1 Receptor (IGF-1R) emerged as an independent favorable prognostic factor, independently of the histological subtype [115].

Other potential biomarkers have been evaluated in mesothelioma: hyaluronic acid (HA), cytokeratin fragment (CYFRA 21-1), carcinoembryonic antigen (CEA), carbohydrate antigen 15-3 (CA 15-3), carbohydrate antigen 15-9 (CA 15-9), tissue polypeptid antigen (TPA) [116]–[118], and more recently osteopontin (OPN), fibulin 3, cancer antigen 125 and high mobility group box 1 (HMGB1). These molecules may have a prognostic value, but they are not sensitive or specific enough to be used in clinic practice [42], [119], [120]. On the contrary, the study on ecto-NOX disulfide-thiol exchanger 2 (ENOX2), as a potential MPM predictive and early detection marker is encouraging [93], [121].

Finally, it is worth to consider that insight into epigenomics is leading to the development of new biomarkers and therapeutic targets in tumor diseases. A hypermethylation pattern of serum DNA was reported for MPM and the combination of three genes (DAPK, RASSF1A, RARb) significantly correlated with survival of malignant mesothelioma affected patients [42], [122]. Moreover, different investigations demonstrated that dysregulated microRNAs, detected in both tissues and biofluids, discriminate MPM patients from health controls with significant accuracy [88]. MicroRNAs as potential MPM biomarkers are discussed in the dedicated paragraph of this thesis.

Summarizing, none of the biomarker candidates (Figure 2), which have been studied till now, is characterized by the necessary combination of sensitivity and specificity. Consequently, investigators are searching for new biomarkers and are evaluating the advantages of combining different biomarkers chosen among the most promising ones [123].

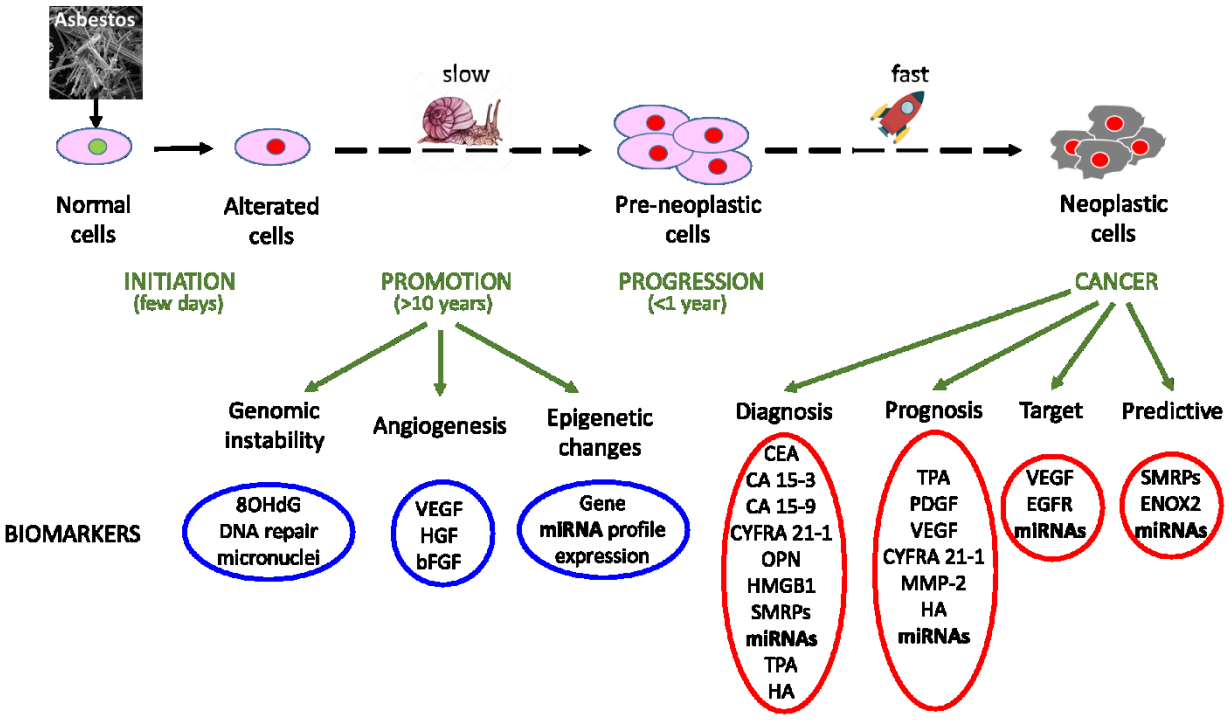

Figure 2: *Schematic presentation of some biomarkers that can be detected at the different phases of the malignant disease development, evaluated from asbestos exposure to malignant mesothelioma onset. Adapted from* [42].

1.1.6 Current therapeutic approaches to MPM

To date there is no curative treatment for MPM. Treatment options include chemotherapy, radiotherapy, surgery and targeted therapy, delivered separately or as part of multimodality treatment [5], [124]. Despite the toxic effects of chemical drugs, chemotherapy for MPM is still the main treatment modality that has been shown to improve survival in MPM, in particular in most advanced stage MPM patients, who are not candidates for surgery [5], [125], [126]. The combination of pemetrexed with cisplatin or carboplatin remains the standard first-line systemic treatment for MPM patients. However, there is an important issue in MPM chemotherapy: the lack of reliable biomarkers to identify potential responders and consequently the impossibility to predict which patients will respond to treatment. An evaluation of over 1700 patients who received pemetrexed with either cisplatin or carboplatin demonstrated low response rates of 26.3% and 21.7%, respectively [127].

Nevertheless there are still many unanswered questions with regard to chemotherapy agents in MPM, chemotherapy remains a central pillar of MPM systemic treatment and the goal today is to develop novel targeted chemotherapy drugs, to be used either alone or in combination to increase efficacy and to prevent or at least minimize the side effects [93], [128].

Surgery is controversial and limited to patients with early stage disease and good functional status. However, surgery is an essential option to help the patient relieve symptoms by reducing pain and by controlling pleural effusions [124]. Radiotherapy, relatively common for MPM, is administrated either pre- or post-operatively. Radiotherapy, in combination with other treatments or alone, is useful to control pain and limit tumor spreading. Moreover, it has been demonstrated *in vivo* that short course of high-dose non-ablative radiation could promote an antitumor immune response [129].

In the context of targeted therapy, phase I and phase II trials, which tested inhibitors of receptors with tyrosine kinase activity in MPM patients with dysregulated EGFR and VEGFR pathways, have given poor results [130]. Clinical trials are also being carried out to test effectiveness of immune checkpoint inhibitors in MPM patients. Safety of pembrolizumab, an antibody against PD-L1 (programmed cell death ligand 1), an inhibitor of immune response expressed on cancer cells, has been tested in MPM. It appears to be well tolerated, and it might confer antitumor activity in MPM, PD-L1-positive patients. However, further investigations are needed with regard to response, durability, and efficacy of this kind of immunotherapy agents [131]. A promising type of immunotherapy based on adoptive cell transfer employs chimeric antigen receptor (CAR) T cells, in which T cells are generated to recognize specific antigen receptors on cancer cells. Numerous trials are currently being explored for MPM. Specifically, phase I clinical trials for genetically modified T cells to recognize specific antigens on MPM cells, such as mesothelin and fibroblast activation protein, are being carried out [132]. However, with regard to the immune-therapeutic approach to MPM, it is necessary to take in account that MPM is a pool of independent clones that would need to be simultaneously targeted for an effective immunotherapy [133]. Preclinical studies are demonstrating that combination treatment of chemotherapy/ radiotherapy and immunotherapy with immune checkpoint inhibitors could lead to

better outcomes for MPM [129]. Summarizing, for more effective results, therapy options should include the combination of two or more different methods of treatment. However, timing, type of agent, and modality are still debated [5].

1.2 MICRORNA

1.2.1 MicroRNAs (miRNAs): biogenesis and functions

MicroRNAs (miRNAs) are small, single-stranded, noncoding RNA molecules consisting of about 20-25 nucleotides. MiRNAs negatively regulate gene expression at post-transcriptional level. Indeed, miRNAs align and bind especially to 3'UTR sequences of their mRNA targets, and initiate either mRNA degradation or translational repression, resulting in reduced protein levels [134], [135].

MiRNAs were first discovered in the nematode *Caenorhabditis elegans* in 1993 and were considered as small temporal RNAs [136], [137]. By 2001, miRNAs have been formally named and recognized as a distinct class of RNAs. To date, they have been found in most eukaryotes, including humans [134], [138]. It is predicted that miRNA account for 1-5% of the human genome and regulate at least 30% of protein-coding genes [134], [139].

MiRNAs are generated through a multi-step process: they derive from the transcription of specific sequences by RNA polymerases II (Pol II) and III (Pol III), which results in dsRNA molecules, with a secondary structure (stem-loop), known as pri-miRNAs. According to their genomic position, miRNAs can be classified into intragenic and intergenic miRNAs. Intragenic miRNAs, embedded within introns or exons of genes on the same strand, are believed to be co-regulated with their host genes by Pol II, whereas intergenic miRNAs, located between genes, are transcribed from their own Pol II or Pol III promoters. Recent studies, however, revealed that intragenic miRNAs, even intronic miRNAs, are not always co-transcribed with their host genes [140], [141]. Some intronic miRNAs have been found to interact with their host genes to affect their stability [142].

After the formation of pri-miRNAs in the nucleus, these primary transcripts are processed (cleavage) to form precursor miRNAs (pre-miRNAs). Pre-miRNAs are exported into the cytoplasm for additional cleavage by an enzyme, called Dicer, to produce single-stranded mature miRNAs. Mature miRNAs are then loaded onto the RNA-induced silencing complex (RISC), where they bind to 3′-UTR sequences of mRNA targets. The degree and nature of the complementarity between the miRNA seed site and the 3′-UTR of its target mRNA determine the gene silencing mechanism: (i) if fully complementary, the mRNA target will be degraded; (ii) if partially complementary, mRNA is targeted for translational inhibition [138], [143]. As the complementarity can be partial, an individual miRNA can regulate expression of hundreds of mRNAs and an individual mRNA can be targeted by different miRNAs [144], [145].

Several computational algorithms have been developed in order to predict miRNA targets. Among these: miRWalk, Diana-Tools, miRanda, Microrna.org, mirDB, TargetMiner, TargetScan, PicTar. These programs are able to predict miRNA targets basing on bioinformatics tools, but then each target has to be validated in laboratory, using techniques such as PCR and Western Blot.

1.2.2 Circulating miRNAs

MiRNAs are endogenous oligonucleotides that are found in intra- and extra-cellular spaces. Indeed miRNAs can be released out of the cell and they were found in almost all biological fluids, including whole blood, plasma, serum, urine, saliva, tears, breast milk, sperm, cerebrospinal fluid [146]–[149]. Circulating miRNAs resulted particularly stable compared to other RNA molecules [150]. Their remarkable stability, even in RNase-rich environments as blood, can be explained by their encapsulation in membrane-bound vesicles or association with RNA-binding proteins, which provides stability and resistance to RNase digestion and enables miRNA transfer from one cell to another [151]–[153].

Circulating miRNAs originate from the following mechanisms: (i) active secretion via membrane vesicles; (ii) active secretion in protein-miRNA complexes and lipoprotein complexes (such as high-

density lipoproteins); (iii) active secretion via exosomes when a multivesicular body (MVB) fuses with the cell membrane; and (iv) passive secretion originating from cell necrosis and apoptosis [154]–[157] (Figure 3).

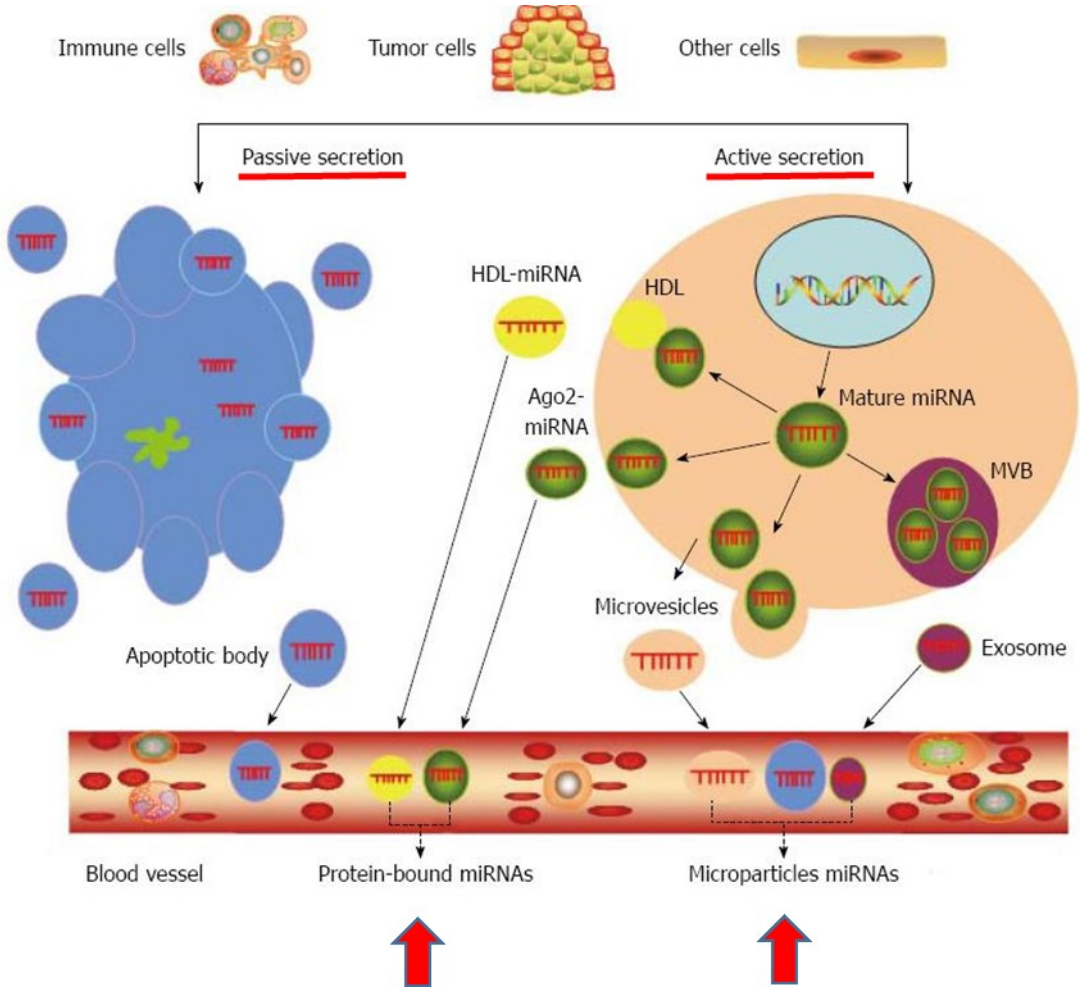

Figure 3: *Mechanisms of microRNAs secretion into blood vessels; origins and types of circulating microRNAs. Adapted from* [151]

1.2.3 MiRNAs in inflammation

The precisely regulated, spatial-specific and temporal-specific expression of miRNAs is crucial for different biological functions including immune homeostasis, normal innate and adaptive immune response and inflammatory processes [158]–[160]. Specific miRNAs were found dysregulated in inflammatory and autoimmune diseases; this implies their potential as both biomarkers and therapeutic targets for this kind of pathologies [159]–[161]. Some small compounds and nucleic acids targeting miRNAs, abnormally expressed in inflammatory diseases, have shown promise in preclinical development; however this kind of treatment is not yet in clinical practice [159].

Interestingly, feedback loops between miRNAs, cytokines and growth factors have been described as contributors in the pathogenesis of inflammatory conditions, such as psoriasis [162], [163]. Moreover, miRNAs secreted in the tumor microenvironment activate a paracrine cross-talk between cancer cells and surrounding immune cells. Indeed, miRNAs act not only as gene-expression regulators, interacting with the mRNA targets, but they can also bind directly proteins [164]. In particular, it was reported that circulating miRNAs are able to bind toll-like receptors (TLR) expressed by the immune cells, acting as agonists of these single stranded RNA-binding TLRs. The interaction between miRNAs and TLRs leads to NF-kB signaling activation and secretion of interleukin (IL)-6 and TNF-α, thus promoting inflammation, which eventually favors cancer cell growth and metastatization [165]–[167].

1.2.4 MiRNAs in tumorigenesis

MiRNAs are reported to regulate more than 30% of the human genome and they may potentially affect any cellular life aspect. Indeed, miRNAs were found to be involved in almost all fundamental biological processes, including cell proliferation, differentiation, apoptosis, migration and metabolic processes [135], [168]–[170]. Moreover, 50% of the known human miRNAs are located at cancer-associated regions of the genome. For all these reasons, it is not surprising that miRNAs

exhibit aberrant expression under different pathological conditions, including cancers [171]–[173]. Many investigations indicated that miRNA expression is dysregulated in tumor tissue and body fluids from several neoplastic diseases. These evidences suggest the potential role of miRNAs as oncogenes or tumor suppressor genes [144], [174].

The discovery of the involvement of miRNAs in human cancers originated from the studies on Chronic Lymphocytic Leukemia (CLL) [175]. Since then, many research groups have identified aberrant expressions of miRNAs in the different types of human cancers, both in hematopoietic malignancies [168], [176], and in solid tumors such as breast, ovarian, lung, bladder, thyroid, gastrointestinal and colo-rectal cancers [134], [177], [178]. Many studies demonstrated the importance of miRNAs in cancer biology. Indeed miRNAs, through the modulation of their mRNA target expression, can play a role in tumor growth, invasion, angiogenesis and immune surveillance escape [169], [179], [180]. Moreover, circulating miRNAs exhibit paracrine effects on tumor growth and they may play a novel role as regulators of intercellular communications during tumor formation. Among the several miRNAs, the cluster miR-17-92 resulted to be associated with tumorigenesis. In humans, this cluster encodes for six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1), which in physiological state are involved in heart, lung and immune system development. It was found that miR-17-92 cluster is activated by c-Myc, a transcription factor which is often hyperactive in cancer cells, and the miRNAs of this cluster resulted over-expressed in B cell lymphomas and CLL [168], [174], [181], [182], as well as in MPM cells, compared to normal mesothelial cells [183].

1.2.5 MiRNAs as potential biomarkers

The discovery of miRNAs has revolutionized not only the traditional view of gene expression, but also the approach to diagnosis and therapy of many diseases, including cancer [183].

MiRNAs, both cellular and circulating, have been proposed as promising biomarkers for several kinds of diseases, such as inflammatory [184], autoimmune [160], [161], metabolic [185] and

cardiovascular diseases [173], [186], [187], as well as for tumors [169], [188], [189]. This is explained by the evidence that the expression of specific miRNAs is dysregulated in the pathological conditions mentioned above, compared to healthy controls.

Furthermore, cell-free miRNAs are particularly interesting as biomarkers candidates for non invasive testing [169], [190]. Indeed, cellular miRNAs can be released in biofluids and were detected and quantified in liquid biopsy, such as serum and plasma. Circulating miRNAs exhibit several evident advantages as potentially ideal biomarkers, including significant stability in different types of biological samples, resistance to environmental conditions, such as freezing, thawing or enzymatic degradation, low cost, ease of analysis, and sensitive detection methods, such as real-time quantitative polymerase chain reaction (qRT-PCR) and droplet digital PCR (ddPCR) [151], [191], [192]. In addition, they are more easily sampled than tissue-derived miRNAs [190].

Altered levels of circulating miRNAs have been found in every type of cancer analyzed, and increasing evidence suggests that they may participate in tumorigenesis by acting as cell-to-cell signalling molecules [190], [193]. Specifically, many research groups have focused on the identification of dysregulated miRNAs as potential non-invasive biomarkers in several kinds of cancer, in particular the most challenging ones, including:

(i) Lung cancer: serum miR-152, miR-148a, miR-148b, and miR-21 were proposed as novel biomarkers in non-small cell lung cancer screening [194];

(ii) Ovarian cancer: several miRNAs were found dysregulated in serum, plasma and urine of ovarian cancer patients [195];

(iii) Glioblastoma multiforme (GBM): the serum expression levels of two miRNAs, miR-320 and miR-574-3p, were significantly associated with a GBM diagnosis [196];

(iv) Melanoma: specific circulating miRNAs or panels of circulating miRNAs were reported to be useful in discriminating melanoma from benign cutaneous lesions [197];

(v) Pancreatic cancer: profiling of deregulated miRNAs in pancreatic cancer can correlate to diagnosis, staging, predict response to therapy and suggest optimal treatment [198].

In summary, miRNAs exhibit a great potential value as biomarkers for diagnosis [199], [200], disease progression and prognosis [198], drug sensitivity [201], therapy and surgical treatment responses [200], [202], and cancer stratification [203], [204].

Despite the interesting data reported by published studies, it is necessary to take into account some issues including the non-homogeneity of detection and normalization methods, which require further investigations on miRNAs function and validation of their biomarker utility in large cohorts. In the future, prospective wide and well-designed clinical trials will be needed to validate the potential of some of the miRNA candidates in clinical practice [145], [197].

Despite these limitations, it is worth to note that there is still a worrying lack of reliable diagnostic markers and methods for early detection and screening for many tumors. Consequently, miRNAs could answer to the urgency of new biomarkers, non-invasive and quantifiable, more specific and sensitive, in particular for some types of dismal cancers, often diagnosed at an advanced and incurable stage, as characterized by asymptomatically development. The addition of specific miRNAs or panels of miRNAs to current testing regimens may improve diagnosis accuracy [195].

1.2.6 MiRNAs as therapeutic targets

Besides their potentialities as biomarkers, miRNAs were proposed not only for therapy monitoring, but as targets themselves for innovative therapeutic approaches for more targeted and effective therapies and personalized medicine [205]. Targeted drugs based on mimic and antago-miRNA were developed for the cure of different diseases [159], including cancer [206].

A severe lack of early diagnosis coupled with resistance to most available therapeutic options renders many cancers still incurable and characterized by poor prognosis. The limited efficacy of current treatments highlights the urgency for the development of novel therapeutic strategies that are based on a deeper understanding of the molecular mechanisms involved in cancer onset and progression. Nowadays, many of the monoclonal antibodies (mAbs) and small molecule inhibitors serve as effective cancer therapeutics in the clinic. However, there are some limitations regarding the

specificity of inhibitors and capability of antibodies to access intracellular targets [207]. Furthermore, some cancer cells develop compensation mechanisms by activating other survival signaling pathways to overcome the targeted cancer treatment [208]. For these reasons, neither inhibitors nor monoclonal antibodies can successfully treat cancer, an extremely heterogenic disease, by suppressing a single target. Since miRNAs can regulate multiple cancer-related genes simultaneously, the use of miRNAs as a therapeutic approach could play an important role in cancer therapy [206], [207].

To date, researchers have developed various methods and strategies to specifically reverse the aberrant expression of miRNAs implicated in different types of diseases. These methods are mainly based on the sequence complementarity of nucleic acids and are therefore highly specific. Several preclinical studies have given encouraging results, giving hope for the development of miRNA-based therapeutics in the near future [159], [206]. However, one of the major challenges of miRNA-based cancer therapy is to achieve specific, efficient and safe systemic delivery of therapeutic miRNAs in vivo. Many efforts are being made for the development of effective miRNA carriers and strategies to systemically deliver miRNAs to cancer without induction of toxicity [207]. To date, although no miRNA-based drugs have yet reached the pharmaceutical breakthrough, several phase 1 and phase 2 clinical trials are ongoing to investigate miRNA drug candidates [209], [210]. In this context, encouraging results were obtained in patients with recurrent MPM or non-small cell lung cancer, using an innovative technology, termed “TargomiR”, for the targeted delivery of miR-16 [210]–[213].

1.2.7 MiRNAs in asbestos exposure and MPM

MiRNAs have well established diagnostic value in cancer and toxic agent exposure, for example some miRNA signatures have successfully been applied in lung cancer screening, diagnosis, and follow-up [214], and miRNA combinations may also be sensitive to the effects of pollution [215]. Similarly, several studies reported a multitude of miRNAs differentially expressed in specimens from MPM, asbestos-exposed and healthy subjects. Indeed, asbestos fibers, inhaled and accumulated in the lung, cause a variety of adverse effects, including epigenetics changes, through deregulation of methylation status and alteration of miRNA expression [62], [216].

Guled and colleagues were the first to document the deregulation of several miRNAs in MPM samples, both compared with normal tissue and among the epithelioid, sarcomatoid, and biphasic subtypes [217]. Since then, many investigations, with different approaches, have identified miRNAs as potential MPM biomarker for:

- diagnosis: i.e., miR-1 [218] and miR-145 [219], which are able to distinguish MPM from normal mesothelial tissue;
- differential diagnosis: specific miRNAs, i.e., miR-200b, miR-200c, miR-141 and miR-429, resulted useful tools in distinguishing MPM from adenocarcinoma, but not among different histological types [220]. Moreover, it has been reported that miR-625-3p levels, in plasma and serum, are able to differentiate, with fairly high specificity, accuracy, and sensitivity, MPM from asbestosis patients [221];
- prognosis: miR-29c-5p, has been shown to be a prognostic factor in MPM, for both time to progression and survival after surgical cytoreduction. Indeed, high levels of this miRNA have been found in forms of epithelial mesothelioma and associated with improved patient survival as it acts on gene methylation and other over-regulated pathways in MPM [222]. On the other hand, miR-31 up-regulation is significantly associated with a worse prognosis in patients with sarcomatoid MPM [223].

In a previous study, our research group identified a group of 22 miRNAs which were significantly dysregulated in MPM cells compared to normal mesothelial cells. Some of these miRNAs belong to the onco-miR-17-92 cluster, which is induced by c-Myc oncogene [183].

Furthermore, Micolucci et colleagues systematically searched through the main biomedical databases for miRNA expression signatures related to asbestos exposure and MPM. As a result, a pool of deregulated circulating and tissue miRNAs, with biomarker potential for MPM, was identified. Referring to tissue miRNAs, the most consistently described, miR-16-5p, miR-126-3p, miR-143-3p, miR- 145-5p, miR-192-5p, miR-193a-3p, miR-200b-3p, miR-203a-3p, and miR-652-3p, were found to provide a diagnostic signature and should be further investigated as possible therapeutic targets. Moreover, comparing data from asbestos-exposed subjects and MPM patients, it was found that the most promising candidates for a multimarker signature were circulating miR- 126-3p, miR-103a-3p, and miR-625-3p in combination with mesothelin [224].

The characteristics of circulating miRNAs, reported in the previous paragraphs, and the limitations of currently available non-invasive methods to diagnose and monitor MPM, indicate that circulating miRNAs are promise biomarkers for early diagnosis and stratification of high-risk patients, as well as for monitoring asbestos ex-exposed subjects, in particular asymptomatic high-risk individuals.

Such miRNAs which are able to discriminate MPM patients from ex-exposed asbestos and healthy subjects, could also help to design targeted therapies for MPM [120], [205], [225], through the use of mimic or antago-miRNA [183], to substitute the lost miRNA in cancer or silence the overexpressed oncomiRNAs, respectively [5], [226], [227]. Out of all the miRNA candidates for therapeutic approach, miR-31 and miR-16-5p emerged for their tumor suppressor activity. It has been shown that in MPM-derived cell lines which do not express miR-31 due to the loss of the 9p21.3 portion gene, the reintroduction of miR-31 is able to suppress the cell cycle and inhibit the expression of multiple factors involved in DNA replication and cell cycle progression, including the phosphatase PPP6C, which had previously been associated with resistance to chemo- and radio-therapy [228].

An innovative mesothelioma treatment based on miR-16 mimic loaded EGFR targeted minicells (TargomiRs) was performed by Van Zandwijk and colleagues [211], [229], [230]. It was the first-in-human use of a miRNA-based therapy for pleural mesothelioma patients, and it showed that it is a safe strategy with promising activity [231]. It has been demonstrated that several members of the miR-16 family are downregulated in MM [212], [232]. Furthermore, it was reported that the most abundant miRNAs in MM cancer exosomes were tumor suppressors and among these, in particular, miR-16-5p. Recently, Munson et colleagues [233] formulated the hypothesis that MM tumor cells preferentially secrete the tumor suppressor miR-16-5p via exosomes. It was demonstrated that miR-16-5p levels can be rebuilt by inhibiting exosome secretion. The restoration of miR-16-5p resulted in cancer cell death and reduction in the oncoproteins targeted by this miRNA, i.e., CCND1 and BCL2 [233]. In addition, there was evidence that the RNA binding protein HuR can interact with miR-16-5p and it is at least partly involved in the packaging of this miRNA into MM cancer exosomes [233]–[235]. Overall, this innovative study of Munson et colleagues [233] suggested that MM tumor exosomes can be used to counteract tumorigenesis. Another recent study, published by Guo et colleagues [236] supports the hypothesis that autologous tumor cell-derived particles, including exosomes, can be a promising therapeutic target. A limitation of this potential therapeutic approach is that exosomes contain lot more than just tumor suppressor miRNAs. Further investigations are needed to elucidate the role of exosomes-associated miRNAs *in vivo* models.

1.2.8 MiR-197-3p, miR-1281 and miR-32-3p

MiR-197-3p, which gene maps in the chromosome 1p13.3, was investigated in different kinds of tumors. With regard to studies on cell cultures, it was reported that miRNA-197-3p promotes metastasis of hepatocellular carcinoma by activating Wnt/β-catenin signaling [237] and induces epithelial-mesenchymal transition in pancreatic cancer cells by targeting p120 catenin [238]. Moreover, during nephroblastoma tumorigenesis, miR-197-3p mediates the cell overgrowth and anti-apoptotic effects by downregulating Insulin-Like Growth Factor-Binding Protein-3 [239]. Another

investigation, on bladder cancer, demonstrated a regulatory relationship between miR-197-3p and the long noncoding RNA (LncRNA) LINC00641, through luciferase reporter assay and RT-qPCR [240]. Specifically, it was reported that i) LINC00641 interacted with and down-regulated miR-197-3p and ii) the target of miR-197-3p was KLF10, a tumor suppressor, member of krüppel-like transcription factor family [240]–[242]. KLF10 was showed to inhibit epithelial-to-mesenchymal transition and metastasis, in lung adenocarcinoma [243], and to inactivate PTEN/PI3K/AKT pathway in myeloma [241]. LINC00641 was found significantly down-regulated in bladder cancer tissues and it was demonstrated that LINC00641 overexpression suppresses proliferation, migration and invasion of bladder cancer cells, and, more specifically, that LINC00641 up-regulates KLF10 expression by inhibiting miR-197-3p and consequently suppresses the PTEN/PI3K/AKT pathway [240]. An interaction between miR-197-3p and another lncRNA, LINC00312, was identified in bladder cancer [244] and in thyroid cancer [245], too. In both studies, miR-197-3p expression was higher, while LINC00312 expression was lower, in tumor tissues, compared with adjacent normal tissues. The overexpression of LINC00312 inhibited cell migration and invasion abilities of both bladder cancer cells and thyroid cancer cells, by down-regulating miRNA-197-3p [244], [245]. Specifically, in the investigation regarding thyroid cancer, P120 was confirmed as a direct target of miR-197-3p, using the dual luciferase reporter gene assay [245]. According to the above mentioned studies, it seems that miR-197-3p has a pro-tumorigenic role. However, other research groups identified miR-197-3p as a tumor suppressor. Indeed, it was found that miRNA-197-3p inhibits gastric cancer progression by directly targeting metadherin [246]. Moreover, in human glioblastoma, the up-regulation of miR-197-3p, by FUS1, display an anti-tumoral activity [247]. Furthermore, interestingly, it was reported that miR-197-3p is down-regulated in platinum-resistant NSCLC samples, resulting in the promotion of chemoresistance, tumorigenicity, and pulmonary metastasis *in vitro* and *in vivo*. Fujita et colleagues [248], demonstrated that miR-197-3p regulates lung cancer drug resistance and tumor progression by directly targeting the cyclin-dependent kinase CKS1B, as well as by indirectly targeting the transcription factor STAT3. Mechanistic investigations reveal that a miR-197-mediated

CKS1B/STAT3 axis exerts tumor progression regulated by various oncogenic genes (Bcl-2, c-Myc, and cyclin D1). In particular, it was reported that the miR-197/CKS1B/STAT3-mediated signaling drives tumor PD-L1 expression. PD-L1 is a T-cell co-inhibitory molecule expressed in various types of cancers that leads to the immune escape of tumor cells. Interestingly, expression levels of miR-197-3p are inversely correlated with PD-L1 expression and are associated with worse overall survival [248]. Consistently, other studies have highlighted that the down-regulation of miR-197-3p may contribute to multidrug resistance in colon cancer cells [249] and head and neck cancer cells [250]. Overall, these data suggest that miR-197-3p plays an essential role in regulating several signaling pathways, including those ones involved in tumor progression and chemo-resistance (Figure 4). Furthermore, miR-197-3p was studied at circulating level, too. This miRNA, together with three other miRNAs, miR-29b-2, -155, and -205, was proposed as a potential diagnostic biomarkers in serum of breast cancer females [251]. Moreover, in other serum samples, from patients with lung cancer, pulmonary tuberculosis, and pneumonia, miR-197-3p exhibited an aberrant expression [252]. In addition, miR-197-3p seems to have a role also in non-tumor diseases, including metabolic, i.e. metabolic syndrome and type 1 diabetes mellitus, as well as inflammatory diseases. In particular miR-197-3p, circulating in serum of patients with symptomatic coronary artery disease, resulted predictive marker of cardiovascular death [186]. Finally, among the predicted targets of miR-197-3p, there are genes significantly involved in inflammation (i.e. CD53 and TNFSF14) (http://www.targetscan.org/) [253] and a crosstalk between miR-197-3p and IL-22 signaling was demonstrated [163]. These data suggest an involvement of miR-197-3p in inflammation process (Figure 4).

MiR-1281, which gene maps on chromosome 22q13.2, was investigated in human cancers both in tissues and in biological fluids. This miRNA was studied in cellular specimens in the investigation of Pignot and colleagues. They found miR-1281 down-regulated in two groups of different types of bladder tumor samples [177]. Other literature is mainly based on the study of circulating miR-1281. In plasma samples of patients affected by adrenocortical tumors, miR-1281 was found differentially expressed, by microarray, but it could not be validated by quantitative real-time PCR [254]. In sera

from patients with non-131I-avid lung metastases of papillary thyroid carcinoma, miR-1281 was found up-regulated, compared to 131I-avid lung metastases [255]. In serum and bile of patients with primary sclerosing cholangitis (PSC), miR-1281 was investigated as potential diagnostic marker of development of cholangiocarcinoma (CC). As a result, miR-1281expression was found significantly higher in serum of PSC patients compared to sera of healthy subjects [256].

Some research groups have investigated the role of circulating miR-1281 in non-tumor pathologies. In chronic kidney disease (CKD), miR-1281 resulted up-regulated in both urine and plasma of patients with decreased eGFR [257], whereas it was found down-regulated in patients with immune thrombocytopenic purpura (ITP) and it was suggested as biomarker to improve the diagnosis of this disease [258]. In summary, miR-1281 has been investigated in tumor and non-tumor pathologies and it was found dysregulated in many of these diseases, but there is no common message: miR-1281 can be down- or up-regulated and it is not yet clear whether it could be used as reliable diagnostic marker.

MiR-32-3p, which gene maps in chromosome 9q31.3, has not been well investigated, yet. So far, little information has been reported regarding this miRNA. Li et colleagues [259] found miR-32-3p dysregulated in serum samples from acute ischemic stroke subjects and bioinformatics approach revealed that this miRNA seems to be involved in important signaling pathways. In particular computational algorithms for miRNA target prediction, like DIANA tools [260], reported KRAS gene among the miR-32-3p predicted targets, while according to miRWALK [261], this miRNA seems to be involved in cancer pathways, including Wnt signaling pathway.

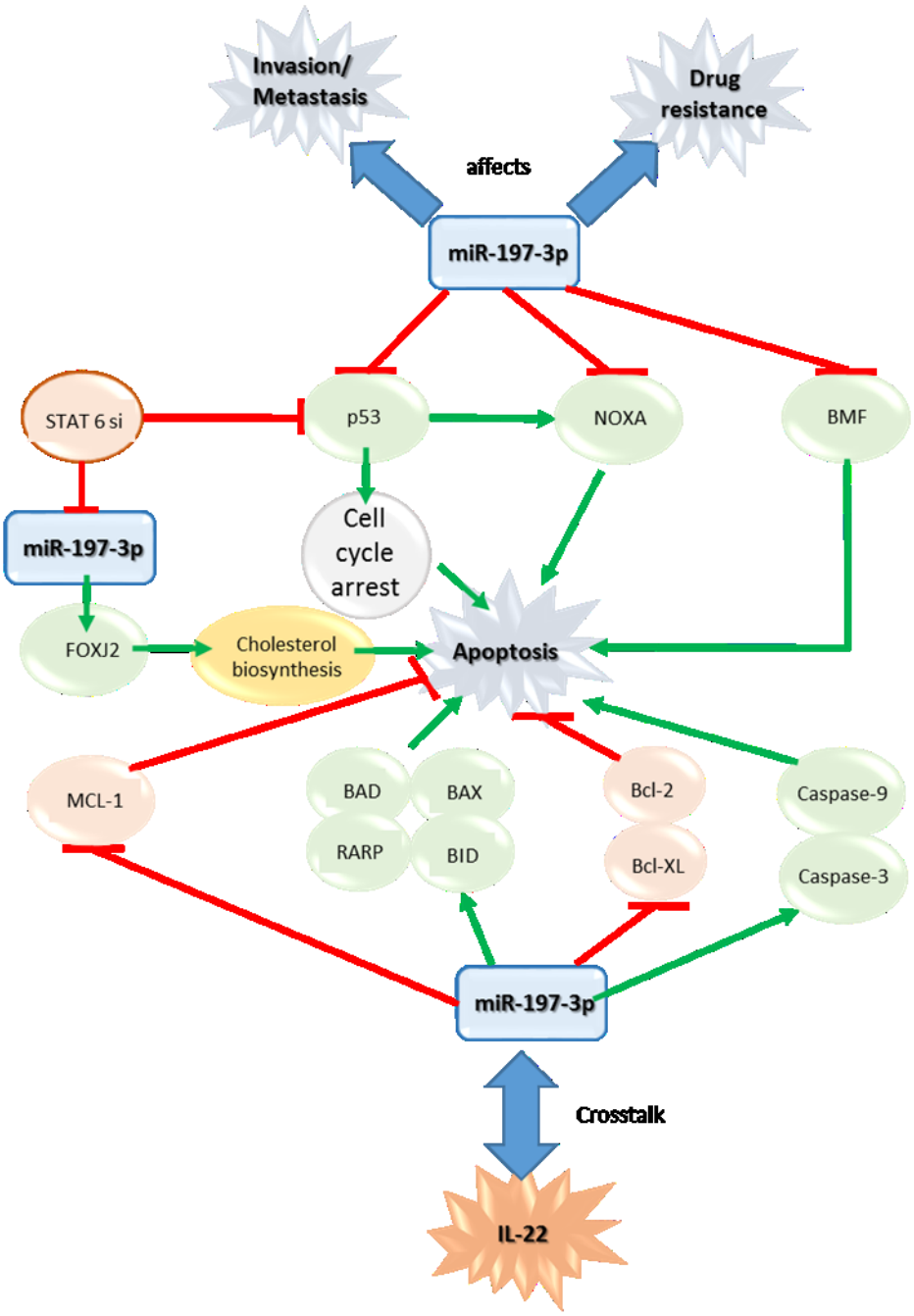

Figure 4*: targets and molecular pathways of miR-197-3p. Adapted from* [163], [172].

NOXA, p53 and BMF are direct targets of miR-197-3p. Consequently, up-regulation of miR-197-3p inhibits the expression of NOXA, p53 and BMF, resulting in decreasing cell apoptosis. On the other hand, miR-197-3p influences the expressions of MCL-1, Bcl-2, Bcl-XL, BAD, BAX, RARP, BID, caspase-3 and caspase-9 increasing apoptosis. CD82/KAI 1, p120 catenin and NLK are other targets of miR-197-3p. Among those, p120 represses EMT and NLK inhibits Wnt/beta-catenin pathways.

Consequently, miR-197-3p targeting and inhibiting p120 catenin and NLK, could promote cell invasion and metastasis. Moreover, miR-197-3p targets and inhibits the functions of MAPK1, TYMS and CKS1B. Among these, CKS1B enhances phosphorylation of STAT3, resulting in drug resistance through Bcl, c-Myc and cycline D1. In addition, miR-197-3p resulted involved in inflammation processes, targeting IL-22 receptor subunit, IL22RA1. A crosstalk between IL-22 signaling and miR-197-3p was demonstrated. IL-22 induces miR-197-3p, which in turn, negatively regulates IL-22 receptor. IL-22 drives proliferation and inhibits the apoptosis, thus favoring, during the chronic inflammation process, the onset of hyperplasia.

2. AIM

Despite the big efforts put into basic and clinical research in the attempt to find a cure for MPM, this tumor is still fatal and it is considered a major public health issue due to its aggressiveness and its resistance to available treatments [5], [7].

Asbestos exposure is the main risk factor for MPM onset and its peculiarity is the long latency period between the asbestos exposure and the appearance of the disease [41], [262]. For this reason, the MPM incidence is predicted to be increasing worldwide, based on both the asbestos exposure of past years as well as the present exposure in several countries, where the use of asbestos is still permitted and not well regulated [4], [18]. In the early phase of the disease, clinical signs are absent or not specific. For this reason, MPM is frequently diagnosed only in the advanced stages. The histopathological diagnosis is difficult and very complex. Only 60% of true positive cases can be identified using cytological examination of the pleural fluid. The confirmatory diagnosis test is based on qualitative analysis of markers in pleural tissue by immune-histochemical staining, but it requires pleural tissue samples obtained by invasive techniques [77]–[79]. To date, no known factor can predict the onset and evolution of MPM with certainty, and there is no adequate treatment or survival prognosis for patients after little more than one year since the diagnosis [7], [75]. For all these reasons, it is of crucial importance to define new biomarkers and therapeutic targets through a deeper knowledge and understanding of the molecular pathways involved in the onset / progression of this cancer. In this scenario, the role of circulating miRNAs is becoming increasingly relevant as minimally invasive, specific and sensitive biomarkers, which hopefully could have an effective translation in the current clinical practice [156], [263], [264].

Recently, my research group investigated circulating miRNAs from serum samples of malignant pleural mesothelioma patients (MPM), workers ex-exposed to asbestos fibers (WEA) and healthy subjects (HS), by microarray and RT-qPCR technologies [265]. This investigation allowed to detect (i) miR-197-3p, miR-1281 and miR-32-3p up-regulated in MPM compared to HS; (ii) miR-197-3p and miR-32-3p up-regulated in MPM compared to WEA; (iii) miR-1281 up-regulated in both MPM and WEA compared to HS. However, the limitation of these interesting results was the small sample

size. The aim of my thesis study was to verify if miRNAs mentioned above may be statistically validated as new biomarkers of MPM and/or asbestos exposure. To this purpose, sera (n=206) from MPM affected patients (n=69), workers ex-exposed to asbestos (WEA; n=75) and healthy subjects (HS; n=62), were investigated using PCR methods, such as RT-qPCR and droplet digital PCR techniques.

3. MATERIALS AND METHODS

3.1 Study groups

Serum samples from Malignant Pleural Mesothelioma (MPM) patients (n=69), and workers ex-exposed to asbestos fibers (WEA; n=75) were from the Mesothelioma BioBank, Pathology Unit, City Hospital of Alessandria, and Occupational Medicine Unit of the University of Ferrara, respectively. Serum samples from healthy subjects (HS; n=62) were collected at the Clinical Laboratory Analysis of the University Hospital of Ferrara (Table 1). Hospital records indicated that the HS were in good health at the time of the blood sampling. Anonymously collected sera were coded with indications of age, gender and pathology if any. For the comparative analyses, sera from MPM, WEA and HS were from subjects with a similar age. Informed written consent was obtained from all subjects. The County Ethics Committee of Ferrara approved the study.

Sera, in small aliquots, were stored at −80°C until the time of the analysis.

Clinicopathologic characteristics, anamnestic, occupational and non-occupational information, were collected for WEA and MPM cohorts (Tables 2 and 3). It was not possible to obtain all information for all subjects. To check the possible effects of asbestos and tobacco exposure, the collective was divided into subgroups with nearly the same number of subjects. With regard to WEA cohort, the following subgroups were formed: (i) three subgroups for asbestos exposure duration (years): <21, 21-30 and >30; (ii) four subgroups for the years from the last asbestos exposure: <11, 11-20, 21-30 and >30.; (iii) four subgroups for the cumulative asbestos exposure (measurement unit: ff/cc, fibers/cm^3): <10, 10.1-50, 50.1-140, >140; (iv) four subgroups for tobacco exposure (measurement unit: P/Y, pack years): 0, 0.1-10, 10.1-20, >20. In addition, the influence of other clinical variables was tested. Regarding the WEA cohort: (i) asbestosis or other asbestos related pathologies, (ii) other diseases, not related to asbestos, (iii) tobacco smoking status and (iv) pharmacological therapies (Table 2). Referring to MPM group: (i) MPM histological subtypes, (ii) asbestos exposure (degree of certainty), (iii) tobacco smoking status, (iv) surgery interventions, including interventions for metastasis, (v) pharmacological therapies, (vi) concurrent diseases, not related to asbestos (Table 3).

Table 1. Sera from MPM patients, WEA and HS subjects.

Cohort	Sera (n)	Median Age (y±SD)	Male (%)
MPM	69	69.1 ± 11.3	68.1
WEA	75	66.3 ± 6.6	97.3
HS	62	65.6 ± 17.0	53.2

Table 2. Clinicopathologic data for WEA cohort

Cohort WEA ***Clinicopathologic characteristics***		n (%)
Cumulative asbestos exposure	Range 0-10	23 (30.7)
	10.1-50	16 (21.3)
	50.1-140	13 (17.3)
	>140	11 (14.7)
	N/A	12 (16.0)
Years of asbestos exposure	Range 0-20	14 (18.7)
	21-30	24 (32.0)
	>30	26 (34.7)
	N/A	11 (14.7)
Years since the last asbestos exposure	Range 0-10	14 (18.7)
	11-20	25 (33.3)
	21-30	17 (22.7)
	>30	8 (10.7)
	N/A	11 (14.7)
Tobacco smoking status	Smoker	11 (14.7)
	Ex-smoker	30 (40.0)
	Non-smoker	19 (25.3)
	N/A	15 (20.0)
P/Y	0	23 (30.7)
	0.1-10	16 (21.3)
	10.1-20	13 (17.3)
	>20	12 (16.0)
	N/A	11 (14.7)
Asbestosis or other asbestos related pathologies	Pleural thickening	2 (2.7)
	Pleural plaques	3 (4.0)
	Fibrosis	1 (1.3)
	Bronchiectasis	1 (1.3)
	Emphysema	1 (1.3)
	Not related to asbestos	54 (72.0)
	N/A	13 (17.3)
Other diseases, not related to asbestos Musculoskeletal diseases	With	7 (9.3)
	Without	57 (76.0)
	N/A	11 (14.7)
Cardiovascular diseases	With	29 (38.7)
	Without	35 (46.7)
	N/A	11 (14.7)
Gastrointestinal diseases	With	10 (13.3)
	Without	54 (72.0)
	N/A	11 (14.7)
Endocrine diseases	With	20 (26.7)
	Without	44 (58.7)
	N/A	11 (14.7)
Respiratory diseases	With	15 (20.0)
	Without	49 (65.3)

	N/A	11 (14.7)
Neurological diseases	With	5 (6.7)
	Without	59 (78.7)
	N/A	11 (14.7)
Genitourinary diseases	With	11 (14.7)
	Without	53 (70.7)
	N/A	11 (14.7)
Neoplastic diseases	With	10 (13.3)
	Without	54 (72.0)
	N/A	11 (14.7)
Therapy	With	57 (76.0)
	Without	18 (24.0)

Clinicopathologic characteristics, occupational e non-occupational information, collected for WEA cohort. It was not possible to obtain all information for all subjects (N/A: data not available).

Note: cumulative asbestos exposure expressed as fibers/cm^3 (ff/cc). Tobacco smoking history expressed as pack/year (P/Y).

Table 3. Clinicopathologic data for MPM cohort

Cohort MPM ***Clinicopathologic characteristics***		n (%)
MPM histological subtypes	Epithelioid	38 (55.1)
	Sarcomatoid	9 (13.0)
	Biphasic	18 (26.1)
	N/A	4 (5.8)
Asbestos exposure	Professional, documented	11 (15.9)
	Professional, possible	6 (8.7)
	Domestic/ para-occupational	2 (2.9)
	Enviromental	4 (5.8)
	Improbable	1 (1.4)
	N/A	45 (65.2)
Tobacco smoking status	Smoker	3 (4.3)
	Ex-smoker	9 (13.0)
	Non-smoker	10 (14.5)
	N/A	47 (68.1)
Surgical interventions	Pleurectomy	6 (8.7)
	Peritonectomy	1 (1.4)
	Extra-pleural pneumonectomy	1 (1.4)
	No intervention	47 (68.1)
	N/A	14 (20.3)
Therapy	Just one combination treatment	
	Pemetrexed	7 (10.1)
	Cisplatin + pemetrexed	5 (7.2)
	Carboplatin + pemetrexed	18 (26.1)
	More than one treatment/cycle	11 (15.9)
	N/A	28 (40.6)
Concurrent diseases, not related to asbestos	Hypertension	10 (14.5)
	Hypertension + Other	7 (10.1)
	No concurrent disease	26 (37.7)
	N/A	26 (37.7)

Clinicopathologic characteristics, occupational e non-occupational information, collected for MPM cohort. It was not possible to obtain all information for all subjects (N/A: data not available).

3.2 RNA extraction

Total RNA, including miRNAs, was extracted from 200 µl of serum using miRNeasy Mini Kit (Qiagen cod. 217004) according to the manufacturer's supplementary protocol (*Qiagen. Purification of total RNA, including small RNAs, from serum or plasma using the miRNeasy Mini Kit),* with some minor modifications. Specifically, soon after adding 1 ml Qiazol Lysis Reagent (Qiagen), synthetic cel-miR-39 miRNA (2.5 µl of 5 nM) was added to the serum sample, as a control. This process is necessary when miRNAs are extracted from serum because, although circulating miRNAs detected in human plasma/serum samples appear to be remarkably stable, the final amount of miRNAs may be influenced by miRNA extraction efficiency or presence of inhibitors. RNA was eluted in 30 µl RNAse-free water and quantified by Nanodrop 2000 [265].

3.3 Reverse transcription of miRNAs

RNA was reverse transcribed using miRCURY LNA RT kit (Qiagen cod. 339340) following the manufacturer's protocol. Specifically, 2 µl of total RNA was reverse transcribed after adding 2 µl of 5X reaction buffer and 1 µl of enzyme mix in a final volume of 10 µl; synthetic RNA spike-in UniSp6 was employed as an exogenous control. Reactions were carried out on the SimpliAmp Thermal Cycler (Life Technologies) using the following conditions: 42°C for 60 min, 95°C for 5 min, and then held at 4°C. RT products were stored at −20°C until the time of qPCR analysis. Before the amplification, cDNA was diluted 1:60 to avoid interferences between the two enzyme mixes. Cel-miR-39 assay and Unisp6 assay were performed to monitor the RT reaction efficacy [265].

3.4 MiRNAs quantification

Hsa-miR-197-3p, hsa-miR-1281 and hsa-miR-32-3p concentrations were analyzed by qPCR techniques using miRCURY LNA miRNA PCR Assays. Specific miRNA assays were carried for (i)

miR-197-3p, with Qiagen cod. 339306- YP00204380, (ii) hsa-miR-1281, with Qiagen cod. 339306-YP02118520, and (iii) hsa-miR-32-3p, with Qiagen cod. 339306- YP00205648.

3.4.1 Specific synthetic LNA oligonucleotides for calibrations and controls

Standard curves were generated for the three miRNAs under investigation, i.e., hsa-miR-197-3p, hsa-miR-1281 and hsa-miR-32-3p, using synthetic Locked Nucleic Acid (LNA) oligonucleotides with the following sequences:

- synthetic hsa-miR-197-3p: 5'-rUrUrCrArCrCrArCrCrUrUrCrUrCrCrArCrCrCrArGrC-3' (Qiagen cod. 339411-YCO0082026);
- synthetic hsa-miR-1281: 5'-rUrCrGrCrCrUrCrCrUrCrCrUrCrUrCrCrC-3' (Qiagen cod. 339411-YCO0082027);
- synthetic hsa-miR-32-3p: 5'-rCrArArUrUrUrArGrUrGrUrGrUrGrUrGrArUrArUrUrU-3' (Qiagen cod. 339411-YCO0082028).

Stock solutions (100 μM) of synthetic oligonucleotides in RNase-free/DNase-free water were prepared according to the concentrations and sample purity, which are based on spectrophotometry analysis, quoted by the manufacturer. 1:10 serial dilutions were performed from 10^{13} copies/ μl to 10^{0} copy/ μl. These standards of synthetic oligonucleotides were used for the absolute quantification of miRNAs by RT-qPCR [266], and as controls in ddPCR.

3.4.2 Real Time Quantitative PCR (RT-qPCR) analysis

RT-qPCR reactions were performed using the miRCURY LNA SYBR Green PCR Kit (Qiagen cod. 339346) according to the manufacturer's instructions. Briefly, 3 μl of cDNA template, diluted at 1:60, was added to 0.5 μl of miRCURY LNA miRNA PCR assay, specific for hsa-miR-197-3p and hsa-miR-1281, and to 5 μl of miRCURY LNA SYBR Green, in a total reaction volume of 10 μl.

Samples were run in triplicate using the thermocycler CFX96 Touch (Bio-Rad) with the denaturation temperature at 95°C 2 min, followed by 40 cycles at 95°C 10 s and 56°C 1 min. A final melting curve analysis, from 56°C to 95°C, was carried out to verify the presence of a single amplified product. Standard curves were run in parallel with samples, enabling an absolute quantification of miR-197-3p and miR-1281 to be calculated.

3.4.3 Droplet Digital PCR (ddPCR) analysis

Digital droplet PCR (ddPCR) is an improvement of conventional PCR technique, which allows a more sensitive and accurate quantification of target nucleic acids [191], [192], [267]–[270]. DdPCR is considered to be able of overcoming problems relating to potential discrepancies in PCR analyses. In particular the ddPCR technique, compared to the traditional qPCR, exhibits some advantages: (i) ddPCR performs absolute quantification based on the principles of sample partitioning and Poisson statistics, thus overcoming the normalization and calibrator issues [266], [271], [272]; (ii) it is relatively insensitive to potential PCR inhibitors [273]–[275]; (iii) it has shown increased precision and sensitivity in detecting low target copies [267], [276], [277]; (iv) it directly provides the result of the analysis expressed as number of copies of target per microliter of reaction [266], [268], [278]. Specifically, in this study Locked Nucleic Acid (LNA)-based miRNA-specific primers (Qiagen) are used employing QX200 ddPCR System (Bio-Rad). Briefly, ddPCR was performed in a 22 µl volume containing 11 µl of 2X EvaGreen supermix (Bio-Rad cod. 1864035), 10 µl of diluted cDNA template, diluted 1:60, and 0.5 µl (for miR-197-3p and miR-1281) or 1 µl (for miR-32-3p) of miRCURY LNA PCR primer set (specific for each one of the three miRNAs). After droplet generation inside the Automated Droplet Generator (Bio-Rad), the 96-well PCR plate was heat-sealed with foil and placed into a conventional thermal cycler. Thermal cycling conditions for EvaGreen assays were as follows: 95°C for 5 minutes, then 40 cycles (50 cycles just for miR-32-3p) at 95°C for 30 seconds and 56°C for 1 minute (ramping rate reduced to 2 °C/sec), and three final steps at 4°C for 5 minutes, 90°C for 5 minutes and a 4°C indefinite hold to enhance dye stabilization. A no template control (NTC), a

negative control for each reverse transcription reaction (RT-neg) and a positive control, synthetic LNA oligonucleotides as shown above, were included for every assay.

QuantaSoft software was used to determine the number of positive and negative droplets at the end of 40 cycles using assay-specific intensity thresholds. These results were exported into a spreadsheet for further analyses.

3.5 Statistical analysis

The statistical analysis was performed using Prism 8.0 statistical software (GraphPad software, La Jolla, CA, USA). The differences in age and percentage of male subjects in the three cohorts, MPM, WEA and HS, were analyzed by ANOVA, followed by t-test, and Chi-square, respectively.

In order to test differences in the mean miRNA values in MPM, WEA and HS cohorts, and in each comparison, ANOVA test and t-test, with or without Welch's correction, were used if the distribution of the results was normal. If distribution was abnormal, the Kruskal–Wallis test and Mann–Whitney test, were employed. Data for the dysregulated miR-32-3p are presented as a percentage of positive analyzed sera in the three groups. Differences among proportions were calculated using the Chi-square test for independence in the contingency tables. Receiver Operating Characteristic (ROC) analysis was used to assess the accuracy of RT-qPCR and ddPCR results in discriminating comparative data in the three cohorts studied. Correlation between the RT-qPCR and the ddPCR output analyses was tested using the linear regression model. Means and standard deviations were calculated for miRNAs levels (copie/ul of cDNA) in the total study groups and in all subgroups. ANOVA test and t-test, with or without Welch's correction, were used to test differences in the mean miRNA value between subgroups. Multiple regression analysis was performed to assess potential correlations between miRNA quantity and independent variables.

P value <0.05 was considered to be statistically significant.

4. RESULTS

4.1 Sample analysis

Serum samples, from the three cohorts, include subjects with the same median age (MPM: 69±11, WEA: 66±6, HS:65±17, years±SD, ANOVA: p>0.05). The gender composition was similar for MPM and HS (MPM: 68,1% and HS: 53.2 % of male, Chi-square: p>0.05), while it was different for WEA with a male percentage of 97.3% (Chi-square both vs MPM and vs HS: p<0.0001****) (Table 1).

4.2 MiRNA analysis

MiRNAs analysis was carried out by two quantitative PCR techniques in sera (n=206) from MPM affected patients (n=69), workers ex-exposed to asbestos fibers (WEA, n=75) and healthy subjects (HS, n=62).

4.2.1 RT-qPCR analysis

In the first step of my investigation, miRNAs analysis was carried out using the RT-qPCR technique. RT-qPCR data confirmed that the recovery of each spike-in, i.e., Cel-miR-39 and Unisp6, was not different among the samples of the three cohorts (ANOVA: p>0,05). RT-qPCR data regarding the investigated miRNAs indicate that miR-197-3p was detectable in all 206 samples, with a significant dysregulation in the three groups, i.e., MPM, WEA, HS (ANOVA: p=0.0009***). Specifically, in the WEA cohort a mean of 336.0 copies of miR-197-3p/ul was detected. The quantity of miR197-3p in the WEA was significantly lower than in the MPM (777.7 copies/ul cDNA, t-test: p=0.0009***) and HS groups (870.1 copies/ul cDNA, t-test: p=0.0001***). The difference in the quantity of miR-197-3p between MPM and HS groups was not statistically significant (p=0.6272) (Figure 5A). Receiver Operating Characteristic (ROC) analysis was used to quantify the accuracy of RT-qPCR results in discriminating data in significant comparisons. The ROC AUC for miR-197-3p in MPM vs WEA was 0.682 (p = 0.0002; 95% confidence interval 0.594 to 0.771) (Figure 5B); whereas in the comparison between WEA vs HS, it was 0.727 (p <0.0001; 95% confidence interval 0.641 to 0.813)

(Figure 5C). These ROC analyses indicated that miR-197-3p was able to significantly discriminate MPM from WEA and WEA from HS.

RT-qPCR analysis for miR-1281 was performed on the same samples described above. It turned out that miR-1281 was detectable in 187/206 (90.8%) sera from the three cohorts, i.e., MPM (n=63), WEA (n=70) and HS (n=54), whereas the number of positive samples was not statistically different among the three cohorts. MiR-1281 was found to be significantly dysregulated in the three cohorts analyzed by RT-qPCR (ANOVA: p=0.0088**). Specifically, in the WEA cohort a mean of 384.6 copies of miR-1281/µl was detected, which was higher than in MPM and HS. The amount of miR-1281 in WEA was significantly higher compared to MPM (194.7 copies/µl cDNA, t-test: p=0.003**), whereas it was not statistically significant compared to HS, although the p value was close to the significance level (250.9 copies/µl cDNA, t-test: p=0.0592). The difference in the quantity of miR-1281 between MPM and HS was not statistically significant (p=0.3622) (Figure 5D).

ROC analysis was used to quantify the accuracy of RT-qPCR results in discriminating data in the significant comparison. The ROC AUC for miR-1281 ratio MPM vs WEA was 0.666 (p = 0.0009** 95% confidence interval 0.573 to 0.759) (Figure 5E). The ROC analysis indicated that miR-1281 was able to significantly discriminate MPM from WEA.

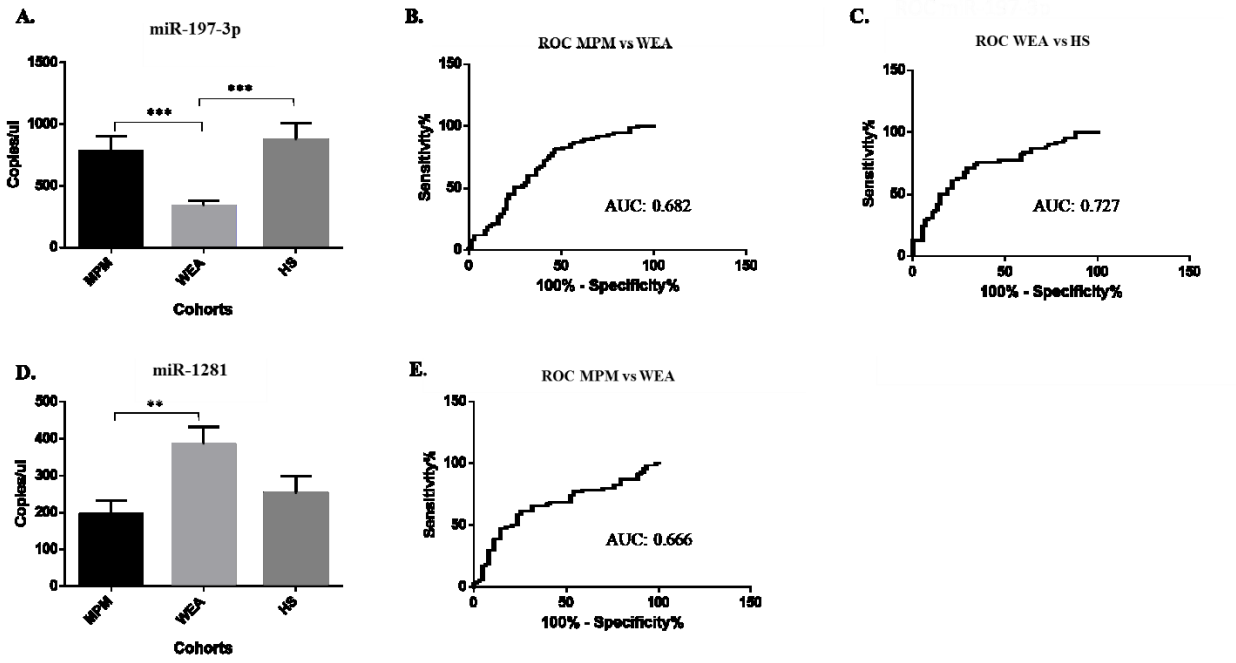

Figure 5: *(A) MiR-197-3p expression in MPM, WEA and HS cohorts detected by RT-qPCR. The amount of miR-197-3p is represented as copies/µl of cDNA analyzed. (B-C) Schematic representations of the Receiver Operating Characteristic (ROC) curves referring to the comparisons in which the differences in miR-197-3p expression are statistically significant. (D) MiR-1281 expression in MPM, WEA and HS cohorts detected by RT-qPCR. The amount of miR-1281 is represented as copies/µl of cDNA analyzed. (E) Schematic representation of the Receiver Operating Characteristic (ROC) curve referring to the comparison in which the difference in miR-1281 expression is statistically significant.*

4.2.2 ddPCR analysis

In the second step of my investigation, miR-197-3p, miR-1281 and miR-32-3p were analyzed in the same 206 sera, using ddPCR. MiR-197-3p was detectable in all the samples (n=206) analyzed. DdPCR analysis showed a significant dysregulation of miR-197-3p in the three groups (ANOVA: p=0.0004***). In WEA, a mean of 525.0 copies of miR-197-3p/µl was detected and it was significantly lower than in MPM (1170 copies/µl cDNA, t-test: p=0.0004***) and HS (1316 copies/ul cDNA, t-test: p<0.0001****). The difference between MPM and HS was not statistically significant (p=0.5748) (Figure 6A).

ROC analysis was used to quantify the accuracy of ddPCR results in discriminating data in significant comparisons. The ROC AUC for miR-197-3p in MPM vs WEA was 0.677 (p = 0.0002**; 95% confidence interval 0.589 to 0.764) (Figure 6B); whereas in the comparison between WEA vs HS it was 0.755 (p <0.0001***; 95% confidence interval 0.675 to 0.835) (Figure 6C).

MiR-1281 investigated by ddPCR was detected in 187/206 sera analyzed, i.e., 63 MPM, 70 WEA and 54 HS (Table 4). DdPCR analysis showed a significant dysregulation of miR-1281 in the three groups (ANOVA: p=0.0436*). The quantity of miR-1281 in WEA was 124 copies of miR-1281/µl, which was higher than in MPM and HS. The difference was statistically significant in WEA compared to MPM (60.8 copies/µl cDNA, t-test: p=0.0111*), whereas the differences between WEA vs HS (p=0.3511) and MPM vs HS (p=0.1320) were not statistically significant (Figure 6D).

ROC analysis was used to quantify the accuracy of ddPCR results in discriminating data in the significant comparison. The ROC AUC for miR-1281 ratio MPM vs WEA was 0.674 (p = 0.0006, 95% confidence interval 0.581 to 0.766) (Figure 6E).

In order to check that the differences found in WEA cohort compared to the other groups were not due to a different composition of genders, data-sets were divided according to gender and the differences in miR-197-3p and miR-1281 expression, in the three groups, were studied in the female data-set and in the male data-set, separately. The same significance, as in the complete data-set, was found in the two separate data-sets.

MiR 32-3p was expressed at a very low level and in very few samples in the previous study [265]. For this reason and considering that (i) ddPCR is more analytical than RT-qPCR, and (ii) in this study ddPCR analyses for miR-197-3p and miR-1281 confirmed RT-qPCR data, miR-32-3p was investigated by ddPCR only. Analyzing the 206 samples described above, miR-32-3p was detected in MPM n= 15 (21.7 %), WEA n=15 (20.0 %) and HS n=8 (12.9 %). Applying Chi-square test in the contingency tables, no significant differences emerged in the comparisons of each cohort with the other two ones (Table 5).

In particular, a mean of 13.7 and 17.9 copies of miR-32-3p/µl of cDNA were found in MPM and WEA groups, respectively. The lowest value was detected for the HS cohort, with a mean of 10.9 copies of miR-32-3p/µl of cDNA, and this was significantly lower than in the WEA (p= 0.0374*) (Table 5).

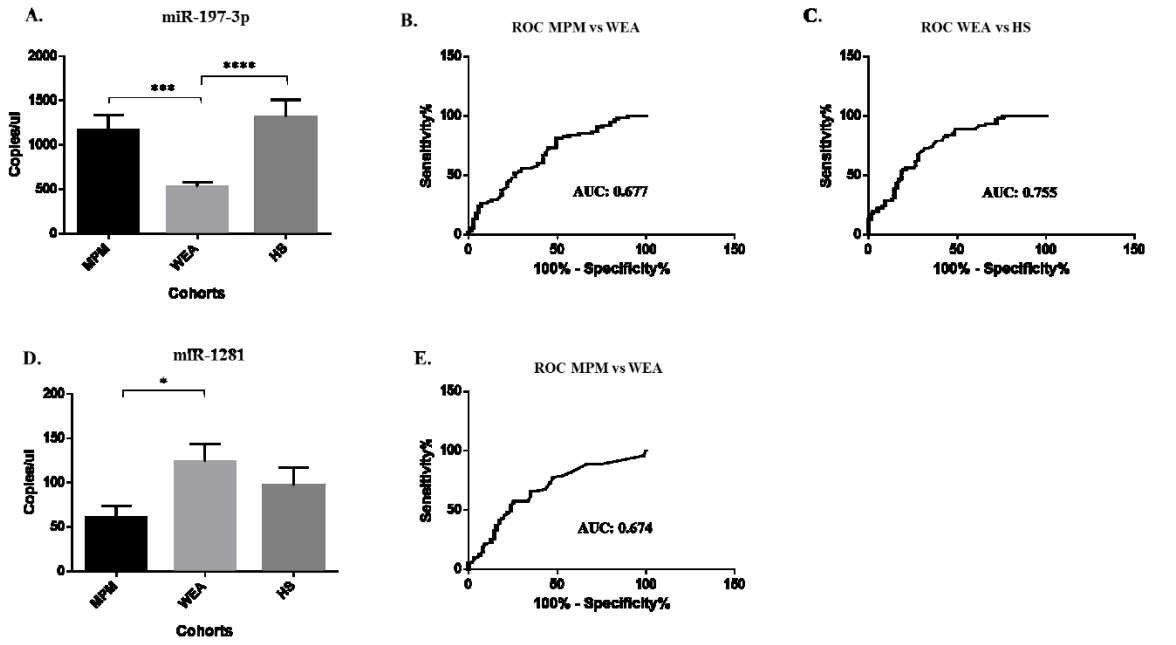

Figure 6: *(A) MiR-197-3p expression in MPM, WEA and HS cohorts detected by ddPCR. The amount of miR-197-3p is represented as copies/µl of cDNA analyzed. (B-C) Schematic representations of the Receiver Operating Characteristic (ROC) curves referring to the comparisons in which the differences in miR-197-3p expression are statistically significant. (D) MiR-1281 expression in MPM, WEA and HS cohorts detected by ddPCR. The amount of miR-1281 is represented as copies/µl of cDNA analyzed. (E) Schematic representation of the Receiver Operating Characteristic (ROC) curve referring to the comparison in which the difference in miR-1281 expression is statistically significant.*

Table 4: Qualitative analyses of circulating miR-197-3p, miR-1281 and miR-32-3p

	miR-197-3p	miR-1281	miR-32-3p
Cohort	Number of positive samples/samples analyzed (%)	Number of positive samples/samples analyzed (%)	Number of positive samples/samples analyzed (%)
MPM	69/69 (100)	63/69 (91)	15/69 (21.7)
WEA	75/75 (100)	70/75 (93)	15/75 (20.0)
HS	62/62 (100)	54/62 (87)	8/62 (12.9)
Total	206/206 (100)	187/206 (91)	38/206 (18.4)

Number of samples analyzed for miR-197-3p, miR-1281 and miR-32-3p, and % of samples in which each miRNA was detected by ddPCR.

Table 5: Analysis of miR-32-3p in each comparison

Comparison	Pos/tot (% pos)	Mean (copies/µl)
MPM vs HS	15/69 (21.7) vs 8/62 (12.9)	13.7 $\pm$ 8.3 vs 10.9 $\pm$ 3.1
MPM vs WEA	15/69 (21.7) vs 15/75 (20.0)	13.7 $\pm$ 8.3 vs 17.9 $\pm$ 11.4
WEA vs HS	15/75 (20.0) vs 8/62 (12.9)	17.9 $\pm$ 11.4 vs 10.9 $\pm$ 3.1 *

The results of ddPCR analysis for miR-32-3p were reported as % of positive samples and mean $\pm$ SD expressed as copies/µl of cDNA.

**p<0.05, significant difference between the two groups.*

4.3 Correlation studies

4.3.1 Correlation between RT-qPCR and ddPCR results

Linear regression analysis indicated a significant correlation between qPCR and ddPCR results (Figure 7). The R-square was 0.900 for miR-197-3p and 0.764 for miR-1281.

Although significant, the correlation was less good for miR-1281; this difference may be due to the fact that the assays used are designed specifically for RT-qPCR, therefore efficiency in ddPCR could be different between the two assays, i.e. assay for miR-197-3p and assay for miR-1281.

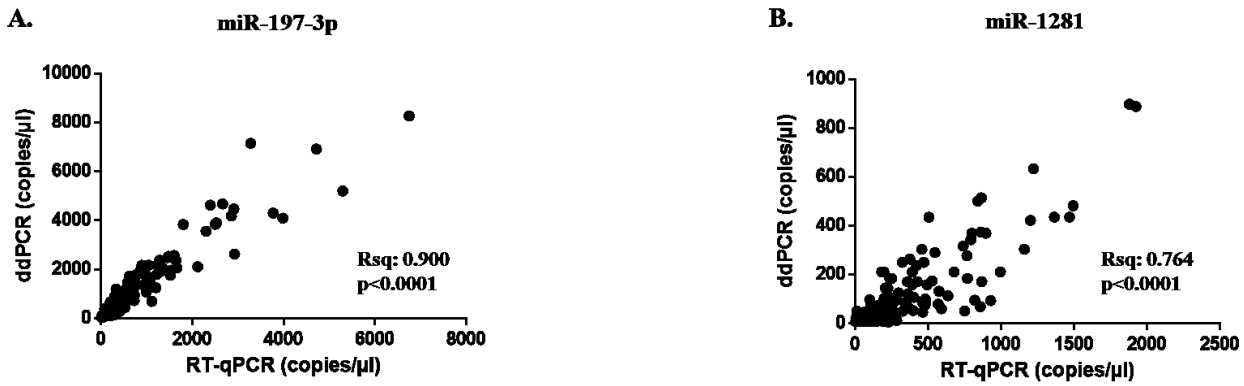

***Figure** 7: Correlation between RT-qPCR and ddPCR measurements. (A) MiR-197-3p and (B) miR-1281 levels (copies/µl) were measured by both PCR techniques in 206 samples.*

4.3.2 Correlation studies between miRNA expression and clinicopathologic characteristics

In reference to the WEA cohort, the level of miR-197-3p, in both PCR analyses, resulted more down-regulated in the subjects with a higher cumulative asbestos exposure (>140 ff/cc), compared to subject with lower values (ranges 0-10, p=0.0218*, and 10.1-50, p=0.0303*). Considering the years since the last asbestos exposure, a peak of miR-1281 expression was found in the range 11-20 years, while a decreasing trend was observed after 20-30 years, since the last exposure, even if it was not statistically significant (p=0.06). Regarding the MPM cohort, a significant difference in miR-197-3p levels emerged between patients with epithelioid MPM and subjects with biphasic histological subtype (p=0.0366*).

Moreover, Spearman correlation coefficients were calculated between miRNA quantity and the independent variables (clinicopathologic characteristics) evaluated. In WEA cohort, a negative correlation was observed between miR-197-3p and the cumulative asbestos exposure. MiR-1281 negatively correlated with cardiovascular diseases (Table 6). As far as concerns the MPM cohort, miR-197-3p positively correlated with MPM subtypes. A negative correlation was observed between miR-1281 and the tobacco smoke status of MPM patients (Table 7). Whereas no correlations were found between miR-32-3p and any of the clinicopathologic characteristics evaluated, neither in WEA nor in MPM (Tables 6 and 7).

Table 6: Correlation coefficients according to Spearman between miRNA expression and independent variables of WEA cohort.

		miR-197-3p		miR-1281		miR-32-3p
		ddPCR	RT-qPCR	ddPCR	RT-qPCR	ddPCR
	Age	-0.223	-0.112	-0.121	-0.102	0.385
	Gender	0.215	0.208	0.154	0.131	0.189
	Smoke status	0.154	0.127	0.041	0.106	-0.242
	P/Y	-0.105	-0.077	0.007	0.015	-0.450
Asbestos exposure	Cumulative exposure	-0.389*	-0.197	-0.059	-0.114	-0.064
Asbestos exposure	Years of exposure	-0.003	0.002	-0.214	-0.191	0.049
Asbestos exposure	Years since the last exposure	-0.140	-0.085	0.174	0.110	-0.147
Asbestos exposure	asbestos related pathologies	-0.028	0.047	-0.212	-0.036	0.367
Other diseases, not related to asbestos	Musculoskeletal	0.114	0.131	-0.046	-0.003	0.231
Other diseases, not related to asbestos	Cardiovascular	-0.056	-0.101	-0.278*	-0.265*	0.126
Other diseases, not related to asbestos	Gastrointestinal	-0.091	-0.065	0.111	0.020	-0.315
Other diseases, not related to asbestos	Endocrine and diabetes	-0.256	-0.120	-0.213	-0.147	-0.033
Other diseases, not related to asbestos	Respiratory	-0.091	-0.049	0.094	0.080	0.096
Other diseases, not related to asbestos	Neurological	0.129	0.099	0.168	0.147	-0.189
Other diseases, not related to asbestos	Genitourinary	-0.126	-0.124	-0.029	-0.086	0.378
Other diseases, not related to asbestos	Neoplastic	0.022	0.121	0.064	0.146	-0.050
Other diseases, not related to asbestos	Therapy	0.110	0.051	-0.177	-0.026	0.278

Correlation coefficients were determined according to Spearman test. The levels of miRNAs were expressed as copies/µl of cDNA analyzed. Correlations with $p < 0.05$(*) were considered statistically significant.

Table 7: Correlation coefficients according to Spearman between miRNA expression and independent variables of MPM cohort.

	miR-197-3p		miR-1281		miR-32-3p
	ddPCR	RT-qPCR	ddPCR	RT-qPCR	ddPCR
Age	-0.1319	-0.1595	0.0469	0.0840	0.4385
Gender	0.0848	0.0776	-0.1393	-0.1071	-0.2565
MPM subtype	0.3008*	0.3145*	0.1938	0.1631	0.0145
Asbestos exposure	-0.0127	-0.0330	-0.0074	-0.0649	-0.4204
Smoke status	0.0785	0.0909	-0.4069*	-0.3639*	0.1271
Surgery intervention	0.1206	0.1138	-0.1197	-0.1319	-0.0260
Therapy	-0.0430	0.0013	-0.0336	-0.0247	-0.2086
Hypertension only	0.0999	0.1128	-0.1875	-0.1651	-0.2685
Hypertension + other	0.1482	0.1691	-0.0998	-0.0850	-0.3072

Correlation coefficients were determined according to Spearman test. The levels of miRNAs were expressed as copies/µl of cDNA analyzed. Correlations with $p < 0.05$(*) were considered statistically significant.

5. DISCUSSION

MPM is a fatal cancer, in which asbestos-related chronic inflammation has been well documented [279]. Although banned or regulated in several countries, asbestos is still broadly widespread, indicating that MPM will remain a major public health concern [4], [14]. Despite significant progresses made in elucidating the molecular mechanisms of MPM pathogenesis, current diagnostic and prognostic tools are still inadequate. Consequently, MPM is diagnosed in advanced stages with very poor overall survival [5], [7]. For these reasons, the identification of more sensitive and specific MPM biomarkers is of paramount importance.

Different data have pointed to miRNAs as biomarkers in inflammatory disorders, tumors, as well as in toxic agent exposure [166], [169], [215]. In my study, I investigated the potential value of three circulating miRNAs, miR-197-3p, miR-1281 and miR-32-3p, as biomarkers of MPM and/or asbestos exposure.

MiR-197-3p is known to play a pivotal role as an oncogene or tumor suppressor in different cancers, targeting key pathways [172]. MiR-197-3p has been reported as being involved in cell proliferation, apoptosis, differentiation, metastasis and drug resistance, as well as other cellular processes [280]. Furthermore, some investigations revealed that a miR-197-mediated CKS1B/STAT3 axis exerts a tumor progression activity regulated by different genes, such as Bcl-2, c-Myc, and cyclinD1 [248]. MiR-197-3p was detected significantly dysregulated in sera/cells of a wide range of human tumors, including lung [252], [281], breast [251], pancreatic [238], thyroid [245], [282], bladder [240], hepatocellular [237], [283], and Wilms [239] cancers. Out of all the different miR-197-3p functions, this miRNA was found also to be involved in inflammation, including the endovascular inflammation and endothelial dysfunction [186], [284]. Moreover, IL22R1 and IL-17A subunits, which are involved in inflammatory responses, are among the validated targets of miR-197-3p [162], [163]. Furthermore, a reciprocal regulation between miR-197 and the IL-6/STAT3 inflammatory signaling pathway was reported in hepatocellular carcinoma [283]. In my investigation, expression profile of miR-197-3p was analyzed in sera from malignant pleural mesothelioma patients (MPM), workers ex-exposed to asbestos (WEA) and healthy subjects (HS). In a previous study [265], miR-197-3p was

found significantly dysregulated by microarray and RT-qPCR, in MPM vs HS (up-regulated) and in MPM vs WEA (up-regulated). Conversely, miR-197-3p resulted down-regulated in WEA vs HS, but not significantly. Herein, miR-197-3p was found to be significantly dysregulated, by RT-qPCR and the more analytical ddPCR, in sera from WEA compared to both MPM and HS. In MPM vs WEA, miR-197-3p was significantly up-regulated, thus confirming the previous data, while it resulted significantly down-regulated in WEA vs HS. On the other hand, the difference between MPM and HS was not significant. These data suggest that miR-197-3p may be involved in pathways dysregulated in WEA. I may speculate that these pathways are altered due to asbestos-induced inflammation. It is also possible that miR-197-3p level increases again in MPM, after the trigger of carcinogenesis process, which further modifies the complicated networks in which miR-197-3p is implicated. Indeed, as reported above, miR-197-3p has been found involved in both inflammatory and tumorigenic pathways. It is known that asbestos fibers can induce alterations of the immune system and inflammation response [24], [49], [285] and, consistently with my results of miR-197-3p in asbestos-exposed subjects, different studies showed that miR-197-3p is down-regulated in inflammatory conditions [163], [286], [287]. In the context of inflammation, the demonstrated crosstalk between miR-197-3p and IL-22 signaling is particularly interesting, in which IL-22 induces miR-197-3p, which in turn, negatively regulates IL-22 receptor subunit, IL22RA1, as indicated by luciferase reporter assay and western blot analysis [163]. Specifically, IL-22 was found to activate miR-197-3p expression through the binding of phosphorylated STAT3 to sequences in the putative promoter of miR-197-3p [163]. It has been reported that during acute inflammatory events IL-22 plays a protective role, while this interleukin is pathogenic during the chronic inflammation. Indeed, IL-22 drives proliferation and inhibits the apoptosis, thus favoring the onset of hyperplasia during the long term process [288]. IL-22, secreted by activated Th1, Th17 and NK-cells [289], interacts with its receptor also in the respiratory system [290]. In particular, in epithelial cells, IL-22 induces STAT3 activation and consequently the transcription of specific genes, involved in the anti-apoptotic process [289], [290]. It is tempting to speculate that low levels of miR-197-3p, like those I found in the WEA

group, may lead to an up-regulation of IL-22 activity and consequently to a cell proliferation increase and, at the same time, the inhibition of apoptosis, which overall could contribute to the MPM development. Moreover, it is worth recalling that the IL22RA1 subunit, which is a miR-197-3p target, is also part of IL-20 receptor [163]. Consequently, it could be inferred that miR-197-3p, by controlling IL22RA1, can fine-tune several aspects of cytokine signaling pathways [163]. It is known that circulating miRNAs participate in cell to cell communication [153] and it has been reported that miRNAs act not only as gene expression regulators, interacting with the mRNA targets, but they can also bind directly proteins, including receptors like TLRs [164]–[166], [291]. In this way, miRNAs secreted in the tumor microenvironment can activate a paracrine cross-talk between cancer cells and surrounding immune cells [166], [167]. In this context, a link between miR-197-3p and TLR4, which is needed for the activation of the NFκB/NLRP3 inflammasome-mediated inflammatory response, has been proved [292]. Overall, the participation of miR-197-3p in inflammation signaling, involving regulatory relationships with both interleukins and TLRs, suggests that miR-197-3p could act as a modulator, a sort of link between the immune system and target cells, including tumor cells. Taken together, these evidences and considerations point to new lines of investigation for miR-197-3p into the biology of MPM and in particular into the chronic inflammation processes underlying this tumor. Furthermore, expression levels of miR-197-3p have been reported to be inversely correlated with PD-L1 expression [248]. I may suppose that the low level of miR-197-3p in the WEA group could correspond to a higher level of PD-L1, leading to tumor immune escape. In addition, it should be considered that miR-197-3p controls epithelial-to-mesenchymal transition (EMT) and metastasis processes silencing homeodomain interacting protein kinase 2 (HIPK2), a multifunctional tumor suppressor protein that modulates cancer cell growth and apoptosis [293], [294]. In this context, it is worth recalling that EMT plays a role in the MPM development [295], [296]. These dysregulated functions could represent other potential pathways through which miR-197-3p may contribute to the MPM onset.

Moreover, in my investigation a significant difference in miR-197-3p levels is emerged between patients with epithelioid MPM compared to biphasic subtype. This result points out at this miRNA as a potential biomarker to discriminate different MPM histological subtypes. In addition, miR-197-3p negatively correlated with the cumulative asbestos exposure, evaluated in the WEA cohort. This suggests that miR-197-3p could be a potential marker of asbestos exposure and might be useful in screening test of high-risk subjects, characterized by high-dose exposure.

MiR-1281 was found to be significantly dysregulated in sera or tissues of different human cancers, such as bladder cancer [177], cholangiocarcinoma [256], oropharyngeal squamous-cell-carcinoma [297], osteosarcoma [298] and glioma [299]. Among the predicted and validated targets of miR-1281, oncogenes, i.e., TRIM65 [299] and HDAC4 [300], and USP39, which has been reported to promote tumor growth [298], were identified. Furthermore, miR-1281 expression can be increased by p53 tumor suppressor, which directly binds to miR-1281 promoter, in condition of endoplasmic reticulum (ER) stress, which means an imbalance of ER functions caused by many intra- and extracellular stimuli [298]. In my investigation, miR-1281 expression profile analyzed in sera from MPM, WEA and HS, resulted dysregulated when using RT-qPCR and ddPCR analyses. These data, obtained investigating a larger sample set, confirmed the up-regulation of miR-1281 in WEA compared to both MPM and HS cohorts, as reported previously in a limited number of sera [265]. Herein, specifically miR-1281 was significantly down-regulated in MPM vs WEA. Conversely, the differences between MPM vs HS and WEA vs HS were not significant, even if in the latter comparison the p value was close to the statistical significance. These data suggest that, similarly to miR-197-3p, miR-1281 may be implicated in pathways altered in WEA, probably due to the inflammation status induced by asbestos fibers. Consistently with the high level of miR-1281 found in the WEA, this miRNA resulted significantly higher in serum of patients with primary sclerosing cholangitis, in which miR-1281 was investigated as a potential diagnostic marker of development of cholangiocarcinoma [256]. Moreover, miR-1281 was found to be up-regulated both in plasma and urine of patients with chronic kidney disease (CKD), whereas this miRNA could target transforming growth factor-beta (TGF-

β)/bone morphogenic protein (BMP) signaling [257]. TGF-β/BMP signaling is involved in a complex cross-talk with other important signaling pathways, including Mitogen-activated protein kinase, phosphatidylinositol-3 kinase/Akt, Wnt, Hedgehog, Notch, and the interleukin/interferon-gamma/tumor necrosis factor-alpha cytokines [301]. It is tempting to speculate that alterations in miR-1281 levels could affect this delicate equilibrium, triggering pathogenic evolution. This hypothesis is supported by the dysregulation of miR-1281 in several tumors. Herein, the significant dysregulation of miR-1281 in MPM vs WEA induce to suppose that this miRNA might have a role in the MPM tumorigenic process or the alterations occurring during the tumorigenesis could affect miR-1281 levels. Furthermore, in my investigations miR-1281 negatively correlated with both cardiovascular diseases, in WEA, and tobacco smoke status, in MPM patients. These data may support the hypothesis of miR-1281 involvement in inflammatory conditions. On the other hand, these correlations indicate that miR-1281 levels can be affected by different confounding factors.

Overall, the larger sample size analyzed for miR-197-3p and miR-1281 allowed to statistically validate the dysregulation of these two miRNAs. It is worth noting that, for both miRNAs, miR-197-3p and miR-1281, the ddPCR results are consistent with RT-qPCR data and there is a linear correlation between the two techniques. These aspects reinforce the value of the results obtained.

With regard to miR-32-3p, the third miRNA of my investigation, it is interesting to note that miR-32-3p was expressed at a higher level in MPM and WEA compared to HS, with a significant difference between WEA and HS. In the previous study [265], miR 32-3p was detected by RT-qPCR in only 6/49 totally analyzed sera. Specifically, miR-32-3p was detected in 6/20 (30%) MPM sera, but it was absent in WEA and HS. In the present investigation, it was possible to detect miR-32-3p also in WEA and HS samples, probably due to the more sensitivity of ddPCR. It is possible that the absence of miR-32-3p was a consequence of a gene mutation or epigenetic silencing mechanism, or that this miRNA, in the serum, was present below the detection limit. Preliminary data, not reported in this thesis, indicate that miR-32-3p is present at a higher level in mesothelial and mesothelioma cells compared to serum. Overall, compared to intracellular miRNAs, circulating miRNAs are more

challenging to be measured accurately because of their low concentration [157]. This is one of the reasons why the more sensitive and analytical ddPCR technique is preferred for circulating miRNA investigations. In the literature, miR-32-3p data are very poor. In this study and in the previous investigation [265], miR-32-3p expression was assessed, for the first time, in a tumor disease such as MPM. This miRNA has been found to be dysregulated in serum samples from acute ischemic stroke subjects and bioinformatics approach revealed that miR-32-3p seems to be involved in important signaling pathways, including mTOR and MAPK [259]. Among the mechanisms triggering ischemic brain damage, oxidative stress, inflammation, and in particular the activation of NF-kB and p38MAPK were reported [302]–[304]. The same pathways mentioned above seem to be involved in the asbestos-related damage and MPM development [30], [305]. Further investigations are needed to elucidate the potential role of miR-32-3p in asbestos exposure and MPM.

In summary, this study added new data to our previous research [265]. However, some differences were revealed between the two investigations. First of all, it is worth noting that herein a larger sample size (206 vs 49 samples) and the absolute quantification by ddPCR were employed. MiR-197-3p, miR-1281 and miR-32-3p have never been investigated by ddPCR before. Overall, my findings shed light on new potential implications of the investigated miRNAs in inflammation pathways related to asbestos exposure and suggest that these miRNAs could have a role in MPM development and therefore as potential predictive biomarkers of disease.

MPM is frequently diagnosed only in the advanced and incurable stages and the gold standard for MPM diagnosis is an invasive test based on qualitative analysis of markers in pleural tissue by immunohistochemical staining. Tests based on miRNA biomarkers could add further relevant information and increase the probability of reaching an early and effective diagnosis. Moreover, non-invasive and easily quantifiable molecules like circulating miRNAs, would be very useful in a screening test, to monitor the high-risk subjects, as predictive biomarkers of malignant pleural mesothelioma in asbestos-exposed subjects.

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