UNIVERSITÀ DEGLI STUDI DI MILANO

Facoltà di Medicina e Chirurgia

SCUOLA DI DOTTORATO IN MEDICINA DEL LAVORO E IGIENE INDUSTRIALE

XXVII CICLO

Anno Accademico 2013/2014

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MicroRNAs in Exhaled Breath Condensate and Blood as biomarker of Pleural Malignant Mesothelioma and Asbestos Exposure

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ABSTRACT

Malignant Pleural Mesothelioma (MPM) is an aggressive cancer refractory to current therapies, the incidence of which is expected to rise in the next decades. Exposure to asbestos is a well-known MPM risk factor. To date, diagnosis is hard to determine, due to a lack of proper markers to specifically identify malignant pleural mesothelioma, since it might be confused with lung-originated metastatic neoplasm.

MiRNAs are single stranded noncoding RNAs of 20-22 nucleotides that post-transcriptionally regulate gene expression by triggering mRNA cleavage or repressing translation. One single miRNA can regulate hundreds of mRNAs and a single mRNA can be targeted by several different miRNAs. Changes in the expression of miRNAs have been implicated in several diseases, including MPM. miRNAs are stable molecules that can be easily investigated in different specimens (e.g. blood), and used as a disease biomarker.

Exhaled breath condensate (EBC) collection is a standardized, non-invasive technique and collected EBC is representative of lung environment. miRNAs measured in EBC/blood could help evaluating asbestos exposed subjects and MPM development.

Specific aims of the project are:

Aim 1: Determine whether a specific miRNA signature (in plasma or EBC) may help to discriminate between malignant pleural mesothelioma patients and patients affected by other pulmonary diseases, among patients presenting at the Thoracic Surgery Unit with a diagnosis of pleural effusion.

Aim 2: Determine whether a specific miRNA signature (in plasma or EBC) may help to discriminate between Malignant Pleural Mesothelioma (MPM) patients and healthy subjects with a Past Exposure to Asbestos (PEA).

Aim 3: Evaluate a possible correlation between miRNAs in EBC and plasma.

Aim 4: Estimate sensitivity and specificity of altered miRNAs using ROC curves.

To address this hypothesis we investigated a population including 23 MPM patients, 19 healthy subjects with a Past Asbestos Exposure (PEA), and 9 subjects with pleural effusions and affected by other diseases. In this study population we screened 733 miRNAs in blood and EBC by high-throughput Open Array. To investigate the possible miRNAs expression deregulation between the cases and controls, we used a multiple linear regression model adjusted for four principal variables (age, sex, BMI and smoking habits) and identified 6 candidate miRNAs in EBC and 13 in plasma.

After Real Time validation, we identified one miRNA in EBC (hsa-miR-30a-5p) and three miRNAs in plasma (hsa-miR-151-5p, hsa-miR-107, and hsa-let-7e-5p), able to discriminate between MPM and PEA subjects. The combination of the 4 miRNAs gives even a better score, with a ROC curve which shows an AUC = 0.85.

To our knowledge, this is the first study in MPM which investigate miRNA expression in EBC and identify a 4-miRNA signature which might be useful for MPM diagnosis. This signature might help identifying subjects with an increased risk of developing MPM after exposure to asbestos.

1. BACKGROUND

1.1. MALIGNANT MESOTHELIOMA EPIDEMIOLOGY – THE SIZE OF THE PROBLEM

Mesothelioma is a rare tumor originating from the mesothelium. The most common site is the pleura, but it can also arise in the peritoneum, pericardium, and the tunica vaginalis of the testis.

Malignant pleural mesothelioma is an aggressive cancer refractory to current therapies, the incidence of which is expected to rise in the next decades [1]. Recent projections have suggested that malignant pleural mesothelioma mortality rates, estimated using mortality from primary pleural tumors, will continue to increase in the next 20 years in most European countries [2, 3].

In Italy permanent malignant pleural mesothelioma epidemiologic surveillance has been in place since 1993 (mandatory since 2000) by means of a National Mesothelioma Register (ReNaM), kept at the Italian Workers' Compensation Authority (INAIL), and collected more than 15000 incident cases. The incidence rates of malignant pleural mesothelioma in Italy in the last years are 3.55 and 1.35 per 100,000 in men and women respectively [4]. The ReNaM has a regional structure with Operating Centres (COR) operating in almost all Italian regions, so covering almost the whole Italian territory. In Lombardy region the COR was established in 2000 at the Department of Preventive Medicine Fondazione IRCCS Ca' Granda. It includes all incident cases of mesothelioma of the pleura, peritoneum, pericardium, and tunica vaginalis of testis that have been diagnosed among subjects living in the region (total population about 10 million). In Lombardy region, the standardized (Standard: Italian population 2001) incidence rates of malignant pleural mesothelioma (2000-2011) were 4.7 and 1.9 per 100,000 respectively in men and women.

1.2. MESOTHELIOMA AND ASBESTOS EXPOSURE

Asbestos is a trade name encompassing various fibrous silicates that can be classified in two main groups: amphiboles and serpentine (Table 1). The International Agency for Research on Cancer (IARC) classified all asbestos types as human carcinogens. A general consensus exists that amphiboles are more potent in causing MPM than chrysotile although the extent of the difference is still debated [5].

According to the Second Italian Consensus Conference on pleural mesothelioma [6], exposure to asbestos may be classified as:

a) occupational exposure in asbestos mining or first processing of asbestos;
b) occupational exposure in trades using asbestos containing materials;
c) occupational exposure due to jobs in workplaces where asbestos materials are present (e.g. insulation);
d) domestic exposure as family members of asbestos workers;
e) environmental exposure consequent to dwelling in proximity of asbestos mining, industries or deposits of asbestos tailings;
f) environmental exposure to natural occurring asbestos (NOA).

Asbestos was widely used in the construction and shipbuilding industries, especially in thermal and acoustic insulation, thanks to its unusual characteristics of plasticity and resistance, and also for water- and fire-proofing. Up to the end of the 1980's, Italy was the second largest asbestos producer in Europe, after the Soviet Union, and the largest in the European Community. From the end of the Second World War to the asbestos ban in 1992, 3,748,550 tons of raw asbestos were produced, reaching its peak in the period between 1976 and 1980 at about 160,000 tons/year [7]. Because of its previous high consumption, the wide spectrum of industries involved and the number of workers and non-workers exposed, Italy is among countries which are most sensitive to the 'asbestos'

Mineral Group	*Mineral Species*	*Asbestiform Variety*	*Ideal Chemical Formula*[a]
Serpentine	Clinochrysolite	Crhysolite	$Mg_3Si_2O_5(OH)_4$
Serpentine	Orthochrysotile	Crhysolite	$Mg_3Si_2O_5(OH)_4$
Serpentine	Parachrysotile	Crhysolite	$Mg_3Si_2O_5(OH)_4$
Amphibole	Riebeckite	Crocidolite	$Na_2Fe_5Si_8O_{22}(OH)_2$
Amphibole	Grunerite	Amosite	$(FeMg)_7Si_8O_{22}(OH)_2$
Amphibole	Cummingtonite	Amosite	$(MgFe)_7Si_8O_{22}(OH)_2$
Amphibole	Gedrite	Amosite	$(MgFe)_5Al_2(Si_6Al_2)O_{22}(OH)_2$
Amphibole	Anthophyllite	Asbestiform anthophyllite	$(MgFe)_7(Si)_8O_{22}(OH)_2$
Amphibole	Tremolite	Asbestiform tremolite	$Ca_2Mg_5Si_8O_{22}(OH)_2$
Amphibole	Actinolite	Asbestiform actinolite	$Ca_2(MgFe)_5Si_8O_{22}(OH)_2$
Amphibole	Richterite	Asbestiform richterite	$Na_2Ca_2(MgFe)_5Si_8O_{22}(OH)_2$
Amphibole	(Alumino)winchite	Asbestiform winchite	$CaNa(MgFe)_4AlSi_8O_{22}(OH)_2$
Amphibole	Ferriwinchite	Asbestiform winchite	$CaNa(FeMg)_4Fe^{3+}Si_8O_{22}(OH)_2$

[a]Simplified representation of the overall stoichiometry of a mineral species. Mineral species typically have chemical modifications, such as substitutions of similar cations and sometimes anions (common examples are Mg2+<->Fe2+ and Si4+<->Al3+). Substitutions may cause substantiated deviations from the ideal chemical formula.
SOURCE: Gaines et al. (1997), Veblen and Wylie (1993).

Table 1 Asbestos classification by International Agency for Research on Cancer (IARC).

question and it has a specific system of epidemiological surveillance of mesothelioma.

The association between asbestos exposure and malignant pleural mesothelioma is well established. The background incidence of malignant pleural mesothelioma (without asbestos exposure) is estimated to be about 1–2 cases per million per year [8]. Among asbestos-exposed populations, the observed number of cases is much higher than expected. In the industrialized world, about 80% of malignant pleural mesothelioma develop in individuals with higher than background levels of exposure to asbestos [9, 10]. Workers involved in the extraction and manufacturing of asbestos fibers – i.e. miners, asbestos-cement workers, shipyards workers, insulators – have the highest risk of malignant pleural mesothelioma [8-11]. Recently, mesothelioma cases and deaths have been found in other workplaces, where asbestos exposure was at a low level [12, 13]. The mean latency time

for malignant pleural mesothelioma has been repeatedly found to be between 30 and more than 40 years [4, 9]. As a consequence of the strong cause–effect relationship, the incidence and mortality trends observed recently follow the asbestos exposure trends with a mean lag time of about 30–50 years.

In Figure 1 is reported the expected number of malignant pleural mesothelioma deaths in Italy along with data on asbestos consumption [7].

The number of mesothelioma cases reported began to increase around 1950 in some countries, with a steep increase noted during 1960s and 1970s. Although preventive measures have been taken at different time points in Europe, European cancer registries have described constant increases in the incidence rates of malignant pleural mesothelioma in the last few decades [14, 15].

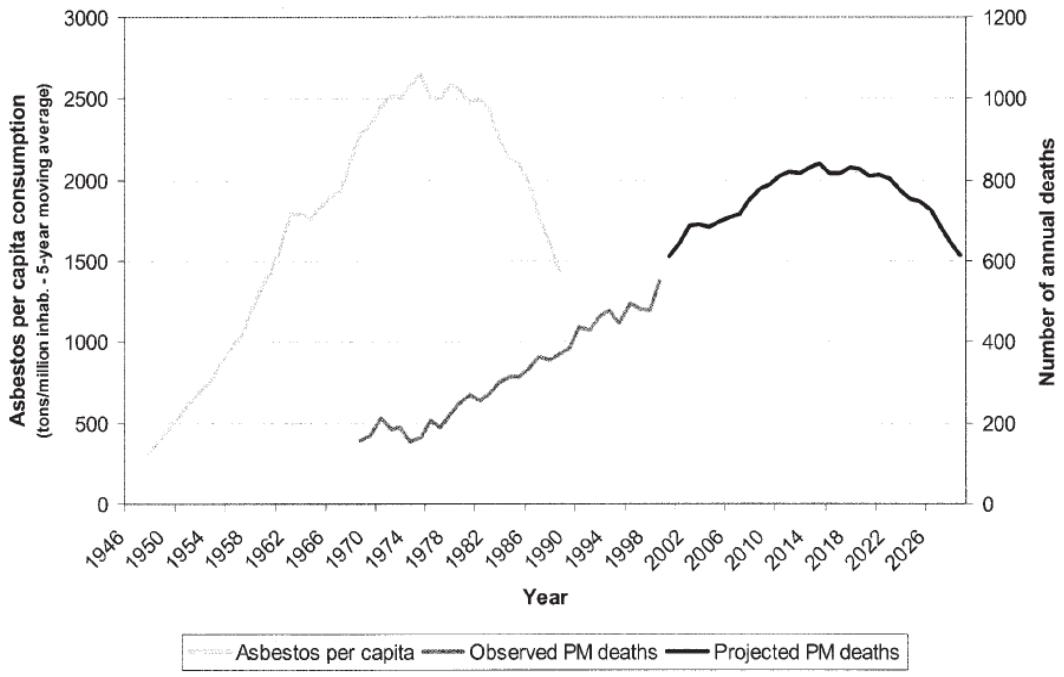

Figure 1 Italian raw asbestos per-capita consumption (5-year moving average; tons per 1,000,000 inhabitants), observed (1970 –1999) and predicted (2000 – 2029) malignant pleural mesothelioma deaths among men aged 25–89 years old in Italy. Pleural mesothelioma deaths = pleural cancer deaths X 0.73

In addition to asbestos group of minerals, there are some other mineral groups that show to have a structure resembling characteristics of asbestos. This minerals are classified as *asbestiform minerals*, since they are formed by fibres with long, thin shapes juxtaposed between themselves, and easily separable one from another [16]. These types of fibres had no or limited industrial use and they include: *erionite*, *fluoro-edenite* and *vermiculite*.

Erionite is a fibrous zeolite contained in the zeolite stones used to build houses in the Cappadocia region of Turkey, where high mesothelioma incidence and mortality were observed [17]. Erionite has been recently reported as possible cause of MPM also in other countries such as North Dakota [18].

An high mortality due to malignant pleural tumor has been observed in Italy in the Sicilian town of Biancavilla in the years 1980-1993. *Ad hoc* epidemiological studies and environmental monitoring revealed an etiological role of an asbestos-like mineral named *fluoro-edenite*. Fluoro-edenitic fibers were found in the materials extracted from a stone quarry located near the city and used in the local building industry, as well as in soils for road paving [19].

Another example of Natural Occurring Asbestos is depicted by exposure to *vermiculite*, a phyllosilicate that expands greatly and exfoliates when heated, in mine workers and inhabitants of Libby, Montana (USA). Here, as in the other places, an high mortality for malignant pleural mesothelioma has been reported [20]. In addition to occupational exposure for miners, vermiculite was extensively used in gardens, driveways, high school track and in the insulation of walls of home by the people living in Libby, thus causing environmental exposure. Local *vermiculite* was contaminated by other kind of fibrous minerals, classified as asbestiform *tremolite-actinolite*, along with *richerite* and *winchite* [21].

1.3. DIAGNOSIS

Most of the patients show to have a fluid accumulation in the pleura by standard radiography and physical examinations. Only between the 10 and 20% of patients present little or no fluid, which in any case tends to diminish as the disease goes on [22]. Usually, pathological confirmation of malignant pleural mesothelioma is represented by imaging tests (e.g. radiography, CT scan, MRI and PET scan), cytology test on pleural fluid, histology and immunohistochemistry staining on biopsy tissue, and thoracoscopy, usually video-assisted.

1.3.1. DIAGNOSTIC IMAGING

First line for the diagnosis of malignant pleural mesothelioma is radiography of the thorax. Generally, thorax radiography could highlight presence of pleural effusions and masses coupled to them. Plaques in the pleura are commonly taken as signs of asbestos exposure, but not as presence of malignant pleural mesothelioma. A limit of standard radiography is the capacity to discriminate the fluid nature from effusion due to hearth failure or other non-malignant diseases. Because pulmonary findings can be obscured by pleural disease on chest radiography, computed tomography (CT) would better evaluate for pulmonary fibrosis and metastases.

CT, being a non-invasive procedure, is preferentially chosen in particular in pre-surgery organization in that subjects who undergoes to surgery. As radiography, CT can identify pleural plaques linked to asbestos exposure. In addition, CT scan can better describe aspects of the lesions defining extension and staging of the disease, since it can help to identify the fluid and to discern the pleural-based masses, lymph nodes, blood vessels, and lung parenchyma from the fluid itself.

Another well used diagnosis imaging is represented by magnetic resonance (MRI). Enabling the differentiation between tumor mass and normal adjacent tissue, MRI is useful in identifying extension of the disease and invasions towards structures close to pleura. In addition, thanks to its characteristics, MRI can be useful in planning radiotherapy for localized disease.

Positron-emission tomography (PET) can be helpful in identifying benignant and malignant effusions, in addition this imaging test can find metastases that are hidden to the other tests. It also appears to be useful for detecting extra thoracic disease, particularly lymph-node involvement, and hence has a role in the staging of tumors. Different patterns of marker uptake are seen, some of which provide additional information about the extent of the disease that is not obvious on CT scans. High standardized uptake values correlate with a worse prognosis and also help to differentiate tumor from fibrosis and necrosis in some patients. It has been suggested that the results of PET combined with CT scanning more accurately reflect the likely response to chemotherapy than do the results of either PET or CT scanning alone.

Anyway, due to similar clinical and radiological features of primitive pleural or peritoneal mesothelioma and secondary metastatic localizations, these tests are not sufficient to give a certainty of diagnosis for malignant pleural mesothelioma. Then, it is recommended to perform cytological analysis and biopsies to enhance confidence index of diagnosis.

1.3.2. BIOPSY PROCEDURE

Biopsy procedures used in diagnosis are thoracentesis (blind percutaneous needle and fine needle aspiration), imaging-guided core biopsy, video-assisted thoracoscopy (VATS) and thoracotomy. The method of choice at present to improve diagnosis accuracy is VATS.

VATS allows to acquire direct pleural biopsy and drainage of effusions, in order to obtain biological material to perform following cytological and histological analysis. In addition, VATS allows to visualize whole hemithorax and to perform, in some cases, complete pleurodesis preventing reaccumulation of the effusion. By sight with VATS, malignant pleural mesothelioma appears to be a firm and greyish tumor that could covers entirely the lung with a thick ring of tumor. In addition, the tumor mass can merge to both visceral and parietal pleural surfaces forming discrete plaques and nodules. Thus, VATS allows to perform accurate visual biopsy and to gain pleural fluid from effusions for cytological exams, and specific tumor specimens for histological assays.

1.3.3. CYTOLOGICAL EXAMINATIONS

Cytological examinations, as mentioned before, are performed on pleural effusion fluid and give some suggestive diagnostic information on the nature of the disease. Alone by itself cytological exams can recognize malignant pleural mesothelioma in less than 85%. Usually cytological exams are performed in consecutive steps. Starting from identifying whether the cells into the fluid are mesothelial, markers, such as calretinin, cytokeratin 5/6, and Wilms' tumor 1 antigen (WT1), are used. The second step is to determine malignancy of cells using markers for epithelial membrane antigen (EMA, also known as CA15-3 and mucin-1), like E29 or MC-5 antibodies, with E29 characterized by an higher specificity than MC-25. Since cytological evidence are not sufficient to determine with certainty the nature of the disease, it is often matched with more accurate histological examination.

1.3.4. HISTOLOGICAL EXAMINATION

Malignant pleural mesothelioma shows to have an extraordinary morphological variety, presenting epithelial peculiarity, with different growth patterns, or sarcomatoid

characteristic; in addition it can shows to have a mix of the two previous characteristics. Accordingly to its morphology malignant pleural mesothelioma can be classified into three main categories: epithelial, sarcomatoid and biphasic. Because of its histological variety, it is extremely problematic to discern malignant pleural mesothelioma form neoplasm and metastasis originated from other cancers. Metastasis from other tumors infiltrated in the serous can show histological morphology often indiscernible from that of the malignant pleural mesothelioma. In addition, secondary metastasis can determine reactive proliferation of mesothelial cells. Thus, bewilderment can be generated during histological diagnosis.

Diagnosis procedure is based on a multidisciplinary approach with different medical experts, i.e. clinician, radiologist, and pathologist. Focusing on the last aspect, pathologists base their anatomical pathology diagnosis on macro and micro criteria backed by histochemetsry and immunohistochemistry to reach a certainty of diagnosis.

Immunohistochemistry, based on antigen-antibody reactions, allows to define immunophenotype of the tumor through the use of mono or polyclonal antibodies which binds in specific manner to given antigens. The most used and principal antigens are listed below categorized for being positive or negative markers of mesothelioma (Table 2):

1. positive markers:
    1.1. keratins and vimentin, when used separately, give limited information for differential diagnosis, because they could be expressed both in primary pleural tumour and metastatic adenocarcinoma. Keratin 5/6 are reported to be expressed in between the 60% and 100% of epithelioid mesotheliomas, depending on the dimension of the biopsy specimen, since staining could be restricted to a focal site, or even in individual cells while adenocarcinomas and serous carcinomas show to have 19% and about 27%, respectively [23-28]. Hence, keratins are quite useful to

Table 2 Immunohistochemical tests for differential diagnosis of malignant pleural mesothelioma.

Test Type	MM ep/m	MMs	CaM	S (p/m)
Keratin 5/6	+	+	+	-
Vimentin	+	+	-/+	+
EMA/HMFG-2	+ membrane	-	+ cytoplasm	-
Calretinin	+	+	-	-
WT	+	+	-	-
CEA	+	+	+	-
B72.3	-/	-	+	-
MOC-31	-	-	+	+
Ber-EP4	-/+	-	+	-

MM ep/m = epithelioid/mixed Malignant Mesothelioma; MMs sarcomatoid Malignant Mesothelioma; CaM = Metastatic Carcinoma (mostly adenocarcinoma); S (p/m) = primitive/metastatic Sarcoma

discriminate mesotheliomas from adenocarcinoma, while these markers are not enough specific to discriminate between mesothelioma and serous carcinomas. To strengthen diagnosis, vimentin is usually coupled to keratins, since a perinuclear staining of both of the two intermediate filaments is suggestive of mesothelioma [29];

1.2. epithelial membrane antigen (EMA) human milk fat globulin 2 (HMFG-2) are related members of a family of high-molecular-weight trans-membranous glycoproteins. Both these two glycoproteins stain positive in mesothelioma and adenocarcinoma of the lung, but with two different patterns of distribution as are respectively expressed on the membrane and in the cytoplasm [30] ;

1.3. Calretinin is one of the preferred immunomarkers as it is reported to be highly specific and sensitive in recognizing malignant pleural mesothelioma. In fact, differently from other highly reactive marker that stain specifically epithelioid

mesothelioma, calretinin shows to be frequently expressed in all mesothelioma subtypes. Even if there are some conflicting about percentage of positive staining in adenocarcinomas and other types of carcinoma, usually calretinin is reported to have a strong, diffuse and clear pattern of expression in epithelioid mesothelioma in both the nucleus and cytoplasm, while in other cancers it appears to be weakly staining and restricted to small focal sites [23, 24, 31-33] ;

1.4. Wilm's tumor 1 (WT1) is another well used marker in identifying mesothelioma. This marker is generally used to discern between mesothelioma and adenocarcinoma, as it shows to have a positive staining in as high as 90% of mesotheliomas and in none of adenocarcinomas of the lung [24]. On the other hand, this immunostaining test has no use in discerning cancer type between mesothelioma and serous carcinoma, since both of the tumors are markedly positive for WT1.

2. negative markers:

2.1. carcinoembryonic antigen (CEA) is one of the first markers used for differential diagnosis of mesothelioma from adenocarcinoma, since CEA shows to be positive in almost 80% of adenocarcinoma of the lung, while is reported to be negative in about 100% of mesothelioma [24, 34]. Conversely, CEA has little use in discriminating mesothelioma from cancer like renal cell carcinoma and serous carcinoma, since these two last tumors show to have a negative immunostaining for the CEA;

2.2. B72.3 is a monoclonal antibody that stains positively in a wide variety of adenocarcinomas (lung, ovary, and peritoneum), while is reported to not stain in epithelioid mesothelioma. Hence, B72.3 is one of the most preferentially used

markers in discriminating between epithelioid mesotheliomas and adenocarcinomas [35];

2.3. MOC-31, a monoclonal antibody that recognize specifically Ep-CAM, is considered to be one of the most effective negative marker for mesothelioma when differential diagnosis is required between mesothelioma and adenocarcinoma of the lung and serous carcinoma of the ovary. On the other hand, MOC-31 has little use for discriminating mesothelioma from renal cell carcinomas [36].

3. Ber-EP4 has a controversial behavior in immunostaining between mesothelioma and other tumors, being positive in a range from 30% and 100% in various carcinomas and adenocarcinomas, while mesotheliomas can be positive to Ber-EP4 stain in as much as one third, even if it appears to be weak and focal. Then, a positivity to this test cannot exclude a diagnosis of mesothelioma, but it is common practice to accept a negative diagnosis of mesothelioma if the reactivity of this marker is wide and intense [37].

At the present immunohistochemistry staining is used to obtain a diagnosis of malignant pleural mesothelioma excluding other neoplasms. Hence, there are no certain positive markers that identify by themselves the disease, but the diagnosis is always performed using different panels of immunomarkers, which stain differentially in different tumor.

In conclusion, the most effective positive markers for mesothelioma are reported to be calretinin, cytokeratin 5/6 and WT1, while most important negative markers are CEA, MOC-31, Ber-EP4 BG-8 and B72.3. Between the mesothelioma subtypes the most accurate immunohistochemical diagnosis is represented by the epithelioid subtype, while sarcomatoid mesothelioma is reported to be the most difficult to discriminate, because its similarity with sarcoma and pulmonary sarcomatoid carcinoma.

What is been exposed here raises two main aspects about malignant pleural mesothelioma diagnosis: a) despite all the advances in imaging technologies and supports of immunochemical staining in determining the nature of the disease, diagnosis for malignant pleural mesothelioma remains a complex process; b) since there are no precise and unequivocal clinic-pathologic guidelines, it is necessary to develop a system that will help clinicians in recognizing the disease with higher certainty and earlier in time.

1.3.5. STAGING

As with all malignancies, proper staging is crucial in malignant pleural mesothelioma for rational treatment planning. Over the years, many staging systems have been proposed. The most widely accepted is the TNM-type system of the International Mesothelioma Interest Group (IMIG) [38].

In brief, malignant pleural mesothelioma is classified into four stages. Each stage describes how far the mesothelioma has spread from its point of origin in the pleura. Stages I and II are considered early-stage mesothelioma. Stages III and IV are considered late, or advanced-stage mesothelioma.

Classification and descriptions of each stage are reported as follows:

Stage I includes lymph node-negative subjects with minimal tumor confined to the parietal pleura (Stage Ia) or with minimal visceral pleural involvement (Stage Ib). Subjects in Stage I show to have the best prognosis, since the disease is more responsive to treatment, because its localization to the pleura. A common curative surgery used for Stage I subjects is the extrapleural pneumonectomy (EPP).

Stage II includes lymph node-negative subjects with confluent superficial tumor on all pleural surfaces or involvement of the diaphragmatic muscle or lung parenchyma. Subjects in Stage II show to have a more favorable prognosis than those diagnosed with an

advanced stage. Stage II subjects are still eligible for potentially curative surgery, like the pleurectomy with decortication (P/D).

Stage III is the most common presenting stage and includes subjects with metastasis to hilar (N1) or ipsilateral mediastinal (N2) lymph nodes, or those with extension of tumor into the soft tissues of the chest wall, the endothoracic fascia, mediastinal fat or pericardium (T3 tumor). The prognosis of subjects diagnosed with Stage III is more favorable than Stage IV. Successful curative surgery may extend a Stage III subject's life expectancy beyond 16 months.

Stage IV includes subjects who have locally advanced tumor invading the spine or ribs, the chest wall extensively, transdiaphragmatic spread, or contralateral pleural spread. Subjects with Stage IV disease also may have contralateral or supraclavicular lymph node involvement (N3) or distant metastases. The average survival rate for Stage IV subjects is 12 months after diagnosis. Stage IV subjects do, however, have treatment options available. Palliative treatment can help ease pain and discomfort caused by advanced-stage mesothelioma.

1.4. OTHER CAUSES OF MALIGNANT PLEURAL MESOTHELIOMA BESIDE ASBESTOS

1.4.1. RADIATION THERAPY

Malignant pleural mesothelioma cases are known to occur in subjects who underwent to thoracic and/or abdominal radiotherapy or received intravenous treatment with Thorotrast [39, 40]. The latency of this mesothelioma has been calculated approximately in 20-30 years after radiation exposure [41, 42]. In children who underwent radiotherapy as treatment for Wilms' tumor, the only causal factor present and ascribable to mesothelioma development was ionizing radiation. [43]. Ionizing radiations were shown to cause

malignant mesothelioma even in experimental studies in rats [44]. Exposure to Thorotrast explains a minimal proportion of MPM occurring in Italy each year (1.7-4.7%).

1.4.2. GENETIC PREDISPOSITION

Familiar predisposition to malignant pleural mesothelioma is reported in the literature. The study of familiar clustering might enlighten a possible causal factor based on individual genetic susceptibility to development of the disease. At any rate, interpretation of familiar clustering has to consider a possible common exposure to asbestos of the subjects under study before claiming a genetic susceptibility [45, 46]. In the study performed by Ascoli et al. on 1954 cases of malignant pleural mesothelioma collected between 1978 and 2005 in three different regions of Italy, the asbestos exposure was confirmed for the major part of the cases, while a familiar causal factor was reported to account for 1.83% of the entire population. Even if the percentage of the familiar cluster is low, it is possible to speculate a genetic origin for malignant pleural mesothelioma. In addition, among entire asbestos exposed population only a small percentage show to develop malignant pleural mesothelioma. Based on these evidences, some studies, in particular in Finland and Italy, had evaluated the existence of a relationship between mesothelioma risk and genetic polymorphisms, in particular genes of the stress oxidative response and DNA repair mechanism, suggesting an interplay of these polymorphisms in the risk of developing mesothelioma. The risk in subjects with these polymorphisms seems to be 2/4 times higher in comparison to non-carrier subjects [47-49]. Recently, genetic susceptibility has been strengthen by various study on BAP1, an oncosuppresor gene often associated to different cancers [50]. Inactivating somatic mutations has been found in 12 tissue pleural samples affected by malignant pleural mesothelioma on 53 total specimens (23%) [51]. Similar findings have been found in other two studies, in which inactivating

somatic mutations showed to be present in 12 cases of malignant pleural mesothelioma of the pleura on 68 total cases, accounting for the 18%, and in 8 mutated cell lines on 25, equal to 32% [52]. Since in both the last two described studies asbestos exposure was frequently confirmed, it has been hypothesized the presence of somatic mutations in BAP1 and asbestos exposure might concur to malignant pleural mesothelioma development [51].

1.4.3. VIRAL INFECTION

The first suggestion of a relationship between malignant pleural mesothelioma and viral infection of SV40 virus traced back to 1994 [53]. Carbone and others observed the presence of SV40 in the majority of studied cases, and concluded that its presence was a an essential phase in inducing the disease. After several years of debating, particularly focused on identifying and confirming the presence of SV40 DNA in malignant pleural mesothelioma samples, recent studies do not confirm this association between virus presence and onset of the disease [54]. In addition, no association between antibody response and malignant pleural mesothelioma has been observed in prediagnostic mesothelioma serum samples [55]; or presence of SV40 DNA in a large population study had not been found [56]; neither SV40 RNA had been found in malignant pleural mesothelioma cells [57].

1.5. MECHANISMS OF ASBESTOS CARCINOGENICITY IN MALIGNANT PLEURAL MESOTHELIOMA

Two important characteristics related to asbestos fiber carcinogenicity are dimensions and surface properties. Experimental studies showed that fibers longer than 5 μm and thinner than 0,1 μm have the higher potential to cause MPM. In particular the early studies by Stanton [58] reported the highest frequency of MPM for fibres longer than 8 μm and thinner than 0,1 μm. However, very short fibres have been detected in pleural and lung

samples of patients thus a possible role also of fibres shorter than 5 μm cannot be excluded [59].

The surface activity of fibres is influenced by numerous factors such as crystal structure, chemical composition, origin of the mineral and metal ions contaminants. In particular, iron ions seem to be the catalytic site for the generation of reactive oxygen species (ROS) and free radicals.

Thin fibres can reach terminal bronchioles and alveoli, where alveolar macrophages can phagocyte the fibres shorter than 14-25 μm. Longer fibres have instead the ability to damage macrophages hindering ("frustrated phagocytosis") one of the main fiber removal mechanism and also leading to the release of ROS.

Bio-persistent fibres (that is fibres neither phagocytised nor altered by physical-chemical actions) may pass through the bronchial epithelium and reach the pleura, the peritoneum and other locations where they can interact with target tissue cells (lung epithelium, mesothelium, interstitial fibroblasts) for very long time [60, 61].

Chrysotile and amphiboles have different estimated lifetime, with shorter period for the first than the amphiboles group. It has been estimated a period of about 9 months to dissolve a fibre with a diameter about 1 μm, whereas smaller particles with 0.1 μm might be totally dissolved in only weeks [62].

The mechanisms of malignant transformation is still incompletely understood. MPM is clinically evident after an average period of 30 or more years during which a complex set of genetic and molecular alterations take place.

The pathogenesis of malignant pleural mesothelioma has been studied by cytological analysis to understand if characteristic abnormal karyotypes are present. Many different abnormalities, like extensive aneuploidy and structural rearrangements, have been

described. The most common changes are the loss of chromosome 22 [63] and structural rearrangement of 1p, 3p, 9p,and 6q [64].

Based on experimental studies, at least six peculiar features of a malignant tissue in active and uncontrolled growth have been reported in MPM and are briefly described (Figure 2).

1. *Growth Advantage*: malignant mesothelial cells show to grow in non-regulated manner via autocrine mechanism, in which mesothelioma cells might produce themselves growth factors needed for proliferation. Growth occurs in response to many different stimuli, such as platelet-derived growth factors (PDGFs) [65], epidermal growth factor (EGF) [66], transforming growth factors β (TGFβ) [67], and *Wnt* protein pathway [68].
2. *Immortalization by the Action of Telomerase*: generally cell division is limited in time, this mechanism is controlled by length of telomeres. It has been shown that telomerases are expressed in the majority of mesothelioma cells, thus allowing the cells to continue proliferation, becoming immortalized. Immortalization brings about to loss of genomic region and aneuploidy [69].
3. *Absence of Tumor-Suppressor Genes*: usually cells avoid to become cancerous cells by the activity of genes called tumor-suppressors. In mesothelioma some important tumor-suppressors gene products are missing. In particular, *p16* and *p14* are commonly absent, and their lack affects the *Rb* and *p53* pathway, which is important in blocking uncontrolled tumor growth [70, 71].
4. *Induction of Antiapoptotic Processes*: on the other hand cells are doomed to die because of the action of apoptotic processes, which lead to the activation of a signal cascade by the release of caspases after binding on membrane receptors signal molecules such as TNF, TNF-related apoptosis-inducing ligand (TRAIL), and Fas

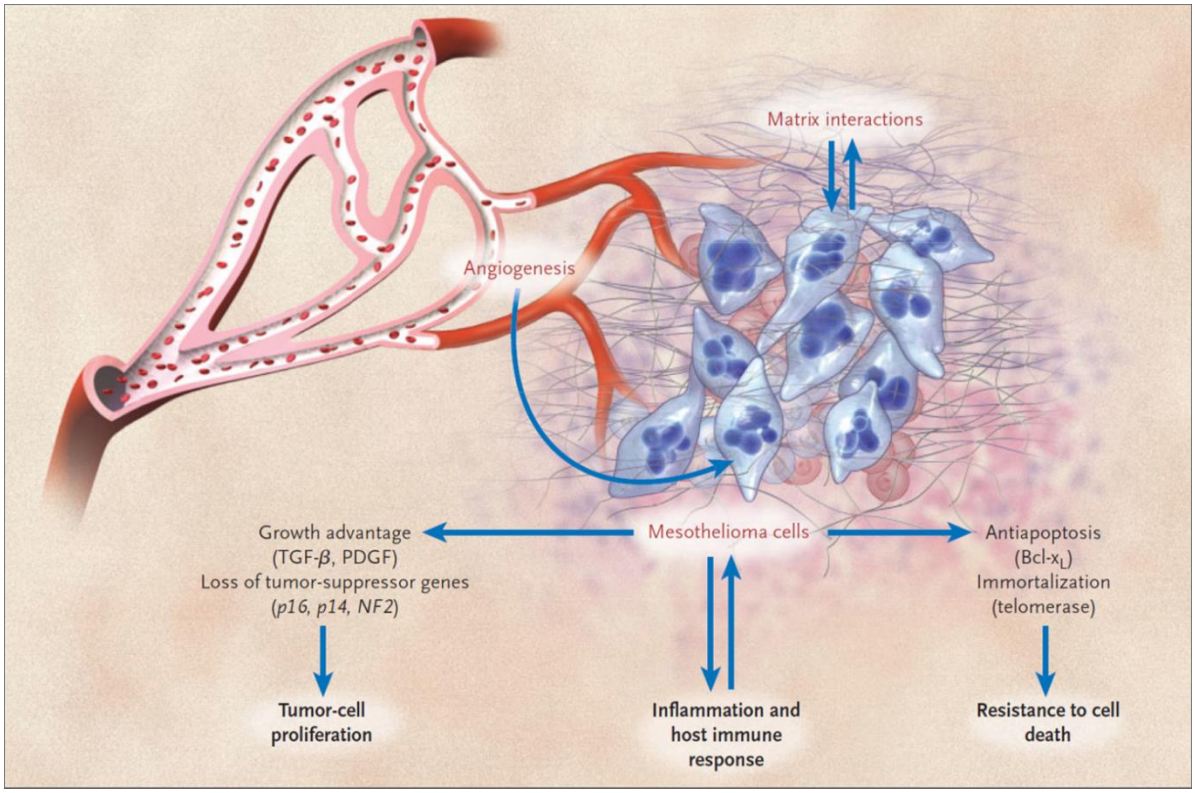

Figure 2 Key biologic features of malignant pleural mesothelioma.

ligand. In mesothelioma cells this apoptotic processes are blocked by the activity of Bcl-x_L, an antiapoptotic molecule often present in tumors.

5. *Increased Angiogenesis*: another important factor for the maintenance of uncontrolled growth is the ability of the cancerous cells to obtain nutrients in large amount. To fulfill this necessity cancers are able to lead formation of new blood vessels. Malignant pleural mesothelioma cells produce a wide range of angiogenic factors, among the other the vascular endothelial growth factor (VEGF). Animal models clearly elucidate the importance of VEGF, in fact in study were this molecule is absent mesothelioma growth results slower [72]. In addition, increase in vascularity is often associated to a worsening of the prognosis in mesothelioma subjects compared to those in which neo-formation of blood vessels is lower [73].

6. *Matrix Interactions*: malignant pleural mesothelioma exists in a collagenous environment, and it is likely that its growth is related to its interactions with and regulation of this environment. Malignant pleural mesothelioma cells make collagen, and the prognosis of mesothelioma appears to be related to the expression of matrix metalloproteinases. Malignant pleural mesothelioma tumors induce responses from their hosts. Chronic inflammation occurs, manifested by the presence of inflammatory cells and cytokines. The inflammation is due both to the asbestos and to the malignant process itself. The host also commonly mounts a weak antitumor immune response to undefined mesothelioma proteins as well as to overexpressed common proteins, such as p53.

All these mechanisms, however, have little specificity and can be activated by a multitude of triggers, thus limiting our capability to provide the groundwork for the development of targeted biomarkers and prevention strategies. The lack of tumor biomarkers for diagnosis and medical survey plays a fundamental role for the development of a universally accepted therapeutic approach and a clearer understanding of the major molecular pathways of malignant pleural mesothelioma is still needed to establish diagnostic, therapeutic and prevention methods [74].

The role of inflammation in causing neoplastic transformation of cells [75] is relevant for mesothelial cells too [76-78]. Overall the processes and the alterations that favor the development of the malignant pleural mesothelioma after asbestos fibres interaction with target cells and with macrophages can be summarized as follows; microenvironment with persistent inflammation and chronic oxidative stress; direct and indirect genotoxic alterations; chromosomal alterations; epigenetic alterations. These alterations cause the activation of the pathways regulating cell cycle, inactivation of oncosuppresor genes, resistance to apoptosis; acquired genomic in stability and neoangiogenis.

1.6. MICRORNA

microRNAs are a relatively recent class of translational regulator. The first findings of small non-coding oligonucleotide sequence are traced back to 1993 [79], when Lee et al. published a study about *C.elegans* development. In this study has been reported that an already known fragment of 700 bp, called lin-4, was able to control timing in nematode larval development without characteristic start and stop codons; therefore without coding for a protein. Once transcribed, lin-4 is folded in a 61 nt long hairpin conformation thanks to the complementarity of its 5' and 3' ends. The hairpin is later cleaved by proteins complex to produce a shorter sequence of 22 nt, which in turn has complementary sites in the 3' UTR of the lin-14 gene. The bound of lin-4 gene product to lin-14 mRNA leads to a significant reduction of the LIN-14 protein, without any change in the total amount of the lin-14 mRNA.

Between the discover of lin-4, in 1993, and another similar small non-coding RNA takes roughly 7 years. Indeed, only in 2000 a study disclose the existence of let-7, a 21 nt long non-coding RNA with a similar timing function in *C.elegnas* development [80]. Conversely to lin-4, the newly called microRNA let-7 was found to be highly conserved across species from nematode to human. Because of this, the existence of other similar RNAs has been hypothesized in other organisms, included human. Indeed, few months later let-7 has been detected at different expression levels in many different tissues and organism [81].

let-7 shows to have a sequence highly conserved not only among species, but also compared to other miRNAs. This highly conserved sequence is called seed sequence. In human 14 miRNAs have been found to share the same seed sequence of let-7, and it is possible to group all of them in a family, in which each different members of the let-7

family can act in redundant manner, cooperating all together to control the same miRNAs target [82-84].

Since 2000, discovery of these ≈22 nt long non-coding RNA has increased exponentially every year (Figure 3), and, together with new miRNAs, our knowledge about their numerous functions and implications in various cellular pathways, such as control of the cell cycle and proliferation [85], cell death [86], signaling pathways [86-88], development [89], and differentiation [90] in physiologic conditions.

More recently it has been proposed that miRNAs may also be involved in several diseases; among them an important role is played in regulation and modulation of gene expression in tumors, including breast, colon, prostate and lung cancers. In neoplasms, miRNAs seem to behave as repressor of tumor suppressor genes or activator of oncogenes, and to be aberrantly in these diseases. Because of their characteristics, miRNAs have been proposed to be used as early biomarkers for cancers and also as a predictor factor of patient survival. Identification of specific miRNA signature not only may be helpful for diagnosis, but would be possible to use miRNAs as target for personal and molecular drug treatment.

1.6.1. MICRORNA BIOGENESIS

In human miRNAs are generally localized within intronic regions of genes or dispersed in non-coding transcripts, rarely miRNAs can be found in exonic regions. Another typical characteristic of miRNAs localization is the organization in polycistronic units, in which different miRNA genes clustered in close proximity to each other. Usually, this clustered miRNAs are transcribed as a single polycistron containing information for all the miRNAs that are singularly post-transcriptionally regulated [91].

Often transcription of intronic miRNA genes are regulated by the same promoter of the host gene. On the other hand, some miRNA genes show to have multiple transcription

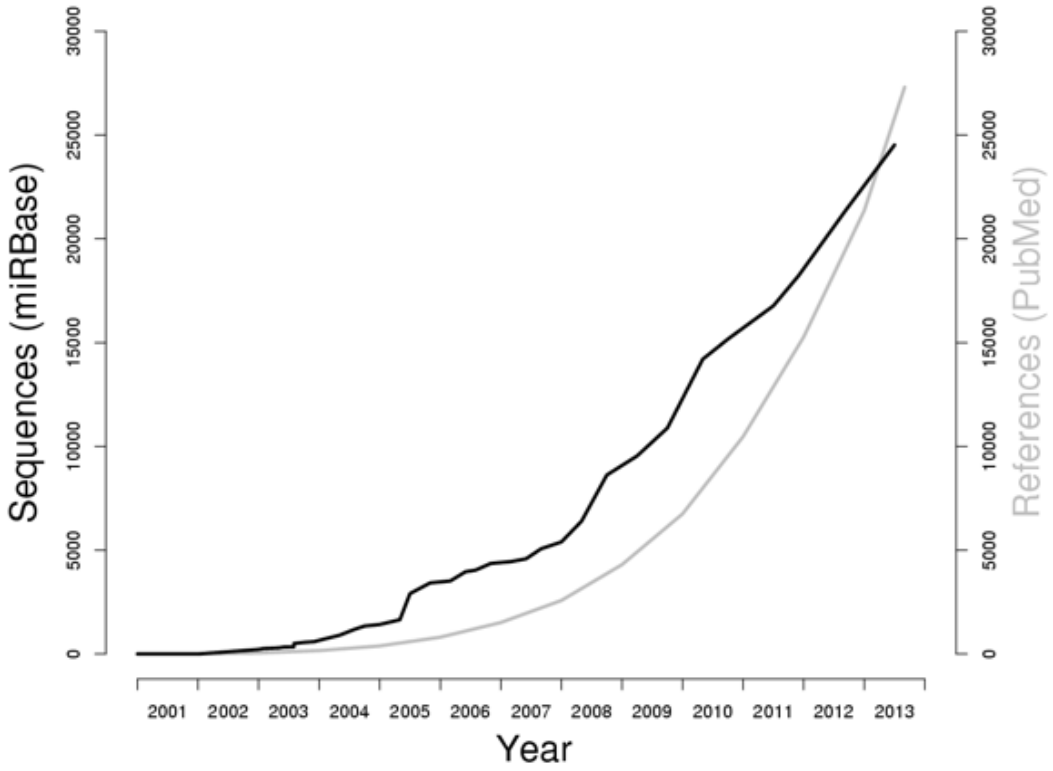

Figure 3 The growth of the number of sequences deposited in miRBase, and the number of papers in PubMed that contain the word "microRNA" in title, keywords, abstract (miRBase R20, June 2013)[http://www.mirbase.org/blog/2014/03/high-confidence-icrornas/]

starting sites with intronic promoters distinct from promoter of their host gene. In both cases, once transcribed, intronic miRNA genes do not interfere with maturation of their host mRNA [92]. Conversely, maturation of a newly transcribed exonic miRNA genes is proven to destabilize the host mRNA [93].

In the nucleus, the transcriptional machinery of miRNAs is normally run by RNA Pol II and its associated transcription factors, together with epigenetic regulators (Figure 4). Then, some transcription factors [94], like MYC, ZEB1 and ZEB2, and epigenetic control [95], such as DNA methylation and histone modification concur to the regulation of miRNA expression. Once miRNA genes are transcribed their primary miRNAs, or pri-miRNAs, are long more than 1 kb and show their characteristic hairpin structure, containing the mature miRNA sequences. Generally, complementary sequences of the same pri-miRNA anneal to form the stem of the hairpin, long 33-35 bp; at the top of the

stem a single-stranded RNA sequence gives rise to the loop of the structure, while at the 3' and 5' ends lay two single-stranded terminals. Then, the maturation process starts with pri-miRNA cleavage by Microprocessor complex (Figure 4), consisting of two proteins Drosha and DRGC8. Drosha is a nuclear RNase III that act specifically on pri-miRNA thanks to its C-terminal double-strand RNA binding domain (dsRBD) and two intramolecular RIIIDs, that dimerize to form one cleavage site for cutting pri-miRNA at its 3' and 5' termini. Since the only Drosha dsRBD isn't sufficient for stabilizing pri-miRNA, DGCR8 is necessary. This protein has two dsRBD close to C-terminal helix and the site of interaction with Drosha. In addition DGCR8 has an important haem-binding domain required for interaction with ferric ions, necessary for an efficient pri-miRNA processing.

Once Microprocessor complex is bound to the pri-miRNA, Drosha is able to recognize a region 11 bp approximately far away from the basal junction, where the pri-miRNA 3' and 5' termini are single-stranded, and 22 bp from the apical junction in the middle of the single-stranded loop. These two regions contain three highly conserved motifs, UG and CNNC motifs in basal junction and UGUG motif in the terminal loop. The interaction between Microprocessor complex is still not totally clear, but deficiency in Drosha causes lethality early in embryogenesis, and lack of DGCR8 in knockout mice arrests development and knockout embryonic cells have impaired proliferation and differentiation.

Therefore, the efficiency of Microprocessor complex is determinant for regulation of miRNAs abundancy and activity. Due to their importance, Drosha and DGCR8 are fine regulated. Actually, while DGCR8 stabilizes Drosha by protein-protein interactions, Drosha acts by enzymatic cleavage on the second exon of DGCR8 mRNA. Thus, this cross-regulatory loop enables the homeostatic maintenance of Microprocessor activity and is deeply conserve throughout the animal kingdom. Other regulations of Microprocessor

activity are determined by post-translational modification, that regulate protein stability, nuclear localization and processing activity [96].

After pri-miRNA is transcripted and processed by Microprocessor complex in the nucleus, it becomes pre-miRNA that is exported to the cytoplasm for its final maturation (Figure 4).

Pre-miRNAs are exported through the nuclear membrane pores by protein transport complexes formed by EXP5 and the GTP—binding protein RAN (Figure 4). So, by GTP hydrolyzation pre-miRNA is released into the cytoplasm and captured by Dicer, an RNase III type endonuclease. C-terminal of Dicer contains two RIIIDs repeated in tandem, forming a catalytic core similarly to those in Drosha (Figure 5). The N-terminal helicase of Dicer recognizes the pre-miRNA loop, increasing the processing of certain pre-miRNAs; while PAZ domain binds preferentially the 3' terminus of pre-miRNAs at two-nucleotide-long overhang left by Drosha cleavage. The recognition and binding specificity make possible to Dicer to produce the pre-miRNA, which are 22-25 nucleotides long. In addition, in mammals and flies, Dicer could bind the 5’ end of the pre-miRNA and cleaves it at ≈22 nucleotides away from the terminus.

The small RNA duplex produced by Dicer is then loaded onto an AGO protein to form an effector complex called RNA-induced silencing complex (RISC), that becomes active after unwinding of the miRNA duplex and removal of the passenger strand (Figure 5). AGO proteins preferentially choose the guide RNA strand (the actual active miRNA) on the base of the relative thermodinamical stability of small miRNA duplexes at their 5’ monophosphate. In facts, the most unstable 5’ terminus is typically selected as the guide strand, especially when the 5’ terminus starts with an U or an A, that are tightly bound by the 5’-phospahate- binding pocket present in AGO proteins. While, passenger miRNA is removed easily when the duplex has central perfect matches. More often, small miRNA

duplexes show central mismatches, then miRNA unwinding occurs without cleavage of the passenger. As the selection of the guide strand doesn't follow a strict process, the passenger strand could become active, even if its relative abundancy is lower than that of the guide strand.

Once pre-RISC is activated to functional RISC, it can affect mRNA translation by two different mechanisms. a) When miRNA loaded onto AGO protein shows to have a perfect complementarity to the target 3'-UTR mRNA, the slicer activity of AGO2 is able to cleave the target between the positions 10 and 11 of the mRNA. Thus, the cleaved mRNA is rapidly degraded. b) In most cases miRNA and its target do not have a perfect complementarity, then RISC starts an inhibitor cascade. Firstly, mRNA translation is likely blocked by the presence of the miRNA. Then, AGO-binding partners are summoned to the RISC-mRNA complex, and the target is deadenylased and decaped at its 5'. In the end, a 5'-to-3' exonuclase is enabled to degrade the mRNA. On the basis of this second inhibition process, it has experimentally described that different miRNA members of the same seed sequence family are able to interfere with translations of different target mRNAs, thus production of a protein is under control of many different miRNA, which concur to the regulation of its translation.

In addition to this regulation, a single mRNA may possess multiple sites that are recognized by miRNA members of different seed family.

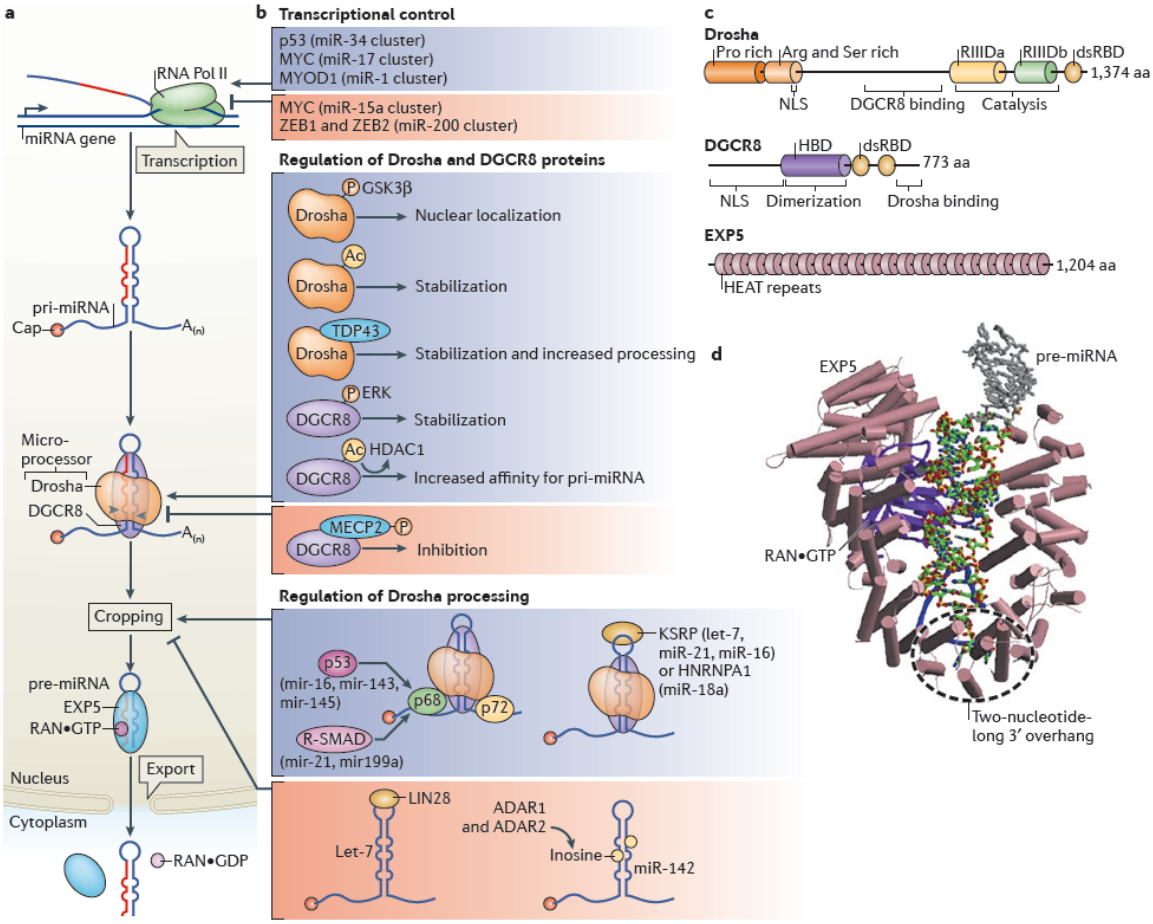

Figure 4 Nuclear events in the miRNA biogenesis pathway. a | Schematic model of miRNA transcription by RNA polymerase II (Pol II), nuclear processing by the Microprocessor complex (comprising Drosha and DGCR8) and export by exportin 5 (EXP5) in complex with RAN•GTP. b | Examples of how miRNA transcription and primary miRNA (pri-miRNA) processing are regulated. p53, MYC and myoblast determination protein 1 (MYOD1) transactivate the miR-34, miR-17 and miR-1 clusters, respectively. MYC transcriptionally suppresses the miR-15a cluster, and ZEB1 and ZEB2 transcriptionally suppress the miR-200 cluster. Various post-translational modifications of Drosha and DGCR8 control the activity and/or localization of these proteins; the protein responsible for the modification, and the effect on Drosha or DGCR8, is shown in each case. The RNA-binding protein TAR DNA-binding protein 43 (TDP43) also interacts with Drosha. RNA-binding proteins, such as p68, p72, KH-type splicing regulatory protein (KSRP), heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) and LIN28, also regulate pri-miRNA processing. Finally, ADAR1 and ADAR2 mediate RNA editing from adenosine to inosine, which interferes with Drosha processing. c | Domain organization of Drosha, DGCR8 and EXP5 in humans. d | Structure of EXP5 (pink) complexed with RAN•GTP (violet) and pre-miRNA83. The position of the two-nucleotide-long 3′ overhang, which interacts with a basic tunnel-like structure on EXP5, is indicated with a circle. aa, amino acid; Ac, acetyl; dsRBD, double-stranded RNA-binding domain; GSK3β, glycogen synthase kinase 3β; HBD, haem-binding domain; HDAC1, histone deacetylase 1; HEAT, huntingtin, EF3, PP2A and TOR1; MECP2, methyl-CpG-binding protein 2; NLS, nuclear localization signal; P, phosphate; RIIID, RNase III domain; R-SMAD, receptor-activated SMAD. Part d from Okada, C. et al. A high-resolution structure of the pre-microRNA nuclear export machinery. Science 326, 1275–1279 (2009).

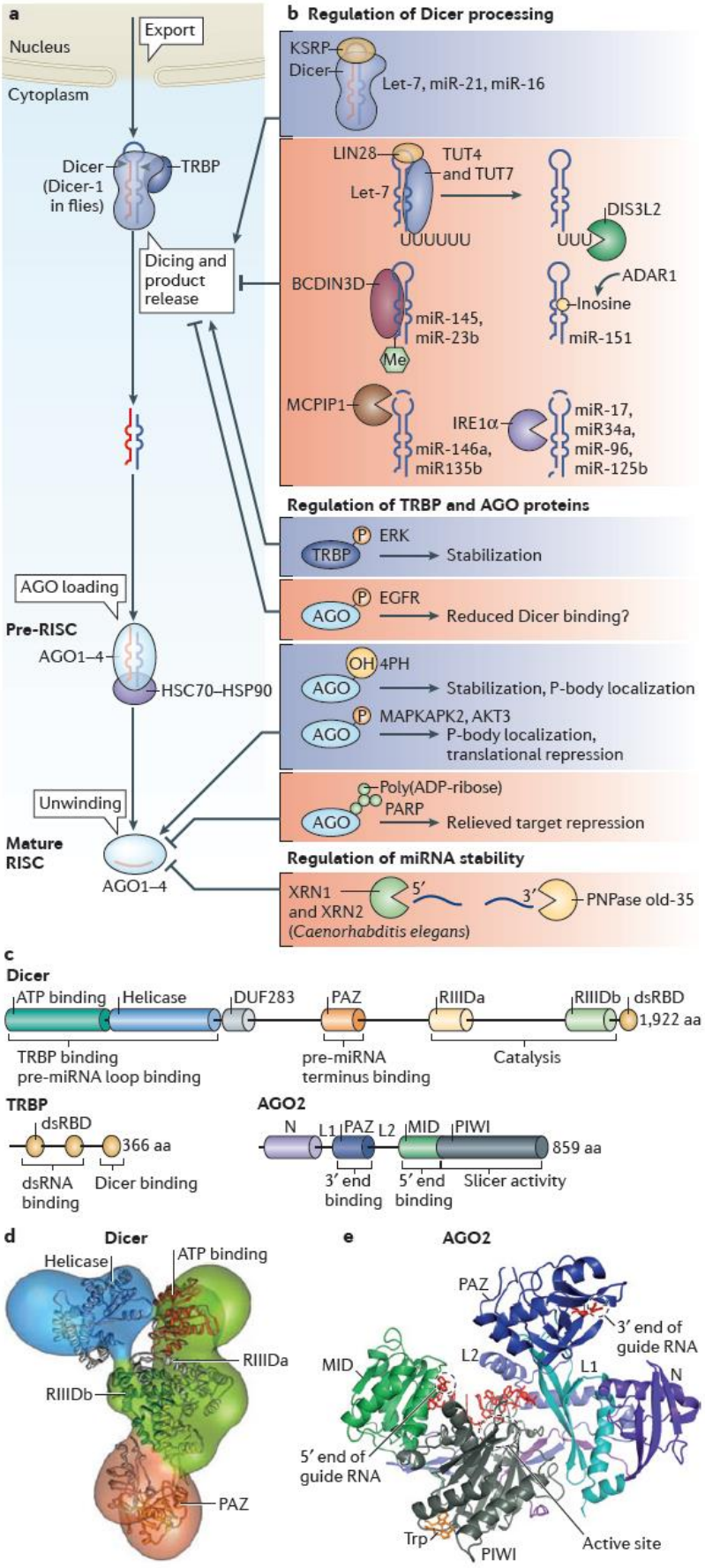

Figure 5 See caption Page 34.

Figure 5 Cytoplasmic events in the miRNA biogenesis pathway. a) Schematic model of Dicer-mediated processing and Argonaute (AGO) loading. Dicer interacts with a double-stranded RNA-binding domain (dsRBD) protein (TAR RNA-binding protein (TRBP) in humans and Loquacious (Loqs) in flies). Following Dicer processing, the RNA duplex is released and subsequently loaded onto human AGO1–4. A heat shock cognate 70 (HSC70)–heat shock protein 90 (HSP90) complex hydrolyses ATP to load the RNA duplex. The 'passenger' strand is discarded and the 'guide' (mature) microRNA (miRNA) remains in one of the AGO proteins. b) TRBP and AGO proteins are subject to post-translational modifications, which influence their ability to regulate Dicer processing, RNA-induced silencing complex (RISC) formation and miRNA activity. miRNA tailing by TUT4 and TUT7, RNA methylation by BCDIN3D, RNA editing by ADAR1, a decrease in RNA stability mediated by MCP-induced protein 1 (MCPIP1) and Ser/Thr protein kinase/ endoribonuclease IRE1α also inhibit Dicer processing. A decrease in miRNA stability can downregulate miRNA activity. c) Domain organization of Dicer, TRBP and AGO2 in humans. d) Cryo-electron microscopic reconstruction of human Dicer108. e) Structure of human AGO2 and the guide RNA (red)136. The 5′ monophosphate of the guide RNA is tightly anchored to the 5′-phosphate-binding pocket at the interface between the MID (middle) and PIWI domain. 4PH, type I collagen prolyl 4-hydroxylase; aa, amino acid; EGFR, epidermal growth factor receptor; KSRP, KH-type splicing regulatory protein; L, linker; MAPKAPK2, MAPK-activated protein kinase 2; N, amino-terminal domain; OH, hydroxyl; P, phosphate; P-body, processing body; PARP, poly(ADP-ribose) polymerase; PAZ, PIWI–AGO–ZWILLE; RIIID, RNase III domain. Figure part d from REF. 108, Nature Publishing Group. Part e from Schirle, N. T. & MacRae, I. J. The crystal structure of human Argonaute2. Science 336, 1037–1040 (2012).

1.7. MESOTHELIOMA AND MIRNAS

As miRNAs are relatively a new discovery in molecular biology, great efforts are especially focused in studying their role in regulation of gene expression, and especially as oncogenic molecules. On the basis of their ability of interfering with gene expression, miRNAs are involved in many important cellular processes, like proliferation, development, differentiation, apoptosis, and stress response. Thus, miRNAs show to have aberrant expression in many different cancers, hence they can play an important role in oncogenesis. An important characteristic of miRNAs is their specificity of expression in normal and cancerous tissues, due to this miRNAs might be used as biomarkers for early detection and diagnosis of diseases, as well as to differentiate diagnosis between a primary or secondary neoplasm forms. In addition, miRNAs are present not only in the site of their production, but from there they are able to spread into in many different human body fluids, like in blood, exhaled breath condensate (EBC), saliva, and urine.

Due to their short sequences, miRNAs are much more stable and resistant to degradation by RNases than mRNA, hence analysis of miRNAs expression are more reliable and reproducible than genes expressions analysis.

To extensively study miRNAs expression profile two technologies are generally used: microarray analysis and quantitative real-time PCR (RT-qPCR). The first is a useful high-throughput method that allows quantification of various miRNA in a single reaction containing specific probes for all the target miRNAs. In addition, microarrays use low volume and amount of starting samples. On the other hand, microarray results need a validation, as quantification reads are not sensitive as RT-qPCR. This second technology is, in fact, highly sensitive and able to quantify in high fidelity the amount of a specific miRNA.

To date, only a few studies have been performed on relationships between miRNAs deregulation and malignant pleural mesothelioma. One of the first studies about miRNA differential expression [97] found a subset of deregulated miRNAs in frozen malignant pleural mesothelioma samples collected during surgery, compared to normal human pericardium. In addition, some of these miRNAs resulted to be specific for each morphological subtype of malignant pleural mesothelioma, suggesting a scope of miRNAs not only in diagnosing malignant pleural mesothelioma, but also in detecting subtype, making their use available as prognostic factors and choice of treatment. In the same article, researchers reported a target genes analysis performed by web-based computational approach to screen predicted target of the deregulated miRNAs. They concluded that among those genes deregulated by miRNAs they found some of the most affected genes in malignant pleural mesothelioma, like CDKN2A, NF2, JUN, HGF and PDGFA [97].

Other fascinating aspects of the use of miRNAs in medicine, in addition to diagnosis markers, are their possible role as predictors of prognosis and cancer therapeutics.

Pass et al. [98] reported a differential regulation of hsa-miR-29c-5p among different histo-morphological type of malignant pleural mesothelioma. In this study, 129 surgically treated snap-frozen malignant pleural mesothelioma tissues, 9 malignant pleural mesothelioma cell lines and three normal mesothelial cell lines were tested by miRNA microarray platform, and further validate with RT-qPCR. Conclusion stated that hsa-miR-29c-5p has an higher expression in the epithelial form of the disease than in non-epithelial malignant pleural mesothelioma, resulting in a more favorable prognosis and a longer median survival of epithelioid-affected patients.

Recently, cell-free miRNAs in plasma of MPM patients has been investigated by several research groups [99-102] but those investigations have been done only on a few patients and/or for a few candidate miRNAs, and results need confirmation.

1.8. EXHALED BREATH CONDENSATE AND BLOOD

Since miRNAs are stable and resistant to RNases-mediated degradation, and do not show any particular post-transcriptional modifications, they are thought to be good molecules to use in diagnosis of many different disease. Moreover, miRNAs have been found not only in cell cytoplasm, but also in extracellular environment such as plasma, serum, saliva and urine [103]. Some studies have positively correlated the level and composition of these biological matrices with diseases or other pathological conditions [103, 104]. On the base of these findings, the investigation of miRNAs as specific biomarker of pathological conditions is intriguing. By definition, a good and useful biomarkers has to be characterized by eager of accessibility, non-invasive procedures of extraction, detection of a specific disease earlier than the symptoms occurrence. Also proteins might be used as good biomarkers [105], but differently from miRNAs, they show an higher complexity of composition due to their many post-translational modifications, in addition proteins have a low abundance in most of the biological samples and this influences greatly the reliability of their use as biomarkers. However, miRNAs could present the same obstacles, but, conversely to proteins, methods like PCR and other amplification strategies might overcome this kind of problems, making miRNAs much easier to detect and to quantify with high-fidelity.

In this project we have chosen two biological matrices from which isolate extracellular miRNAs: blood and exhaled breath condensate (EBC).

Previous studies demonstrate that miRNAs are present and could be detected in blood [103, 104]. Their release out of the producing cells have been hypothesized to follow three possible mechanisms: passive release from cells[106]; active secretion in response to different stimuli as protein-miRNAs complex [107]; and active release through microvescicles [108].

Recently, EBC has been used in clinical studies as a biological matrix where miRNAs can be analyzed and quantified by qPCR to investigate their role in non-small-cell lung cancers [109]. EBC presents the advantages to be easily collected in a non-invasive way causing no discomforts in patients of any age and any health status. In addition, EBC might represent a potential surrogate of the lung environment and the lower respiratory tract, avoiding the need of tissue removal from the same body district.

2. AIM OF THE STUDY

Understanding the molecular mechanisms responsible for malignant pleural mesothelioma development, as well as finding a non-invasive disease biomarker, would be fundamental to better monitor and to early diagnose malignant pleural mesothelioma in affected patients and previously exposed subjects, enhancing prognosis and therapy.

Malignant pleural mesothelioma diagnosis is not easy, as it is often hard to distinguish between inflammatory/reactive diseases and malignant forms. In addition, the long latency between asbestos exposure and disease onset, makes the follow up of exposed individuals quite challenging.

MiRNA expression levels vary greatly among tissues, and it is believed that dysregulation of miRNA can contribute to cancer development. Therefore, miRNA expression in malignant pleural mesothelioma, might have a central role in influencing oncogenic pathways, particularly in EBC produced from pulmonary environment cells, as well as in plasma. Therefore, it is of great interest the analysis of miRNAs as a fundamental part of mechanisms of action mesothelioma-associated gene expression which may have relevance in etiology, progression and treatment of malignant pleural mesothelioma.

Specific aims of this project are:

Aim 1: Determine whether a specific miRNA signature (in plasma or EBC) may help to discriminate between malignant pleural mesothelioma patients and patients affected by other pulmonary diseases, among patients presenting at the Thoracic Surgery Unit with a diagnosis of pleural effusion.

Aim 2: Determine whether a specific miRNA signature (in plasma or EBC) may help to discriminate between Malignant Pleural Mesothelioma (MPM) patients and healthy subjects with a Past Exposure Asbestos (PEA).

Aim 3: Evaluate a possible correlation between miRNAs in EBC and plasma.

Aim 4: Estimate sensitivity and specificity of altered miRNAs using ROC curves.

3. MATERIAL AND METHODS

3.1. STUDY POPULATION

The study population includes 23 malignant pleural mesothelioma (MPM) cases (group A), 9 subjects with pleural effusion due to diseases others than MPM (group B) and 19 healthy subjects occupationally exposed to asbestos (control group C).

Group A and B subjects have been enrolled among patients presenting at the Thoracic and Lung Surgery Unit (Ospedale Maggiore Policlinico Fondazione IRCCS Ca' Granda) with pleural effusions. Each subjects underwent to echo-assisted thoracentesis. Samples of pleural fluid were tested by cytology for malignant tumor cells, BK, common germs and chemical-physical examination. Same subjects underwent to a further examinations by total body PET combined with CT of the thorax, these imaging tests help clinician in both preoperative organization and staging of the disease. Video-Assisted Thoracoscopy (VATS) was then performed on the basis of the imaging knowledge to obtain tissue from the site of pleural effusions. After biopsy, specimens has been analysed histologically to describe the ethology of pleural effusion. 23 subjects resulted positive to immunohistochemical staining for malignant pleural mesothelioma composed the group of Malignant Pleural Mesothelioma (MPM) cases, while subjects with a negative response to malignant pleural mesothelioma fell into the group of Non-Mesothelioma (NM) subjects (Group B).

Group C includes subjects presenting at the Occupational Health Unit for clinical surveillance due to Past Exposure to Asbestos (PEA). Each subjects underwent to routine examinations with Pulmonary Function Testing coupled with $D_{LCO,}$ thorax oblique radiography, and Bronco-Alveolar Lavage, to determine quantitatively asbestos exposure by counting number of asbestos corpuscles per milliliter.

The enrolment period ranges from October 2012 to August 2014. The study has been approved by the local Institutional Review Board ("IRCCS Fondazione Policlinico, Ca' Granda")

3.1.1. DIAGNOSTIC CRITERIA FOR MALIGNANT PLEURAL MESOTHELIOMA

The diagnosis of malignant pleural mesothelioma was established based on standardized criteria following the ReNaM criteria [110]. A subject was classified as MPM when the histological examination of paraffin-embedded tissue confirmed a diagnosis of malignant pleural mesothelioma; at least one of the following immunohistochemistry test was positive: keratin LMW/HMW, vimentin, calretinin, and HMBE-1 and VATS confirmed neoplastic lesions in the pleura.

If none of the above criteria was satisfied the subjects were classified in NM group (group B).

3.1.2. DATA COLLECTION

After signing informed consent each subjects was administered with a standardized questionnaire to collect information about asbestos exposure at work, home and in the environment, smoking habits, previous clinical history and demographic data.

Individual, written, informed consent from each participant and approval from the local Institutional Review Board ("IRCCS Fondazione Policlinico, Ca' Granda" review board) had been obtained before the study. All the subjects of the study had been informed about treatment of the data and its disclosure to third parties; aim and rationale of the project; health risks derived from collection of biological samples; and prospects of use on the fringes of biological samples in surplus, also for studies concerning the same aim.

After signing informed consent, each subject was interviewed by a trained interviewer using a standardized questionnaire (ReNaM questionnaire, appendix A to collect information about asbestos exposure at work, home and in the environment, lifestyle habits, in particular smoking habits, personal and family clinical history, drug intake and demographic data.

3.1.3. ASBESTOS EXPOSURE EVALUATION

Once clinical data and occupational information were acquired, an expert panel reviewed the diagnostic accuracy of cases after examining complete clinical records, including radiology and pathology results and evaluate asbestos exposure. The panel is composed of a pneumologist, an oncologist, a pathologist, an occupational health physician and an industrial hygienist, according to the national guidelines.

Asbestos exposure is coded as follows ::

1. certain occupational exposure:

   subjects who had worked in occupational environment with documented presence/use of asbestos. At least one of the following must be reported: explicit declaration of the patient; environmental investigations, or reports from supervisors, or administrative records, or statements of co-workers, or relative/partner. In addition, subjects with occupational exposure Code 2 or 3 showing disease manifestations such as bilateral pleural plaques, asbestosis, and/or positive Broncho-Alveolar Lavage have to be included in this level.

2. probable occupational exposure:

   subjects who had worked certainly with/in presence of asbestos for occupational reason, but exposure documentation/statement is not available.

3. possible occupational exposure:

subjects who had worked in occupational environment belonging to an economic sector in which presence/use of asbestos was generally observed, but no information about exposure of subjects is available.

4. domestic or familiar exposure:

   subjects not occupationally exposed, but exposure due to domestic environment, since living with at least one employee assigned to categories 1 or 2.

5. environmental exposure:

   subjects who had lived in the vicinity of asbestos manufacturing plants (industries, mines, etc.) or who had attended regularly environments with presence of asbestos for non-occupational reasons.

6. non-occupational exposure:

   subjects who had used asbestos or related materials for non-professional reasons (use of asbestos in furnishings, DIY, home repairs, etc.).

7. improbable exposure:

   subjects who can be reasonably excluded from any asbestos exposure above "natural background" levels, on the basis of complete collected information.

8. unknown exposure:

   incomplete collected information or insufficient knowledge of subjects do not allow an assignment into an exposure category.

9. to be defined exposure:

   subjects for whom exposure assessment and information are being evaluated by experts panel.

10. unclassifiable exposure:

    subjects who are not and will not be available information (closed case).

3.2. BLOOD AND EXHALED BREATH CONDENSATE COLLECTION

Peripheral Blood (9 ml) was collected in tubes containing EDTA (Figure 6 A), processed within 3 hours, and centrifuged at 1200 xg for 15 min to separate plasma and buffy coat fraction from red cells fraction. After centrifugation, plasma fraction was aliquoted in two 2 ml criovials (containing 1500 µl each. Aliquots from the same subject were labelled with an unique ID number and stored at –80 °C.

Exhaled Breath Condensate (EBC) was collected with TURBO-Disposable Exhaled Condensate Collection System (DECCS) (Medivac, Parma, Italy) (Figure 6 B). This system is disposable as suggested by ERS/ATS guideline to avoid any contamination by the presence of disinfectants employed to clean used circuits, or even worst by virus. Briefly, subjects were asked to breath into the mouthpiece for 15 min under normal tidal volume condition with a nose clip to avoid nose breathing. In addition to avoid salivary contamination, the mouthpiece is equipped with a special T-trap, placed right after the mouth piece. Depending on patient's health conditions, collection of EBC could be stopped allowing the subject to regain breath or to cough. Samples of EBC were collected in a refrigerated tube at 5 °C. Briefly after collection EBC samples were centrifuged at 3000 xg for 15 min at 4°C. EBC samples were labelled with subject's ID and stored at –80 °C.

3.3. PROCESSING OF BIOLOGICAL SAMPLE

To isolate free miRNAs and microvescicles from plasma, an aliquot of 1.5ml per subject was thawed on ice; samples were then centrifuged three times at increasing speeds (1000 xg, 2000 xg, 3000 xg) for 15 min at 4 °C. After every centrifugation, supernatant was decanted into a new tube and centrifuged again to remove cell debris and aggregates.

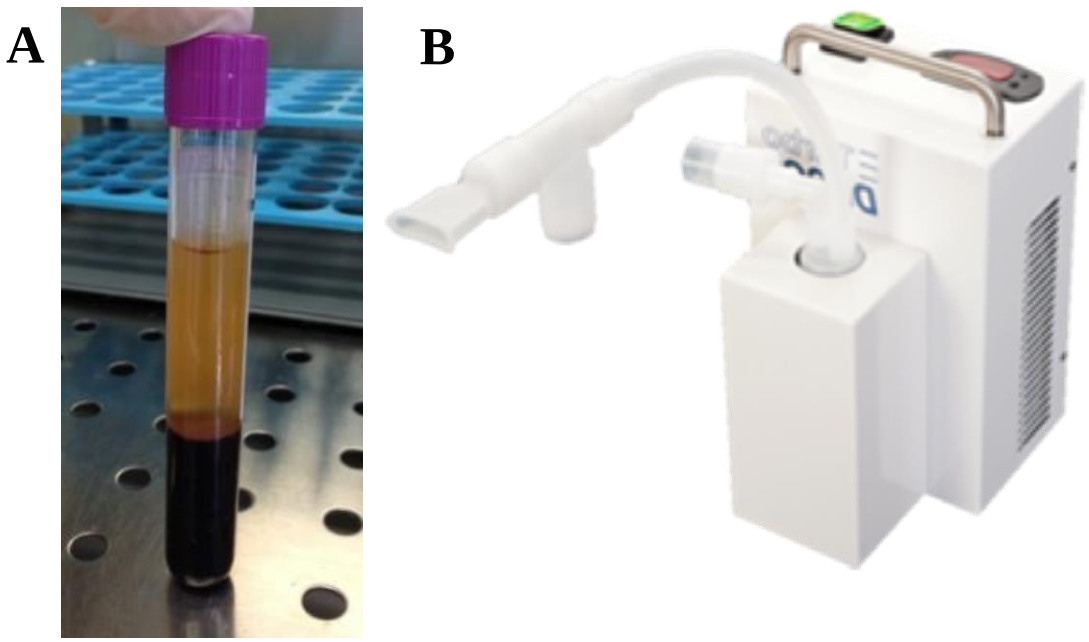

Figure 6 EDTA Tubes with Centrifuged Peripheral Blood. B TURBO-DECCS System.

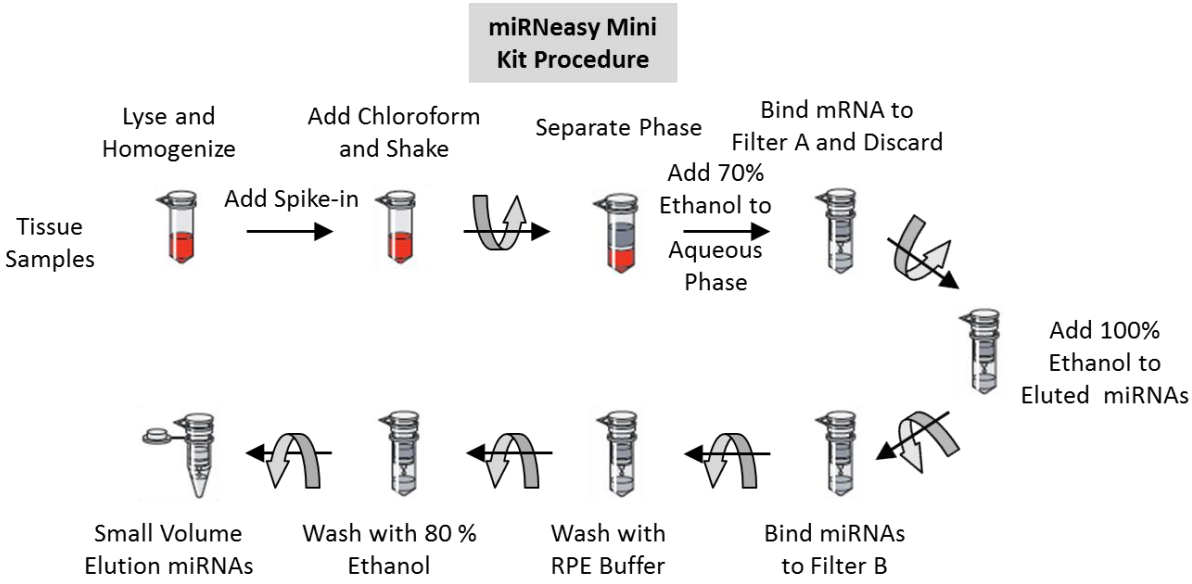

Figure 7 miRNeasy Mini Kit Procedure

EBC samples were thawed on ice and spun down at 4°C to collect all the EBC at the bottom of the vial.

Shortly after centrifugation, all plasma and EBC samples were transferred into ultracentrifuged tubes (Polycarbonate Centrifuge Bottles-Beckman Coulter) and filled up to 10.5 ml total with NaCl 0.9% solution. Ultracentrifugation was performed at 110,000 xg for 2h at 4°C to allow sedimentation of microvescicles.

After ultracentrifugation, supernatant was discarded and tubes were allowed to air dry for 2 min. Then, tubes were stored at -20°C.

3.4. MIRNA PURIFICATION

miRNAs purification was performed using miRNeasy Mini Kit (Qiagen, Düsseldorf, GER)(Figure 7). This method purifies miRNA and other small RNA molecules from human biospecimens, such as Exhaled Breath Condensate and plasma.

The miRNeasy Mini Kit combines phenol/guanidine-based lysis of samples and silica membrane–based purification of total RNA.

Microvescicles were lysed in the 700 µl of reagent QIAzol Lysis Reagent. QIAzol Lysis Reagent is a monophasic solution of phenol and guanidine thiocyanate, designed to facilitate lysis of tissues, to inhibit RNases, and also to remove most of the cellular DNA and proteins from the lysate by organic extraction. After pipetting and vortexing samples, 5 µl of external miRNA spike-in were added to each sample. The external miRNA ath-miR-159a spike-in was then used as normalizer of target miRNAs for following real time PCR experiments (see Statistical Analysis); it consists of an oligonucleotide from *Arabidopsis thaliana* added in a known concentration of 1,2 pg/µl for a total of 6 pg. To allow lysis and RNases degradation, 140 µl of chloroform were added to separate the homogenate into aqueous and organic phases by centrifugation at 12,000 x g for 15 min at 6°C. RNA

partitioned to the upper, aqueous phase, while DNA partitioned to the interphase and proteins to the lower, organic phase or the interphase. The upper phase (~350 µl) was taken and 1 volume of ethanol 70% was added to provide the flow of small RNA molecules through the membrane of the miRNeasy Mini spin column, while RNA molecules from 200 nucleotides upwards remained bound to it. The sample was loaded to a new miRNeasy Mini spin column, and 2/3 volume of ethanol 100% were added to wash efficiently away phenol and other contaminants by centrifugation at 9,700 x g for 15 s at RT. miRNeasy Mini spin column, containing miRNA, were washed with RPE buffer and 80% ethanol by repeated centrifugations, and later 20/25 µl of RNases free water were added to the column to elute miRNAs. Two aliquots per each samples of EBC and plasma were prepared for further Open Array analysis (20 µl Exhaled Breath Condensate and 15 µl plasma) and for qPCR validation (5 µl both).

3.5. miRNA Quality Control

To assess quality of miRNAs purification, samples were measured by 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA)(Figure 8) using Agilent RNA 6000 Small Kit, made to detect a wide range of RNA molecules.

2100 Bioanalyzer is a capillary electrophoresis instrument based on chips used to analyze DNAs, RNAs and proteins.

The chip is composed by 16 wells, 3 of them are for the gel-dye mix, 1 for the ladder ranging from 20 nt to 150 nt, and 12 wells are for samples (1 µl of sample for each well). Firstly, he gel- dye mix is loaded and is spread on the bottom of the chip by a syringe, after this the other two gel-dye mix are loaded, and finally once the gel is ready the 12 samples and the ladder could be loaded.

Figure 8 Agilent Technologies 2100 Bioanalyzer.

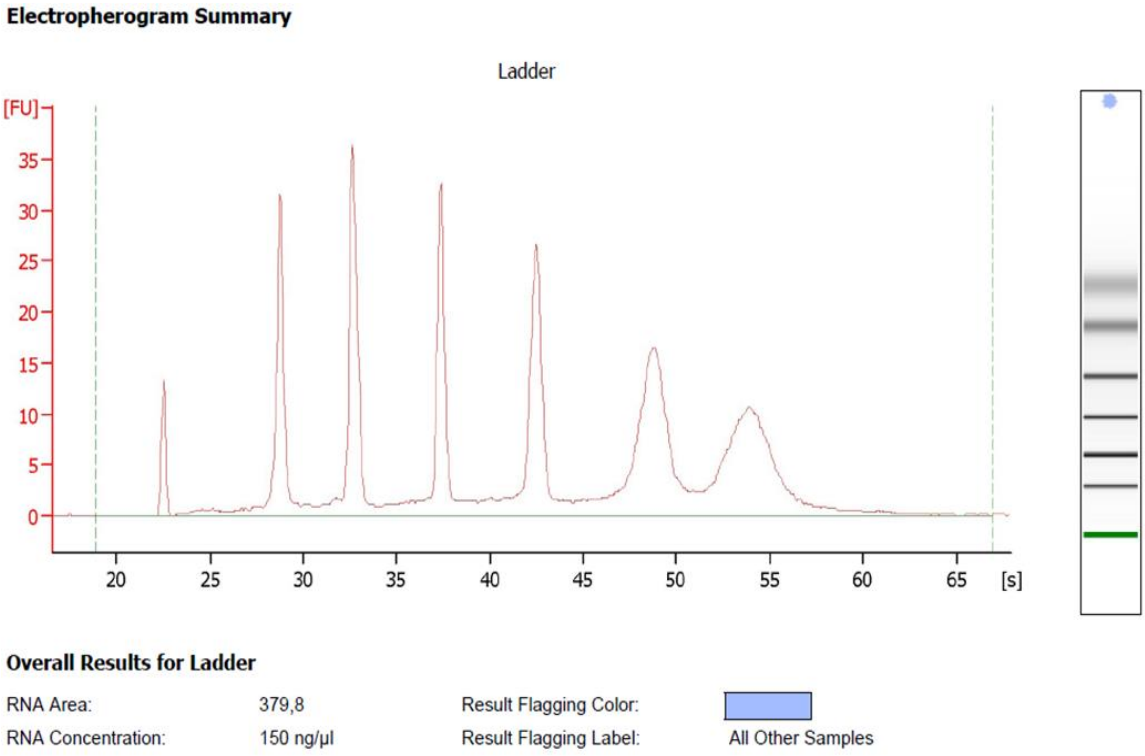

Figure 9 Bioanalyzer ladder.

Charged molecules, as RNA, were leaded electrophoretically by the voltage gradient through the gel on the basis of constant ratio charge-mass. Then, the molecules were separated because of their dimension and detected by laser-induced fluorescence. The analysis software transformed fluorescence data in electropherogramms and compared automatically each sample electropherogramm to the standard ladder (Figure 9) to assess concentration, dimension and quality of each sample.

After quality control, EBC and blood samples, previously purified, were concentrated with Concentrator Plus/Vacufuge Plus (Eppendorf, Hamburg, GER) to 1 µl at room temperature to obtain desired concentration for reverse transcription and Open Array analysis. Directly after concentration, samples were placed on ice.

3.6. REVERSE TRANSCRIPTION AND HIGH-THROUGHPUT QRT-PCR

miRNAs can be reverse transcribed using a reverse transcriptase in combination with specific stem-loop primers (Figure 10). Reverse transcriptases are a class of enzymes that allow to obtain a copy of complementary DNA (cDNA) starting from an RNA template. Since miRNAs are short oligonucleotides, standard random primers are not able to anneal as in long mRNAs sequences allowing the following specific real time assay. While, the stem-loop primers are designed to extend the 3' end of the target miRNA producing an RT primer/mature miRNA chimera template that can be used as in a standard real time PCR. Also, the stem-loop structure in the tail of the primer confers a key advantage to these assays: specific detection of the mature, biologically active miRNA.

Up to 762 miRNAs and controls were reverse transcribed in two separate reactions using two RT Primers pool, containing each one a mixture of miRNA-specific stem-loop primers for an almost comprehensive coverage of the known miRNoma, consistent with Sanger miRBase v14.

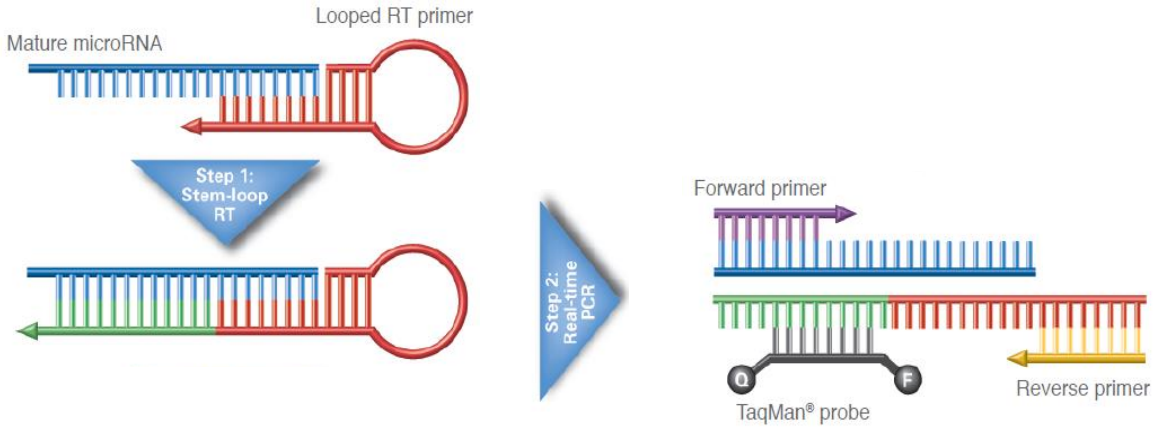

Figure 10 TaqMan MicroRNA Assay mechanism. In a first step mature miRNA is annealed in specific manner to a looped RT primer and reverse transcribed in cDNA. In the second step a normal pair of specific primers are annealed to amplify the miRNA target.

Since the concentration of miRNA in plasma and EBC was low, a uniformed and unbiased amplification was performed starting from 1 ng of total input template, thus all the reverse transcribed miRNAs present in original samples were increased significantly, making assay sensitivity and miRNAs detection better.

For the final quantitation step, a polymerase, with its substrates, was added to each sample and later loaded on Open Array MiRNA panels (Applied Biosystem, Foster City, CA) by AccuFill System (Applied Biosystem, Foster City, CA). The real-time PCR is then run on the QuantStudio 12K System (Applied Biosystem, Foster City, CA).

Briefly (Table 3Table 4), the reactions to reverse transcribe target miRNAs were prepared separately in reaction A and B in a 96-wells plate using 0.75 µl of Megaplex RT Primers Pool A v2.1 and Pool B v3.0 (Life Technologies, Carlsbad, CA), respectively. Both of the two reactions included 0.15 µl of dNTPs 100 mM, 0.75 µl 10X RT Buffer, 0.90 µl of $MgCl_2$ 25 mM, 0.1 µl of RNases Inhibitor 20 U/µl, 1.5 µl of MultiScribe Reverse Transcriptase (Life Technologies, Carlsbad, CA) 50 U/µl. Subsequently, 3.5 µl of miRNA sample were added to reach a total volume of ~7.5 µl. Following reverse transcription

Table 3 Reagents RT Reaction Mix.

Reagents RT Reaction Mix	Volume per sample
Megaplex RT Primers Pool A v2.1/B v3.0	0.75 µl
dNTPs 100 mM	0.15 µl
10X RT Buffer	0.75 µl
$MgCl_2$ 25 mM	0.90 µl
RNases Inhibitor 20 U/µl	0.10 µl
MultiScribe Reverse Transcriptase 50 U/µl	1.50 µl
Volume Reagents	**4.15 µl**
miRNA sample	3.50 µl
Total Volume	**7.65 µl**

Table 4 RT PCR conditions.

Stage	Temperature	Time
	16° C	2 min
40 Cycles	42° C	1 min
	50° C	1 sec
Hold	85° C	5 min
Hold	4° C	∞

consists of 40 cycles at 16°C for 2 min, 42°C for 1 min and 50°C for 1 s, plus one cycle at 85°C for 5 min and final stage at 4°C.

After reverse transcription, each reaction required to be pre-amplified in a 96-wells plate using TaqMan PreAmp Master Mix Kit (Life Technologies, Carlsbad, CA) (Table 5Table 6) with a specific reaction mix for each of the two human miRNA pools. The reaction mix included: 20 µl of TaqMan PreAmp Master Mix 2X, 8.5 µl of Nuclease-free Water and 4 µl of Megaplex PreAmp Primers Pool A/Pool B 10X. Subsequently, 7.5 µl of previously RT miRNA were added to the pre-amplification reaction mix. Pre-amplification thermal conditions consisted in an activation cycle 95° C for 10 min, 55°C 2 min, 72°C for 2 min, 16 cycles of 95°C for 15 s and 60°C for 4 min, 99.9°C for 10 min to inactivate polymerases and final stage at 4°C.

Subsequently, each pre-amplification product is diluted 1:20 with Nuclease-free Water, transferred in 8 wells of a 384-wells plate and mixed with TaqMan Open Array Real Time PCR Master Mix in ratio volume 1:1=MasterMix:cDNA with robot MicroLab STAR Let (Hamilton Robotics, Birmingham, UK).

Open Array Reaction Mix was transferred from the 384-wells plate to a TaqMan Open Array MiRNA Panel with AccuFill System. Each panel was composed of a hydrophobic surface divided in 48 subarrays with 64 hydrophilic through-holes each (Figure 11).

Generally, 16 subarrays cover the detection of the assays obtained by reverse transcription with Megaplex RT Primers Pool A v2.1 and Pool B v3.0. By reverse transcription of 756 miRNAs assays, based on Sanger miRBase v14 for human miRNAs, it is possible to screen the more well-studied miRNAs in 3 different subjects. In addition, the QuantStudio 12 K Flex Open Array manages to run and analyze up to 4 Open Array Panels at the same time.

Table 5 PreAmp Reaction Mix.

Reagents PreAmp Reaction Mix	***Volume per sample***
Megaplex PreAmp Primers Pool A/B 10X	4.0 µl
TaqMan PreAmp Master Mix 2X	20.0µl
Nuclease-free Water	8.5 µl
Volume Reagents	**32.5 µl**
RT miRNA sample	7.5 µl
Total Volume	**40.0 µl**

Table 6 PreAmp PCR conditions.

Stage	***Temperature***	***Time***
Hold	95° C	10 min
Hold	55° C	2 min
Hold	72° C	2 min
16 Cycles	95° C	15 sec
	60° C	4 min
Hold	99.9° C	10 min
Hold	4° c	∞

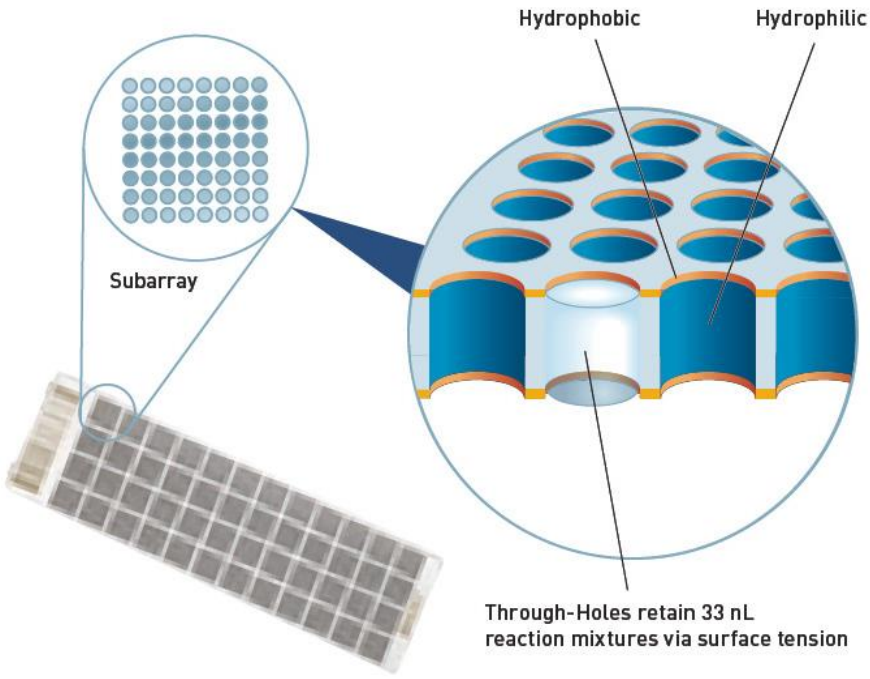

Figure 11 Open Array Plate. Subarray partition and Through-Holes.

Table 7 RT qPCR thermal cycles conditions.

Step	Enzyme Activation	PCR		
	Hold	35 Cycles		
		Denature	Anneal	Extend
Temperature	95° C	95° C	30-60° C	65-70° C
Time	10 min	15 sec	60 sec	

Real-Time qPCR is a method based on PCR amplification to simultaneously quantify of samples of DNA or cDNA. Measure of the quantity for a specific target is measured by the fluorescence generated during PCR reaction. Fluorescence might be caused by different chemistry reaction. In these experiments, hybridization of specific probe was used to target cDNA. TaqMan probes are one of the methods with a more higher rate of fidelity and accuracy, as the TaqMan probes are designed to anneal only to unique target sequence.

A typical Real-Time qPCR cycle is similar to a normal PCR consisting of two main steps as reported in Table 7.

Real-Time PCR is a quantitative method, because data are recorded during exponential growth phase, when quantity of the reaction product is directly proportional to the quantity of the starting nucleic acid.

miRNA quantification is based on fluorescence detected at every reaction cycle. During each cycle cDNA molecules are doubled since they reach a plateau when all the reagents are depleted and enzymes activity is drastically reduced.

Once the reaction is completed, Open Array software gives data for the further analysis. Useful data for analysis must fulfil two main characteristics:

1. Relative Threshold Cycle (C_{rt}): this value represents cycles number of PCR in which reaction efficiency of qPCR is in the beginning of the exponential phase, then the target miRNA is copied at the maximum speed. This data determines the starting quantity of the target miRNA. Briefly, higher C_{rt}s correspond to a low presence of the target miRNA, since that miRNA needs an high number of cycles to enter in its exponential phase and to give a signal detectable by the software. On the other hand a low C_{rt} represents a high amount of starting miRNA. The software keeps trace of C_{rt} from 1 to 35, but a good C_{rt} number falls between the 10th and the 28th cycles. Earlier

than the 10th, the signal might be confused with background noise, then miRNAs, which have lower C_{rt}, are considered undetermined. C_{rt}s higher than 28 are considered as undetermined too, because the exponential phase doesn't reach the plateau phase, so we aren't able to assess the beginning of the exponential phase.

2. AmpScore: this value is calculated automatically by the software and gives a valuation of amplification efficiency and of the exponential curve. By default a good AmpScore has to be higher than 1.24, but it might be decreased to 1.1 if the visualized curve is checked by the operator and it is considered as good as a reference curve with AmpScore of 1.24. For this reason AmpScore values can be used to discriminate between false positives and negatives.

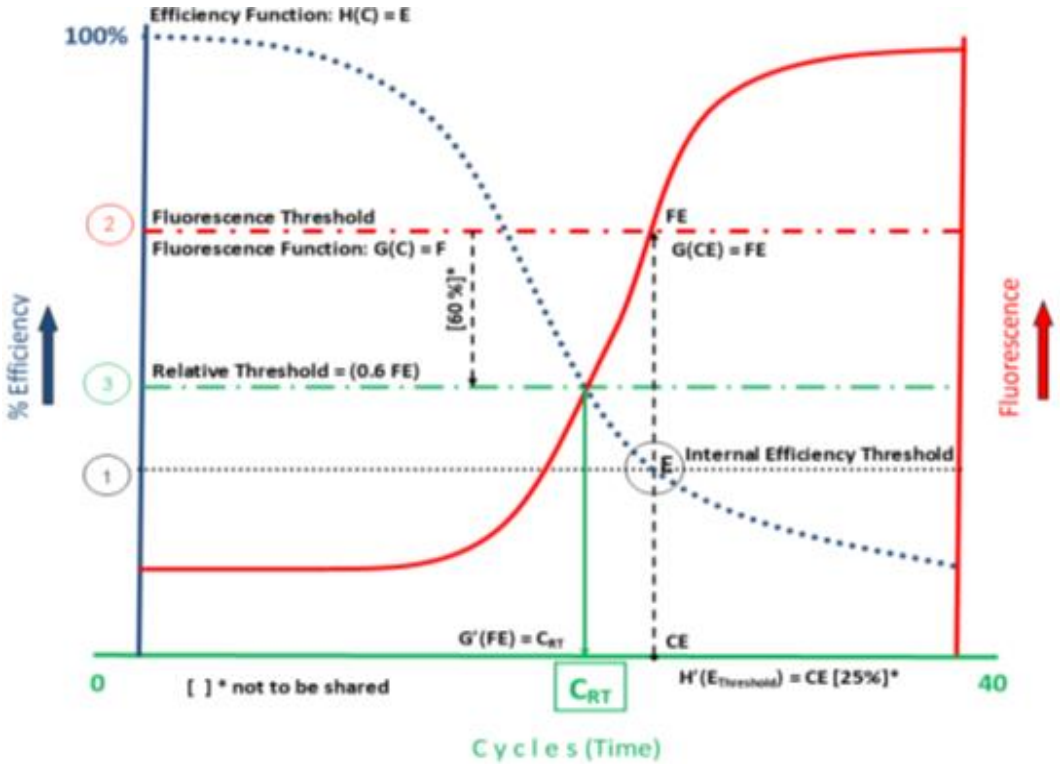

Figure 12 C_{rt} diagram.

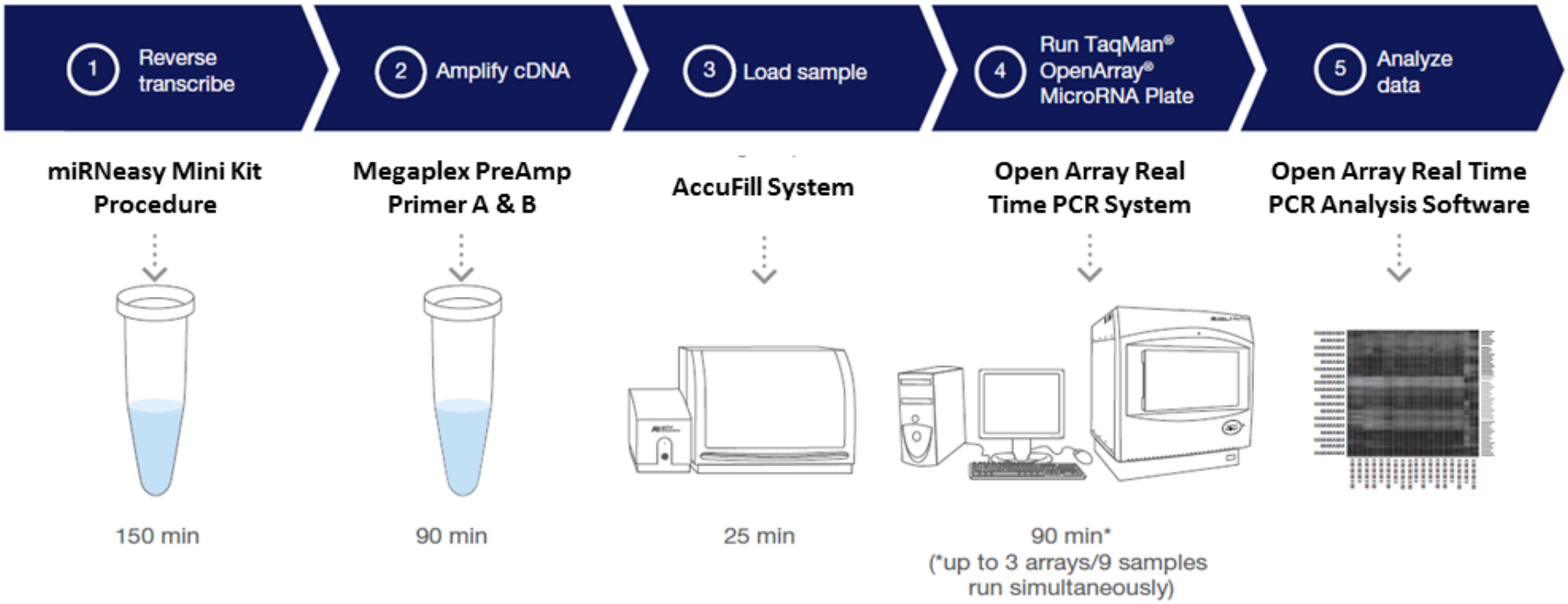

Figure 13 Open Array workflow: from Purified miRNAs to Data Analysis.

3.7. VALIDATION BY RT-QPCR

Validation of previous findings in plasma was performed by using Custom TaqMan Low Density Array on which the following TaqMan miRNA assays were pre-plated in triplicate: hsa-miR-151a-5p, hsa-miR- 107, hsa-let-7e-5p, hsa-miR-664a-3p, hsa-miR-210-3p, hsa-miR-101-3p, hsa-miR-320b, hsa-miR-93-3p, hsa- miR-92a-3p, hsa-let-7c-5p, hsa-miR-378a-3p, hsa- miR-375, and the exogenous spike-in ath-miR159a for normalization. Validation of previous findings in EBC was performed by using Single TaqMan Low Density Array on a 384-wells plate in triplicates for the following miRNAs: hsa-miR-30a-5p, hsa-miR-520b, hsa-miR-646, hsa-miR-378a-3p, hsa-miR-206, hsa-miR-9-5p, and the exogenous spike-in ath-miR-159a for normalization.

3 μL of miRNAs (110 ng) for both plasma and EBC samples were reverse transcribed using Custom RT primer Pool (provided with Custom TL DA). The RT reaction mix of 12 μl included: 6 μl Custom RT primer Pool, 0.3 μl dNTPs (100 nM), 3 μl MultiScribe Reverse Transcriptase (50 U/μl), 1.5 μl RT Buffer (10×), 0.19 μl RNase inhibitor (20 U/μl) and 1.01 μl nuclease-free water. After incubation on ice for 5 min reverse transcription was performed using a C1000 Thermal Cycler (Biorad, Hercules, CA). Thermal cycler conditions were as follows: one step of 16°C for 30 minutes, one step of 42°C for 30 minutes, followed by 1 step of 85°C for 5 minute. The RT reaction was stored at -20°C. All reactions were performed as specified in the protocols of the manufacturer, described in the previous paragraph.

3.8. DATA NORMALIZATION

C_{rt} not detected, with values over 28, or with an AmpScore <1.1 were considered not expressed and then deleted from the statistical analysis. Detected C_{rt} values between 10

and 28 with an AmpScore ≥1.1 were considered well amplified and then normalized. Commonly, normalization is made in combination or with one of the two following methods. (a) The average C_{rt} value obtained from all detected miRNA assays is used to normalize each singular miRNA assay. This strategy required a sufficient large amount of miRNA expressed among the entire study population or at least in a large majority of the subjects. (b) Reference endogenous miRNAs could be used to normalize other miRNA assays. The endogenous approach assumes that a reference miRNA is found stable in its expression among all the study subjects and it isn't affected by any change in the organism. Often a change might affect expression in the expression of the chosen endogenous, then to avoid important data loss more than one endogenous is taken and normalization is made with the average value obtained by the chosen references. Unlike endogenous housekeeping genes, a specific miRNA rarely show to function as a good housekeeping among different tissues, then usually each tissue has its own endogenous miRNA that cannot function as well in other tissues. In addition, our study is focused on two biological matrices, blood and EBC that collect miRNAs from other districts. In facts, these two can be thought as basins inflow (blood like a more unspecified basin, while EBC like a lung district specific basin) where different organs can pour their miRNAs. Because of their characteristics, it is very hard to find and use one or more endogenous miRNA, as for other tissues.

A new strategy for quantifying extracellular miRNAs in serum [111] has been proposed to normalize RT-qPCR miRNAs data to an external RNA spike-in control as it has been shown that the total RNA and total miRNA content of serum is an experimental variable and that normalization to candidate endogenous control miRNAs introduces a systematic bias in miRNA quantification. We used as external spike-in control, introduced during miRNA purification at the lysis phase with QIAzol, a miRNA from *Arabidopsis thaliana*

(ath-miR-159a), as it has no mammalian homologue. In addition to the external spike-in normalization strategy, introduction of an exogenous miRNA has a double effect: a) to monitor the efficiency of miRNA purification and b) to highlight co-purification of PCR inhibitors.

Once data are acquired, all the 8 ath-miR-159a replicates in each Open Array Panel Pool A and B were taken together and the obtained average was used to normalize all the target miRNAs. As discussed before, ath-miR159a showed to remain constant between samples and Open Array run, for this it can be taken as a good reference miRNA, to which other target miRNAs can be normalized. Briefly, normalization was done as the difference (ΔC_{rt}) between a specific C_{rt} miRNA assay of Pool A and B and average C_{rt} of the ath-Mir159a of the same Pool of the target miRNA, or C_{rt}(target miRNA) – C_{rt}(average ath-miR159a). When ΔC_{rt} of a single miRNA assay is greater than 0, it means that the C_{rt} of that specific miRNA is higher than the ath-miR-159a average C_{rt} and its relative quantification is lower than the reference. While, when ΔC_{rt} is minor than 0, the target miRNA C_{rt} is lower than the spike-in ath-miR159a C_{rt} and its relative quantification is higher than the reference.

3.9. STATISTICAL ANALYSIS

Once the ΔCrt was obtained, the Relative quantification (RQ) was calculated as $2^{-\Delta Crt}$. Relative quantification gives information about how much a specific miRNA is up- or down-regulated in a specific group in order to calculate differences between case group and control group.

We performed linear regression model adjusted for age, sex, BMI and smoking habits, to analyze possible expression differences between groups, i.e. MPM subjects vs PEA group, MPM subjects vs NM group. In order to fulfil the assumption of normality of the linear regression model RQ values were previously log2-transformed.

We assumed that a p-value lower than 0.05 was a good marker to identify those miRNAs that showed a change in their expression between different healthy state.

Due to the high number of dependent variable (miRNAs) simultaneously evaluated through separated linear regression models, False Discovery Rate (FDR) was applied to identify the potential top miRNAs. The FDR correction is designed to control the expected proportion of incorrectly rejected null hypotheses and used in multiple-hypothesis testing to reduce Type-1 errors A FDR p-value significance threshold of 0.10 was adopted to select significant top miRNAs.

To categorize the MPM, PEA and NM subjects according to their expression patterns, hierarchical clustering analysis was performed using miRNA expressions with FC higher then 2 and p-value for median comparisons lower than 0.05.

Qlucore software was used for cluster analysis and heat map chart construction for expression profile visualization.

The heat map is a graphical representation of data using colors to indicate the level of espression: red colors indicate down-regulation while green colors indicate up-regulation of miRNA expression.

Volcano plots were obtained to graphically compare the size of the fold change to the statistical significance level of each miRNA in group comparisons (MPM vs NM and MPM vs PEA).

Briefly, every dots coincide with miRNAs found expressed by Open Array analysis, intercepts of every miRNAs on X and Y-axis determine the value of Fold Change (FC) and p-value, respectively. FC was calculated as the ratio between Relative Quantification (RQ) for a given miRNA of MPM group and that of the control group taken in account for the comparison. Hence, when FC was higher than 2, it corresponds to 2-folds up-regulation of

the miRNA in MPM group; while an FC value comprised between 0 and 0.5 reflects a down-regulation of 2-folds or more for the given miRNA. Values of FC are log_2-transformed, hence all the dots at left and right of the drawn vertical lines (intercepting X-axis in ±1) are considered 2-folds negative or positive deregulated, respectively, in MPM subjects in respect of the considered control group. The Y-axis shows negative log10-transformed p-values, hence lower and higher horizontal lines determine values of 0.05 and 0.01, respectively, and all the miRNAs found over those lines are considered significantly differentially expressed in the MPM group in respect of controls group.

We performed ROC curve analysis to evaluate the diagnostic ability of the validated miRNAs found in EBC and plasma. In a ROC curve each point represents a sensitivity/specificity pair corresponding to a particular decision threshold of RQ value. The area under the ROC curve (AUC) is a measure of how well the RQ threshold can distinguish between affected and healthy subjects. A miRNA that discriminate perfectly has a ROC curve that passes through the upper left corner (100% sensitivity, 100% specificity). Therefore the closer the ROC curve is to the upper left corner, the higher the overall accuracy of the miRNA.

4. RESULTS

4.1. STUDY POPULATION

The study population includes :

I. 23 Malignant Pleural Mesothelioma (MPM) cases diagnosed according to the standardized criteria described in section 3.1.1;

II. 19 subjects with Past Exposure to Asbestos (PEA) participating to an health surveillance program due to their past occupational exposure to asbestos;

III. 9 Non-Mesothelioma (NM) cases hospitalized for pleural effusion but with negative VATS and immunohistochemical analyses for MPM .

The main characteristics of the study subjects are reported Table 8.

Most subjects in each group were males (73.9% in MPM , 78.9% in PEA and 88.9% in NM). BMI, age and smoking distributions did not differ across groups (p=0,81; p=0,2; p=0.82 respectively).

Table 9 reports specific characteristics of each MPM, in particular the histological distribution and the main variables related to asbestos exposure such as occupation, period of exposure, latency (years since the beginning of exposure to diagnosis) and years since last exposure to EBC and Blood collection. The more frequent histological types were epithelioid (10 cases) and biphasic (11 cases). 12 cases had certain occupational exposure to asbestos; asbestos exposure was coded as possible in only 1 case with potential exposure due to job as safety system installer in explosion-risk plants. The median latency period was 57 years, and the time since last exposure and test collection ranged between 0 and 62 years.

15 out of 19 subjects with PEA were males (Table 10). These subjects underwent a surveillance program because included in an *ad hoc* registry by the Local Health Unit

(LHU) of their residence. A qualitative judgment about exposure as evaluated by the LHU was available for 17 subjects. 3 were considered at high exposure whereas the remaining subjects had low exposure. 11 PEA subjects had performed Broncoalveolar Lavage (BAL) to verify and quantify possible presence of asbestos bodies (AB): 2 were negative, for the others the number ranged between 1,6/ml and 18/ml. Interestingly, the highest asbestos burden was seen in two females who worked in cured meat packaging . 16 subjects had pleural plaques (a well-known indicator of past exposure to asbestos) at chest x-ray. In addition, two subjects had a radiological diagnosis of asbestosis (the number of AB in BAL was 10 and 6 respectively). The median duration of exposure was 27 years, and the time since last exposure and test collection ranged between 6 and 53 years..

The main characteristic of 9 NM cases are reported in Table 11. A possible exposure to asbestos was detected in 4 of them. 7 had a diagnosis of cancer (4 lung, 1 lymphoma, 1 kidney, 1 breast).

Table 8 Main characteristic of the three study groups.

Variable	***Malignant Pleural Mesothelioma***		***Past Asbestos Exposed***		***Non-Mesothelioma***	
Sex, n (%)	M	F	M	F	M	F
	17 (73.9)	6 (26.1)	15 (78.9)	4 (21.1)	7 (88.9)	2 (11.1)
Age, average (SD) [years][a]						
	70.2 (7.8)		66.5 (6.4)		72.3 (7.4)	
Body Mass Index, average (SD)						
	25.1 (4.4)		26.3 (4.2)		26.7 (6.7)	
Smoking, n (%)						
Current	-		-		-	
Never	6 (26.1)		9 (47.4)		2 (22.2)	
Former	12 (52.1)		7 (36.8)		5 (55.6)	

Table 9 Main characteristics of Malignant Pleural Mesothelioma cases.

ID	Sex	Date of Birth	Age [a]	BMI	Smoking	Asbestos Exposure [b]	Histology	Occupation	Period of Exposure	Date of Diagnosis	Duration of Exposure	Latency (Years)	Years since Last Exposure
MPM01	M	04/10/1950	62	31	Former	UN	Epithelioid	-	-	26/11/2012	-	-	-
MPM02	M	26/03/1956	57	24	Current	PO	Biphasic	Safety System Installer in Explosion-Risk Plants	1980 - 2012	30/11/2012	32	32	0
MPM03	M	28/07/1944	68	30	Former	CO	Epithelioid	Car Maintenance Technician	1976 - 2002	13/12/2012	26	36	10
MPM04	M	22/06/1942	70	35	Former	UN	Biphasic	-	-	17/12/2012	-	-	-
MPM05	F	30/03/1954	59	21	Never	UN	Biphasic	-	-	20/12/2012	-	-	-
MPM06	M	23/11/1934	78	23	Former	CO+EO	Sarcomatoid	Security Service in Asbestos-Caulked Plants	1949 - 1988	21/12/2012	39	63	24
MPM07	M	15/01/1940	73	22	Never	UN	Biphasic	-	-	04/02/2013	-	-	-
MPM08	M	13/12/1936	76	34	Former	CO	Epithelioid	Asbestos-Panels Retailer, Inventory	1980 - 1997	06/02/2013	17	33	16
MPM09	F	23/07/1958	55	18	Former	UN	Epithelioid	-	-	14/02/2013	-	-	-
MPM10	M	08/05/1940	73	22	Former	CO	Epithelioid	Jewel Maker, Abestos-Panel User	1956 - 1986	22/02/2013	30	57	27
MPM11	F	09/02/1941	72	19	Current	CO	Epithelioid	Textile Industry, Laundry Worker, Seamstress	1960 - 1967	12/03/2013	7	53	46
MPM12	M	19/09/1936	77	25	Former	CO	Biphasic	Asbestos Brake Lining Repairing Service	1951 - 1992	04/04/2013	41	62	21
MPM13	M	28/07/1931	82	25	Former	CO	Biphasic	Asbestos-Products Reseller	1946 - 1951	02/07/2013	5	67	62
MPM14	F	23/02/1955	58	22	Never	UN	Biphasic	-	-	09/07/2013	-	-	-
MPM15	M	22/09/1938	75	26	Former	CO	Epithelioid	Asbestos Brake Lining Production Electric Oven Operator in Metal Industry	1953 - 1954 1964 - 1989	18/07/2013	1 25	60	24
MPM16	F	26/07/1937	76	21	Never	CO	Biphasic	Medal Maker, Asbestos-Panel User	1952 - 1958	29/07/2013	6	61	55
MPM17	M	07/09/1942	71	23	Current	CO+EO	Epithelioid	Jewel Maker, Abestos-Panel User	1956 - 2003	22/08/2013	47	57	10
MPM18	M	21/02/1937	77	25	Never	ENV	Epithelioid	-	1955 - 1956	14/11/2013	1	58	57
MPM19	F	22/09/1938	75	30	Never	UN	Epithelioid	-	-	10/12/2013	-	-	-
MPM20	M	03/02/1951	63	25	Current	ENV+EO	Biphasic	-	1971 - 1972	26/02/2014	1	43	42
MPM21	M	28/02/1935	79	24	Former	CO+EO	Biphasic	Construction Industry Worker, Plumber, Cable and Tube Layer	1949 - 1969	13/03/2014	20	65	45
MPM22	M	03/04/1947	67	28	Current	CO	Biphasic	Construction Industry Worker, Plumber, Cable and Tube Layer	1963 - 2013	22/04/2014	50	51	1
MPM23	M	11/07/1944	70	25	Former	UN	-	-	-	08/05/2014	-	-	-

a) Age at diagnosis.

b) Classification of Asbestos Exposure: CO, certain occupational; PO, probable occupational; EO, extra-occupational; ENV, environmental; and UN, unknown.

Table 10 Main characteristics of subjects with Past Asbestos Exposure.

ID	Sex	Date of Birth	Age [a]	BMI	Smoking	Asbestos Exposure [b]	Exp Level [c]	BAL [d]	Diagnosis [e]	Occupation	Period of Exposure	Date of Sampling	Duration of exposure	Years since Last Exposure
PEA01	F	23/03/1948	65	20	Former	CO	High	-	PP	Gunny Sack Recycler	1961 - 1963	16/01/2013	2	50
PEA02	F	06/03/1946	67	26	Never	CO	High	-	PP	Gunny Sack Recycler	1959 - 1960	16/01/2013	1	53
PEA03	M	08/06/1948	65	27	Never	CO	Low	-	PP	Construction Industry Bricklayer (Roofs)	1963 - 2007	18/01/2013	44	6
PEA04	M	16/04/1957	56	35	Current	CO	Low	0	PP, PE	Glassworks	1980 - 2000	18/01/2013	20	13
PEA05	M	09/10/1945	67	28	Never	CO	Low	10	PP, Asb	Caulker	1973 - 1992	22/01/2013	19	21
PEA06	M	04/11/1945	67	28	Never	CO	Low	-	Neg	Welder Balcksmith in Metal Industry	1971 - 1998	24/01/2013	27	15
PEA07	M	01/09/1932	80	22	Former	CO	Low	7	PP	Upholsterer - Fireproof Furniture Production Maker	1948 - 1997	19/01/2013	49	16
PEA08	M	06/06/1937	76	26	Current	CO	Low	2	PP	Boiler Maintenance Technician	1973 - 1999	30/01/2013	26	14
PEA09	M	30/09/1940	72	27	Never	CO	High	6	PP, Asb	Caulker	1963 - 1998	01/02/2013	35	15
PEA10	M	12/05/1949	64	24	Former	CO	Low	2	PP	Metalworker in Metal Industry	1970 - 2001	04/02/2013	31	12
PEA11	M	13/05/1949	64	24	Current	CO	Low	3	PP	Metalworker in Metal Industry	1973 - 2000	04/02/2013	27	13
PEA12	M	26/02/1946	67	27	Never	CO	Low	0.5	PP	Caulker	1968 - 1995	05/02/2013	27	18
PEA13	M	06/11/1949	63	21	Former	CO	Low	1.6	PP	Welder Balcksmith in Metal Industry	1969 - 2001	12/02/2013	32	12
PEA14	M	18/12/1954	58	26	Former	CO	Low	-	Neg	Welder Balcksmith in Metal Industry	1970 - 1991	28/03/2013	21	22
PEA15	M	30/05/1947	66	30	Former	CO	Low	-	Neg	Welder Balcksmith in Metal Industry	1971 - 1996	05/03/2013	25	17
PEA16	F	25/08/1950	63	20	Never	CO	Low	18	PP	Cured Meat Factory	1965 - 1987	17/04/2013	22	26
PEA17	F	03/06/1956	57	35	Never	CO	Low	13	PP	Cured Meat Factory	1971 - 2001	17/04/2013	30	12
PEA19	M	08/03/1940	74	25	Former	CO	-	-	PP	Electrician	1954 - 1998	25/03/2014	44	16
PEA20	M	03/04/1941	73	26	Never	CO	-	-	PP	Rubber Vulcanization Technician	1968 - 1984	01/03/2014	16	30

a) Age at samples taking

b) Classification of Asbestos Exposure: CO, certain occupational.

c) Qualitative Exposure Level to asbestos evaluated by LHO.

d) Number of AB per ml are reported.

e) Diagnosis PP, pleural plaques; PE, pleural effusions; Asb asbestosis; and Neg, negative.

Table 11 Main characteristics of Non-Mesothelioma subjects.

ID	Sex	Date of Birth	Age [a]	BMI	Smoking	Asbestos Exposure [b]	Diagnosis	Occupation	Date of Diagnosis
NM01	F	09/08/1953	59	20	Former	UN	Lung Cancer	-	14/11/2012
NM02	M	29/06/1933	79	24	Former	UN	Inflammatory Status	-	27/11/2012
NM03	M	23/02/1933	80	26	Never	PO	Kindey Cancer	Power Plant, Occasionally Switchboard Wirer	08/05/2013
NM04	M	15/01/1948	66	41	Never	UN	Lung Cancer	-	23/07/2013
NM05	M	10/09/1943	70	21	Former	PO	Lymphoma	Short Time Periods in Asbestos-Caulked Plants	21/08/2013
NM06	M	24/02/1932	82	27	Former	UN	Lung Cancer	-	08/11/2013
NM07	M	12/05/1940	74	34	Former	PO	Silicosis, Bilateral Pleural Thickening	Marble Cutter, Short Exposure Periods To Serpentine	07/03/2014
NM08	M	07/11/1942	71	22	Current	PO	Lung Cancer	Construction Industry Bricklayer	28/03/2014
NM09	F	03/09/1945	69	19	Current	UN	Breast Cancer	-	10/04/2014

a) Age at diagnosis.

b) Classification of Asbestos Exposure: PO, probable occupational; and UN, unknown.

4.2. miRNOME SCREENING WITH OPEN ARRAY

We analyzed miRNA expression profiles in Exhaled Breath Condensate (EBC) and plasma, using Open Array high-throughput technology, that allows to investigate in high-fidelity and short-time 733 well studied miRNAs spotted in single and divided in two pool A and B for both reverse-transcription and quantification.

Since EBC and plasma don't have any endogenous miRNAs which are able to function as normalizer, we used the average of the octuplicate Cts obtained from the external spike-in ath-miR-159a. The usage of an external spike-in has been recently validate as normalizing method for microarrays data in absence of a well-known endogenous miRNA [111].

To consider a miRNA as expressed, it has to fulfil two criteria after analysis with Open Array: 1) Cycle threshold (Ct) cannot be higher than the 28^{th} cycles; 2) AmpScore must be equal or higher to 1.1. Among the 733 analyzed miRNAs in EBC and plasma we found a total of 444 and 256 expressed miRNAs, respectively. In Table 12 are reported how many expressed miRNAs were found in each biological matrices across the three studied groups.

Table 12 Numbers of expressed miRNAs in each group detected by Open Array analysis.

Numbers of expressed miRNAs in each (%)			
	MM	***PEA***	***NM***
EBC	157 (61.3)	215 (84.0)	113 (44.1)
Plasma	369 (83.1)	401 (90.3)	335 (75.5)

4.3. HEAT MAP HIERARCHICAL CLUSTER ANALYSIS

Expression profiles determined by Open Array analysis in 23 MPM, 19 PEA and 9 NM were used for Hierarchical Cluster Analysis utilizing Qlucore software. The Cluster Analysis is an explorative analysis that tries to identify structures within the data. More specifically, it tries to identify homogenous groups of cases, showing a similar expression of a specific microRNA pattern.

Cluster analysis was performed on differential miRNAs defined as those miRNAs with FC higher than 2 or lower than 0.5 and p-value lower than 0.05 (using the Mann-Whitney U test).

In Figure 14Figure 15, hierarchical cluster analyses are reported for expression profile from EBC and plasma, respectively. In both the figures MPM, PEA and NM groups are marked in yellow, blue and red, respectively. In EBC, 31 differential miRNAs were used to hierarchically cluster the three groups, while in plasma deregulated miRNAs were a total of 55.

The EBC Heat map did not show any specific clustering of the samples.

The plasma Heat map, on the contrary, showed an imperfect clustering. While most of the cancer-free samples clustered separately, some were dispersed within the group of the malignant samples. The separation may be due to a specific expression of miRNAs in benign diseases commonly present in PEA and MPM, e.g., asbestosis or pleural plaques.

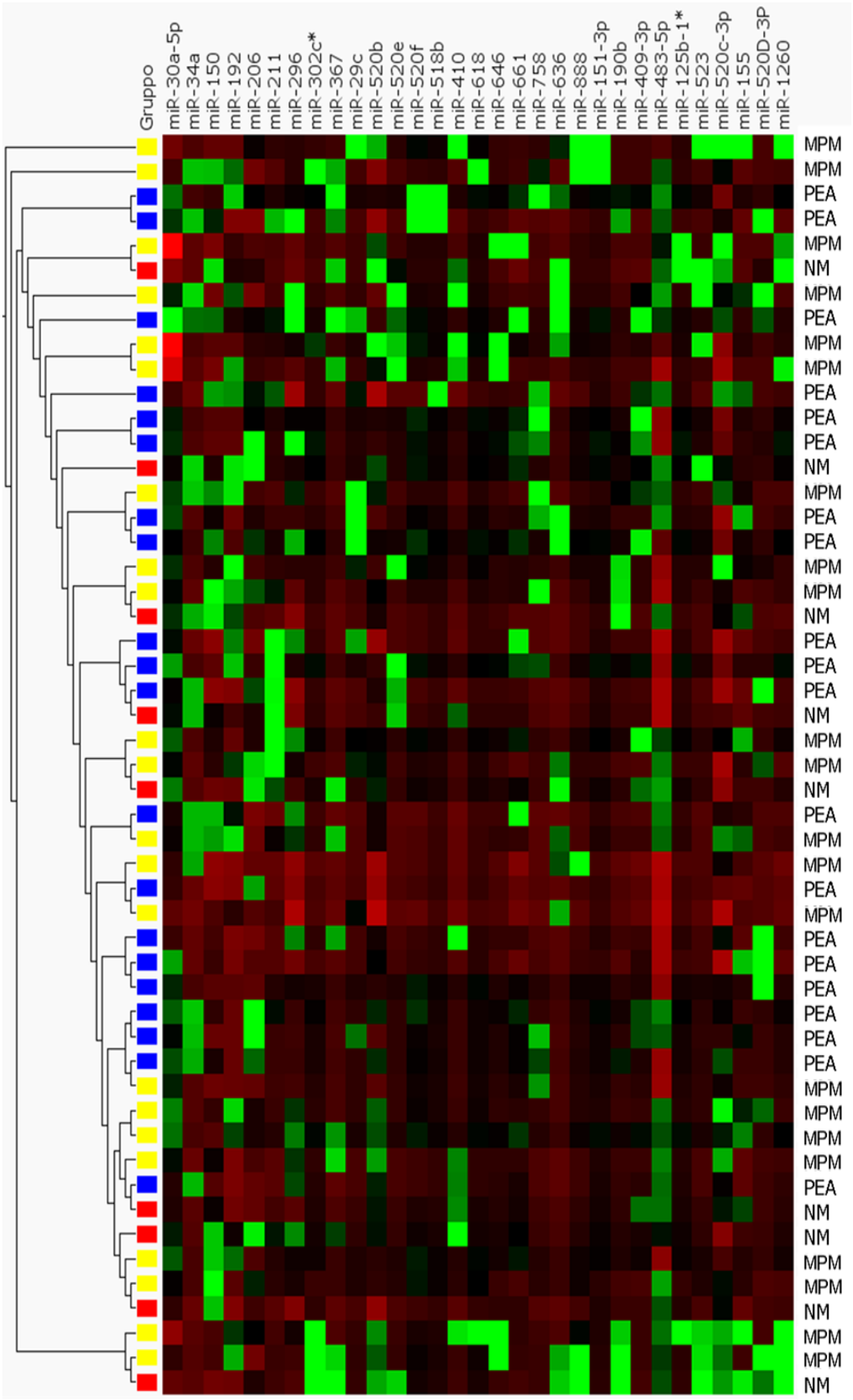

Figure 14 Heat map of differential miRNA expression in EBC of MPM and control groups. Cluster analysis was performed using miRNA expressions with statistical significance (p-value<0.05) and a FC<0.5 (red=down-regulation) or FC>2 (Green=up-regulation). MPM samples are marked yellow, PEA blue , and NM red.

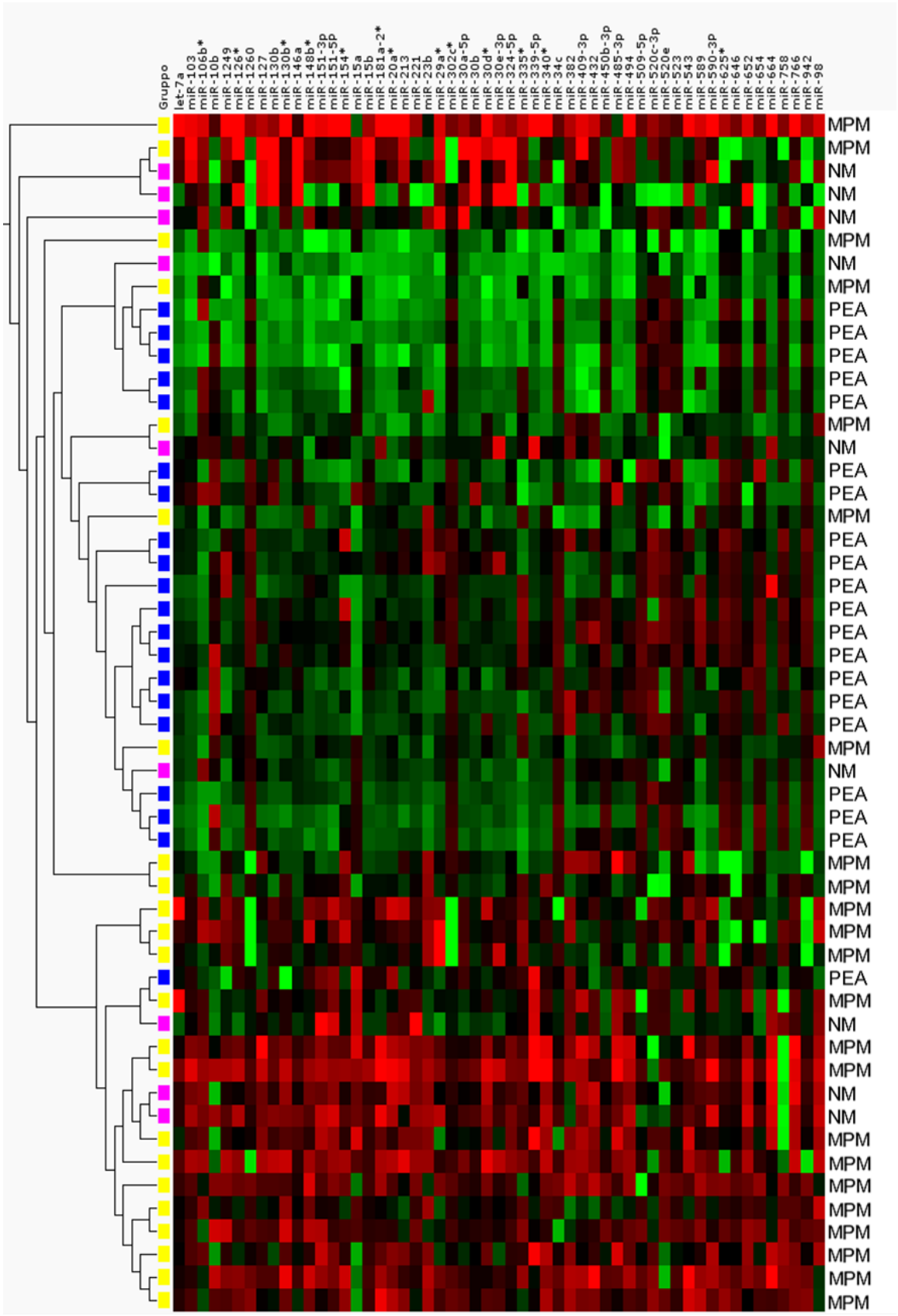

Figure 15 Heat map of differential miRNA expression in plasma of MPM and control groups. Cluster analysis was performed using miRNA expressions with statistical significance (p-value<0.05) and a FC<0.5 (red=down-regulation) or FC>2-folds (Green=up-regulation). MPM samples are marked yellow, PEA blue, and NM in red.

4.4. miRNA Analysis in Exhaled Breath Condensate

We analyzed the expression of 733 miRNAs in EBC obtained from three study groups.

After quality control and filtering steps, we detected 157 miRNAs in MPM, 113 miRNAs in NM and 215 miRNAs in PEA that were considered for subsequent analyses, ie. Multiple linear regression adjusted for gender, age, BMI, and smoking habits.

When we compared MPM and PEA, we found 8 miRNAs having, on average, different expression between the two groups ($p < 0.05$); and all of them had false discovery rate correction (FDR) of < 0.1 (Appendix B - EBC).

When we compared MPM and NM, we found 3 miRNAs having different expression between the two groups ($p < 0.05$); and again all of them had false discovery rate correction (FDR) of < 0.1 (Appendix B - EBC).

In Figure 16, comparisons between MPM vs PEA (Figure 16 A) and MPM vs NM (Figure 16 B) are reported, using a volcano-plot. Briefly, every dots represents a specific miRNA: on X and Y-axis the value of Fold Change (FC) and p-value are reported.

As shown in Figure 16 A among the 131 miRNAs that have passed criteria of $Ct \leq 28$ and AmpScore>1.1, and were expressed in both MPM and PEA, only 7 showed p-value lower than 0.05 and a FC greater than 2-folds. Three of them (hsa-miR-30a-5p, hsa-miR-141 and hsa-miR-518b) were down-regulated in MPM; while hsa-miR-520b, hsa-miR-646, hsa-miR-520c-3p and hsa-miR-888 were up-regulated in MPM group.

Figure 16 B represents the Volcano plot obtained by the comparison between MPM and NM groups. hsa-miR-378a-3p, hsa-miR-206 and hsa-miR-9-5p resulted in a lower regulation in MPM than NM subjects.

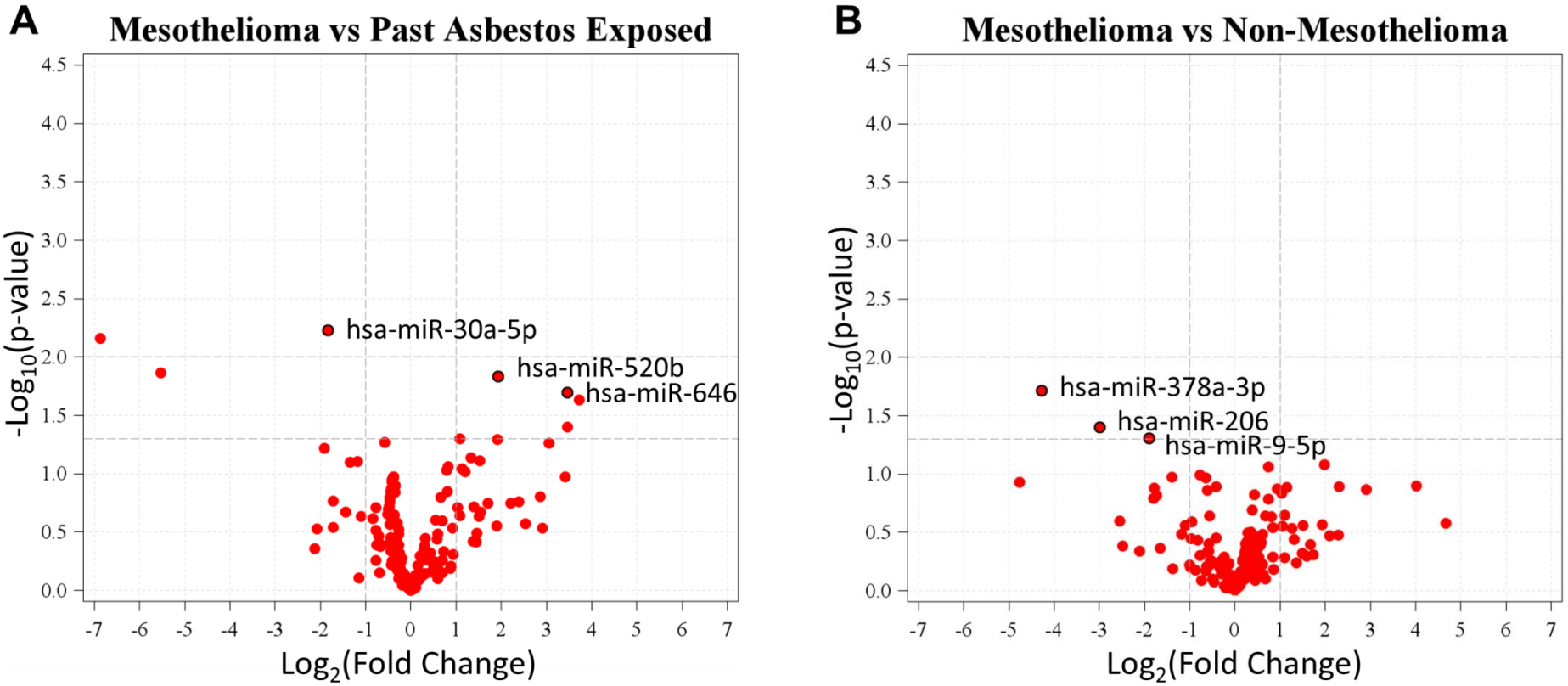

Figure 16 Volcano plot for EBC screening. Every dots correspond to a specific miRNA. X-axis reports FC as Log2, then dots on the left and right of vertical lines are considered to be 2-folds, negatively and positively, deregulated in MPMs than the reference control group.

4.5. miRNA Analysis in Plasma

Using a workflow similar to the one described for EBC analysis, in plasma we detected 369 miRNAs in MPM, 401 miRNAs in NM and 335 miRNAs in PEA that were considered for subsequent analyses.

Comparing MPM and PEA, we found 124 miRNAs which were differentially express between the two groups ($p < 0.05$); and 82 of them passed the false discovery rate correction (FDR) of < 0.1 (Appendix B - Plasma).

Comparing MPM and NM, we found 39 miRNAs having different expression between the two groups ($p < 0.05$); and 1 of them passed the false discovery rate correction (FDR) of < 0.1 (Appendix B - Plasma).

As expected, in plasma we were able to detect an higher number of miRNAs compared to EBC, since plasma collects miRNAs coming virtually from all body districts, while EBC is only representative of pulmonary environment.

Figure 17 A reports the comparison between MPM and PEA subjects. 115 out of 351 miRNAs showed a p-value≤0.05 and FC>2-folds or FC <0.5.

105 resulted to be significantly down-regulated in MPM compared to PEA subjects, whereas 9 miRNAs were upregulated in MPM subjects.

The Volcano-plot comparing MPM and NM subjects is reported in Figure 17 B. 335 miRNAs showed a p-value≤0.05 and FC>2-folds or FC <0.5.

44 miRNAs were significantly down-regulated in MPM subjects compared to NM subjects.

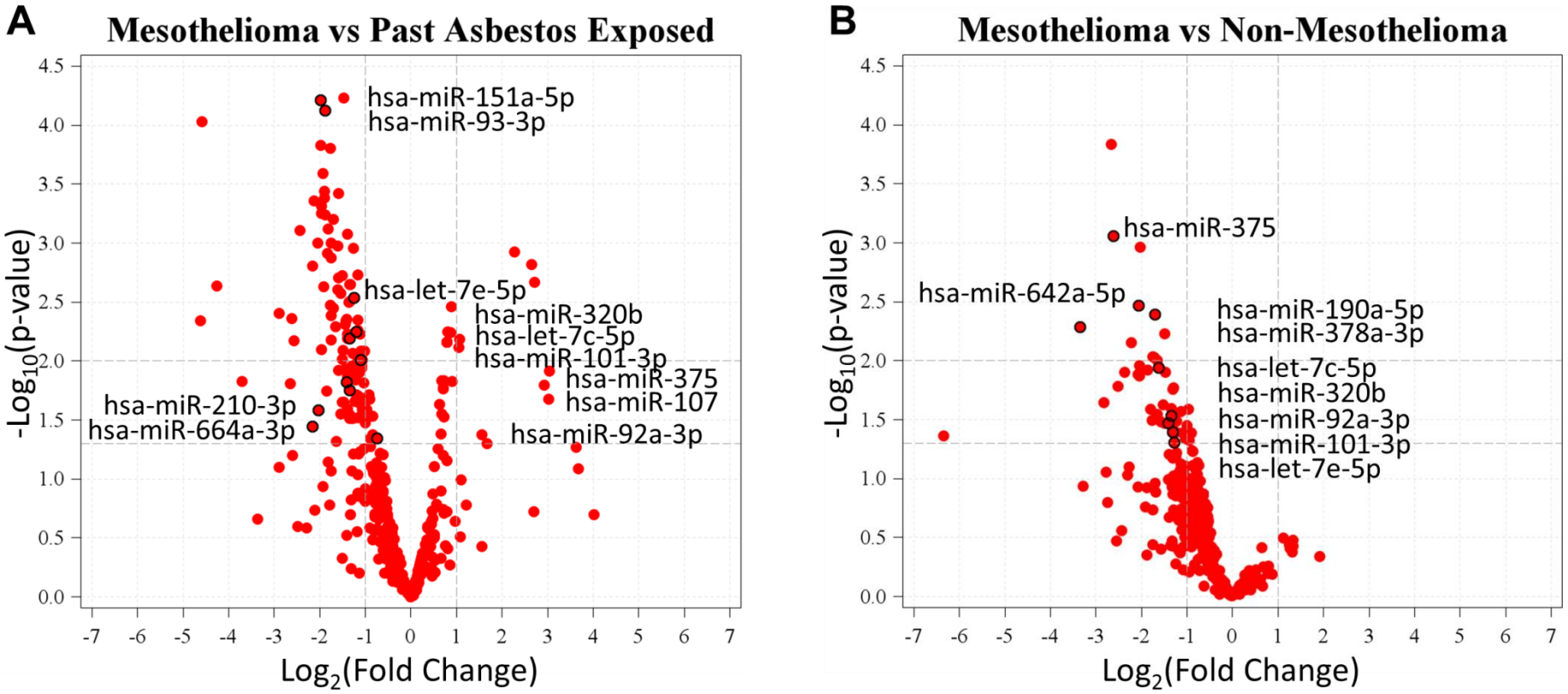

Figure 17 Volcano plot for plasma screening. Every dots correspond to a specific miRNA. X-axis reports FC as Log2, then dots on the left and right of vertical lines are considered to be 2-folds, negatively and positively, deregulated in MPMs than the reference control group.

4.6. SELECTION OF CANDIDATE MIRNAS

To validate candidate miRNAs as molecular biomarkers for malignant mesothelioma, we followed two main criteria:

1. candidate miRNAs should show a strong statistical difference in expression in the compared groups,
2. and be expressed in at least 50% of the examined subjects.

When we consider EBC, hsa-miR-30a-5p, and hsa-miR-520b and hsa-miR-646 satisfied these two criteria in the comparison between MPM vs PEA and the same hold for hsa-miR-378a-3p, hsa-miR-206 and hsa-miR-9-5p in the comparison between MPM vs NM. subjects, and hence were chosen as candidate biomarkers for malignant pleural mesothelioma diagnosis and used for further validation by RT-qPCR.

Among the analyzed miRNAs only three passed the previous described criteria of FC and p-value.

The 6 miRNAs chosen as candidate biomarkers for further validation by RT-qPCR are reported in Table 13 .

Table 13 List of candidate miRNAs in EBC. P-values<0.5 indicate statistical significance for the deregulation of given miRNA in MPM versus one of the reference control group. FC was calculated has the ratio between RQ for given miRNA in MPM and the same miRNA RQ in the reference control group.

EBC	MPM vs PEA			MPM vs NM		
miRNA name	pvalues	FC	Regulation	pvalues	FC	Regulation
hsa-miR-30a-5p	0.0590	0.28	↓			
hsa-miR-520b	0.0147	3.83	↑			
hsa-miR-646	0.0202	10.99	↑			
hsa-miR-206				0.0312	0.11	↓
hsa-miR-378a-3p				0.0148	0.05	↓
hsa-miR-9-5p				0.0438	0.27	↓

In plasma, due to the high number of differentially expressed miRNAs, in addition to the above mentioned criteria, which allowed the selection of 4 candidate miRNAs (hsa-miR151-5p, hsa-miR-93-3p, hsa-miR-190-5p and hsa-miR-642a-5p), we applied further criteria based on:

1. being differentially expressed in both MPM vs PEA and MPM vs NM (hsa-let-7c-5p, hsa-let-7e-5p, hsa-miR-101-3p, hsa-miR-320b, hsa-miR-375 and hsa-miR-92a-3p)
2. having a previously reported role in oncogenic and inflammatory processes (hsa-miR-107, hsa-miR-210-3p and hsa-miR-664a-3p)

Therefore, we selected a total of 13 miRNAs, reported in Table 14.

Table 14 List of candidate miRNAs in Plasma. P-values<0.5 indicate statistical significance for the deregulation of given miRNA in MPM versus one of the reference control group. FC was calculated has the ratio between RQ for given miRNA in MPM and the same miRNA RQ in the reference control group.

Plasma	MPM vs PEA			MPM vs NM		
miRNA name	p-value	FC	Regulation	p-value	FC	Regulation
hsa-miR-151a-5p	0.0001	0.89	↓			
hsa-miR-93-3p	0.0001	0.30	↓			
hsa-miR-190a-5p				0.0036	0.31	↓
hsa-miR-642a-5p				0.0050	0.24	↓
hsa-let-7c-5p	0.0065	0.42	↓	0.0112	0.31	↓
hsa-let-7e-5p	0.0029	0.45	↓	0.0557	0.41	↓
hsa-miR-101-3p	0.0098	0.50	↓	0.0381	0.39	↓
hsa-miR-320b	0.0057	0.51	↓	0.0182	0.37	↓
hsa-miR-375	0.0151	0.36	↓	0.0014	0.16	↓
hsa-miR-92a-3p	0.0452	0.65	↓	0.0403	0.37	↓
hsa-miR-107	0.0178	0.42	↓			
hsa-miR-210-3p	0.0263	0.29	↓			
hsa-miR-664a-3p	0.0362	0.32	↓			

4.7. VALIDATION OF miRNAs IN REAL TIME PCR

Using the previously reported criteria, quantitative real-time PCR was performed to validate OpenArray results on the selected miRNAs.

Specifically, miRNAs chosen for validation test in EBC samples were analyzed by single assays run on 7900HT Fast Real-time PCR system. miRNAs chosen for validation test in plasma samples, were analyzed by Custom TaqMan Low Density Array run on 7900HT Fast Real-Time PCR System.

In EBC, miR-520b and miR-646 were not detectable. Among the remaining miRNAs (miR-30a-5p, miR-206, miR-378a-3p and miR-9-5p), only miR-30a-5p was validated and resulted to be significantly down-regulated in MPM patients compared to PEA (Table 15).

The results pertaining validation in plasma are reported in Table 16: 3 (hsa-miR-151-5p, hsa-miR-107 and hsa-let-7e) out of 13 miRNA candidates were validate for their expression profiles in the comparison between MPM and PEA.

Table 15 Results of miRNA validation in EBC.

EBC	***MPM vs PEA***		
miRNA name	***p-values***	***FC***	***Regulation***
miR-30a-5p	0.0050	0.26	↓
miR-9-5p	0.0986	0.60	↓
miR-206	0.1241	0.55	↓
miR-378a-5p	0.2625	0.54	↓

Table 16 Results of miRNA validation in plasma.

Plasma		MM vs PEA	
miRNA name	p-values	FC	Regulation
hsa-miR-151a-5p	0.0041	0.36	↓
hsa-miR-107	0.0150	0.47	↓
hsa-let-7e-5p	0.0252	0.45	↓
hsa-miR-664a-3p	0.0828	0.52	↓
hsa-miR-210-3p	0.1016	0.58	↓
hsa-miR-320b	0.1465	0.59	↓
hsa-miR-101-3p	0.1502	0.58	↓
hsa-miR-93-3p	0.1564	0.62	↓
hsa-miR-92a-3p	0.1658	0.69	↓
hsa-let-7c-5p	0.4720	0.69	↓
hsa-miR-378a-3p	0.6328	1.15	↑
hsa-miR-375	0.8352	0.86	↓

4.8. ROC CURVE ANALYSIS

To evaluate the accuracy in determining the diagnosis for malignant mesothelioma we performed ROC analysis for the validated miRNAs found in EBC and plasma in the comparison MPM vs PEA.

For the only miRNA validated in EBC (hsa-miR-30a-5p) (Figure 18 A), we obtained an AUC value of 0.77 (95% CI 0.6223 - 0.9232, p-value<0.0005). The best Relative Quantification (RQ) cut-off used in the analysis was 0.0001, with a sensitivity of 82% and a specificity of 70%.

Individual ROC analysis were first performed for the three miRNAs validated in plasma. Results are shown in figure XXX B, C and D. hsa-miR-151-5p showed an AUC value of 0.77 (95% CI 0.6333-0.9144, p-value<0.0001) (Figure 18 B). A proper cut off for hsa-miR-151-5p using a RQ value of 0.09 with a sensitivity of 83% and a specificity of 70%. hsa-miR-107 and hsa-let-7e-5p revealed an AUC value of 0.75 (95%CI 0.6028–0.9059, p-value<0.001) and 0.72 (95%CI 0.5697–0.8825, p-value<0.005), respectively

(Figure 18 C and D). A proper cut off for hsa-miR-107 using a RQ value of 0.01 was determined to discriminate between MPM from PEA revealing a sensitivity of 65% and a specificity of 85%; while hsa-let-7e-5p cut off was calculated in a value of 0.14, and produces a sensitivity of 87% and a specificity of 60%. When we combined the three miRNAs, the AUC value was 0.79 (95%CI 0.65–0.92) with a sensitivity of 78% and a specificity of 70%.

The last step was the calculation of a single ROC curve for the combined 4 validated miRNAs in EBC or plasma (Figure 18 E). The AUC found by this analysis was equal to 0.85 (95% CI 0.72 – 0.97) with 86% sensibility and 80% specificity.

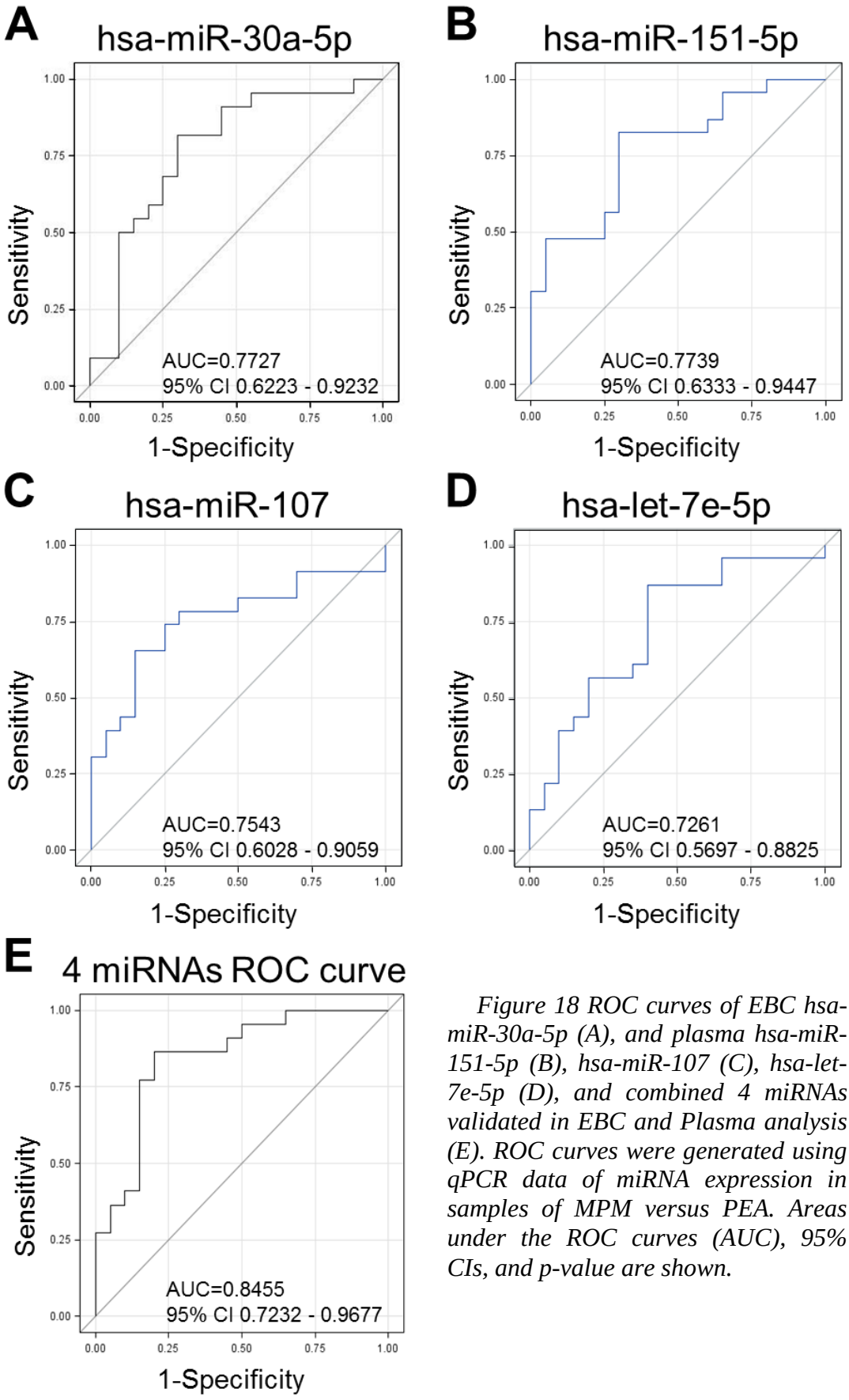

Figure 18 ROC curves of EBC hsa-miR-30a-5p (A), and plasma hsa-miR-151-5p (B), hsa-miR-107 (C), hsa-let-7e-5p (D), and combined 4 miRNAs validated in EBC and Plasma analysis (E). ROC curves were generated using qPCR data of miRNA expression in samples of MPM versus PEA. Areas under the ROC curves (AUC), 95% CIs, and p-value are shown.

5. DISCUSSION AND CONCLUSIONS

Malignant Pleural Mesothelioma (MPM) is an aggressive cancer refractory to current therapies, the incidence of which is expected to rise in the next decades [1]. Recent projections have suggested that MPM mortality rates, estimated using mortality from primary pleural tumours, will continue to increase in the next 20 years in most European countries [2, 3].

MPM is often difficult to distinguish from lung adenocarcinomas and accurate diagnosis require multiple time-consuming tests (i.e. light microscopy with a panel of immunohistochemical stains, as gold standard [38]) as well as invasive procedures (i.e. thoracoscopy [112]). The lack of efficient tumor biomarkers for MPM diagnosis and medical survey plays a fundamental role for the development of a universally accepted therapeutic approach and a clearer understanding of the major molecular pathways of MPM is still needed to establish diagnostic and therapeutic methods.[74]

With a median survival of less than 1 year and a 5-year survival rate of less than 5% [113], prognosis of MPM remains very poor [114].

In recent years, microRNA expression (for example detected in plasma) have been closely associated with several cancers, including lung diseases [115], as well as MPM [116]. However, only a few studies focused on the investigation of circulating miRNA expression in MPM with inconsistent findings. In addition, the extreme variability of applied techniques hampered , a reliable comparison among studies .

The possibility of measuring miRNA expression in EBC of MPM cases has never been evaluated.

miRNAs measured in plasma and EBC might give different information, although complementary. Plasma collects circulating miRNAs coming from all the different body

districts and, thus, plasma miRNAs are more a systemic marker. On the contrary, EBC is representative of the pulmonary environment only, and might be much more tissue specific.

We have performed a screening of 733 miRNAs by high-throughput Open Array analysis in MPM affected-patients and two different control groups; one formed by healthy subjects with a Past Asbestos Exposure (PEA), and the other composed by subjects enrolled among patients with pleural effusions due to other causes (NM). For each subject we analyzed paired samples of miRNAs purified from plasma and EBC.

The comparison between MPM and PEA allowed us to evaluate miRNA expression between diseased and healthy subjects, while the comparison between MPM and NM subjects has been chosen to investigate the ability of differential miRNAs to discriminate between MPM and other pulmonary diseases.

After Real Time validation, we identified one miRNA in EBC (hsa-miR-30a-5p) and three miRNAs in plasma (hsa-miR-151-5p, hsa-miR-107, and hsa-let-7e-5p), able to discriminate between MPM and PEA subjects. The combination of the 4 miRNAs gives even a better score, with a ROC curve which shows an AUC = 0.85. To our knowledge this is the first study in MPM which investigate miRNA expression in EBC and identify a 4-miRNA signature which might be useful for MPM diagnosis.

In the comparison between MPM and NM, we were not able to validate any miRNAs. This may be due the small sample size and to a non-homogeneous composition of the NM group, being composed by 7 subjects affected by different cancers, 1 subject with silicosis and 1 subject presenting an inflammatory status.

Previous studies have suggested a possible role for miRNA deregulation in MPM. There have been some reports on the role of miR-30 family members that may be related

to the pathogenesis of tumors [117]. Mitochondrial fission is involved in the initiation of apoptosis, while apoptosis is relevant to the tumorigenesis. Li et al. reported that the overexpression of miR-30 family members, including miR-30a, miR-30b and miR-30d, was able to reduce mitochondrial fission initiated apoptosis by targeting p53, which is transcriptionally activated by the mitochondrial fission protein, dynamin-related protein-1 (Drp1) [118]. Cell senescence is a major tumor suppressing mechanism. Martinez et al. demonstrated that miR-29 and miR-30 regulate B-Myb expression by binding to its 3'UTR and suggested that these microRNAs play an important role in Rb-driven cellular senescence [119]. It has also been demonstrated the down-regulation of miR-30a in chronic lymphocytic leukemia (CLL) [120], lung cancer [121], colon cancer [122], supporting the oncogenic role of miR-30a-5p, which we found down regulated in EBC of MPM patients.

In MPM, we found miR-151-5p significantly down-regulated. In accordance with this observation, lower expression of circulating miR-151-5p miRNA expression was observed in the malignant pleural effusion (MPE) in non-small-cell lung cancer (NSCLC), and was associated with poor survival [123].

We also observed a down regulation of miR-107 in MPM, according to the previous reported data showing that miR-107 induces cell cycle G1 arrest and inhibits invasion by targeting cyclin dependent kinase 6 (CDK6), thereby inhibiting tumor progression in gastric cancer, pancreatic cancer, and NSCLC cell lines [124-126].

Let-7 has a highly conserved sequence across organisms from *C.elegans* to humans. It regulates the expression of a range of genes through its 5′ seed sequence (5′-UGAGGUA-3′), which binds to corresponding sequences located in the 3′ UTRs of genes. Notably, the loss of let-7 expression is often considered to have prognostic value since it indicates poor

survival in many cancers [127]. In agreement with this data, we observed a down-regulation of let-7e-5p in MPM.

The novel use of EBC is interesting, but at the same time challenging: the amount of miRNAs present in this biospecimen is extremely low and limit our capability of detecting them. To overcome this technical issue, one possibility would be to collect at least two samples for each subject, trying to maximize miRNA yield.

The main limitation of this study is the limited number of subjects, especially for the NM group. In future investigations, it will be fundamental to increase the number of subjects and, for the NM group, to select subjects on the base of a more homogeneous health status.

A second limitation is that the use of endogenous controls to normalize circulating target miRNAs is very controversial. Thus far a real endogenous control for serum-based miRNA detection has not well established. Previous studies reported that the use of a single internal gene for normalization leads to relatively large error; thus, using multiple reference genes may be a better strategy to evaluate target gene expression [128, 129]. In this study, however, it was difficult to apply this strategy, since in EBC the subset of miRNAs expressed by each subject was extremely variable, and the miRNAs expressed by all subjects were too low. Frequently used reference genes, such as U6 and 5S ribosomal RNA, were found a less-stable expression than others or degraded in serum sample [104]. For these reasons, we decided to use an exogenous non-human spike-in control, which was added to each samples and quantified [130].

In conclusion, data obtained in this project, support a possible role of miRNAs in the etiology of malignant pleural mesothelioma but need confirmation in larger samples.

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APPENDIX A

QUESTIONARIO SULLA STORIA DI LAVORO E SULLE ABITUDINI DI VITA

Centro Operativo Lombardia

Tutte le domande si riferiscono a:

Cognome ……..…..…… N. identificazione

Nome …….…….……..…… |__|__|__|__|__|__|

Data di nascita |__|__| / |__|__| / |__|__|__|__| Sesso: |_| M |_| F

Luogo di nascita ……..……..…… Provincia ……..……..

Ultimo domicilio (dove abita): Via .....…….....…….....……..….....

Comune .....……...…… Provincia ……... C.A.P. |__|__|__|__|__|__|

Telefono ……...……..……...

- Se il domicilio è differente dalla residenza, specificare anche la residenza:

Ultima residenza (indirizzo): Via .....…….....….....…….

Comune .....…… Provincia ……... C.A.P. |__|__|__|__|__|__|

Telefono ……..……...……...

Stato civile .....…….....….... Se sposato, anno di matrimonio |__|__|__|__|

Titolo di studio (o ultima classe frequentata): .....……..……

Data intervista |__|__| / |__|__| / |__|__|__|__|

Orario INIZIO intervista |__|__|,|__|__| - Orario FINE intervista |__|__|,|__|__|

Luogo dell'intervista

……..……...…….……

Intervistatore (nome e qualifica)...…….……..……

Ente ……..…….

Recapito telefonico intervistatore ……...

PARTE PRELIMINARE: Fattori di confondimento sul livello di mi-RNA

Peso: ……..Kg

Altezza …….cm. BMI ……..

1. È attualmente fumatore? SI NO

- se 'SI', da quanti anni fuma? ……..

- quante sigarette fuma in una giornata? ……..

- quante sigarette ha fumato oggi? ……..

2. Soffre di qualche malattia cronica? SI NO

- se 'SI', quale? ……

3. Le sono mai stati diagnosticati enfisema polmonare/bronchite cronica?

SI In quale anno? …….....

NO

4. Attualmente è in terapia con cortisone o farmaci antinfiammatori?

SI (indicare i nomi dei farmaci) ……..…….....

NO

- se 'SI', per quale motivo? ……..……..…….……..

……..……..…….……

- ha assunto la terapia oggi? SI ……...…..…….……..

NO

5. Abitualmente assume altri farmaci?

SI (indicare i nomi dei farmaci) ……..…….....

NO ……..…….....

6. Ha mai sofferto di rinite o asma allergico? SI NO

- se 'SI', sa indicare a quali allergeni è sensibilizzato?

…...……...

- ha avuto disturbi su base allergica nell'ultima settimana? SI NO

…...……...

7. Soffre di reflusso gastroesofageo, ernia jatale, o in generale di disturbi della digestione?

SI ……...…..…….……

NO

8. Nell'ultima settimana ha sofferto di uno dei seguenti disturbi?

- gengivite o aftosi del cavo orale
- ascesso o granuloma dentale
- epistassi (sangue dal naso)
- rinite / croste dolorose nel naso
- mal di gola / tonsillite / infezioni delle vie aeree

9. Utilizza protesi dentarie? SI NO

PARTE A: ABITUDINI AL FUMO

Al. Fuma?

|_| SI
|_| NO
|_| HA SMESSO
|_| NON SO

A2. Se è o è stato fumatore, cosa fuma/fumava?

|_| Sigarette
- |_| con filtro
- |_| senza filtro
- |_| parte con, parte senza
- |_| non so

|_| Sigari
|_| Pipa
|_| Non so

A3. A che età ha cominciato a fumare? ……...……...… |_| NON SO

A che età ha smesso? ……...……...…… |_| NON SO

A4. Quante sigarette fuma/fumava al giorno? ……...…… |_| NON SO

PARTE B: STORIA PROFESSIONALE - PARTE GENERALE

(In base alle risposte, integrare con le schede generiche previste e, per attività di tipo industriale o artigianale, anche con eventuali schede specifiche)

B1. Svolge attualmente un'attività professionale? |_| SI |_| NO

Se no, indicare il motivo:

……..……..……..……..…….....……

B2. Storia professionale completa.

N.	Inizio		Fine		Nome, attività dell'Azienda, Città	Mansione
	Età	Anno	Età	Anno		

N.	Inizio		Fine		Nome, attività dell'Azienda, Città	Mansione
	Età	Anno	Età	Anno		

N.B.: in caso di lavoro discontinuo o saltuario specificare la durata complessiva (< 6 mesi o 1 volta alla settimana).

- E' eventualmente disponibile il libretto di lavoro? |_| SI |_| NO |_| NON SO

B3. Svolge o ha mai svolto lavori saltuari? |_| SI |_| NO

Se si, li descriva nella storia professionale completa……..

B4. SERVIZIO MILITARE

- Ha fatto il militare? |_| SI
|_| NO

Se NO, specificare il motivo dell'esonero:

……...

……

……...

……

……...

Se SI, quando? Dal …….. al ……...….. Di leva |_| Di carriera |_|

In tempo di guerra? |_| SI
|_| NO

- In quale arma ha fatto il servizio militare?

.....……...……

……..

Dove? ……..…...……..….. Dal ……..…...…… al

.……...…..…

Con quali mansioni?

.....……..……...……

- Durante il servizio militare ha mai lavorato alla riparazione di mezzi meccanici? |_| SI |_| NO

Se si, specificare il tipo di mezzo e di

intervento:…….……..…….

……...……..……...……

……

- Ha mai guidato carri armati o mezzi blindati? |_| SI |_| NO

Se si, si ricorda con cosa erano coibentati:

…….……..……..…….

……...……...……

……...

- Ha mai fatto parte di squadre antincendio? |_| SI |_| NO

Se si specificare sa ha fatto uso di materiali in amianto o contenenti amianto (tute, guanti, etc.): ……..

…...…….……..……

……..

- Era addetto a depositi di polvere da sparo o depositi di munizioni? |_| SI |_| NO

Se si, ricorda con che materiale erano coibentate le pareti:

...……...……..……..……...

……..……

…….……..

- Se ha fatto il militare in marina specificare:

Era imbarcato? |_| SI |_| NO

Se si, su che tipo di unità navale? ……..……..... Anno di costruzione

……...……..

Con quali mansioni?

……..……..……..……..……...

Ha svolto anche mansioni di manutenzione?

Se si , specificare quali:

……..……..…….

NOTA: Se è stato prigioniero, chiedere se ha svolto attività lavorative particolari e descriverle sulla scheda per attività lavorative.

PARTE C: ESPOSIZIONE EXTRA-LAVORATIVA

C1. STORIA LAVORATIVA DEL FAMILIARI E CONVIVENTI

(Riferita al periodo in cui hanno vissuto nella stessa casa del soggetto in esame)

COMPOSIZIONE DELLA FAMIGLIA (solo i conviventi)

Grado di Parentela	Periodo di convivenza (dal… al…)	Sommaria descrizione del lavoro (attività lavorativa svolta per più tempo)		a *	Abiti **
		Nome e attività dell'azienda	Mansione		
Padre					
Madre					
Coniuge					
altro parente convivente					

*Il lavoro comportava l'utilizzo di amianto o manifatture contenenti amianto? **Gli indumenti di lavoro venivano portati a casa?

- In caso di risposta positiva, o di incertezza, nella colonna **a** compilare la seguente scheda:

Congiunto: Grado di parentela ……..……...……..

Cognome e Nome ……..……

Deceduto per ……..……

(Elencare se possibile solo i lavori svolti durante il periodo di convivenza con il soggetto in esame)

Periodo	Azienda	Principali prodotti o servizi	Attività del reparto	Mansione e descrizione dell'attività svolta	Abiti (a)	Lavag. (b)

(a) Barrare nel caso che rientrando dal lavoro portasse con sé gli indumenti di lavoro.
(b) Barrare nel caso che gli indumenti di lavoro venissero spazzolati o lavati a casa.

Il suo congiunto è in possesso di rendita INAIL per malattia professionale?

|_| SI |_| NO |_| NON SO

Se si, per quale malattia e da quando? ……..…….

……..……...……..……

……..……...……..……

……..……...……..……

C2. **STORIA RESIDENZIALE**

Periodo Dal … al …	Comune	Prov.	Indirizzo	Tipo di abitazione (*)

(*) Tipi di abitazione: 1 = Rurale o Cascina
2 = Casa unifamiliare o Villa
3 = Condominio
4 = Comunità (pensionati, caserme, collegi, ricoveri)
5 = Altro (specificare)

Nelle abitazioni successive al 1945 erano presenti manufatti in cemento amianto (coperture in *eternit*, controsoffittature, serbatoi d'acqua, altro)? ……..……

……..……..……...

…….

…….

Vicino alla sua abitazione, erano presenti (considerare tutte le abitazioni):

|_| impianto chimico o petrolchimico nel periodo ……..…… distanza……..
|_| impianto siderurgico o fonderia nel periodo ……..…… distanza……..
|_| centrale termoelettrica nel periodo ……..…… distanza……..
|_| porto nel periodo ……..…… distanza……..
|_| cantiere navale nel periodo ……..…… distanza……..
|_| cementificio nel periodo ……..…… distanza……..
|_| impianto produzione *eternit* nel periodo ……..…… distanza……..
|_| impianto produzione/riparazione rotabili ferroviari nel periodo ……..…… distanza……..
|_| inceneritore o discarica nel periodo ……..…… distanza……..
|_| cava o miniera nel periodo ……..…… distanza……..
|_| altro nel periodo ……..…… distanza……..

C3. STORIA EXTRA-PROFESSIONALE

Faceva riparazioni in casa? |_| SI |_| NO |_| NON SO Periodo ……..
Riparazioni di idraulica o di elettricità? |_| SI |_| NO |_| NON SO Periodo ……..
Lavori di isolamento termico? |_| SI |_| NO |_| NON SO Periodo ……..
Lavori di muratura? |_| SI |_| NO |_| NON SO Periodo ……..
Altre riparazioni (specificare di che tipo e il periodo): …..……..……..
……..…….…….
……..…….…….
……..…….…….

Ha mai usato attrezzi domestici come: - guanti per forni |_| Periodo ……
- pannelli di protezione dal fuoco |_| Periodo ……
- asse da stiro con copertura in amianto |_| Periodo ……
- appoggiaferro da stiro |_| Periodo ……

Ha mai riparato la sua auto o altri mezzi meccanici (freni, frizione, tamburo, guarnizioni della testa)?

|_| SI |_| NO |_| NON SO Periodo ……

Ha mai praticato attività nel suo tempo libero che comportassero l'uso di manufatti in amianto?

|_| SI |_| NO |_| NON SO

Quali? ……..…… Periodo……..…….
Quali? ……..…… Periodo……..…….
Quali? ……..…… Periodo……..…….
……..……..……..

Quanto assiduamente veniva svolta tale attività?

|_| RARAMENTE

|_| SPESSO (nel caso di secondo lavoro, inserirlo nella scheda di cui alla domanda B2)

Ha fatto uso di talco cosmetico? |_| SI |_| NO

Se si, specificare:

Quando? ……..……..

Quanto? ……..……...

Ha frequentato assiduamente locali di uso collettivo o mezzi di trasporto come:

	Periodo	Durata	Città
Teatri ……..		…….	……
Cinema ……..		…….	……
Palestre ……..		…….	……
Piscine ……..		…….	……
Treno/metro/traghetto ……..		…….	……
Altro ……..		…….	……

PARTE D: SALUTE

D1. E' stato sottoposto a radiodiagnostica una delle seguenti sedi?

	SI	NO	NON SO	MOTIVO	N°
Testa	|_|	|_|	|_|	……	….....
Torace	|_|	|_|	|_|	……	….....
Addome		|_|	|_|	|_| ……	….....
Rene, vescica, ureteri	|_|	|_|	|_|	……	….....
Colonna vertebrale		|_|	|_|	|_| ……	….....
Altre sedi (specificare)	|_|	|_|	|_|	……	….....
……				……	….....

D2. E' mai stato sottoposto a trattamenti terapeutici con raggi X? |_| SI |_| NO |_| NON SO

Sede	Motivo	Durata (mesi)	Ospedale	Anno di inizio
……	….....	……	……	….....
……	….....	……	……	….....
……	….....	……	……	….....

D3. E'/è stato in possesso di rendita INAIL per malattia professionale dell'albero respiratorio?

| _ | SI | | _ | NO | | _ | NON SO

Se si, per quale malattia e da quando? ……

……

D4. Ha mai sofferto di TBC pleuro/polmonare? |_| SI |_| NO |_| NON SO
D5. Ha mai subito importanti traumi toracici? |_| SI |_| NO |_| NON SO
D6. Nella sua famiglia ci sono stati casi di patologia tumorale? |_| SI |_| NO |_| NON SO
Se si, specificare:

Grado parentela	Tumore	Anno di inizio
……	……	……
……	……	……
……	……	……

ATTENDIBILITÀ DELLE RISPOSTE

---

1. Completezza delle informazioni

□ Scarsa □ Parziale □ Sufficiente

2. Atteggiamento nei confronti dell'intervista

□ Favorevole □ Con qualche riserva □ Negativo

3. Motivazione a collaborare

□ Buona □ Discreta □ Bassa

4. Stato di salute del paziente

□ Buono □ Compromesso □ Intervista non applicabile (intervista a sostituto)

5. Il paziente è a conoscenza della patologia

□ Completamente □ Parzialmente □ Non è a conoscenza

6. I famigliari sono a conoscenza della patologia del paziente

□ Completamente □ Parzialmente □ Non sono a conoscenza

Esistono altri parenti o compagni di lavoro del soggetto che potrebbero dare risposte più approfondite rispetto ad alcuni particolari periodi? Se si, riportare se possibile indirizzo e numero di telefono.

……..……..……..

……..……..……..

……..……...

……..……..

NOTE: ……

……..……...

……..……...

Data |__|__| / |__|__| / |__|__|__|__|

Luogo dell'intervista Firma dell'intervistatore

……..……..……..…… ……..……..……...

Sono stati redatti Primo certificato e Denuncia/Referto di Malattia Professionale? |_| SI |_| NO

Dal Dr./Dr.ssa..... In data |_|_| / |_|_| / |_|_|_|_|

APPENDIX B –EBC DIFFERENTIAL MIRNAS

MPM vs PEA		
miRBase ID(s)	**FC**	**p-values**
hsa-miR-30a-5p	0.280753	0.005943
hsa-miR-520d-3p	0.008541	0.006966
hsa-miR-518b	0.021609	0.013594
hsa-miR-520b	3.831813	0.014748
hsa-miR-646	10.99091	0.020164
hsa-miR-520c-3p	13.18051	0.02331
hsa-miR-888-5p	11.02651	0.039851
hsa-miR-376a-3p	2.117156	0.04988

MPM vs NM		
miRBase ID(s)	**FC**	**p-values**
hsa-miR378	0.049168	0.014848
hsa-miR206	0.110251	0.031217
hsa-miR9	0.265293	0.043812

APPENDIX B – PLASMA DIFFERENTIAL MIRNAS

MPM vs PEA		
miRBase ID(s)	**FC**	**p-values**
hsa-miR-628-3p	0.37568	0.000218
hsa-miR-151a-5p	0.286561	0.000295
hsa-miR-93-3p	0.300425	0.000321
hsa-miR-98-5p	0.049642	0.000341
hsa-miR-30d-5p	0.328762	0.000617
hsa-miR-148b-5p	0.286644	0.000628
hsa-miR-589-3p	0.179872	0.00081
hsa-miR-340-3p	0.284145	0.000907
hsa-miR-130b-5p	0.284536	0.001071
hsa-miR-505-5p	0.34804	0.00113
hsa-miR-103a-3p	0.271125	0.001192
hsa-miR-625-3p	5.184904	0.001387
hsa-miR-766-3p	0.297441	0.001499
hsa-miR-942-5p	6.791041	0.001671
hsa-miR-409-3p	0.268254	0.001673
hsa-miR-126-5p	0.327779	0.001687
hsa-miR-30a-5p	0.287003	0.001689
hsa-miR-146b-3p	0.421028	0.001756
hsa-miR-1260a	7.259183	0.00225
hsa-miR-20a-3p	0.309987	0.002301
hsa-miR-151a-3p	0.31479	0.002386
hsa-miR-127-3p	0.271098	0.002422
hsa-miR-183-5p	1.921576	0.003084
hsa-miR-223-5p	0.425485	0.003181
hsa-miR-181a-2-3p	0.358715	0.003329
hsa-miR-23a-3p	0.329772	0.003419
hsa-miR-654-5p	2.305953	0.0035
hsa-miR-154-3p	0.319475	0.003509
hsa-miR-543	0.329703	0.003581
hsa-miR-26b-3p	0.455529	0.004025
hsa-miR-383-5p	2.25916	0.004616
hsa-miR-30e-3p	0.255835	0.004796
hsa-miR-646	11.45214	0.004834
hsa-miR-147a	1.810541	0.005134
hsa-miR-23b-3p	0.060484	0.005269
hsa-miR-494-3p	0.290693	0.00604
hsa-miR-142-3p	0.427734	0.006093
hsa-miR-106b-3p	0.134365	0.006446
hsa-miR-335-3p	0.168747	0.006659
hsa-miR-510-5p	1.879752	0.006786

MPM vs NM		
miRBase ID(s)	**FC**	**p-values**
hsa-miR-489-3p	0.155677	0.000188
hsa-miR-375	0.164129	0.001423
hsa-miR-483-3p	0.246724	0.001587
hsa-miR-190a-5p	0.307291	0.003612
hsa-miR-642a-5p	0.239922	0.00498
hsa-miR-25-3p	0.35171	0.007322
hsa-miR-378	0.106803	0.007725
hsa-miR-365a-3p	0.217752	0.009835
hsa-miR-144-5p	0.303538	0.011222
hsa-let-7c-5p	0.313274	0.011242
hsa-miR-193a-5p	0.294463	0.012173
hsa-miR-886-3p	0.266727	0.013006
hsa-miR-200a-3p	0.244418	0.013385
hsa-miR-450b-3p	0.194443	0.014373
hsa-miR-218-5p	0.328296	0.014559
hsa-miR-455-3p	0.36225	0.016966
hsa-miR-31-5p	0.165954	0.017124
hsa-miR-885-5p	0.238934	0.017673
hsa-miR-122-5p	0.245242	0.017978
hsa-miR-320b	0.371009	0.018165
hsa-miR-193b-5p	0.402687	0.022523
hsa-miR-135b-5p	0.409626	0.022817
hsa-miR-125b-1-3p	0.139646	0.024671
hsa-miR-99a-5p	0.34446	0.027242
hsa-miR-1255b-5p	0.507204	0.02998
hsa-miR-500a-5p	0.392921	0.030402
hsa-miR-654-5p	0.328002	0.033774
hsa-miR-193b-3p	0.292076	0.034166
hsa-miR-455-5p	0.460077	0.035092
hsa-miR-125b-5p	0.315456	0.035174
hsa-miR-1275	0.315488	0.035651
hsa-miR-101-3p	0.394766	0.038064
hsa-miR-92a-3p	0.371828	0.040274
hsa-miR-16-5p	0.427593	0.040558
hsa-miR-660-5p	0.351997	0.040896
hsa-miR-202-3p	0.50283	0.040991
hsa-miR-34b-3p	0.530273	0.043945
hsa-miR-27a-5p	0.419498	0.048065
hsa-miR-216b-5p	0.467026	0.048246

MPM vs PEA		
miRBase ID(s)	**FC**	**p-values**
hsa-miR-618	1.775793	0.006933
hsa-miR-504-5p	1.775793	0.006933
hsa-miR-450b-3p	1.775793	0.006933
hsa-miR-455-3p	1.775793	0.006933
hsa-let-7e-5p	0.446778	0.007211
hsa-miR-29a-5p	0.380887	0.007232
hsa-miR-19b-1-5p	0.444879	0.007279
hsa-miR-1249	0.422312	0.007541
hsa-miR-324-5p	0.330102	0.007572
hsa-miR-145-5p	0.318175	0.007834
hsa-miR-671-3p	0.416829	0.007884
hsa-miR-590-3p	0.382217	0.009052
hsa-miR-29b-3p	0.398221	0.010212
hsa-miR-181a-3p	0.327098	0.010286
hsa-miR-30d-3p	0.416286	0.010381
hsa-miR-22-5p	0.494648	0.011048
hsa-miR-382-5p	0.054696	0.011306
hsa-miR-18a-5p	0.474983	0.011415
hsa-miR-361-5p	0.405237	0.011898
hsa-miR-221-3p	0.450861	0.011941
hsa-miR-181c-5p	0.345878	0.012318
hsa-miR-519a-3p	1.717979	0.013458
hsa-miR-302c-5p	8.984328	0.013772
hsa-miR-744-3p	0.421244	0.013926
hsa-let-7c-5p	0.417543	0.01407
hsa-miR-191-3p	0.504484	0.014885
hsa-miR-27a-3p	0.459145	0.015183
hsa-let-7f-5p	0.485009	0.015216
hsa-miR-375	0.356058	0.015341
hsa-miR-744-5p	0.412421	0.015574
hsa-miR-589-5p	1.685922	0.015658
hsa-miR-450b-5p	1.637586	0.015666
hsa-miR-199a-5p	0.427968	0.016023
hsa-miR-508-3p	1.918199	0.016167
hsa-miR-548b-3p	1.708638	0.016865
hsa-miR-136-3p	0.338666	0.017635
hsa-miR-136-5p	1.663488	0.017848
hsa-let-7a-5p	0.199848	0.01785
hsa-miR-125a-5p	0.372199	0.018199
hsa-miR-130b-3p	0.395922	0.018232
hsa-miR-526b-5p	1.670495	0.018637
hsa-miR-432-5p	0.376884	0.019793
hsa-miR-142-5p	0.442703	0.020104

MPM vs PEA		
miRBase ID(s)	**FC**	**p-values**
hsa-miR-15b-5p	0.350969	0.020833
hsa-miR-320b	0.50541	0.020848
hsa-let-7d-5p	0.386217	0.021384
hsa-miR-485-3p	0.304979	0.021825
hsa-miR-30a-3p	0.432392	0.02269
hsa-miR-101-3p	0.503494	0.022767
hsa-miR-128-3p	0.511282	0.023593
hsa-miR-127-5p	1.578706	0.025101
hsa-miR-125a-3p	1.69695	0.026312
hsa-miR-135a-5p	0.483027	0.026349
hsa-miR-29c-3p	0.512421	0.027172
hsa-miR-30b-5p	0.292208	0.028398
hsa-miR-326	0.53148	0.028812
hsa-miR-15a-3p	0.490211	0.029044
hsa-miR-301b	0.475846	0.029758
hsa-miR-15a-5p	0.091591	0.030228
hsa-miR-579-3p	0.4953	0.030328
hsa-miR-373-3p	1.685615	0.030388
hsa-miR-503-5p	0.520033	0.030929
hsa-miR-652-3p	0.436477	0.031566
hsa-miR-181a-5p	0.359646	0.032169
hsa-miR-27b-3p	0.503086	0.032922
hsa-miR-148b-3p	0.479335	0.03421
hsa-miR-107	0.422768	0.034922
hsa-miR-199b-5p	0.449518	0.035547
hsa-miR-629-3p	0.556286	0.035786
hsa-miR-758-3p	7.214694	0.036851
hsa-miR-222-3p	0.467657	0.039743
hsa-miR-328-3p	0.538681	0.041034
hsa-miR-339-5p	0.204598	0.041149
rno-miR-301a-3p	0.481937	0.041396
hsa-miR-99b-5p	0.498538	0.041484
hsa-miR-374a-5p	0.568027	0.044481
hsa-miR-520e	20.6435	0.046595
hsa-miR-324-3p	0.533766	0.046734
hsa-miR-551b-3p	0.413438	0.048161
hsa-miR-32-5p	0.499282	0.048162
hsa-miR-331-3p	0.545767	0.048407
hsa-miR-376b-3p	0.504434	0.048735
hsa-miR-208a-3p	1.56183	0.048768
hsa-miR-146a-5p	0.369494	0.049217