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in
SALUTE DELL'UOMO

MIRNA SIGNATURE TO IDENTIFY LUNG ASBESTOS-RELATED MALIGNANCIES.

Relatore: Chiar.mo

Prof.ssa Lory Santarelli

Dottoranda:

Dr.ssa Simona Gaetani

Correlatore:

Dott. Marco Tomasetti

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ABSTRACT

Introduction. Exposure to asbestos results in serious risk of developing malignant mesothelioma (MM) and lung cancer (LC). The association between asbestos exposure and lung adenocarcinoma is well established. Nevertheless, precise histopathological data are poorly considered when investigating the asbestos-cancer link in compensatory approach. Occupational lung cancer generates high mortality and is the most common compensated in France. Although, the Helsinki criteria for identifying individuals with high risk of asbestos exposure at work have been accepted, it is insufficiently proved and specific asbestos-related parameters are needed. The presence of pleural plaques is not considered a pre-cancerous condition, while asbestosis, as well smoking is associated with an increased risk of lung cancer. MicroRNAs (miRNAs) have rapidly become an attractive method for profiling cancer. MiRNA expression is early altered by exposure to occupational/environmental carcinogens, thus, useful to identify a novel asbestos-related profile able to distinguish asbestos-induced cancer from cancer with different etiology.

Methods. Consequential study phases have been performed to identify miRNAs associated to the development of asbestos-induced malignancies. Four groups have been included: patients with asbestos-related malignancies ($NSCLC^{Asb}$ and MM), non-asbestos-related lung cancer (NSCLC), and disease-free subjects (CTRL). Next, the selected miRNAs were evaluated in an asbestos-exposed population, and an in 'in vitro' model was performed to identify the mechanism of asbestos-induced miRNA regulation.

Results. Four serum asbestos-related miRNAs consisting of miR-126, miR-205, miR-222 and miR-520g were found to be involved in asbestos-related malignant diseases. MiR-222

and age best depicted the asbestos-related malignancies. The association of miR-222 with miR-222/miR-126 best characterised the non-asbestos related NSCLC group. We found that miR-126 and miR-222 were strongly associated with asbestos exposure and both miRNAs were involved in major pathways linked to cancer. To elucidate the role of mediation effect of these miRNAs between asbestos-exposure and the tumour development, a disease-free population exposed to asbestos, stratified as currently-exposed and ex-exposed with and without asbestos-related diseases (ARDs), was evaluated for the selected miRNAs. Notably, increased expression of miR-126 and miR-222 including miR-126/miR-205, miR-222/miR-205, and miR-222/miR-520g ratios were found only in currently exposed subjects. It had been reported that miRNA expression changes in response to environmental carcinogens exposure are transient and revert to normal levels after recovery from exposure. Accordingly, ex-exposed subjects to asbestos did not show any changes in miRNA expression (***cf*** **Fig.26**). Increased expression of EGFR was found in the asbestos-initiated cells, causing activation of the down-stream effector AKT and p38 MAPK signalling. Asbestos-mediated activation of EGFR-AKT pathway resulted in miR-126 and miR-222 upregulation associated with miR-520g downregulation. Both miR-222 and miR-520g were reversed by inhibiting EGFR (AG1478), suggesting its involvement in asbestos-induced miRNA regulation. Moreover, the upregulation of miR-126 and miR-222 was enhanced when asbestos-induced pre-cancerous cells were cocultured with tumour stromal cells, such as fibroblasts and endothelial cells. Thus, suggesting that cancer stroma cross talk induced the expression of miR-126 and miR-222 to facilitate angiogenesis and invasion growth of lung malignancies.

Conclusions. This study uncovers miRNAs that are potentially involved in asbestos-related malignancies and their expression outline mechanisms whereby miRNAs may be involved in asbestos-induced pathogenesis.

1.INTRODUCTION

Asbestos-related diseases are still a major public health problem. The World Health Organization (WHO) has estimated that 107,000 people worldwide die each year from mesothelioma, lung cancer, and asbestosis (WHO, 2010). Although worldwide consumption of asbestos has decreased, consumption is increasing in many developing countries in which exposures may also be high in these areas. All forms of asbestos have been considered carcinogenic by several Health Organizations including the International Agency for Research on Cancer (Int. Agency Res. Cancer (IARC). 1977. Asbestos, Vol. 14. Lyon, Fr.: IARC), and in United States by the Environmental Protection Agency (Int. Programme Chem. Saf. (IPCS). 1988. Chrysotile. Geneva: WHO). At the IARC meeting in March 2009 scientists concluded that all forms of asbestos (chrysotile, crocidolite, amosite, tremolite, actinolite, and anthophyllite) are associated with an increased risk of mesothelioma and lung, laryngeal, and ovarian cancers (IARC, 2012; *A Review of Human Carcinogens: Arsenic, Metals, Fibres, and Dusts;* Straif, 2009). The use of asbestos has now been banned in 55 countries worldwide. Nevertheless, asbestos exposure is still widespread in the world: beside active occupational exposure that is estimated to affect about 125 million men and women (IARC. IARC monograph on the evaluation of carcinogenic risk to human, vol. 100 C, Arsenic, Metal, Fibres and Dusts. Lyon: IARC Press, 2012.), a large number of people are exposed to asbestos in areas that may remain in place for decades after the enforcement of asbestos bans. Because of its widespread past use, the epidemic of asbestos-related diseases is known to be nearly worldwide and thus may be described as a pandemic rather than just an epidemic (**Fig.1**).

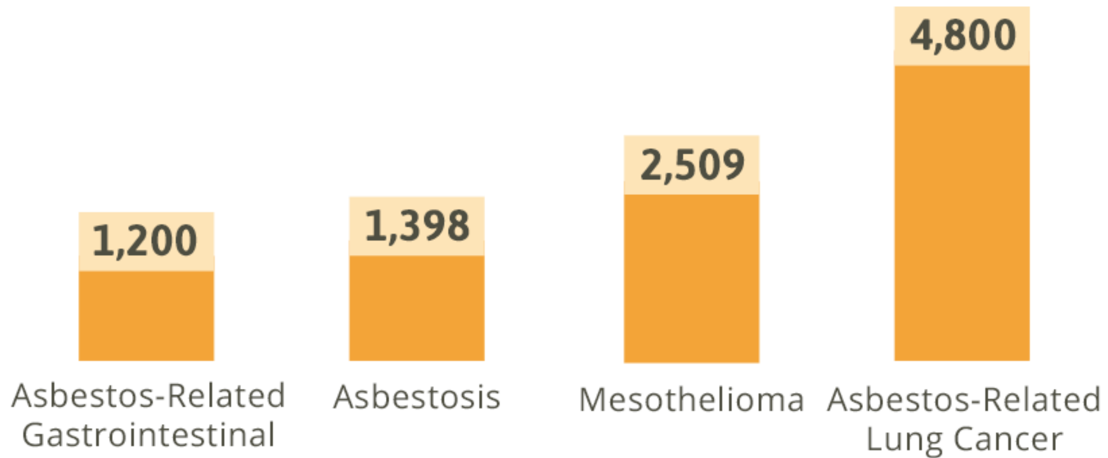


Figure 1. The Environmental Working Group (EWG) states that mesothelioma, asbestosis and asbestos-related lung and gastrointestinal cancers claimed as many as 230,000 lives between 1979 and 2001. This chart reflects the EWG's yearly morbidity estimates for three primary asbestos-associated cancers as well as asbestosis, a noncancerous condition that is sometimes diagnosed in asbestos cancer patients.

Asbestos-related diseases (ARDs) resulting from exposure to asbestos include mostly lung cancer (LC) and malignant mesothelioma (MM). Most data on mortality from ARDs came from MM, an aggressive cancer whose association with asbestos exposure is well established. *Driscoll et al*. (Driscoll et al., 2011) estimated that 43,000 people worldwide die each year by MM although the real cases of MM have been underestimated. Although the proportion of MM attributable to asbestos exposure varies, a fraction of 80% has been reported (Tossavainen, 1997).

In **Figure 2** is shown the distribution of age MM incidence rated for males by country between 1998-2002 and these data, which include only cancer registries that had at least 15 years, come from IARC report, *Cancer in Five Continent* (IARC, 2007).

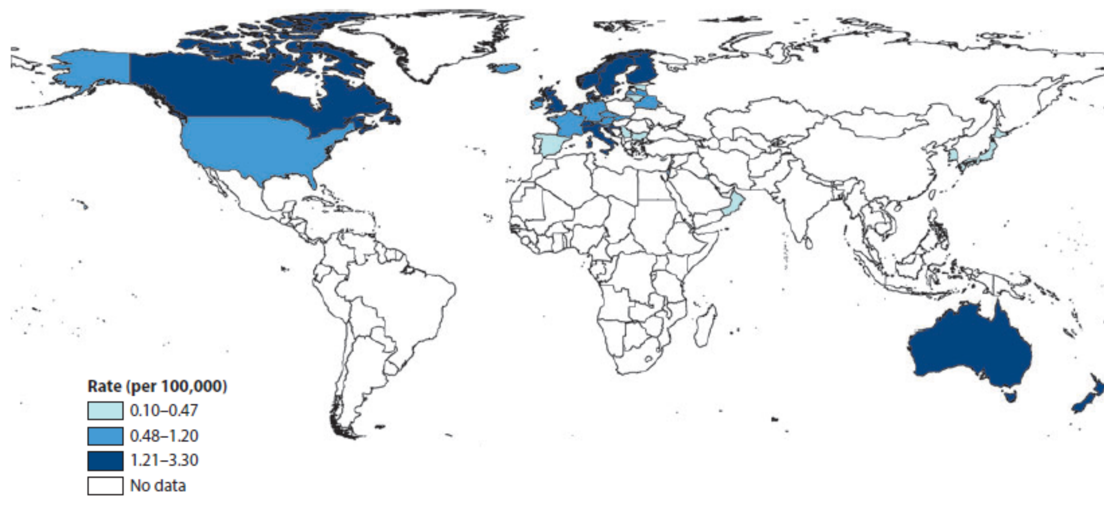


Figure 2. Worldwide age standidardized mesothelioma incidence rates (per 100,000) for males in 1998-2002. Data from Reference (IARC.2007. *Cancer Incidence in Five Continents*: Vol. IX, ed.)

Bianchi C. underlined a wide area of the world for which there is not any information on MM incidence (Bianchi and Bianchi, 2007). The highest incidence of MM in the world was reported in the Italian Genoa Province (5.8 per 100,000). Other areas of the world reporting high rates include the West Cape of Australia (4.7 per 100,000), the Northern Yorkshire (4.2 per 100,000) area of the United Kingdom, Northern Ireland (4.0 per 100,000), and Scotland (3.6 per 100,000). Approximately 95% of the participating cancer registries have reported cases of MM to the IARC program. *Delgermaa et colleagues* (Delgermaa et al., 2011) established a total of 92,253 MM deaths by 83 countries between 1994 and 2008. The worldwide age-adjusted rate for mesothelioma mortality was 4.9 per million. The United Kingdom was found to have the highest age-adjusted mortality rate (17.8 per million), followed by Australia (16.5 per million), and Italy (10.3 per million).

Malignant mesothelioma is the cancer mainly associated with asbestos exposure. However, epidemiologic studies reported several cases of LC in asbestos-exposed workers for all types of asbestos except for crocidolite (Henderson et al., 2004; Stayner et al., 1996).

Although the major risk factor for LC is tobacco smoke, an estimated 5-7% of all these cancers are attributable to asbestos exposure (LaDou, 2004). Asbestos-related LC is considered one of the most important occupational cancers (Karjalainen et al., 1994).

Hazardous occupational exposures to asbestos fibers have occurred in a variety of industrial operations, including mining and milling, manufacturing, shipbuilding and repair and construction (Current intelligence bulletin 62, 2011).

Current exposures to commercial asbestos occur predominantly during maintenance operations and remediation of older buildings containing asbestos. In addition to occupational asbestos exposure, environmental exposure is highly relevant for the risk to develop asbestos-related malignancies. This has significant health and economic implications that have been well documented. The 20-40-year latency periods of ARDs (Lanphear and Buncher, 1992) and their low incidence rates in the general population make preventive strategies and early treatment extremely challenging. The availability of well-validated diagnostic biomarkers of asbestos exposure would greatly facilitate both prevention and early treatment strategies. Biomarkers are crucial in the screening of patients with a high risk of developing cancer, diagnosing patients with suspicious tumours at the earliest possible stage, establishing an accurate prognosis, predicting and monitoring the response to specific therapies. Biomarkers of response, which reflect a change in biologic function in response to asbestos exposure, have proved to be more useful. MM is the major biological response to asbestos that can be readily monitored and numerous studies have used this disease as confirmation of a previous asbestos exposure. Epigenetic alterations are innovative biomarkers for cancer, due to their stability, frequency, and non-invasive accessibility in body fluids. Recently, circulating microRNAs (miRNAs) were

found to be particularly attractive as biomarkers for early diagnosis and prognosis of many cancer types (Song et al., 2017).

Therefore, the identification of asbestos-related molecular-signature has long been a topic of increasing research interest. MiRNA expression is early altered by exposure to occupational/environmental carcinogens, thus, useful to identify a novel asbestos-related profile able to distinguish asbestos-induced cancers from cancers with different etiology.

1.1. Asbestos-lung disorders

The exposure to asbestos induces the formation of both benign lung disorders such as, asbestosis (diffuse interstitial pulmonary fibrosis due to asbestos inhalation), pleural plaques (PPs), diffuse pleural thickening (DPT), benign asbestos pleural effusion (BAPE), rounded atelectasis (RA), and malignancies, including LC and MM. The clinical characteristics of ARDs are summarized in **Tab.1.**

Disease	Presenting symptoms	Chest X/ray findings	Treatment
Asbestosis	Dysponea and dry cough	Bilateral,irregular reticulo/nodular opacities at the lung bases	No effective therapies,supportive and symptomatic care
PPs	Asymptomatic	Discrete elevated areas of hyaline fibrosis from parietal pleura,white shaggy appearance	No effective therapies
BAPE	Usually asymptomatic	Small-to-moderate size and unilateral pleural effusion, may be massive or bilateral	Drainage to alleviate symptoms, spontaneously resolve completely
RA	Usually asymptomatic	Rounded mass-lile opacity in the peripheral lung	Restoring the pulmonary capacity/breathing comfort
DPT	Dysponea	Countinous,smooth pleural shadowing extending over at least one quarter of one or more costophrenic angles	No effective therapies,supportive and symptomatic care
LC	Cough,dysponea and chest pain	A mass in the lungs +/- enlarged lymph nodes	Multimodality treatment including surgery,radiotherapy and chemotherapy
MM	Cough,dysponea and chest cough	Unilateral pleural effusion,occasionally multiple pleural masses or with pleural thickening	Chemotherapy, multimodality treatment

Table 1. Clinical characteristics of the asbestos-related diseases. Abbreviations: **PPs**, pleural plaque; **BAPE**, benign asbestos pleural effusion; **RA**, rounded atelectasis; **DPT**, diffuse pleural thickening; **LC**, lung cancer; **MM**, malignant mesothelioma.

1.1.1. Asbestosis

Asbestosis is defined as diffuse interstitial pulmonary fibrosis due to inhalation of asbestos fibres. The latency period for disease development is usually 15 years or more, and is influenced by duration and intensity of exposure. Relatively high levels of asbestos inhalation are required to produce asbestosis (cumulative exposure ≥25 fibres /mL-years), although there are some reports that record asbestosis following lower levels of asbestos exposure (Roggli et al., 2010). Asbestosis is interstitial fibrosis that is sub-pleural and initially affects the lung bases. Diagnostic criteria have been published (American Thoracic Society, 2004) and include a compatible exposure history, clinical and radiographic features. Asbestosis usually presents with gradual and progressive dyspnoea and accompanying dry cough. The histological diagnosis of asbestosis requires an appropriate pattern of interstitial fibrosis and demonstration of asbestos bodies in histological section. High-resolution computed tomography (HRCT) is more sensitive and specific than chest radiography (Akira et al., 2003).

1.1.2. Pleural plaques

Another benign ARDs are pleural plaques (PPs) which the commonest manifestation of asbestos exposure affecting up to 58% of asbestos-exposed workers and up to 8% of general environmentally exposed populations, with a latency period of 20-30 years. They are hypo cellular lesions composed of thick collagen bundles arranged in a 'basket-weave' pattern covered by a single layer of normal mesothelial cells (Chapman et al., 2003).

PPs are variable in size and number with white or pale yellow appearance, typically distributed on the posterolateral chest wall, the dome of the diaphragm and the mediastinal pleura (**Fig.3A,B**) (Roach et al., 2002).

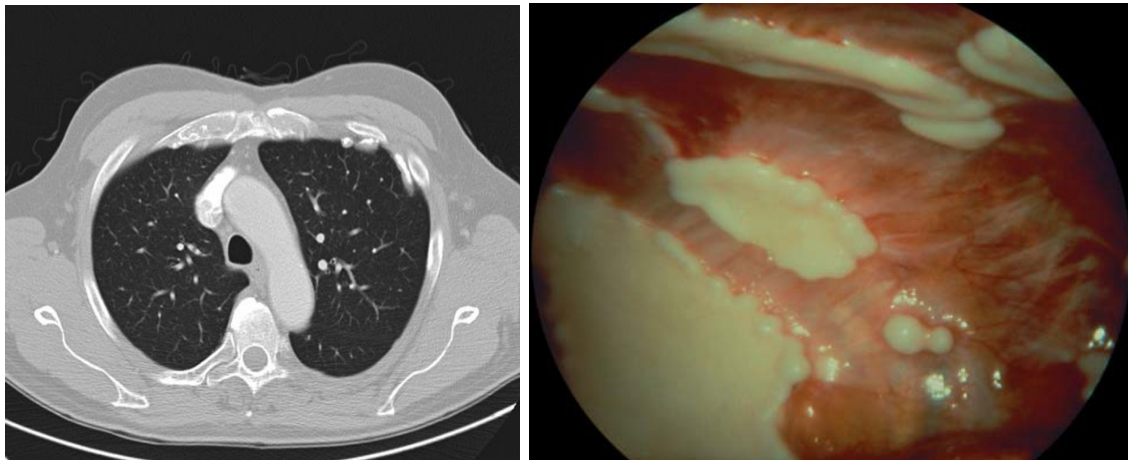


Figure 3. (A) Radiological and computed tomography scan of the thorax demonstrating asbestos-related pleural plaques. (**B**) Thoracoscopic view of PP on the parietal pleura. Pleural biopsies have done at the level of non-specific inflammation to rule out an early-stage malignant mesothelioma.

They are sharply demarcated from sub-pleural tissue, and calcification is a common late finding (Huggins and Sahn et al., 2004). The diagnosis relies on radiographic findings and a compatible history of exposure. The CXR is a standard diagnostic tool utilizing ILO classification guidelines, although false-positive, false-negative and inter observer variability rates are relatively high. HRCT is more sensitive and specific for making the diagnosis, but because of its high radiation exposure and unavailability, it is inappropriate for screening (American Thoracic Society, 2004). Pleural plaques do not undergo malignant degeneration into MM, and their presence does not increase the risk of developing asbestos-related malignancies (Harber et al., 1987; Weiss, 1993).

1.1.3. Benign asbestos pleural effusion (BAPE)

Benign asbestos pleural effusion (BAPE) is an exudative and often haemorrhagic pleural effusion following asbestos exposure and is a diagnosis of exclusion after other possible causes such as malignancies, tuberculosis and other infections (Hillerdal and Ozesmi, 1987). It usually occurs within 10 years after exposure, earlier than other ARDs, although this is not variable (American Thoracic Society, 2004).

BAPE is often unilateral with left-side predominance and usually resolve completely with a mean duration of 3-4 months, but may recur (30%-40%) and usually occur within 3 years of the initial presentation (Epler et al., 1982; Robinson et al., 1981).

A prolonged follow-up is necessary for asbestos-exposed patients with pleural effusions. BAPE does not have specific prognostic implications respect to the subsequent development of MM (Robinson et al., 1981; Gaensler and Kaplan, 1978), but is the witness of exposure to the main risk factor of this malignancy.

1.1.4. Diffuse pleural thickening (DPT)

DPT is characterized by extensive thickening of the visceral pleura, often with adherence to the parietal pleura, and obliteration of the pleural space (American Thoracic Society, 2004; Yates et al., 1996). Many studies showed that DPT is a consequence of BAPE (Epler et al., 1982; MacLoud et al., 1985; Lilis et al., 1985; Fridriksson et al., 1981). However, DPT is not a pathognomonic marker of asbestos exposure: many other causes of exudative pleural effusion can lead to DPT.

DPT accounts for 22% of all ARDs (Hannaford-Turner et al., 2010). It can develop within a year of exposure but can also take up to 40 years. PPs often coexist. DPT may be associated with dyspnoea and chest pain (Yates et al., 1996; Jeebun and Stenton, 2012). Although symptoms are generally mild, severe restrictive lung disease with hypercapnic respiratory failure and death can rarely occur (Miles et al., 2008). DPT can cause significant restrictive ventilatory impairment. On a chest radiograph, DPT presents as continuous, irregular pleural shadowing that often extends up both chest walls and with blunting of one or more cost phrenic angles (**Fig. 4**).

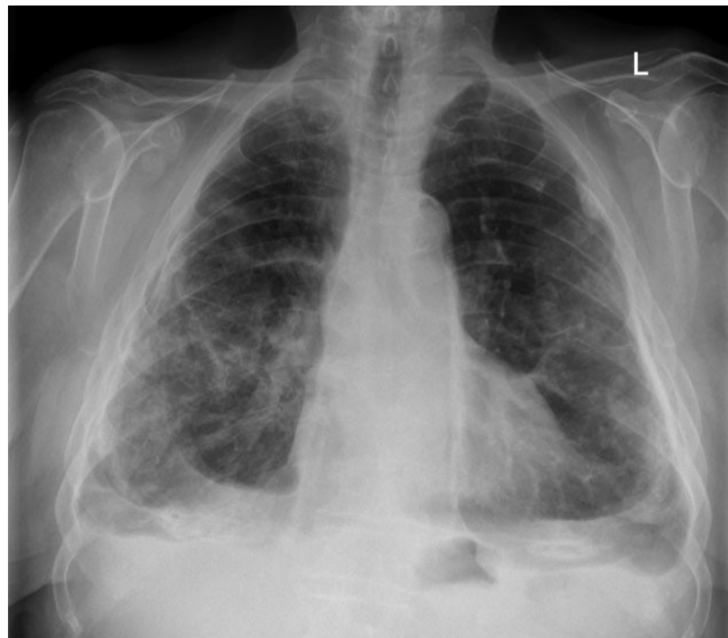


Figure 4. Posteroanterior chest radiograph demonstrating asbestos-related diffuse pleural thickening.

HRCT is more sensitive than CXR for detection of early pleural thickening (i.e. 1–2 mm in thickness) (American Thoracic Society, 2004) (**Fig. 5**).

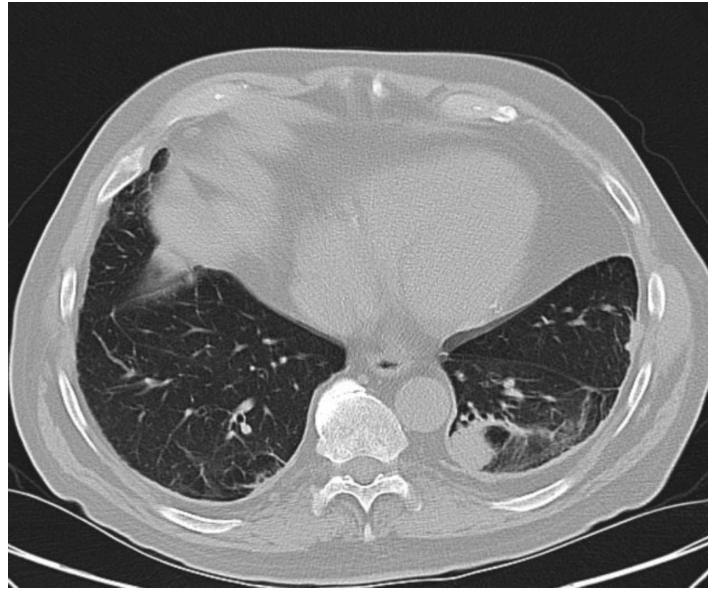


Figure 5. Computed tomography scan of the thorax demonstrating asbestos-related diffuse pleural thickening. Note the rounded atelectasis on the left and the sub-pleural interstitial pulmonary fibrosis bilaterally.

Treatment is largely limited to supportive and symptomatic care.

1.1.5. Rounded atelectasis (RA)

Rounded atelectasis (RA) (Blesovsky's syndrome) may occur with DPT. Pleural adhesions and fibrosis cause deformation of the lung with bending of some small bronchi (Hillerdal, 1989). On CXR, this presents as a rounded opacity in the peripheral lung adjacent to the thickened pleura, with curvilinear opacities (the comet tail sign) extending from the site of atelectasis towards the hilum (Batra et al., 1996). Exposure to asbestos is the principal cause today, but any type of pleural inflammatory reaction can cause RA (Hillerdal, 1989). RA is usually asymptomatic, but may be accompanied by breathlessness.

1.1.6. Malignant mesothelioma (MM)

MM is an aggressive and incurable tumour arising from mesothelial cells of the pleura, peritoneum and rarely elsewhere. Although cases due to environmental and para-occupational asbestos exposure have been described, most MMs are occupational in origin. MM can develop even after short and low exposure, but a dose-effect relationship has been demonstrated. Median latency is approximately 40 years (range 15-67). MM has a poor prognosis with median survival of 8-14 months (British Thoracic Society, 2007; Musk et al., 2011).

Patients usually present with chest pain (60-70%), dyspnoea (50-70%), cough (20-30%) (Fuhrer and Lazarus, 2011) and restrictive gas exchange abnormality. The radiographic manifestation is usually a unilateral pleural effusion or pleural thickening. CT is the primary modality for the diagnosis, staging and response assessment of MM **(Fig. 6A, B)**.

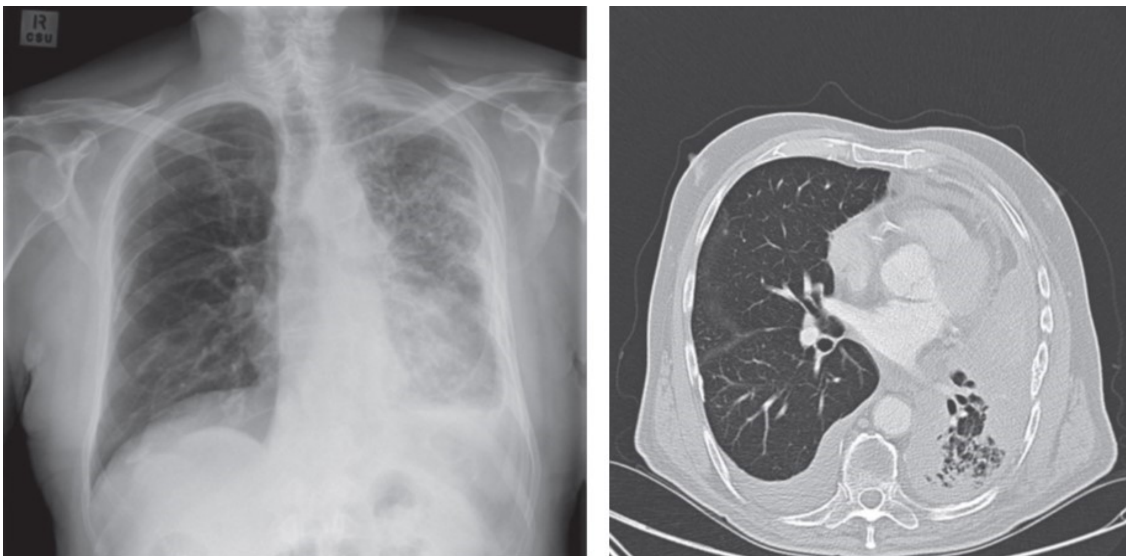


Figure 6. (A) Posteroanterior chest radiograph of a patient with left-sided malignant mesothelioma.**(B)** Computed tomography scan of the thorax of a patient with left-sided malignant mesothelioma showing opacification and very little lung aeration.

Fluorodeoxyglucose PET/CT (FDGPET/ CT) has been shown to be a useful diagnostic tool for detection of distant metastasis and to differentiate MM from benign pleural disease (Sharif et al., 2011; Basu et al., 2010).

Staging relies on the International Mesothelioma Interest Group TNM (tumour, nodes, and metastases) staging system (NCCN, 2013). Survival depends on the stage of the disease when diagnosed. Stage I has approximately 12 months of survival, Stage II 4 months, and about 3 months in Stages III–IV (Rusch,1995). The definitive diagnosis of MM requires a tissue sample; however, negative results do not exclude it as sampling problems can occur and the diagnosis is a difficult one histologically.

MM occurs in three main histological subtypes: epithelioid, sarcomatoid and biphasic. These have prognostic significance, with epithelioid being the most common and sarcomatoid subtype predicting the worst outcome (Safe Work Australia, 2011; Musk AW et al., 2011). Several tumour biomarkers measured in either the serum, plasma or the pleural fluid have been evaluated for diagnostic purposes. Biomarkers could also be useful to detect the development of MM at an early, potentially resectable stage, although no randomized data currently exist that confirm prolonged survival with surgical resection. Promising biomarkers include soluble mesothelin-related protein (SMRP) (Wheatley-Price et al., 2010; Park et al., 2008), osteopontin (Pass et al., 2009; Park et al., 2009), and more recently, fibulin 3 (Pass et al., 2012) and integrin-linked kinase (ILK) (Watzka et al., 2013). Although initial results appear promising, most of these biomarkers have yet to be evaluated in prospective studies and primarily apply to epithelial type MM. SMRP has been evaluated in this manner and unfortunately was found to have a high rate of false-positives (Park et al., 2008). High SMRP levels have been shown to correspond with

disease volume, suggesting that SMRP may be a useful for detecting the progression of MM and monitoring MM during treatment (Wheatley et al., 2010; Franko et al., 2012). Serum osteopontin levels are elevated in MM but also in patients with non-malignant ARDs (Pass et al., 2005; Park et al., 2009) resulting in rather low specificity and sensitivity (Creaney et al., 2008; Grigoriu et al., 2007), limiting its value as a screening tool. Plasma fibulin-3 levels discriminated between Stage I or II mesothelioma and asbestos exposure without MM, with a specificity of 94% and a sensitivity of 100% (Pass et al., 2012). Integrin-linked kinase (ILK) serum levels have been shown to be significantly higher in patients with MM compared with healthy asbestos-exposed workers (Watzka et al., 2013). There is potential for the use of ILK as a marker of disease progression, as its levels are increased in advanced stages of MM in comparison with early stages (Watzka et al., 2013), but more work in this area is needed. Novel biomarkers such as volatile organic compounds measured in exhaled breath (Chapman et al., 2012), microRNAs (Kirschner et al., 2012) may prove useful in the future and/or a combination of biomarkers.

Many strategies have been tried for treating MM, including surgery, chemotherapy and radiation.

The principal role of radiotherapy is as an adjuvant following surgical resection (Scherpereel et al., 2010). Chemotherapy plays an integral role in multimodality treatment and is also recommended alone as a treatment for inoperable patients (NCCN, 2013), but no highly effective agents are yet available. A first-line regimen of pemetrexed or gemcitabine in combination with a platinum agent (cisplatin or carboplatin) is currently regarded as the best available treatment for MM (NCCN, 2013). Pemetrexed and raltitrexed in combination with cisplatin have been shown to improve survival, global

quality of life and pulmonary function, when compared with cisplatin alone, but the survival effect is only approximately 12 weeks (van Meerbeeck et al., 2005; Vogelzang et al., 2003). Currently, chemotherapy has at best only a modest benefit for these patients. Further improvements in drug development and better-designed clinical trials are strongly needed. In recent years, advances in knowledge of molecular and biological mechanisms of MM have led to development of immunologically based and targeted therapies, but these are still under evaluation.

1.1.7. Asbestos-related lung cancer

Lung cancer (LC) has continued to be the most common type of cancer worldwide for several decades, with the highest incidence and mortality rates (Ferlay et al., 2012). Only 13% of patients with lung cancer survive for > 5 years (Balgkouranidou et al., 2013). As the second leading risk factor for lung cancer, asbestos exposure is responsible for an estimated 5-7% of all these cancers (LaDou, 2004.). Asbestos-related lung carcinoma is considered one of the most devastating occupational cancers (Kettunen et al., 2011). In 1997, the Helsinski criteria for identifying individuals with a high risk of asbestos exposure at work were accepted (Tossavainen, 1997). Although the use of asbestos has been banned in many developed countries, asbestos-related lung cancer still poses a great public health threat due to the long latency period from asbestos exposure to the incidence of asbestos-induced cancer (Lin et al., 2007).

Heavy asbestos exposure produces an increased risk of LC, with a latency period of approximately 15-20 years Among asbestos-related lung cancer, non-small cell lung cancer (NSCLC) accounts for at least 80 % of these cases (Balgkouranidou et al., 2004).

In 1997 the Helsinki criteria, which have been established that all four major histological types (squamous, adenocarcinoma, large-cell and small cell) can be related to asbestos, have been updated: the current classification (WHO 1999) includes two additional types of lung cancer (sarcomatoid and adenosquamous). These are included as type of lung malignancies that may occur because of asbestos exposure. (Consensus report, Wolff et al., 2015). In the Helsinki criteria, it is reported that there is no threshold of concentration of fibers of airborne asbestos below which the risk of asbestos-related LC is null (Consensus report, Wolff et al., 2015).

Asbestos-related LCs account for about 3-8% of all LCs and is similar in both the type of cancer and in its signs and symptoms in asbestos-exposed and unexposed individuals. Older studies found inconsistent results regarding the lobe of origin and histology of asbestos-related lung cancer (ARLC) (Nielsen et al., 2014).

Some studies showed an upper lobe location similar to tobacco-related lung cancer, whereas other investigators found a lower lobe location (Auerbach et al, 1984; Hiraoka et al., 1990; Johansson et al., 1992; Warnock and Isenberg, 1992; Anttila et al., 1993; Hillerdal et al., 1983; Sluis-Cremer, 1980; Weiss, 1981). Although adenocarcinoma was found to be the most prevalent in some studies, a recent review of the literature by *Nielsen et al* (Nielsen et al., 2014) showed that there was no difference in location and cell type between ARLC and non-ARLC. They concluded that cell type and location of lung cancer were not useful for differentiating ARLC from other lung cancers. Prognosis of ARLC was not different from that of other lung cancers (Nielsen et al., 2014). Because pleural plaques may be associated with low levels of exposure, the attribution of LC to asbestos must be supported by an occupational history of substantial asbestos exposure or measures of

asbestos fibre burden. Presence of pleural plaques demonstrated a previous asbestos exposure but was not a precancerous condition. Pleural plaques alone are insufficient for the attribution of LC to asbestos. A possible indicator for attributing LC to asbestos exposure is "bilateral diffuse pleural thickening" which is often associated with moderate or heavy exposure, as seen in cases of asbestosis and should be considered accordingly in terms of attribution. Asbestosis argued for high asbestos exposure and was associated with an increased risk of LC (Nielsen et al., 2014). All asbestos types were associated with LC (Nielsen et al., 2014). The risk of developing LC is linearly related to cumulative asbestos exposure. The issue of whether asbestosis is a necessary precursor for LC is still controversial, but recent consensus statements have concluded that heavy asbestos exposure of ≥25 fibres/mL-years rather than asbestosis is needed (Henderson et al., 2004). A minimum lag time of ten years from the first asbestos exposure is required to attribute the LC to asbestos. Epidemiological evidence indicates that the combined effect of smoking and asbestos exposures on LC incidence appears to be more than additive and is probably multiplicative. Smoking cessation has major health benefits and should be recommended in all patients with past asbestos exposure.

One recent British study showed an inverse relationship between time since smoking cessation and LC mortality risk in asbestos workers (Frost et al., 2011). Former smokers who had stopped smoking for 40 and more years had same risk of LC as asbestos workers who had never smoked (Frost et al., 2011).

The relative lack of symptoms during the early stages of LC frequently results in a delayed diagnosis. However, early diagnosis and treatment can result in long-term survival, improving the 5-year survival to approximately 70%. The treatment of LC and survival is

very dependent on disease stage and the presence of co-morbidities and is identical in asbestos-exposed and non-exposed patients. Treatment includes surgical removal of the cancer, chemotherapy, radiotherapy or a combination, and several excellent reviews are available (Rossi et al., 2013; Saintigny et al., 2012). CT screening has been evaluated in a population exposed to asbestos and shown to be feasible, however, with a high incidence of incidental findings (Vierikko et al., 2007). CT screening presents an opportunity for early diagnosis and treatment in asbestos-related LC and possibly also for MM.

1.1.7.1. The attribution of lung cancer to asbestos exposure: a difficult topic

It is extremely difficult to correlate exposure to asbestos and the onset of lung cancer.

According the Helsinki criteria updated in 2014, the “attribution of causation requires a reasonable medical certainty on a probability basis that the agent (asbestos) has caused or contributed materially to the disease” (Consensus report, Wolff et al., 2015). Among the causative factors for asbestos-related lung cancer, it is necessary to consider:

- **Occupational exposure**

  Epidemiological studies indicate that asbestos exposure is associated with an increased risk of lung cancer, (Lash et al., 1997; Hodgson et al., 2000; Berry and Gibbs, 2008) accounting for an estimated 2-5% of new lung cancers (Doll and Peto, 1981; Gustavsson et al., 2003; Marinaccio et al., 2008). An increased proportion of lower-lobe tumour localization (Craighead and Mossman, 1982) of adenocarcinomas (Patz et al., 2000) has been reported among workers exposed to asbestos, but these associations have not been confirmed in other studies (Lee et al.,

1998). The risk of developing lung cancer is linearly related to cumulative asbestos exposure, with an estimated increase of 1% for each fiber/mL-year of exposure (Boffetta et al., 1998). However, the strength of the association and the slope of the cumulative dose-response relationships vary considerably across studies and occupations (Mossman and Gee, 1989). The risk appears to be smaller in miners (McDonald et al., 1980), friction product manufacturers, (McDonald et al., 1984), intermediate in asbestos cement (Finkelstein,1983) and product manufacturers (Weill et al., 1979) and highest in textile workers (McDonald et al., 1983; McDonald et al., 1982).

The slope is steeper in asbestos cement manufacturers (Finkelsteinm, 1983) much less in friction product manufacturers (McDonald, 1984) and intermediate in the textile industry (McDonald et al., 1983; McDonald et al., 1982). The Helsinki Report estimates a cumulative exposure of 25 fibers/mL-years to increase the risk of lung cancer by two-fold (Consensus report: Helsinki criteria, 1997). However, a subsequent reevaluation concluded that probably the exposures associated with increased risk of lung cancer are higher (Tossavainen et al., 2000). A recent risk analysis of 14 studies identified a cumulative “no effect” exposure to crysotile for lung cancer in the range of 25-1,000 fibers/mL-years (Pierce et al., 2008). However, these dose estimates have been challenged for accuracy, mainly because of crude surrogates of exposure (Gibbs et al., 2007; Greenberg, 2007; Roggli et al., 2008). Lower-level occupational exposures are not associated with an increased risk of lung cancer (Weiss, 1999; Berry and Gibbs, 2008; Camus, 1998).

- **<u>Asbestosis and ARLC</u>**

  There is a controversy on relationship between asbestos-related lung cancer and asbestosis. Several authors hold the notion of an increased risk of LC in asbestos-exposed workers in the absence of evidence of asbestosis (Egilman and Reinert, 2005; Reid et al.,1991), but others conclude that the risk only increases if asbestosis is present (Hughes and Weill, 1991; Jones et al., 2009). However, a high correlation between asbestosis and LC rates was observed in 38 cohorts of workers (Weiss, 1999) and the risks of developing asbestosis and lung cancer are in the same range of cumulative dose (Loomis et al., 2009). The progression of asbestosis was shown to predict the development of lung cancer (Oksa et al., 1998) and this condition is a good indication that exposure of the patient was high enough to put him at risk of lung cancer. In addition to asbestosis, also pleural plaques were put in correlation with greater risk of LC (American Thoracic Society, 2004; Hillerdal and Henderson, 1997), but this conclusion should be challenged because plaques manifest themselves at asbestos exposures much smaller than those associated with increased risk of cancer (Weill and Weill, 2005). When both high asbestos body burden and fibrosis are present, the attribution of LC is clear, but it is also acceptable in the absence of fibrosis (Consensus report: Helsinki criteria, 1997).

- **<u>Custom tobacco and asbestos interaction</u>**

  Epidemiological evidence indicates that smoking and asbestos exposures taking place together have more than additive and less than multiplicative effects on LC incidence (Wraith and Mengersen, 2008). Hypotheses consider the mutual influences of asbestos and smoke in the delivery to epithelial cells of smoke

carcinogens and fibers, and there is some evidence in humans that smoking increases the penetration of fibers into the bronchial mucosa where the effect is greater for chrysotile than for the amphiboles (Nelson and Kelsey, 2002; Churg and Stevens, 1995). Therefore, extrapolations can hardly be made at levels of asbestos exposure that do not increase the risk of lung cancer.

2.1. Characterization of asbestos exposure

An important point for asbestos-related malignancies that no safe threshold for asbestos exposure has been established. Indeed, *Hodgson and Darton* suggested that any threshold for mesothelioma is at a very low level and they observed that the proportion of mesothelioma cases in population studies from whom no likely source of asbestos exposure can be identified is often quite high.

These observations suggest that relatively brief exposures may carry a low, but non-zero, risk of causing mesothelioma (Hodgson and Darton, 2000).

Some individuals develop mesothelioma following exposure to small amounts of asbestos, while others exposed to heavy amounts do not (Carbone et al., 2012), suggesting that genetic factors influence risk of this disease. Indeed, in a study *Testa et al.* reported that the presence of germline *BAP1* mutations in membres of families that have a high incidence of mesothelioma, despite very modest exposure to asbestos. These authors showed that *BAP1* mutations are associated with a novel hereditary cancer syndrome that predisposes to mesothelioma, uveal melanoma and potentially other cancers and they hypothesized that when individuals with *BAP1* mutations are exposed to asbestos, mesothelioma

predominates. Alternatively, *BAP1* mutation alone may be sufficient to cause mesothelioma (Testa et al., 2012).

In addition, it is important to take a comprehensive occupational and environmental history to identify people at risk because of previous exposure. Factors to establish include the specific occupation, the duration of that occupation, and the intensity of exposure (e.g., was the dust visible or not). A significant exposure can be defined as at least several months' exposure to visible dust that began more than 10 years earlier. Although much attention is focused on industrial exposure, environmental sources also play a role. These include residence near asbestos and prolonged exposure in buildings with open sources of contamination. Few studies have examined the incidence of asbestos related-disease (ARDs) associated with distinct sources of asbestos exposure (occupational, familial, or environmental).

While the association between occupational exposure to asbestos and asbestos-related disease has been established, the other source of asbestos exposure is less studied. Despite the decrease in asbestos production and use, there is still the possibility for environmental exposure (EE), whose impact on MM occurrence is less know (Carbone et al., 2016; Lemen, 2016). Quantification of the ARDs burden associated with EE to asbestos is further limited by the lack of reliable assessment of type and amount of exposure, smaller sample numbers, and smaller effect sizes compared to those seen in occupational studies. (Lin et al., 2007; Mc Donald, 1985; Goldberg and Luce, 2009; Bourdes et al., 2000). In a recent review, *Liu et al.* (Liu et al., 2017) classified non-occupational exposure to asbestos as environmental exposure (EE).

This exposure was grouped into the following four categories:

1) “non-occupational asbestos exposure” (NOA) to in areas where geological structure has shown the presence of asbestos but asbestos-related industries are absent;

2) “neighbourhood exposure” based on residence near industrial/mining sources of asbestos;

3) household exposure for family members of occupationally exposed subjects, which includes what some authors call familial exposure (Mirabelli et al., 2010; Corfiati et al., 2015);

4) other non-occupational exposures, which includes home-related (Mensi C. et al., 2015) or domestic exposure (Girardi et al., 2014; Goldberg et al., 2010 Gogali et al., 2012). For example, exposure occurring during hobby/leisure activities (Gogali et al., 2016; Marinaccio et al., 2015) do-it-yourself (DIY) projects during home maintenance and renovations (Mensi et al., 20016; Olsen et al., 2011) or residence in urban or polluted areas (Olsen et al., 2011; Tarrés et al., 2013).

Other authors used the term “familial” to refer studies of genetic susceptibility to MM (Ascoli et al., 2014; de Klerk et al., 2013). For example, *Ascoli et al.* (Ascoli et al., 2014) used “familial MM” to define a proband or index case in which MM was diagnosed in at least one first-degree relative (parent or sibling), regardless of the type of exposure, in an attempt to disentangle the genetic component of MM from the familial component deriving from a shared environment. Different definitions of EE have been used. For example, some studies referred to any non-occupational exposure (Jung et al., 2012), whereas others referred to non-occupational exposure as any type of exposure other than those occurring in domestic, household, or neighbourhood situations (Mirabelli et al., 2010; Ascoli et al.,

2014; Corfiati et al., 2015; Mensi et al., 2015; Mensi et al., 2016). *Camiade et al*. (Camiade et al., 2013) classified EE as the exposure occurring while living in a town where asbestos-processing plants were located. *Bianchi and Bianchi* considered "domestic exposure" that occurring in women, who washed clothes of occupationally exposed family members. (Bianchi and Bianchi, 2009).

Another aspect to consider is that different exposure categories tend to overlap. For example, neighbourhood and household exposure often occur simultaneously, because family members of occupationally exposed workers are likely to live in close proximity to asbestos factories or mines. *Méndez-Vargas et al*. (Méndez -Vargas et al., 2010) used the term "para-occupational exposure" to combine subjects classified as neighbourhood exposure with workers who had intermittent occupational exposure. These authors also classified residents of large polluted cities as environmentally exposed. *Mensi et al.* (Mensi et al., 2016) divided non-occupational asbestos exposure into three groups: para-occupational (exposure through cohabitants), home related (e.g., exposure through ironing on asbestos-coated ironing boards, repair works, thermal insulation, and use of asbestos-containing products), and environmental.

Other authors grouped EE into three categories: those with domestic and para-occupational exposure to asbestos-containing material (for the most part, these were people living with asbestos workers or near asbestos mines and manufacturing plants), those with EE from NOA, and those exposed to asbestos-containing material in buildings (Goldberg et al., 2009).

There are also non-identifiable sources of asbestos exposure. In the literature there is a considerable amount of cases which have a non-identifiable source of asbestos exposure

(non-IAE i.e. no known, an unknown, or an unspecified/unclassified source). It is possible that these are situations in which not all sources of EE can be properly captured by the traditional exposure questionnaires. Two studies in France attempted to quantify the contribution of different types of sources of asbestos exposure. In the first study, *Goldberg et al*. (Goldberg et al., 2010) reported that approximately 27% of MM cases diagnosed during the decade 1998-2008 were cases with no IAE; they had an M/F ratio of 0.9:1. Further examination of geographic patterns revealed that cases with no IAE were geographically correlated with cases with an identifiable occupational source of asbestos exposure (with the correlation coefficient b= 0.69 [95% CI: 0.14-0.84]). This correlation was likely driven by female cases, as the positive association was restricted to women (for women, b =0.59 [95% CI:-0.05, 0.84]; for men, b= 0.06 [95% CI: -0.40, 0.52]). The results suggest that the cases with no IAE were likely associated with neighbourhood and domestic EEs. In the second study by *Camiade et al.* (Camiade et al., 2013) the authors used a hybrid clustering analysis of exposure, demographics, and diagnostic variables in 318 French cases in which MM was diagnosed in female patients between 1998 and 2009. They found that more than half of the subjects (59.4%) were clustered together and most of the cases within these clusters had no known asbestos exposure (e.g., no occupational, para-occupational, environmental, or non-occupational exposures to asbestos). These results suggest that there is a large proportion of cases, mostly involving women, for which a clear exposure to asbestos cannot be identified with the commonly used epidemiologic instruments, such as questionnaires. Higher proportions of females among those with no IAE were also reported in other studies. In studying the lifetime exposure of 622 British patients with MM and 1,420 population controls, *Rake et al.* (Rake et al., 2009) observed

that the disease in 14% of male and 62% of female patients could not be attributed to occupational or domestic asbestos exposure.

2.1.1. Assessment of different exposure routes

Most studies reported the overall incidence of ARDs with both occupational and environmental exposure combined, with only a few studies reporting the incidence of these diseases specifically associated with EE. Individuals often had multiple exposure circumstances (occupational and not occupational-related) and the exposure assessment took into account their whole exposure history and computed a single exposure index, reflected the contribution of all sources. As already mentioned, non-occupational exposure included living in proximity to industrial or natural sources of air-bone asbestos (environmental exposures), sharing home with individuals occupationally exposed to asbestos (familial exposure), having asbestos-containing materials installed at home or handling such materials during home repairs or leisure-time activities (domestic exposure). In a study, *Ferrante et al*. (Ferrante et al., 2016) have defined the asbestos exposure routes (occupational, environmental and domestic) using estimated quantitative exposure and not on a qualitative basis. They observed that a cumulative exposure trend in the risk of asbestos-related diseases (particularly MM) increased with non-occupational exposure as well as with occupational exposure and there is an increased risk of MM for domestic exposure from asbestos exposed family members. The authors have highlighted as any exposure circumstance may entail multiple exposure patterns, which have been separately assessed.

Occupational asbestos exposure was primarily identified through questionnaires collecting exposure history, completed by either participants or their proxies. The most appropriated reference value for fibre concentration in each exposure pattern was chosen from collections of fibre measurements organized by job, industry and calendar period available from the literature and the web (Anonimus 2007 and 2015).

The exposure was assessed along the four axes of probability, frequency, intensity and duration. For occupational exposure, the probability was classified as definite, probable, possible and unlikely. The authors used the "definitive probability" when people report that they had work that involves the use of asbestos or materials containing asbestos. Instead, the "probable exposure" refers people who have worked in a firm where asbestos was certainly used, but whose exposure cannot be documented because exposure neither reported nor denied at interview but his prevalence in the specific job, industry and period was estimated to be high. The "possible exposure" refers to people whose have worked in firms in an economic sector where asbestos has been used but the prevalence of exposure was estimated to be low. It was classified as "unlikely" when it had not been reported at interview and the rather had no knowledge of its occurrence under the specific circumstance being evaluated.

The other parameter that the authors used to define the extent of occupational exposure to asbestos is the frequency. Frequency was assessed as the time spent under the exposure pattern under evaluation relative to the duration of a standard 8h work-shift and it is obtained with question on the amount of time that people spent carrying out the different tasks entailed by each job.

Then the parameter "intensity" was defined according to an ordinal scale arranged in eight increasing step. The lowest level correspond to fibre concentrations in areas without man-made or natural sources (< 0.3 fibres/L (f/L)) and the highest ones to those typical of unprotected tasks, in presence of very powerful sources of air-bone fibres (30-300 fibres/mL (f/mL)) and eventually, under the influence of other critical factors (≥ 300 f/mL). Finally, the "duration" of exposure was assessed as the difference between the year of start and the year of end of people's job reported at interview.

For every occupational exposure pattern, the exposure index was computed by multiplying frequency, intensity and duration. The resulting exposure index had the dimension of fibre per millilitre years (f/mL-years) and intensity correspond to a lifetime cumulative exposure of 0.03 f/ mL-years.

The authors applied the same procedure to define non-occupational exposure circumstances. Assessment of "environmental exposures" was based on the residential distance from identified neighbouring source(s) and from the source characteristics that may determine its emissions. For non-industrial source, such as spaces paved with finely broken AC tailing, assessment was based only on the distance from home and this surface; instead, for industrial sources the exposure assessment was based on areas that involving direct use of asbestos or of asbestos-containing materials and level of production, asbestos consumption. These evidence contributed to define two values: a near field exposure level (30-300 f/L) or a far field (3-30 f/L); instead for the diffuse presence of individually unidentifiable sources the authors used a value of 0.3-3 f /L.

For familial/domestic exposures, the intensity was assessed according to: characteristics of the asbestos-containing material reported at interview to be present at home (asbestos type

and content, friability, surface damage, enclosure), type of contact (active, passive) and potential for mechanical damage associated with interventions.

3.1. MicroRNA

It is well established that occupational/environmental carcinogens induce epigenetic alterations (Chappell et al., 2016), thus affecting gene expression, and alteration of microRNAs (miRNAs) expression. Accordingly, altered miRNAs levels can be proposed as biomarkers for early biological effects. MiRNAs are small noncoding RNA, single strand gene products of about 22-24 nt processed from precursors with a characteristic secondary structure (Ambros et al., 2003). They can play important regulatory roles by targeting mRNAs for cleavage or translational repression (Bartel, 2004). Actually, they are the most studied classes of molecules for their involvement in numerous processes, such as growth, differentiation, cell proliferation, maintenance of homeostasis, establishment of disease, regulation of apoptotic mechanisms (Staton and Giraldez, 2008; Kloosterman and Plasterk, 2006).

MiRNAs act as modulators of gene expression programs in different diseases, particularly in cancer, where they act through the repression of genes, which are critical for carcinogenesis. Also these small RNAs have proved to be more efficient in distinguish between tumour histology, classifying undifferentiated tumours and predicting patient outcome compared with traditional gene expression profiling of mRNAs (Wang Q et al., 2009). MiRNAs are fundamental for the life: an organism devoid of any mature form of miRNA cannot neither survive nor reproduce (Barnstein et al., 2003).

3.1.1. Discovery and features

The miRNAs discovery is recent and dates only to 1993 when Lee R.C. and her group, investigating the role of LIN-14 protein in *Caenorhabditis elegans*, found two transcripts of 61 and 22 nt able to regulate LIN-14 translation after interaction with the corresponding mRNA. The 61 nt transcript is the precursor of the mature lin-4 microRNA of 22 nt and the regulation of LIN-14 expression takes place through an antisense RNA-RNA interaction. Lin-4 has a small sequence complementary to a repeated sequence element in the 3' UTR of lin-14 mRNA and, increasing the lin-4 expression, lin-14 mRNA and protein levels decrease (Lee et al., 1993). For years the discovery made by Lee et al. was ignored, until the work published by Reinhart B.J. et al. They discovered the let-7 microRNA, an ssRNA of 21 nt with sequences complementary to the 3'UTR region of lin-14, lin-28, lin-41, lin-42 and daf-12 mRNAs and involved in the temporal regulation of C. elegans differentiation (Reinhart et al., 2000). Since then, many groups of scientists have discovered thousands of genes coding for microRNAs in eukaryotes such as human, animals, plants, yeasts (Lagos-Quintana et al., 2001; Vaucheret, 2006), and even viruses (Pfeffer et al., 2004; Cullen, 2006). Identified miRNAs may be species-specific or evolutionarily conserved across species: the mechanism depends on the level of conservation of the microRNA gene, on the miRNA expression that varies spatially and temporally among species, and on the conservation of the mRNA target region (Mor and Shomron, 2013). To date, many microRNA families phylogenetically conserved have been identified from nematodes to humans (Altuvia et al., 2005; Arteaga-Vasquez et al., 2006; Lee et al., 2007) and some researchers have proposed their use in phylogenetic studies as an additional line of evidence (Tarver et al., 2013).

MiRNAs are also tissue-specific and time-specific: different tissues of the same organism have their own miRNA expression profile (Basso et al., 2009; Lagos-Quintana et al., 2002). It changes in relation to the development phase (Lee et al., 1993; Reinhart et al., 2000) and to the surrounding environment (Kalman et al., 2014; Poy et al., 2004).

3.1.2. Biogenesis

Most of the genes coding for miRNAs are located within intronic regions of known genes (about 80%), suggesting the presence of a convenient mechanism for the coordinated expression of the miRNA and the target protein; the remaining part is positioned in exon or intergenic regions of about 1kb (Rodriguez et al., 2004). Moreover, approximately 50% of the known miRNAs is located in close proximity to other miRNAs: generally, they form clusters of 2-7 genes and present a high similar expression profile (Lagos-Quintana et al., 2001). These groups of miRNAs can be individually transcribed through its own specific promoter and regulated by the same enhancer, or transcribed in the form of a single polycistronic units drive by a single promoter and divided during the maturation process (Lee et al., 2002). The clusters of miRNA genes are the result of segmental duplication events, forming the repeated-derived miRNA families (RdmiRs) (Yuan et al., 2011).

Over 500 miRNA genes have been experimentally validated and over 1000 miRNA genes predicted through computer programs in human, representing about the 2% of the whole genome (Li and Zhang, 2015). Recently, scientists have isolated few microRNA genes transcribed by RNA polymerase type III (Borchert et al., 2006) but in the majority of the cases, the RNA polymerase type II transcribes genes coding for miRNAs, as demonstrated by the work of *Lee and his group*. The primary transcripts, known as “pri-miRNA”, can

reach sizes > 1kb: they present the poly-A tail at the 3'-end and the cap of 7-methyl guanosine at the 5'-end, in addition to a complex tertiary structure with different hairpins and loops (Lee et al., 2004) (**Fig. 7**).

Pri-miRNA undergo to a complex process of maturation in two steps, highly compartmentalized. The process begins in the nucleus through a Microprocessor complex, a protein multi-enzymatic complex of about 650 kDa (Gregory et al., 2004). The complex presents different copies of a protein that binds dsRNA in proximity of the loops: this protein is known as DGCR8 in mammalian and Pasha in *Drosophila* and *C. elegans* (Han et al., 2004). Once bound, the pri-miRNA is processed by Drosha, a Microprocessor complex protein characterized by two Rnasi III domains. Drosha cleaves the transcript 11 bp distance from the stem-ssRNA junction, generating a 2 nt overhang on the 3' end, which is recognized by the successive protein of the maturation process (Han et al., 2006). The product of the cleavage is a hairpin-shaped precursor of dsRNA of about 70 nt, known as "pre-miRNA" (Lee et al., 2003) (**Fig. 7**).The second step of the maturation process takes place in the cytoplasm; therefore, the pre-miRNA must be transferred from the nucleus to the cytoplasm. The enzyme complex Exportin-5, located in the inner membrane of the nuclear pore, mediates the passage. The protein belongs to a family of receptors able to bind cooperatively the molecule to be exported and a cofactor Ran linked to a molecule of GTP from which derive the energy required for the cytoplasm transfer (Bohnsack et al., 2004). Exportin-5 recognizes the pre-miRNA by the presence of the stem-loop and the overhang of two nucleotides at the 3' (Lund et al., 2004).

Once in the cytoplasm, Dicer processes the pre-miRNA, an RNase type III very similar to Drosha (Knight et al., 2001). Through PAZ domain, Dicer recognizes the 2 nt overhang at

the 3’ end and cleaves the pre-miRNA 22 nt distance from the protrusion. The product is miRNA duplex of about 22 nt without any stem loop (Ma et al., 2004) (**Fig. 7**). The miRNA duplex immediately becomes part of the ribonucleoprotein complex miRISC (miRNA-containing RNA-induced silencing complex) that contributes to complete the maturation process and supports the miRNA regulation of gene expression.

The pre-RISC complex is composed by Dicer, the core of the complex, TRBP and PACT, two dsRNA binding proteins, Argonaute proteins (Ago 1-4 in mammalian cells) and many other proteins (Redfern et al., 2013).

TRBP and PACT bind the miRNA duplex, recall in site Ago 2 and the other Argonaute proteins and participate to the silence of the gene expression (Thimmaiah et al., 2005; Lee et al., 2006). In particular, Ago1 is responsible for the last step of the miRNA maturation process: it is a RNA endonuclease, which cleaves the passenger strand of the duplex (indicate as miRNA*) (Baumberger et al., 2005), while the helicase Gemin3 separated the two miRNA strands (Mourelatos et al., 2002).

The presence of mismatches, internal loops or G:U pairs could influence the choice of the miRNA guide strand: in most cases, the guide strand is the one whose 5’ end is less tightly paired to its complement. The passenger strand miRNA* can be removed by the helicase or degraded (Schwarz et al., 2003). The guide ssmiRNA of about 22 nt becomes part of the definitive miRISC complex and represents the mature form of the miRNA able to silence the gene expression (**Fig. 7**).

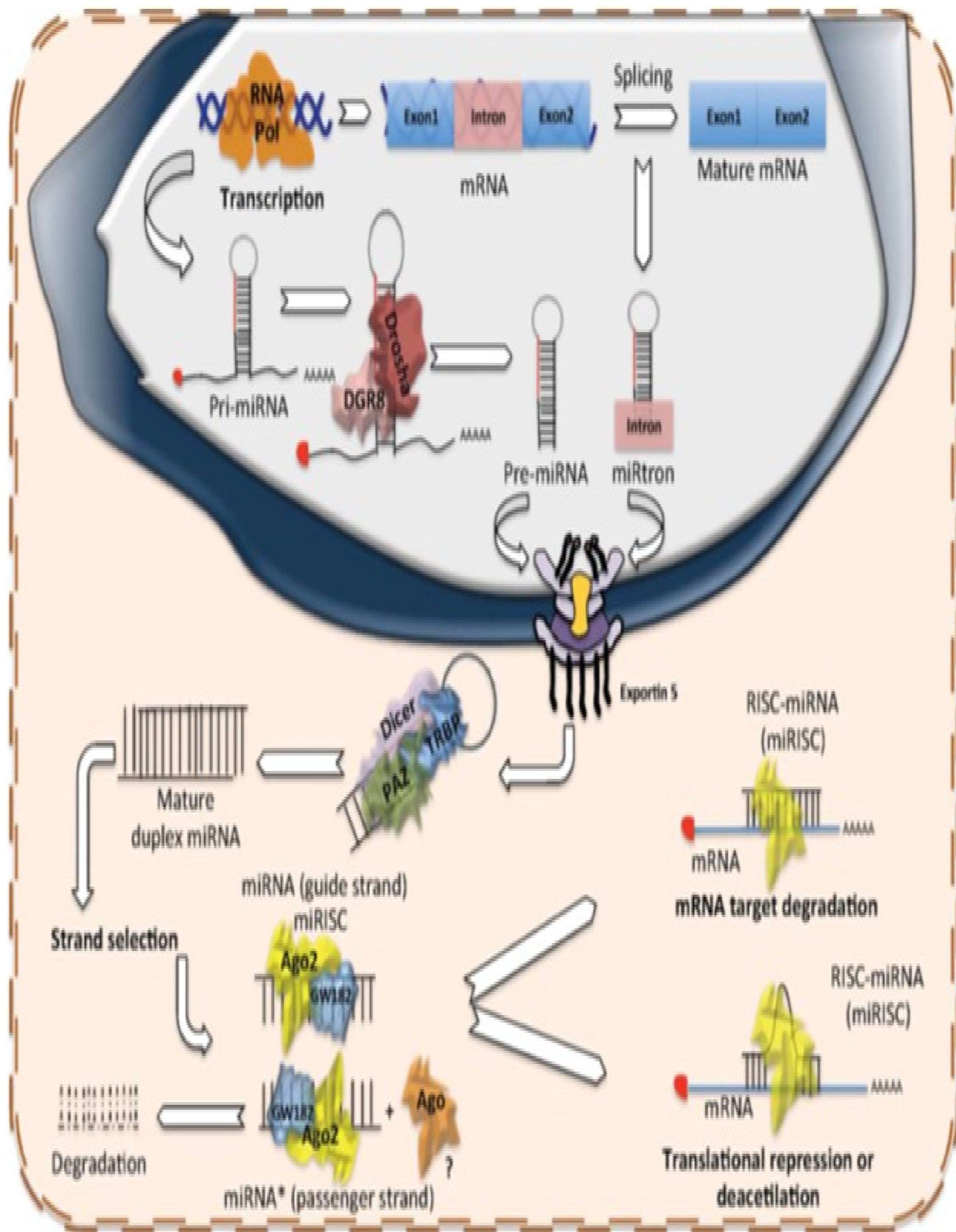


Figure 7: **Biogenesis of miRNAs**.Production of miRNAs starts in the nucleus with the polymerization of the primary hairpin miRNA transcript (pri-miRNA) by RNA polymerase II or III, followed by the cleavage and digestion of the pri-miRNA by the microprocessor complex (Drosha–DGCR8). The resulting transcript is the pre-miRNA, which is exported to the cytoplasm by Exportin-5–Ran-GTP. Once in the cytoplasm, Dicer, TRBP and Paz proteins cleave the pre-miRNA hairpin and digest it to produce a mature duplex miRNA. Then, one of the strands is loaded onto the RISC complex and finally this guides the miRNA to its mRNA target to silence it by direct degradation or by translational repression.

3.1.3. MiRNA structure

The regulation of gene expression by miRNA is carried out by its pairing with the target mRNA. Experimentally scientists have observed that the miRNA structure regulates the pairing mode (**Fig. 8**):

- at the 5’ end, miRNAs present the "seed" region, corresponding to the first 2-8 nt of the molecule, which are characterized to be perfectly complementary to the region 3' UTR of the target mRNA, coating a fundamental role in silencing (Lewis BP et al, 2003),

- the downstream region contains "bulges" and "loops" derived by the formation of structural mismatch in the miRNA-mRNA pairing (Lewis et al., 2003),

- the miRNA 3’ end shows a poor complementarity to mRNA but it can provide compensating sites for the binding of the target molecule, including thanks to the formation of G:U pairs (Brennecke et al., 2005).

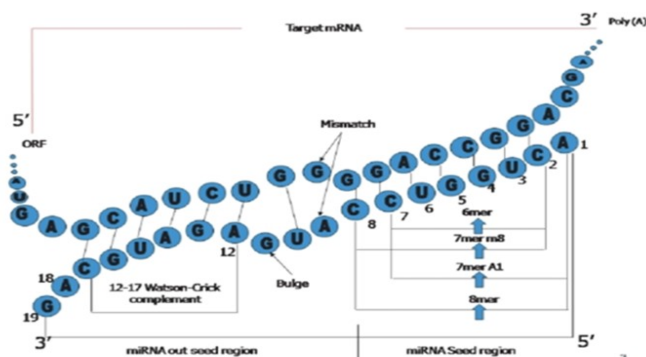


Figure 8: Structure of microRNAs and pairing with the target m RNA.

These characteristics have been used for the construction of the predictive computer programs of microRNA target, such as miRANDA, Targetscan and Targetminer: these

programs have shown that about 30% of human genes is a possible target of the silencing action of microRNAs (Lewis et al., 2005).

3.1.4. MiRNAs are gene expression mediators

MiRNAs are involved in regulating gene expression of the cells via gene silencing of target mRNAs. Generally, miRNAs act at the post-transcriptional level, pairing to specific sequences present on the target mRNA called MRE (miRNA Recognition Element), place most frequently in the 3'UTR region (Lytle et al., 2007). The regulatory mechanism essentially depends on the degree of complementarity between miRNA and mRNA (Hutvagner and Zamore, 2002).
When the complementarity between the two molecules is imperfect and rather unstable, miRNA carries out a translational repression mechanism. The process can take place in different ways and proteins of the Argonaute family of the miRISC complex, Ago1 and Ago2, coat a key role:

- the silencing can be implemented in the initial phase of translation after the bond between Ago2 and eIF4E, protein involved in the recognition of the 7-methyl-guanosine cap at the 5' end of the mRNA, a necessary step for the recruitment of messenger in the ribosome, or, following the ATP-dependent deadenilation. Ago1 can recognize the cap through the support of the GW182 protein, going to prevent the assembling of small and large subunits of the ribosome (Iwasaki and Tomari, 2009) (**Fig. 9A-B**);

- the repression can also be performed during the elongation of the polypeptide chain by inducing a premature termination signal or promoting the degradation of nascent polypeptides (Petersen et al., 2006) (**Fig. 9C**).

The translationally repressed mRNAs are sequestered in P bodies or stress granules in association with several proteins and can be translated later (Liu et al., 2005).

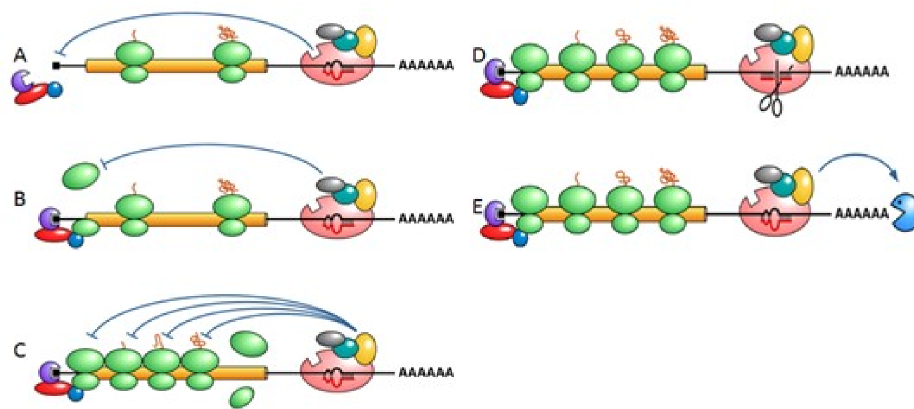


Figure 9. Gene expression silencing by miRNAs associated with miRISC via two different mechanisms: (**A**) translational repression in the early stage of the translation with the binding of eIF4E protein. (**B**) The repression through the association impediment of ribosomal subunits (**C**) or in the phase of elongation of the polypeptide; the degradation of the messenger thanks to an endonucleotidic cut of the mRNA (**D**) or following the deadenilation (**E**).

On the contrary, when the complementarity between miRNA and mRNA is sufficiently high thus leading to the formation of a stable interaction, the mRNA is degraded. The process could happen in three main ways:

- miRNA can promote the mRNA cleavage by the miRISC protein complex: the cut, generally made by Ago 2, takes place between the 10th and the 11th residue and generates two RNA fragments with unprotected ends, easily attacked by exonucleases (Zamore et al., 2000) (**Fig. 9D**);

- alternatively, the mRNA degradation may be promoted by the poly-A tail removal at the 3’ end mediated by the CAF1:CCR4:NOT complex: the deadenilation involves the loss of the protective binding proteins and also triggers the removal of the cap, exposing the filament and the information it contains to the fatal action of 5’-exonuclease (Eulalio et al., 2009) (**Fig. 9E**);
- miRNA can recruit the decapping DCP1: DCP2 complex: after removal of the cap at the 5’ end, mRNA is degraded by the exonuclease XRN1 (Rehwinckel et al., 2005).

The degradation leads to a reduction in the concentration of transcript and its protein counterpart, and represents an irreversible mechanism of gene expression inhibitory regulation. In addition to the role of miRNAs in gene silencing, several groups have discovered some miRNAs able to promote the translation of the messenger, increasing the mRNA stability (Vasudevan et al., 2007), and miRNAs that regulate gene transcription rather than perform post-transcriptional repression. An mRNA can contain more MRE sequences recognized by the same miRNA, strengthening the regulatory effect. The same mRNA may have MRE recognized by different miRNAs undergoing to a fine regulation of gene expression. A miRNA can have multiple target mRNAs; these are mainly genes involved in the same metabolic pathway or the same process.

MicroRNAs can perform their silencing action in the cell from which are produced (autocrine), in the neighbouring cells (paracrine) or systemically (endocrine). In the paracrine mechanism, the miRNAs especially exploit the gap junctions shuttling, a high specific transfer attested in stem cells, cardiac cells, macrophages and many other types

(Lemcke et al., 2015). It is well known that miRNAs may be found in plasma, serum (Chen et al., 2008) and in many other biological fluids (Park et al., 2009; Hanke et al., 2010). The main transport system of miRNAs discovered is represented by exosomes, small vesicles containing proteins, nucleic acids and other molecules involved into cell communication (Valadi et al., 2007). A few years later, researchers reported that about 90% of miRNAs could be found free in the fluids. The protection from nucleases is ensured by the association with proteins, in particular those of the family of Argonaute, as Ago2 (Arroyo et al., 2011). Recently, microRNAs associated with apoptotic bodies or HDL have been also found (Chen et al., 2012; Wagner et al., 2013). The miRNA-transport systems are of interest to evaluate suitable methods of miRNA administration in cancer therapy.

3.1.5.MiRNAs in tumour development and progression

Proliferation and apoptosis are cellular processes under strict control to maintain tissue homeostasis and their impairment may result in the development of cancer, because of the uncontrolled proliferation and the survival of damaged cells. Many genes switched on/off to directly control cell proliferation and differentiation. These genes, known as tumour suppressor genes and oncogenes, play a critical role in the development of cancer. Recent evidence, reported that about 50% of the miRNA-coding genes are located in areas of the genome known as "fragile site" and associated with cancer (Calin et al., 2004), and their mutations or changes in their expression are correlated with different cancer types. MiRNAs associated with cancer are clustered in the so-called "oncomirs" group (Esquela-Kerscher and Slack, 2006).

MiRNAs encoded by the let-7 family were the first group of "oncomirs" that has been shown to regulate the expression of oncogenes; in particular, it negatively regulates the RAS genes. The RAS proteins are small GTPases, which are signalling proteins associated with the membrane and involved in the regulation of cell growth and differentiation; an increase in their expression induce cell transformation (Johnson et al., 2005). The let-7 family, referred to as tumour suppressors, is mapped in fragile sites, and is under-expressed in lung, breast, urothelial and cervix cancer (Calin et al., 2004).

MiRNAs can act also as oncogenes. The 13q31 locus is amplified in many cancers, particularly lymphomas, and its transcripts include C13orf25 and miR-17-92, from which origin several miRNAs such as miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1 (Ota et al., 2004). These miRNAs have been shown able to regulate MYC, a transcription factor involved in the regulation of cell growth, capable of inducing proliferation and apoptosis. By grafting hematopoietic stem cells with MYC and miR-17-92 in wild-type mice depleted of bone marrow and subjected to radiation, the development of malignant lymphomas was faster compared to mice that received only stem cells with MYC.

MiRNAs work cooperatively as oncogenes by targeting MYC-induced apoptotic factors, thus inducing uncontrolled cell proliferation with the development of cancer (He et al., 2005). So far, overexpression of miRNA oncogenes has been correlated with the development and progression of tumour.

New miRNAs involved in the establishment and regulation of cancer are continually discovered. Some miRNAs are involved in metabolic reprogramming of the transformed

cells. They contribute to the metabolic switch from mitochondrial respiration to glycolysis (Jiang et al., 2012, Tomasetti et al., 2014), de novo synthesis of fatty acids (Davalos et al., 2011), as well the regulation of tricarboxylic acid (TCA) cycle (Rathore et al., 2012), interacting with the other pathway activated by the tumour transformation. Other miRNAs regulate the communication between the cancer cells and the surrounding microenvironment represented by the stroma composed of Cancer Associated Fibroblasts (CAFs), macrophages, T and B cells, endothelial cells (ECs), and pericytes (Zhang et al., 2015; Liu et al., 2013; Ghosh et al., 2009; Zhou et al., 2014). Through miRNAs, tumour can modulate important process involved in cancer, including inflammatory response, angiogenesis, proliferation and invasion of other tissues to form metastases (Fabbri et al., 2012; Liu et al., 2011; Chen et al., 2016; Cai et al., 2016).

3.1.6. Epigenetic alterations at miRNA loci

Epigenetic mechanisms are also important for miRNA transcriptional regulation. Different approaches have shown that DNA methylation and histone deacetylase inhibitors can modify the expression of several miRNAs (Lujambio et al., 2008; Lujambio et al., 2007). The characterization of CpG island content in genomic regions harbouring miRNAs reveals that such regions share a similar DNA and chromatin context, for example, the promotion of a closed chromatin configuration defined by CpG island hypermethylation and covalent histone modifications (Lujambio et al., 2010; Urdinguio et al., 2010).

The identification of miRNAs undergoing DNA methylation in a broad set of tumours pointed out the importance of this process in miRNA down-regulation and in the

establishment of cancer programs. miR-124 and miR-34, well defined tumour suppressors, are subject to epigenetic silencing by aberrant DNA hypermethylation affecting cell cycle pathways in tumours (Lujambio et al., 2010; Silber et al., 2008; Agirre et al., 2009); while down-regulation of miR-34 affects the Notch pathway involved in cell invasion and apoptosis (Pang et al., 2010). Furthermore, DNA methylation profiles in miRNA promoter regions can be useful as a diagnostic and prognostic marker. For example, miR-23b, a miRNA with tumour suppressor activity in prostate cancer, is down-regulated through DNA hypermethylation of its promoter region and its expression level is correlated with overall survival and recurrence-free survival (Majid et al., 2012). Deregulated expression of miRNAs in cancer is also a consequence of alteration in histone marks, which occur primarily due to the aberrant action of histone deacetylases and the Polycomb Repressor Complex 2 (PRC2). For example, over expression of PRC2 in prostate cancer contributes to the repression of miR- 101 and miR-205 by increasing the levels of H3K27me3 at their promoters. These alterations result in an increased rate of cell proliferation. In colorectal cancer, chromatin at promoter regions of tumour-suppressor miRNAs shows a closed configuration, producing a repressed transcriptional state (Goel and Boland, 2012).

Moreover, BRCA1, a well-known tumour suppressor, in addition to its canonical function, can also epigenetically repress the oncomiR miR-155 via its association with HDAC2, which deacetylates histones H2A and H3 on the miR-155 promoter (Chang et al., 2011). CTCF, another epigenetic factor, acts as a border that delimits the propagation of DNA methylation and histone repressive marks over different regulatory regions controlling gene expression. In different cancers, CTCF is lost, promoting repressive epigenetic mechanisms (Soto-Reyes et al., 2012). Recent studies have shown that CTCF regulates

miRNAs such as the tumour suppressor miR- 125b and the oncomiR miR-375 in breast cancer cells (Zhang et al., 2011).

3.1.7. MiRNAs in diagnosis and therapy

Given the large and growing number of evidences attesting the interactions between miRNAs and cancer, many studies have been finalized to evaluate miRNAs expression profiles to classify tumours and define useful biomarkers to diagnose the cancer and devise possible therapies. Research is focused in finding miRNAs that can distinguish malignant tumours at early stages (Pal et al., 2015), effectively differentiate tumours from benign or inflammatory conditions (Von Brandenstein et al., 2012), and define the cancer histological type (Calura et al., 2013). The miRNA profile can also be suggestive of tumour progression (Lu et al., 2005), useful as prognostic biomarkers to evaluate patient outcome (Li et al., 2015) and as predictive biomarkers to evaluate the efficacy of the treatment (Dreussi et al., 2016). Although the currently gold standard of cancer diagnosis is represented by tissues biopsy, it is spreading the possibility to use the “liquid biopsy” (Larrea et al., 2016). In this context, miRNAs can be detected in biological fluids, primarily blood (plasma or serum), but also in saliva, cerebrospinal fluid, urine, and milk (Weber et al., 2010; Mitchell et al., 2008). Through the liquid biopsy, circulating miRNAs delivered by exosomes can be detected (Wang et al., 2016; Thind and Wilson, 2016).

Increasing evidence supporting the essential role of miRNAs in cancer, points to the possibility of using miRNAs as treatment. Therefore, microRNA-based therapy is one of the challenges in the future. The therapy consists of the restoration of normal levels of miRNAs involved in carcinogenesis: the miRNA replacement can correspond to the

silencing of their oncogene targets (Melo and Kalluri, 2012; Yang and Yin, 2014). 'MiRNA mimics' are an effective alternative to restore the normal function of tumour suppressive miRNAs by replacing or substituting the lost miRNA using synthetic miRNA-like molecules. These are small, chemically modified (2′-O′methoxy) RNA duplexes that can be loaded into RISC and achieve the downstream inhibition of the target mRNAs (Barh et al., 2010; Liu et al., 2009). Numerous studies have validated the efficiency of miRNA replacement therapy in in *vitro* and in *vivo* models. For example, introduction of miRNA mimics for miR-15a in prostate cancer cell lines induced marked apoptosis and blocked proliferation (Bonci et al., 2008). Intranasal administration of let-7 in a K-ras mutant mouse effectively restrained the growth of the tumours by repression of proliferation and cell cycle pathways (Trang et al., 2010).

Oncogenic miRNAs are frequently overexpressed in human cancers, and they need to be silenced to restore the normal expression and function of their target tumour suppressive genes. MiRNA inhibitors are essentially complementary single stranded oligonucleotides that sequester the endogenous miRNA in an unrecognized conformation. The silencing of miRNA oncogenes can occur in two ways. One is the addition of antagonistic molecules, especially "antagomir", miRNAs complementary to the targeted miRNAs able to silence its activity (Krutzfeldt et al., 2005). When intravenously administered to mice, antagomir-122 induced a marked, specific, and persistent (up to 23 days) reduction of endogenous miR-122 gene expression in liver, lung, kidney, heart, intestine, fat, skin, bone marrow, muscle, ovaries and adrenals (Krutzfeldt et al., 2005). Another example of anti-miRNA oligonucleotides is the Locked Nucleic Acid (LNA) anti-miRNAs, in which an extra methylene bridge connecting the 2′-O atom and the 4′-C atom 'locks' the ribose ring in a

C3′-endo or C2′-endo conformation (Elmen et al., 2008). LNA-modified oligonucleotides exhibit higher thermal stability and high-affinity Watson-Crick hybridization with their RNA target molecules, with improved mismatch discrimination. Alternatively, ‘miRNA sponges’ or ‘miRNA decoys’ contain multiple artificial miRNA binding sites that compete with the endogenous miRNA targets for miRNA binding (Ebert et al., 2007). Inhibition of miR-9, which is upregulated in breast cancer cells and directly targets CDH1, using a ‘miRNA sponge’ inhibited metastasis formation (Ma et al., 2010; Tay et al., 2015). Small molecule inhibitors of miRNAs (SMIRs), which are small molecules that primarily function by inhibiting miRNA biogenesis or by actively impeding miRNA-target interaction can be used (Monroig et al., 2015).

The cell delivery of miRNA-mimicking or miRNA antagonist molecules can be carried out by viral or non-viral approaches. Viral methods are effective given the small size of the miRNAs, the ability to adjust the tropism of the virus to specific tissues, and the studies carried out with the latest generation of vectors showed no side effects (Geisler and Fechner, 2016). Non-viral methods encompass different strategies. One is represented using liposomes or nanotechnology-based systems. These technologies showed satisfied results in vitro but with immunogenic potential in vivo (Tyagi et al., 2016). In recent years, it is getting off the possibility of using exosomes: they represent a natural transport system of microRNAs, are not immunogenic, and can be recognized specifically by the target cells (Ha et al., 2016).

3.1.8. MiRNA as molecular biomarkers

Biomarkers define disease states. In cancer, biomarkers can subtype tumours and contribute in monitoring of therapeutic interventions (Ratain and Glassman, 2007). Useful biomarkers can contribute in dismantling pathways of pathogenesis and are especially in need for development and optimization of new and improved personalized molecular therapies. Not yet clinically established, but recently discovered and investigated is the potential role of mRNA and miRNAs as biomarkers in oncology (Calin and Croce, 2006). As an attempt to establish whether miRNA could be used for tumour classification, diagnosis and prognosis, different platforms to assess the global expression of miRNA genes in normal and diseased tissue were developed (Calin and Croce, 2006). Genome-wide profiling showed that miRNA expression signatures allowed different types of cancer to be discriminated with high accuracy (Volinia et al., 2006) and the tissue of origin of poorly differentiated tumours to be identified. By contrast, miRNA profiles were highly inaccurate indicators of tissue or cancer type. Indeed, miRNA expression profiling has proven to be helpful in early disease detection, distinguishing different cancer types, such as sub-classification of breast cancer subtypes and prostate cancer subtypes, identifying the tissue of origin in cancer with unknown primary and contributing to the postoperative adjuvant situation. Predictive miRNA signatures have been established for a number of tumours, including lung, prostate, cervical and colon cancers (Calin and Croce, 2006). One study, for example, developed a classifier of 48 miRNAs from a sample of 336 primary and metastatic tumours, and could use this classifier to accurately predict the tissue origin in 86% of a blind test set, including 77% of the metastatic tumours (Rosenfeld et al., 2008). Given that cancers of undefined origin account for approximately 4% of all malignancies

and are associated with a poorer prognosis, the continued development of miRNA classifiers has foreseeable benefits for clinical management. miRNA profiles can distinguish not only between normal and cancerous tissue and identify tissue of origin, but can also discriminate different subtypes of a cancer, or even specific histopathological abnormalities. Gene expression profiling has already demonstrated its effectiveness at subtyping various cancers: miRNAs for example, are differently expressed between basal and luminal breast cancer subtypes (Sempere et al., 2007), and can specifically classify estrogenic receptor, progesterone receptor and HER2/neureceptor status (Mattie et al., 2006). Other examples are the differential expression of miRNAs according to specific histotypes of ovarian carcinoma (Iorio et al., 2007) and the ability of miR-205 expression to discriminate squamous from non-squamous non-small cell lung carcinoma (Lebanony et al., 2009). Although more validation that is comprehensive needs to be done to address inconsistencies between different studies, which might be based on such challenges, this is unlikely to represent an obstacle to the development of miRNA in diagnostics. Another major clinical issue is clearly represented by the need of biomarkers for an early diagnosis; extremely important considering that prognosis of patients is closely linked to the stage of the tumour at the time of detection. miRNAs have great potential as early diagnostic biomarkers in oncology: overexpression of miR-205 and miR-21 in ductal adenocarcinoma as an example are reported to precede morphologic changes of the ducts, thus suggesting the possibility for an early detection of this neoplasm (du Rieu et al., 2010). By analysing plasma samples of lung cancer patients collected 1-2 years before the onset of therapy, miRNA signatures were found with strong predictive diagnostic and prognostic potentials (Boeri et al., 2011). Although predicting survival might be important in a more general

sense, the prediction of response to a specific therapy is of far greater clinical value. It is very interesting to note that miRNAs have potential not only for longitudinal monitoring during treatment in general, but more specifically for contributing to rationally select patients for specific subtypes of targeted therapies (Calin and Croce, 2006). In addition, miRNAs have been correlated with a poor response to specific treatments. In various cancers, increased miR-21 expression is an indicator of poor outcome (Schetter et al., 2008) and is also sufficient to predict poor response to adjuvant chemotherapy in adenocarcinomas. High levels of miR-125b in breast cancer predict poor response to Taxol-based treatments in vitro (Zhou et al., 2010), and a similar finding has been reported for miR-21 in pancreatic cancer patients treated with gemcitabine (Giovannetti et al., 2010). In a clinical context, miRNAs can be extremely useful in disease diagnosis and prognosis and in prediction of therapeutic response. In 2004, *Takamizawa and co-workers* were the first to pinpoint the prognostic value of miRNAs by showing that let-7 expression was reduced in lung cancers and that lung cancer patients with low let-7 expression levels have a significantly shorter survival after potentially curative resection (Takamizawa et al., 2004). In 2005, *Calin et al.* (Calin et al., 2005) reported the first study showing the diagnostic/prognostic importance of miRNAs at the genome-wide level. These authors found that miRNA expression profiles could be used to distinguish normal B cells from malignant B cells in patients with CLL. In fact, a unique miRNA expression signature is associated with prognostic factors such as ZAP-70 expression (predictor of early disease progression) and mutational status of IgVh. In addition, these authors found nine miRNAs that were differently expressed between patients with a short interval from diagnosis to initial therapy and patients with a significantly longer interval. Furthermore, this study also

highlighted the fact that one mechanism of miRNA deregulation is mutation: a germline mutation in the precursor of miR-16-1- miR15a caused low levels of miRNA expression in both vitro and in vivo (Calin et al., 2005). Currently, the clinical utility of miRNAs as diagnostic/prognostic biomarkers has been demonstrated in several types of cancer by numerous studies using tumour samples removed during surgery or biopsies (Garzon and Calin, 2009).

3.1.8.1. MiRNAs as biomarkers in plasma or serum

Current techniques for cancer diagnosis commonly involve a biopsy of the cancer tissue. Because this technique is invasive and unpleasant for patients, some studies have been focused on the search for biomarkers in human fluids such as plasma/serum, urine, or saliva. Blood samples from patients are usually readily available and many biological molecules, as circulating nucleic acids, can be found in blood serum/plasma, including miRNAs (Wittmann and Jäck, 2010; Cortez and Calin, 2009). These small non-coding RNAs in the blood are incorporated into microparticles and exosomes (50- to 90-nm) membrane vesicles that prevent their degradation, conferring an advantage to the use of miRNAs as markers in serum (Wittmann and Jäck, 2010). In addition, detection of miRNAs in serum is easy owing to highly sensitive PCR detection methods, the lack of post-processing modifications of miRNAs, and simple methods of miRNAs extraction from serum (Wittmann and Jäck, 2010). *Chim et al*. published the first report addressing the utility of miRNAs as diagnostic tools in biological fluids in 2008. In the study the detected placental miRNAs in the maternal plasma (Chim et al., 2008). In the same year,

Lawrie et al. (Lawrie et al., 2008), by comparing serum from patients with diffuse large B-cell lymphoma and healthy controls; found that miR-155, miR-210, and miR-21 levels were significantly upregulated in patients. Interestingly, these miRNAs have been shown to be deregulated in tumours. Moreover, high expression of miR-21 in patient's serum was correlated with improved relapse-free survival times (Lawrie et al., 2008). To date, miRNAs deregulation in serum of cancer patients have been described for several cancers, including leukaemia, lymphoma, and gastric, colorectal, lung, oral and squamous cell, breast, ovarian, prostate, pancreatic, and hepatocellular cancers (Cortez and Calin, 2009).

3.1.9. MiRNA in asbestos-lung malignancies: state of art

Several studies, mostly based on initial screening by miRNA microarrays and validation by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), have explored the expression of miRNAs in MM (Guled et al., 2009; Busacca et al., 2010; Balatti et al., 2011; Benjamin et al.,2010; Gee et al., 2010; Andersen et al., 2012; Andersen et al., 2014). The first study demonstrating deregulated miRNA expression in MM was carried out by *Guled et al* (Guled et al., 2009) who screened the expression of 723 human miRNAs in MM samples both compared with normal tissue (pericardium from healthy subject) and among the different subtypes of MM. The MM tissue displayed overexpression of 12 miRNAs and underexpression of nine microRNAs. Further studies distinguished 7 MM-specific miRNAs including the members of the miR-200 (miR-200 a/b/c), miR-141, miR-429,miR-192,miR-193a-3 as useful tools for differential diagnosis from pulmonary adenocarcinoma (Gee et al., 2010). The combination of analysis of miRNA expression patterns and functional assay has highlighted that miR-1 is down regulated in MM

compared with normal mesothelium (Xu et al., 2013). In addition, miR-145 loss has been seen to distinguish MM from normal mesothelial tissue (Cioce et al., 2013). *Anderson et al*. in a study have identified 4 miRNAs (miR-126, miR-143, miR-145, and miR-652) capable of differentiating MM from non-cancer samples (Andersen et al., 2014). *Ramírez-Salazar et al*. have found that 19 miRNAs are differentially expressed in MM, chronic pleural inflammation and mesothelial hyperplasia compared with non-cancer/ non-inflammatory tissue (Ramírez-Salazar et al., 2014) and that the expression of 6 miRNAs enabled predicting survival in MM patients (Kirschner et al., 2015). *Ak G and colleagues* identified eleven significantly upregulated miRNAs in MM compared with benign asbestos-related pleural effusion (Ak et al., 2015). For lung cancer, asbestos-related *Nymark P et al*. identified thirteen novel asbestos-related miRNAs and inversely correlated target genes by an integrative analysis of miRNA, mRNAs and copy number alterations of chromosomal regions in tissue samples from lung cancer patients with high asbestos exposure and without exposure (Nymark et al., 2011). For avoid the problem of collecting suitable numbers of MM tissue the studies about miRNA profiling has been undertaken about MM cell lines. A microarray-based miRNA profiling in vitro by *Busacca et al.* displayed upregulation of 10 miRNAs and downregulation of 19 miRNAs in two commercially available MM cell lines as compared to immortalized human mesothelial cells (HMC) (Busacca et al., 2010). Ivanov and co-workers suggested that miR-31 could serve as a prognostic factor because its loss *in vitro* had a pro-tumorigenic effect on MM cell lines (Ivanov et al., 2010). The first studies that shifting the search of the deregulated miRNAs from tissues to the circulation were conducted by *Santarelli et al.* (Santarelli et al., 2011) and *Tomasetti et al.* (Tomasetti et al., 2012). These authors has highlighted the

clinical significance of miR-126 in sera from MM patients suggesting that circulating miR-126 is a sensitive disease marker that should however be used in combination with other biomarkers, such as mesothelin, to increase its specificity (Tomasetti et al., 2012). For assay of circulating miRNAs, a new approach, based on the evidence that tumours generate a characteristic miRNA fingerprint in the cellular fraction of peripheral blood (Häusler et al., 2010) has shown that miR-103 levels were able to discriminate MM patients from asbestos-exposed subjects and healthy controls (Weber et al., 2012). Combining miR-103a-3p with mesothelin has improved diagnostic performance (Weber et al., 2014). The first miRNA profiling study in plasma/ serum was reported by *Kirschner et al.*, who demonstrated that miR-625-3p levels showed high specificity, accuracy, and sensitivity in differentiating MM from asbestosis patients (Kirschner et al., 2012). Finally, the most recent study has identified two different serum miRNA signatures correlating respectively with MM histological subtype and clinical outcome (Lamberti et al., 2015).

3.1.10. Effect of environmental carcinogens on the microRNA machinery.

It is well established that the expression of miRNAs was dramatically altered in cancer cells. However, a body of evidence has accumulated concerning the early alteration of the miRNA machinery (before the onset of cancer) in healthy organisms exposed to environmental carcinogens. Accordingly, altered miRNA levels can be proposed as biomarkers of early biological effects. A recent study reported that the miRNA alterations induced by environmental carcinogens that occur in healthy organisms are predictive of the future appearance of cancer only when these miRNA alterations are irreversible (Izzotti et

al., 2011). Conversely, reversible miRNA alterations represent adaptive rather than pathogenic mechanisms. The irreversibility of a miRNA alteration is reflected in the inability of the cell to restore the physiological miRNA expression level despite the cessation of exposure to the environmental carcinogen. The change from reversibility to irreversibility of miRNA alteration mainly depends on the duration of the exposure. Indeed, only long-term exposures are able to induce an irreversible alteration of the miRNA machinery (Izzotti et al., 2011). These findings were obtained in a study of the lungs of mice that were exposed to mainstream cigarette smoke (CS), in which lung cancer was induced according to the perinatal carcinogenic model (Balanskyet et al., 2012). The exposure dose also influences miRNA alterations. Indeed, in a recent study, the expression of lung miRNA was not significantly altered after 1 month of exposure to low doses of CS, whereas it was dramatically altered by exposure to high doses (Izzottiet al., 2011). These findings indicate that early miRNA alterations reflect both the intensity and the duration of the exposure and that, the threshold mechanisms affect these biomarkers. The early sensitivity of miRNA to environmental carcinogens can be explained on a mechanistic base. It has been established that irreversible loss of miRNA function in cancer cells is a result of the homozygous deletion of miRNA genes (Calin et al., 2004). However, similar genetic damage does not occur in non-cancerous cells. Several hypotheses have been raised to explain the early sensitivity of the miRNA machinery to environmental carcinogens. These hypotheses mainly include the activation of miRNA gene expression because of DNA damage (Suzuki and Miyazono, 2010) and alterations in the miRNA-processing machinery (Ligorio et al., 2011). These studies provided evidence that early miRNA alterations can be interpreted as adaptive mechanisms that increase the expression

of the defensive genes involved in metabolic detoxification, DNA and protein repair, and apoptosis activation. Indeed, the early miRNA downregulation induced by CS is paralleled by increasing messenger RNA (mRNA) and protein expression (Izzotti et al., 2009a). Initially, experimental evidence indicated a strict inter-relationship between exposure to environmental carcinogens and early miRNA alterations in CS-exposed rodents and humans (Izzotti et al., 2009b; Schembri et al., 2009). Further studies demonstrated that this phenomenon occurs in cases of exposure to a wide variety of environmental carcinogens and mutagens. Thus, environmental carcinogens alter miRNA expression by downregulating onco-protective miRNA and upregulating oncomiRNA. For each carcinogen, there is a miRNA alteration signature and this can be used as early markers of the biological effects of exposure to environmental carcinogens in healthy organisms.

In a study *Izzotti* and *Pulliero* (Izzotti and Pulliero, 2014) proposed the mechanisms by which miRNA expression is altered by exposure to environmental carcinogens. In the first mechanism, (**Fig. 10A**) the authors underlined that the alteration of miRNA expression by genotoxic agents, such as ionizing radiation, has been ascribed to p53. It interacts with the Drosha/DGCR8 processing complex through an association with RNA helicase p68, which modulates the processing of pri-miRNAs to pre-miRNAs (Suzuki et al., 2009; Suzuki and Miyazono, 2010). Indeed, p53 enhances the post-transcriptional maturation of several miRNAs with a growth-suppressive function, including miR-16-1, miR-143, and miR-145, in response to DNA damage. So, miRNA expression can be modulated via a p53-dependent mechanism in which Dicer has a central role and modifies the expression of miRNA genes in the nucleus. In the second mechanism, electrophilic metabolites of environmental carcinogens bind to nucleophilic sites of miRNA precursors, thus forming

miRNA adducts and modify the structure of miRNAs. This binding blocks the access of miRNA to the catalytic pockets of Dicer and arresting miRNA maturation process. This evidence has been obtained evaluating the amount of adducts of the lungs of mice exposed to CS which was 5.67-fold higher in the miRNAs than in the nuclear DNA (**Fig. 10B**). In the third mechanism, metabolites of environmental carcinogens preferentially bind to Dicer and these mutagens affect miRNA maturation by competing with pre-miRNA for Dicer binding (**Fig. 10C**).

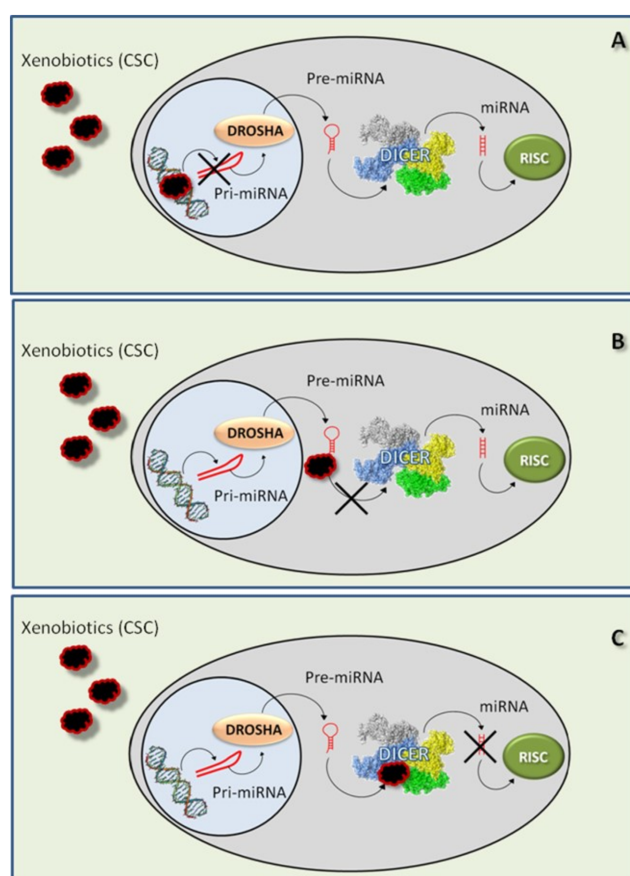


Figure 10. Mechanisms of miRNA-expression alteration as induced by environmental carcinogens. (**A**)In response to DNA damage, the p53/miRNA interconnection modifies the expression of miRNA genes in the nucleus; (**B**) Electrophilic metabolites of environmental carcinogens bind to nucleophilic sites of miRNA precursors thus forming miRNA adducts, which cannot access the catalytic pockets of DICER in cytoplasm; (**C**) Metabolites of environmental carcinogens bind to DICER in the proximity of miRNA catalytic sites thus blocking maturation of miRNA precursors.

Therefore, experimental evidence has demonstrated that exposure to chemical and physical environment carcinogens results in the early alteration of miRNA expression in the target organs, as triggered by genotoxic or epigenetic mechanisms. The genotoxic mechanisms include p53-network activation in response to DNA damage. The epigenetic mechanisms include the binding of carcinogenic metabolites to Dicer.

Another factor that affects the miRNA response to carcinogens is the duration of the exposure. Indeed, altered miRNA expression persists after the discontinuation of exposure only in the case of long-term exposure (Izzotti et al., 2011). Accordingly, the dose and duration of exposure are critical factors in the miRNA response to carcinogens.

Then, the miRNA response plays a critical role in defining the phenotypic consequences of exposure to environmental carcinogens. Indeed, the miRNA response is crucial for the following process:

1) activation the defensive machinery that triggers the adaptive response in case of short-term exposure (Izzotti et al., 2011);
2) commission the cells to lung carcinogenesis in the case of long-term exposure (Izzotti et al., 2011; Schembri et al., 2009);
3) induction the phenotypic alteration in embryos that are exposed to teratogenic agents (Gueta et al., 2010).

Due to the importance of these mechanisms, cancer cells undergo irreversible miRNA alterations through miRNA gene deletion, which typically occurs in human cancers (Calin et al., 2004). Definitely, the alteration of miRNA expression is an established mechanism by which chemical carcinogens induce alterations in target cells. These alterations occur

early during exposure, initially representing adaptive events that aim to defend the cells by activating detoxifying mechanisms. The protective mechanisms, at least in continuously dividing cells, include the p53-related activation of DNA-repair processes, cell-cycle arrest, and apoptosis that are induced by changes in members of the miR-34 family. When oncogene mutations occur due to early genotoxic events, their phenotypic consequences are blocked by the activation of onco-protective miRNAs, as observed for k-ras, the expression of which is hampered by the expression of let-7 family members. However, when exceedingly high exposure doses or long-term exposure overwhelms the miRNA-based adaptive response, the irreversible alterations that occur play a pathogenic role in cancer. The miRNAs have the potential to be used as biomarkers for identifying genotoxicity and carcinogenicity of chemicals and indicating exposure of carcinogens.

4. Purpose of the thesis

Exposure to asbestos results in serious risks of developing lung malignancies such as MM and LC. Approximately one-half of all deaths due to occupational cancer are estimated to be caused by asbestos. The most accurate data on the burden of asbestos-related diseases are the estimated cases of MM due to its highly specific association with previous exposure to asbestos. Lung cancer, for its high incidence and mortality, is the most cause of death for cancer in many developed countries. Although the major risk factor for LC is tobacco smoke, accumulated studies indicate that approximately 4 to 10% of LC in most developed countries is due to asbestos exposure. Lung cancer caused by asbestos is generally more frequent than MM, even if cases of LC are far more likely to be recognized as being caused by asbestos. The underestimation of lung cancer caused by asbestos is widely reported, thus contributing to a significant health and economic implications. As occupational diseases, the asbestos-related malignancies can be compensed by the National Institute Against Occupational Injury Insurrance (INAIL). Therefore, the reliable attribution of asbestos exposure is of great importance. Currently, the asbestos exposure is established according to defined statutory criteria (LGs 257/92, LGs 277/91 and LGs 81/08). Although well accepted, these criterias are insufficient to best define the exposure, and most of cases of LC are not recognized as asbestos-related disease. Therefore, the identification of asbestos-related molecular changes has long been of great interest. In recent years, microRNAs (miRNAs) have been widely studied for their ability to regulate numerous physiological processes through the regulation of gene expression. Changes in miRNA expression patterns are associated to disease states and cancer progression. A body

of evidence has accumulated concerning the early alteration of the miRNAs machinery in subjects exposed to environmental carcinogens. Accordingly, changes in miRNA levels can be proposed as biomarker for early effects, as well as biomarkers of cancer ethiology.

The purpose of my thesis is to identify a specific miRNA signature that characterizes the lung malignancies from asbestos exposure from other origins in order to identify asbestos-related lung diseases to detect their incidence and establish a unique criterion of etiologic attribution to asbestos. In addition, this miRNAs signature could be useful in discriminating the two main pathologies related to a previous exposure to asbestos (malignant mesothelioma and lung cancer) and to be extended for screening of currently or ex-exposed subjects who have benign asbestos-related pathologies.

5. Materials and Methods.

5.1. Study design

A multiphase and case-control study was designed to identify serum miRNAs as surrogate biomarkers in discriminating NSCLC (Non- Small Cell Lung Cancer; NSCLC) patients, asbestos-related NSCLC (NSCLC^{Asb}), and MM.

In the discovery phase, a retrospective study based on subjects financially compensated by occupational INAIL was carried out to identify patients with asbestos-related lung diseases. Formalin-fixed, paraffin-embedded (FFPE) tissue of the selected subjects affected by NSCLC (n=4), NSCLC^{Asb} (n=4) and MM (n=4) were collected from the Archive of the Pathological Anatomy Unit of the University Hospital of Ancona, Italy. FFPE samples were cut into 5 μm sections and stored at room temperature until analysis. The adjacent non-cancerous tissue was used as a control. These samples were respectively subjected to miRNA profiling and miRNAs with significant differences in expression levels among three groups were identified.

In the verification phase, candidate miRNAs were first tested by RT-qPCR in an independent cohort of serum samples from patients with NSCLC (n=16), NSCLC^{Asb} (n=11), MM (n=19), and controls (CTRL, n=14). This phase was used to verify whether the selected miRNAs in the FFPE samples can be detected in serum. Next, the detected serum miRNAs have been tested in an independent cohort (Training phase, consisting of patients affected by NSCLC (n=24), NSCLC^{Asb} (n=19), MM (n=22) and controls (n=21). A binary logistic regression on the miRNAs levels detected in the training phase was

performed to establish a miRNA classifier differentiating asbestos-related malignancies from that non-asbestos related.

The predictive “miRNAs panel” was, then, evaluated in a validation cohort.

In the validation phase, serum samples from another independent cohort of patients with NSCLC (n=20), $NSCLC^{Asb}$ (n=15), MM (n=33) and controls (n=36) were prospectively entered into the discriminatory model to validate the predictive accuracy of the selected “miRNAs panel”.

In this phase patients affected by sinonasal cancer (SNC, n=16), as malignancy associated to occupational exposure to wood dusts and leather, have been included to evaluate the predictive model specificity.

Finally, the miRNA-panel was tested in a population of asbestos-exposed subjects (testing phase), which, according to the period of exposure, were stratified as currently exposed, ex-exposed with and without benign asbestos related diseases (ARDs).

The phases of the study are summarized in **Fig.11**.

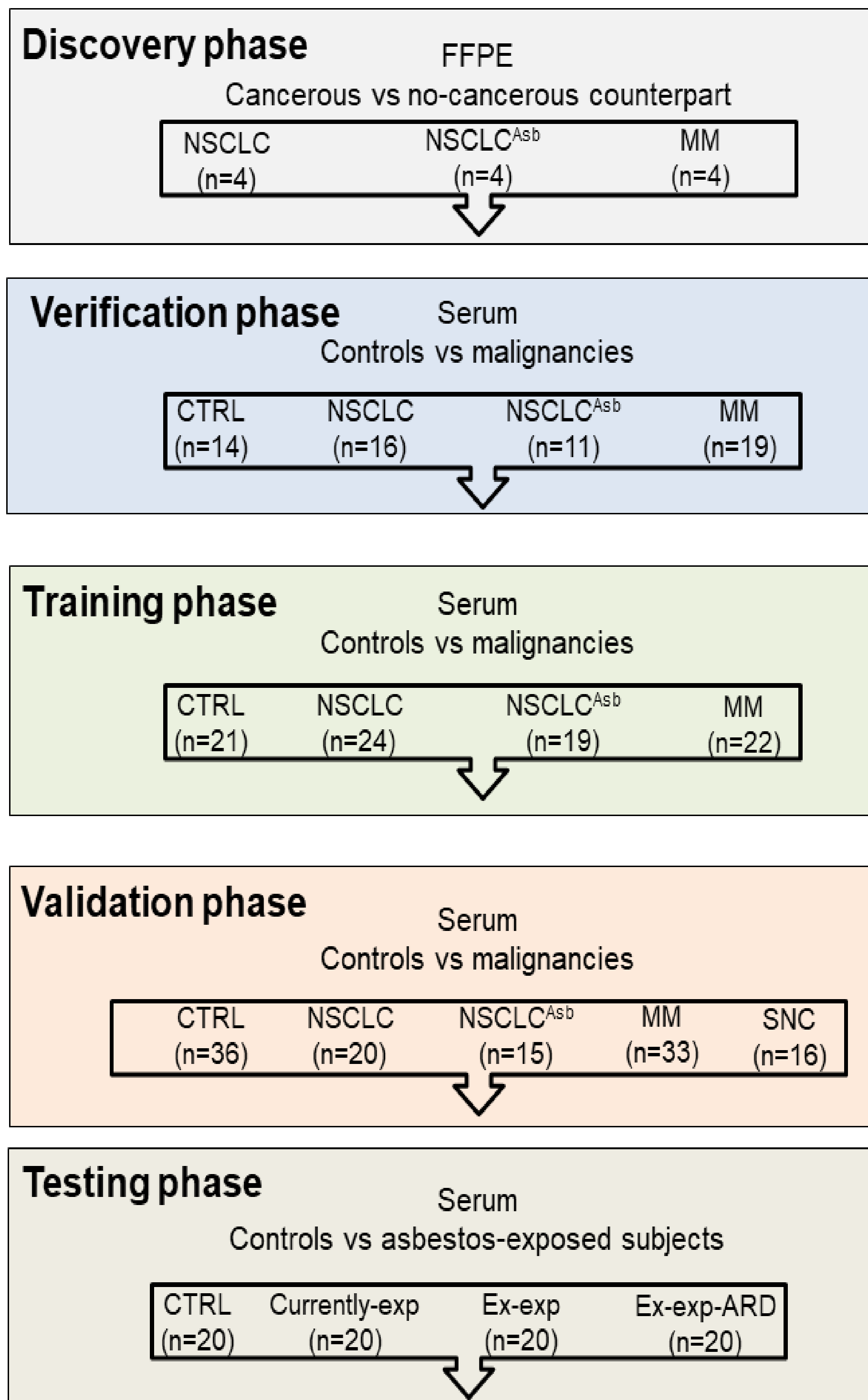


Figure 11. Schematic representation of the multipurpose study.

5.2. Study population

The lung cancer cohort included 60 patients affected by NSCLC (squamous, adenocarcinoma and large cell carcinoma) without any signs of previous exposure to asbestos. Patients were recruited from January 2011 to March 2017 at the Clinic of Pneumology and Thoracic Surgery of the University Hospital of Ancona, Italy. All patient diagnoses were confirmed histologically, and tumour stage was determined by biopsy and imaging technology. Tumour staging was performed according to the Sixth Edition of American Joint Commission on Cancer tumour-node-metastasis (TNM) staging system.

In the $NSCLC^{Asb}$ group were included 45 patients with lung cancer who had submitted a complaint of occupational disease to INAIL as exposed to asbestos. These subjects were selected based on the presence of evidences of occupational and non-occupational (environmental, familial, domestic) exposure to asbestos. In this group were included shipbuilders, machine operators, electricians, pipe fitters, and construction workers, all subjected to passive exposure at construction sites or their family members.

MM patients (n = 74) were recruited from November 2008 to January 2013 at the Clinics of Oncology, Pneumology and Thoracic Surgery of the University Hospital of Ancona, Italy. Pathological diagnosis was performed on pleural biopsies obtained by thoracoscopy or thoracotomy. Tumours were classified as epithelial in 54, sarcomatoid in 16, and mixed-type in 4 cases.

The patients affected by sinonasal cancer (SNC) were enrolled at the UOC-ORL Budrio-Metropolitan Hospital Bologna, Italy.

The participants were interviewed by trained personnel and answered a detailed questionnaire that included information on the gender, age, histology, neoadjuvant chemoradiation and therapy administration (before surgery), smoking status and the pathologic staging, as well the duration of asbestos exposure and occupational tasks. Each patient underwent lung function analysis, chest radiography and high-resolution computed tomography. Radiographic evidence of asbestosis and/or pleural plaques was found in 39% of asbestos-exposed patients. The selection of these patients was based on the following inclusion criteria: age 50-70 years; absence of other neoplastic, inflammatory, and metabolic pathologies such as diabetes. According to *Ferrante et al.* (Ferrante et al., 2016), a 'fiber-year' exposure metric was calculated for each asbestos-exposed subject, assigning to each person an arbitrary coefficient of 'inhaled fibers (ff)' indicating the occupational hazard. The 'cumulative fibers' (*Cf*) are interpreted as the cumulative dose of asbestos fibers in the workplace of (ff/L) ×yrs.

The asbestos-exposed cohorts consisting of 80 subjects (age 57.5±12.2) with a history of asbestos exposure (asbestos cement, rolling stock, shipbuilding) were enrolled at the Institute of Occupational Medicine, University of Trieste, Trieste, Italy. According to the period of asbestos exposure, the population was stratified as currently exposed (commercial asbestos occurs predominantly during maintenance operations and

remediation of older buildings containing asbestos) and ex-exposed with and without benign asbestos-related diseases.

The control group consisted of healthy subjects (n = 78) recruited from November 2008 to January 2017 at the Institute of Occupational Medicine, Polytechnic University of Marche, Ancona, Italy and at Occupational Medicine of University of Trento, Trento, Italy. The subjects were undergoing screening radiography at the Pneumology Clinic of the University Hospital of Ancona, Italy. None of them had ever been exposed to asbestos as documented by their occupational histories and they presented with normal chest radiographs. Controls were matched to MM, NSCLC, $NSCLC^{Asb}$ patients based on demographic characteristics, family history of MM, NSCLC, smoking status and occupational and non-occupational exposure.

The demographic and pathological characteristics of the subjects are summarized in **Tab.2.**

	FFPE			SERUM												
	Discovery			Verification				Training				Validation				
	NSCLC (n=4)	NSCLC^{Asb} (n=4)	MM (n=4)	CTRL (n=14)	NSCLC (n=16)	NSCLC^{Asb} (n=11)	MM (n=19)	CTRL (n=21)	NSCLC (n=24)	NSCLC^{Asb} (n=19)	MM (n=22)	CTRL (n=36)	NSCLC (n=20)	NSCLC^{Asb} (n=15)	MM (n=33)	SNC (n=16)
Age (yrs)	65±7	69±11	72±8	41±11	68±12*	70±7*	73±8*	56±17	71±10*	74±9*	71±7*	56±9	71±9	69±7	71±7	64±14
Gender (M/F %)	100/0	100/0	100/0	92/8	69/31	64/36	84/16	79/21	60/40	80/20	83/17	89/11	55/45*	80/20	82/18	69/31
Smoking (%)																
No	25	25	25	40	38	33	50	42	29	20	38	22	16	13	29	62
Yes	75	75	75	20	50	42	0	26	26	35	12	25	21	33	12	38
Former	0	0	0	40	12	25	50	32	45	45	50	53	63	53	59	0
Asb-exp (%)																
No	100	0	0	100	100	0	0	100	100	0	0	100	100	0	0	100
Occupational	0	100	100	0	0	100	100	0	0	45	59	0	0	67	74	0
Environmental	0	0	0	0	0	0	0	0	0	55	41	0	0	33	26	0
Cf (ff/L) x yrs										6.4±3.7	4.2±3.3	0	0	6.6±4.3	8.0±7.3	0
ARD (%)	0	100		0	0	40	83	0	0	55	81				74	
Histotypes (%)	0(SQ) 0(LC) 100(AD)	25(SQ) 0(LC) 75(AD)	90(EP) 0(BF) 20(BF)		43(SQ) 0(LC) 57(AD)	40(SQ) 20(LC) 40(AD)	90(EP) 0(BF) 20(BF)		35(SQ) 10(LC) 55(AD)	36(SQ) 18(LC) 46(AD)	75(EP) 0(BF) 25(SA)		13(SQ) 12(LC) 75(AD)	67(SQ) 0(LC) 33(AD)	59(EP) 11(BF) 30(SA)	

Table2.**Demographic characteristics and clinic parameters of study population. NSCLC-histotypes**: SQ, Squamous; LC, Large Cell; AD, Adenocarcinoma; **MM-histotypes**: EP, Epithelioid; BF, Biphasic; SA, Sarcomatoid *CTRL vs NSCLC, NSCLC^{Asb}, MM, SNC.

5.3. Asbestos exposure assessment

Information on lifetime asbestos exposure, in both occupational and environmental settings, has been collected through a standardized questionnaire administered to each subject by trained interviewers. Based on standardized criteria asbestos exposure is classified as occupational (definite, probable, possible), domestic, environmental, unlikely or unknown (Marinaccio et al., 2012). Thus, for each subject enrolled in the study has been attributed for occupational exposure a value of fibres concentrations between 30 fibres/mL (minimum dose) and 300 fibres/mL (maximum dose). By multiplying for the duration of work a minimum and maximum cumulative dose at the workplace have been obtained and expressed as ff/L x years. The non-occupational exposure (environmental, familial/domestic exposure) for each subject has been calculated by attributing three range of values:

1) 0.3-3.0 fibres/mL to indicate the diffuse presence of individually unidentifiable sources as in major urban areas or lastly, background exposure;
2) 3-30 fibres/mL to indicate a far field exposure level relative at a specific asbestos exposure source;
3) 30-300 fibres/mL to reference a near field exposure level at neighbouring source(s) and the source characteristics that may determine its emissions. These values were also multiplied by the duration of exposure expressed in years.

5.4. Ethics statement

All subjects filled a questionnaire including their informed written consent. The study was carried out according to the Helsinki Declaration and the samples were processed under

approval of the written consent statement by Ethical Committee of the University Hospital of Marche, N. 51/DG 05/02/2009, Italy.

5.5. Clinical specimen collection

Whole blood samples were collected in serum separator tubes (Becton Dickinson) and centrifuged at 3.500 rpm at room temperature for 10 min within 4 h after collection. Serum was aliquoted and stored at -80 °C until use. For all cancer patients, blood samples were collected at the time of diagnosis, before tumour resection or treatments.

5.6. MiRNA qPCR analysis profiling in FFPE tissue samples

Total RNA was extracted from formalin-fixed, paraffin-embedded (FFPE) sections (10μg) using the Ambion RecoverAll Total Nucleic Acid Isolation Kit (item no. AM1975; Life Technologies, Grand Island, NY), according to the manufacturer′s instructions. The concentration and integrity of RNA samples were determined measuring absorbance ratio at 230, 260 and 280 nm by Nanodrop 1000 UV-Vis spectrophotometer (item no. ND1000; Thermo Fisher Scientific Inc., Waltham, MA).

The RT-qPCR screening was performed using ″Human Cancer Pathway Finder 384HC miRNA PCR Array: MIHS-3102Z″, Quiagen (**Fig.12**).

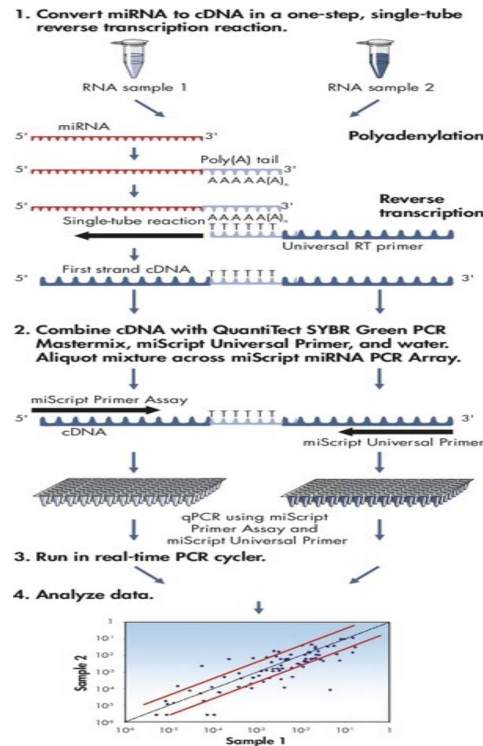


Figure 12. Schematic Representation of miScript miRNA PCR Arrays.

The Human Cancer PathwayFinder 384HC miScript miRNA PCR Array profiles the expression of 372 miRNAs differentially expressed in tumours versus normal tissue. This array provides a convenient way to quickly analyze the miRNAs most relevant to tumourigenesis. A set of controls (6 housekeeping genes, 3 control genes for quality of the retrotranscription and 3 control genes for qPCR) included on this array enables data analysis using the ΔΔCT method of relative quantification, assessment of reverse transcription performance, and assessment of PCR performance. Using SYBR Green real-time PCR, the expression of a focused panel of miRNAs related to cancer research can be easily and reliably analyzed with this miScript miRNA PCR Array.

The gene fold changes were investigated in the study groups (NSCLC, $NSCLC^{Asb}$, MM) by evaluating ΔΔCt between the cancerous tissue and non-cancerous counterpart according to method $2^{-\Delta\Delta Ct}$. Positive fold change values corresponded to over-expressed miRNA, while negative values corresponded to down-expression. The results were reported as

volcano plots to quickly visualizing the significance of the fold change values of single gene.

5.7. Circulating miRNA detection

Total RNA was isolated by adding 750 µL of Tri-Reagent BD (Sigma) plus 200 µL of chloroform; the phase lock gel (Eppendorf) was used to improve the phase separation. The miRNAs were further purified from total RNA using the miRNA isolation kit (SABiosciences). MiRNAs were eluted in the final volume of 40 µL RNase-free water. The RNA quality and quantification were performed by spectrophotometer analysis (Nanodrop 1000 spectrophotometer; Thermo Scientific). The amount of miRNA present in serum may be influenced either by miRNA extraction efficiency or by qRT-PCR robustness (i.e. by the presence of inhibitors). Both of these factors can be controlled by the use of a synthetic non-human miRNA spiked as the control before RNA isolation. In order to adjust for such variations, 1 µL of synthetic Caenorhabditis elegans miRNA (cel-miR-39) from a 1 nmol/µL stock solution were added into 250 µL of the serum samples after the addition of the denaturing solution. The synthetic cel-miR-39 went through the entire RNA isolation process and was ultimately assessed by qRT-PCR in the final RNA eluate, providing an internal reference for normalization (exogenous control) of technical variations between samples.

5.8. Reverse transcription.

Reverse transcription reactions were performed using TaqMan®Advanced miRNA cDNA Synthesis Kit (item no. A25576; Life Technologies, Grand Island, NY). In the first step of

the reaction, mature miRNAs from total RNA are modified by 1) extending the 3' end of the mature transcript through poly(A)addition, then 2) lengthening the 5′ end by adaptor ligation. 3) The modified miRNAs then undergo universal reverse transcription followed by amplification to increase uniformly the amount of cDNA for all miRNAs (miR-Amp reaction). The schematic representation of this reaction is illustrated in **Fig.13.**

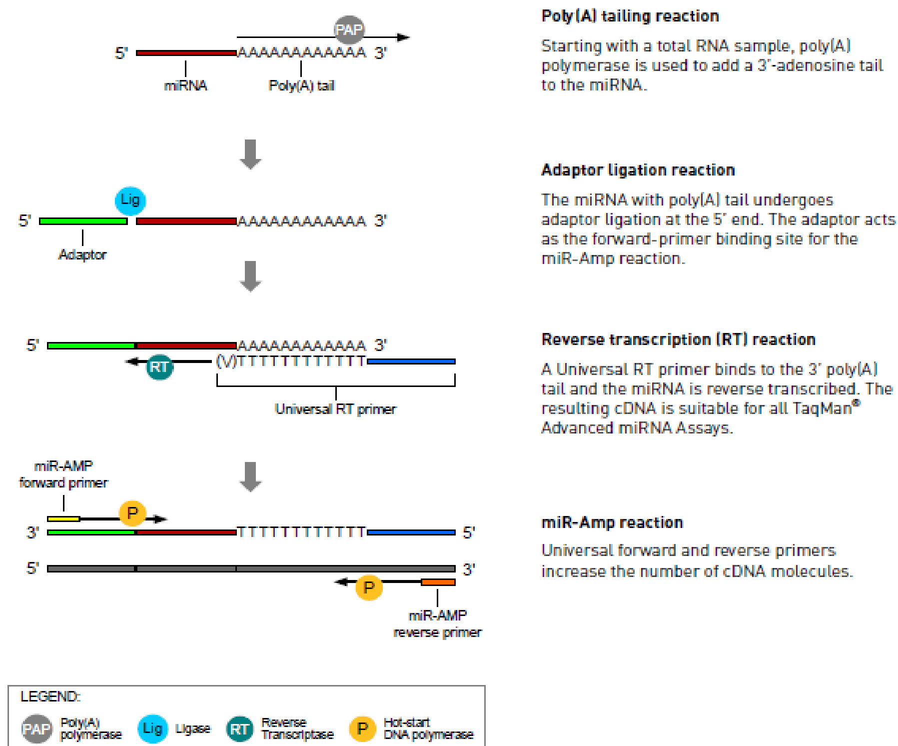


Figure 13. Schematic Representation of TaqMan®Advanced miRNA cDNA Synthesis.

In the poly(A)tailing reaction, 2 µl of sample eluent from each serum is added in 3 µl of poly(A) reaction cocktail (0.5 µl 10X Poly(A) Buffer, 0.5 µl ATP 10Mm, 0.3 µl Poly A Enzime and 1.7 µl RNAse-free water) (5 µl total volume in each tube). Poly (A) tailing reaction was carried out on the Biometra thermal cycler (MMedical) using the following conditions: 37 °C for 45 min, 65 °C for 10 min and then hold at 4 °C. In each tube, after poly (A) tailing reaction, add 10 µl of the ligation reaction (3 µl 5X DNA Ligase Buffer, 4.5 µl 50% PEG 8000, 0.6 µl 25X Ligation Adaptor, 1.5 µl RNA Ligase and 0.4 µl

RNAse-free water). The Ligation reaction was carried out on the thermal cycler using the following conditions: 16 °C for 60 min and then hold at 4 °C. Next, reverse transcription reaction was performed as follows: add 15 µL of RT reaction cocktail (6 µl 5X RT Buffer, 1.2 µl dNTP mix 25 mM each,1.5 µl 20X Universal RT primer, 3 µl 10X RT Enzyme Mix and 3.3 µl RNAse-free water) in each tube. This reaction was carried out on the thermal cycler using the following conditions: 42 °C for 15 min, 85 °C for 5 min and then hold at 4 °C. The last step is miR-Amp reaction that performed as following conditions: add 5 µL of RT reaction product in 45 µL of the miR-Amp reaction cocktail (25 µL 2X miR-Amp Master Mix, 2.5 µL 20X miR-Amp Primer Mix and 17.5 µL RNAse-free water) in each tube. The miR-Amp reaction was carried out on the thermal cycler using the following conditions: 95 °C for 5 min, 95°C for 3 seconds and 60°C for 30 seconds (these two steps were repeated for 14 cycles), 99°C for 10 minutes and then hold at 4 °C. miR-Amp reaction products were stored at 20 °C prior to running the real-time PCR.

5.9. Quantitative RT-qPCR analysis.

The qRT-PCR reactions were performed in duplicate, in 10 µL reaction volumes using 0.5 µL TaqMan® MicroRNA Assay (20X) specific primer/probe mix, 5 µL TaqMan® Fast Advanced Master gene expression (2X) (Applied Biosystems), 3.83 µL Nuclease-free Water H_2O and 0.67 µL miR-Amp product. The mix was aliquoted in duplicate into RNase-free strip-tubes and sealed with an optical plug. The qRT-PCR reactions were carried out using the Realplex Mastercycler epgradient S (Eppendorf) using the following conditions: 50 °C for 2 min, 95 °C for 20 s, followed by 40 cycles of 95 °C for 1s and 60 °C for 20s, followed by a hold at 4 °C. The raw data were analyzed using the automatic cycle threshold (Ct) setting for assigning the baseline and the threshold for Ct

determination. The samples were run in singlet and miRNAs with a Ct value >35 were excluded from the comparison. The relative expression fold change was calculated by using the $2^{-\Delta\Delta Ct}$ method (Schmittgen TD et al., 2008).

5.10. Fibres and chemicals.

Crocidolite asbestos fibres were provided from Union International Contra Cancer (UICC), and these fibres were characterized (chemically and physically) in detail (Governa et al., 1995). The crocidolite samples showed a chemical composition of SiO_2, 48.7%; Al_2O_3, 0.1%; FeO, 36.9%; MgO, 3.8%; CaO, 1.0%; Na_2O, 4.4%; TiO_2, 0.7%. The fibres were examined using a scanning electron microscope (Philips XL30, Monza, Italy) equipped with energy-dispersive X-ray analysis (EDAX) apparatus. The length and diameter of 300 fibres were measured by the scanning electron microscope at a tension of 20 kV and magnification of 2000X. The specific surface area of crocidolite asbestos fibres (AFs) was measured using the nitrogen absorption isotherm technique (Ghio et al., 1992). The samples were degassed at 90 °C for 16 h and then examined with a Sorpty 1750 (Fison Instruments, Milan, Italy), and free space was determined with helium. The fibre amount was expressed as ratio of specific surface area of fibres and surface area of well (cm^2/cm^2). All fibres used were endotoxins free.

Sodium arsenite ($NaAsO_2$), potassium dichromate ($K_2Cr_2O_7$), the EGFR inhibitor, AG1478, phorbol myristate acetate (PMA, P1585), Lipopolisaccaride (LPS) were purchased from Sigma-Aldrich (St Louis, MO). Dichlorodihydrofluoresceine acetate (DCFA) and dihydroethidium (DHE) were from Molecular Probes (Thermo Fisher Scientific).

5.11. Cell cultures and treatments.

Immortalized Human bronchial epithelial cells (BEAS-2B ATCC® CRL9609™) were obtained from American Type Culture Collection (ATCC; Rockville, USA, www.ATCC.org). BEAS-2B cells were grown in the Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 1% of penicillin and 10% of streptomycin and regularly checked for absence of mycoplasma contamination using the PCR Mycoplasma Test. The cells have been cultured not more than six passages within 1 month after resuscitation.

Human umbilical vein endothelial cells (HUVEC) were obtained from ATCC and were grown in EGM™-Plus Growth Media following the company's instruction (Lonza CC-2935, NJ, USA). The human monocyte U937 cell line (ATCC) was grown in RPMI 1640 medium containing 10% FBS with penicillin (50U/ml) and streptomycin (100µg/ml) at 37°C in a humidified incubator with 5% CO_2 and 95% air atmosphere. The U937 cells were differentiated into macrophage-like cells by being treated with 50 nM phorbol myristate acetate (PMA) for 24h. Differentiated macrophage-like cells were exposed to LPS 1µg/ml for 72h to stimulate the production of proinflammatory cytokines. The normal human lung fibroblast cell line IMR90 was obtained from ATCC and were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 1% of penicillin and 10% of streptomycin. All cells were maintained in culture in a humidified incubator at 37°C and in presence of CO_2 at 5%.

The crocidolite asbestos fibres were suspended in PBS and diluted in complete culture medium with a final concentration of 5µg/cm^2. Sodium arsenite ($NaAsO_2$) and potassium dichromate ($K_2Cr_2O_7$) were used for *in vitro* carcinogenesis induction. The carcinogens

were suspended in PBS and diluted in complete culture medium with a final concentration of 1 μM for sodium arsenite and 0.5 μM for potassium dichromate.

The EGFR inhibitor, AG1478 (Sigma, St Louis, MO) was added to the culture medium at 10 μM for 24 h from a 10mM stock solution in DMSO.

5.12. Pre-cancerous cells selection

To obtain cells with acquired pre-cancerous phenotype (initiated cells), BEAS-2B cells ($1x10^4$) were treated with sodium arsenite ($NaAsO_2$, 1 μM), crocidolite asbestos fibres (5μg/cm^2), or potassium dichromate ($K_2Cr_2O_7$, 0.5 μM) for 4 weeks. Next, the carcinogens-exposed cells were seeded in low melting point agar (0.7%) in 24-well plates, overlaid with 0.35% low melting point agar, and cultured at 37°C in 5% CO_2 for 3 months (Clancy et al., 2012). Every 7 days, 0.5 ml of fresh complete medium was replaced to each well. The colonies formed greater than 0.1 mm diameter were collected and grown in complete DMEM medium. No colony formation was found in non-exposed BEAS-2B cells used as controls.

5.13. Cell proliferation assay and mitochondrial-reducing activity

Pre-cancerous cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml in PBS) at 37°C for 3h. After removing the media, 200μL of isopropanol was added to dissolve the crystals. Absorbance was read at 550nm in an ELISA plate reader (Sunrise, Tecan, Männedorf, Swiss). The proliferation index was expressed as relative change with respect to the controls set as 100%. The MRA

(mitochondrial-reducing activity) was assessed in the Pre-cancerous cells as the reduction of resazurin with being based on the mitochondrial metabolic activity (Abu-Amero KK and Bosley TM, 2005; Atamna H et al., 2008; van de Loosdrecht AA et al., 1994). For the resazurin assay, cells were incubated with resazurin (6μM). Fluorescence intensity was read at 0-240 min in a fluorescence plate reader (Infinite F200 PRO, Sunrise, Tecan, Männedorf, Swiss). The excitation and emission filters were set at 485 and 530 nm, respectively. The results were normalized to the total protein detected by Bradford assay (Sigma).

5.14. Assessment of ROS and mitochondrial membrane potential

Intracellular ROS levels were estimated using the fluorescent dye 2′7′-dichlorofluorescein diacetate (DCFDA; oxidized by hydrogen peroxide to DCF). Pre-cancerous cells (2×10^5) were seeded in six-well plates, supplemented with 20 μM DCFDA for well for 30 minutes. After treatment, the florescent probe was removed. The cells were washed, resuspended in PBS and analyzed by flow cytometry (FACS Calibur, Becton Dickinson). The level of ROS was expressed as fluorescence increment respect to control cells (BEAS-2B). Intracellular superoxide anion was estimated using dihydroethidium (DHE). DHE probe is oxidized by superoxide to form 2-hydroxyethidium (2-OH-E^+) (λ_{ex} 500-530 nm / λ_{em} 590-620 nm). Pre-cancerous (2×10^5) were incubated with 20 μM DHE for 30 minutes and analyzed by cytometry (FACS Calibur, Becton Dickinson). The level of ROS was expressed as fluorescence increment respect to control cells (BEAS-2B).

For mitochondrial membrane potential $\Delta\Psi_{m,i}$ assessment, pre-cancerous cells (3×10^4) were seeded in Corning ®96-well black-bottom plates, and treated for 4 hours with

tetraethylbenzimidazolylcarbocyanine iodide dye (JC1, 5μM). JC1 is a lipophilic, cationic dye that can selectively enter into mitochondria and reversibly change color from green to red as result of membrane potential increase. In healthy cells, JC1 spontaneously forms complexes known as J-aggregates with intense red fluorescence colored emission (590±17.5 nm). In apoptotic or unhealthy cells, JC-1 remains predominantly in a monomeric form that yields green fluorescence with emission of 530±15 nm. Therefore, a decrease in aggregate fluorescent intensity is indicative of depolarization, whereas an increase of fluorescence is indicative of hyperpolarization of mitochondrial membrane ($\Delta\Psi_M$). After treatment, the florescent probe was removed, the cells washed and resuspended in PBS. Fluorescence intensity was read in a fluorescence plate reader (Infinite F200 PRO, Sunrise, Tecan, Männedorf, Swiss). The monomer form was detected by setting excitation and emission filters at 485 and 530 nm, respectivelty. While, the aggregate form was estimated by setting the excitation and emission filters at 530 and 590 nm, respectively. The results were normalized to the total protein analysed by Bradford assay (Sigma).

5.15. Glucose uptake

Pre-cancerous cells were seeded in 96-well black-bottom plates ($3x10^4$ cells per well) in low glucose (1 g/l) DMEM at 5% CO_2 and 37°C. After overnight incubation, the cells were treated with 2-nitrobenzodeoxyglucose (2-NBDG, 50 μM) for 30 min. The level of fluorescent intencity was evaluated at 550/590 nm using a fluorescence plate reader (Infinite F200 PRO, Tecan).

5.16. Detection of glucose, lactate and ATP

Glucose and lactate were evaluated using commercial kits (Abcam) according to the manufacturer's protocol. Intracellular ATP was determined using a luciferase-based assay (Abcam). The results were normalized to the total protein content.

5.17. Macrophages-induced cytokine production

The U937 (1×10^4) cells were differentiated into macrophage-like cells by phorbol myristate acetate (PMA, 50 nM) for 24h. Differentiated macrophage-like cells were co-cultured with carcinogen-induced pre-cancerous cells (1.5×10^5 cells/well), and then exposed to LPS (1µg/ml) for 72h to stimulate the production of proinflammatory cytokines and growth factors. The bottom of the insert chamber has 0.4-µm pores (Corning, NY, USA), allowing cytokines and growth factors produced by macrophages to reach the lower chamber, where pre-cancerous cells are cultured. The cells were kept in coculture up to 72 hours. The **Fig.14** shows the model of coculture realized.

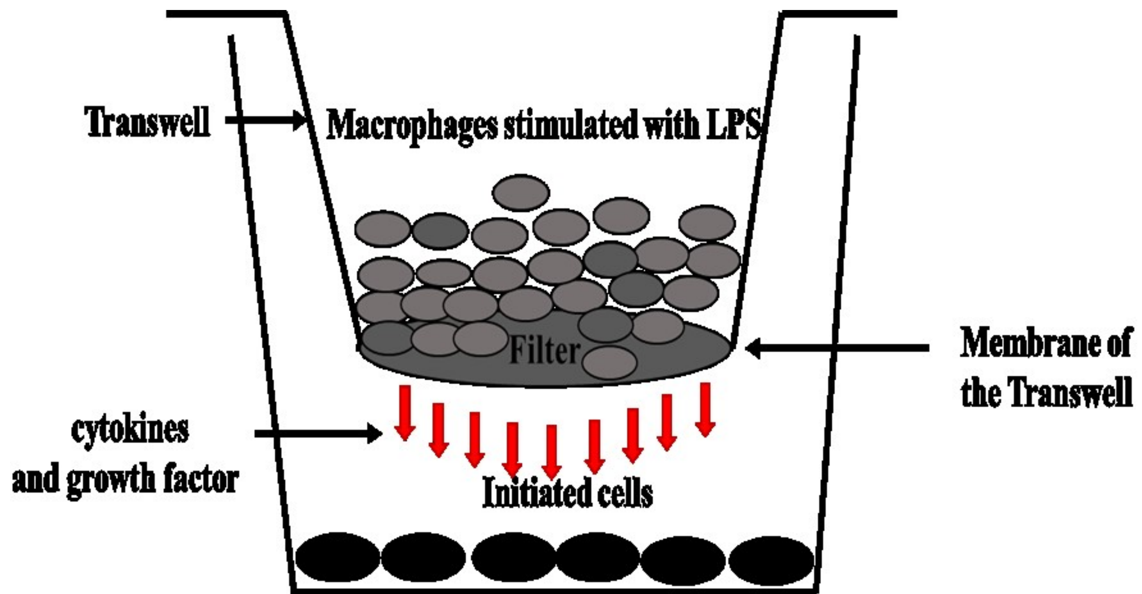


Figure 14. **Model of initiated cells and macrophages coculture**. The initiated cells are grown in transwell culture plate. The stimulated macrophages-like cells are cultivated within the transwell featured a 0.4 µm membrane that allows the passage of cytokines and growth factors to the cells seeded into lower chamber, if any between the two compartments not allowing cell migration.

5.18. Triple co-culture model

A 3-µm Transwell insert (Costar 3452; Corning, NY, USA) was first plated with 10^5 primarily cultured IMR90 cells in an inverted position. After 6 hours of incubation, inserts were flipped and placed into a six-well Transwell plate, where 3 x 10^5 HUVEC cells were loaded on the other side of the insert and cultured for 24 hours. This HUVEC-IMR90 precoated Transwell insert was then placed into another six-well transwell, where 2 x 10^5 pre-cancerous cells had been plated for the same duration of time, and was cultured for additional 2 days before collecting (**Fig.15**).

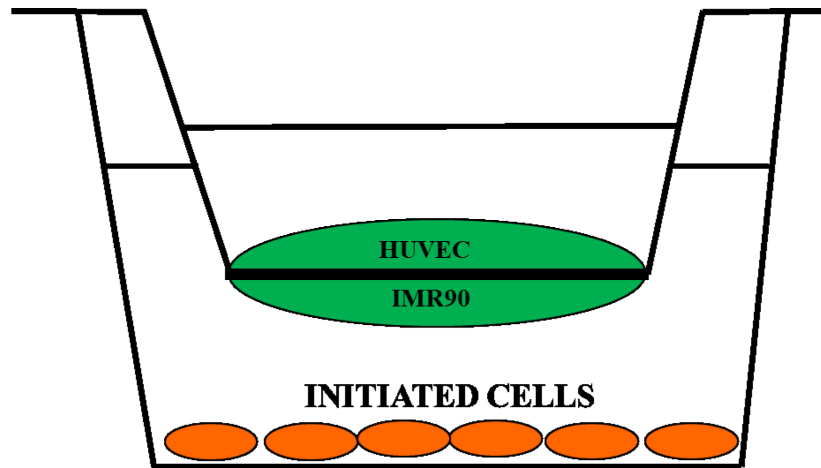


Figure 15. Diagram of a layered triple coculture model. HUVEC and IMR90 cultured on the two surfaces of the transwell insert and the initiated cells cultured at the bottom of the transwell.

5.19. Western blot analysis

Pre-cancerous cells (3×10^5 per well in six-well plates) were lysed in the RIPA buffer containing Na_3VO_4 (1 mM) and protease inhibitors (1 µg/ml). After 20 min incubation on ice, the pellet was centrifuged at 12.000 rpms for 10 min at 4°C, and the supernatant was collected. The protein level was quantified using the Bradford assay (Sigma). For Western blot analysis, the cell lysate protein (50 µg per lane) were resolved using 4–12% SDS-PAGE (Life Technologies), and transferred onto nitrocellulose membranes (Protran). After blocking with 5% non-fat milk in PBS-Tween (0.1%) for 1 hour, the membranes were incubated overnight with antibodies against AKT, phospho-AKT, ERK1/2, phospho-ERK1/2, p38-MAPK, phospo-p38 MAPK, EGFR, phosphor-EGRF, and IRS1 (Cell Signalling Technology, Danvers, MA, USA). After incubation with the HRP-conjugated secondary IgG (Sigma), blots were developed using the ECL detection system (Pierce Biotechnology, Rockford, IL, USA). The band intensities were visualized and quantified with ChemiDoc using the Quantity One software (BioRad Laboratories, MI, Italy).

5.20. Quantitative RT-qPCR analysis

Total RNA from cells were obtained using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The miR-126, miR-205, miR-222, miR-520g first-strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative RT-PCR (qPCR) was performed using the TaqMan Gene Expression Master Mix (Applied Biosystems, Life Technologies) with U6 as housekeeping gene. The qPCR assays were performed using the Mastercycler EP Realplex (Eppendorf). The results were expressed as fold changes or relative miRNA expression using the equations $2^{-\Delta\Delta Ct}$ or $2^{-\Delta Ct}$, respectively.

5.21. Statistical analysis

Results are expressed as mean ± S.D. unless indicated otherwise. Comparisons among groups of data were made using one-way ANOVA with Tukey post-hoc analysis. The two-tailed Student's t-test was used to compare two groups. Differences with $p<0.05$ were considered statistically significant. Correlations were performed according to Spearman′s test. Receiver operating characteristics (ROC) curves were plotted to quantify the marker performance. The area under curve (AUC) indicates the average sensitivity of a marker over the entire ROC curve.

Logistic regression model was used to estimate the probability (P) of the risk to develop NSCLC, $NSCLC^{Asb}$, MM. P was expressed by the logit(P) transformation, which is defined as the natural logarithm of the odds, and odds are defined as the probability of a positive outcome (P) divided by the probability of a negative outcome (1 − P). The logit(P) transformation was predicted by the generalized mathematical expression: $\text{logit}(P) = \beta_0 +$

$\beta_1 \times 1 + \beta_2 \times 2. . .\beta nXn$, which link the logit (P) transformation to a number of n predictor variables and n + 1 regression constants ($\beta_0. . .\beta_n$). All data generated in this study were analysed using the SPSS software.

6. Results

6.1. Discovery phase: identification of miRNA-expression profile

To identify candidate asbestos-related miRNA, 372 miRNAs were analysed in total RNA extracted from four diagnostic biopsy of patients affected by NSCLC, asbestos-associated NSCLC, and MM as asbestos-induced malignancy. All samples were analyzed succefully, with no signs of inhibition of the real-time amplification due to contaminantes. As shown by the volcano plot, of the 372 analysed miRNAs, only 9 miRNAs were found over-expressed and 3 miRNAs were under-expressed in the NSCLC group (**Fig.16 and Tab.3**).

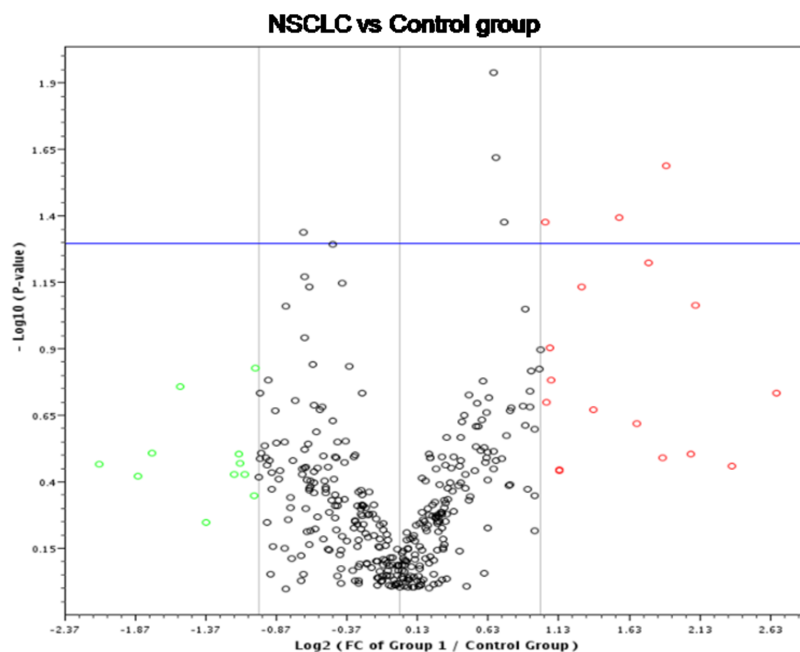


Figure 16. **Volcano plot** showing differential distribution of miRNAs based on the fold change and significance (p-value) in the group NSCLC.

Over expressed	Fold change	p-value
hsa-miR-146a-3p	2.44	0.073
hsa-miR-152-3p	2.04	0.042
hsa-miR-204-5p	3.70	0.026
hsa-miR-485-5p	3.40	0.059
hsa-miR-205-5p	2,20	0.085
hsa-miR-708-5p	2.95	0.040
hsa-miR-10a-5p	2.10	0.120
hsa-miR-31-5p	4.27	0.085
hsa-miR-328-3p	2.06	0.198
Under expressed	**Fold change**	**p-value**
hsa-miR-32-5p	-2.01	0.147
hsa-miR-519a-3p	-2.93	0.172
hsa-miR-126-5p	-2,85	0,062

Table 3. **Deregulated miRNA in the NSCLC group *versus* control.**

The group of NSCLC patients with established exposure to asbestos ($NSCLC^{Asb}$) showed a profile of deregulated miRNAs (**Fig. 17**) consisting of 6 miRNAs over-expressed and 6 miRNAs under-expressed as shown in **Tab 4.**

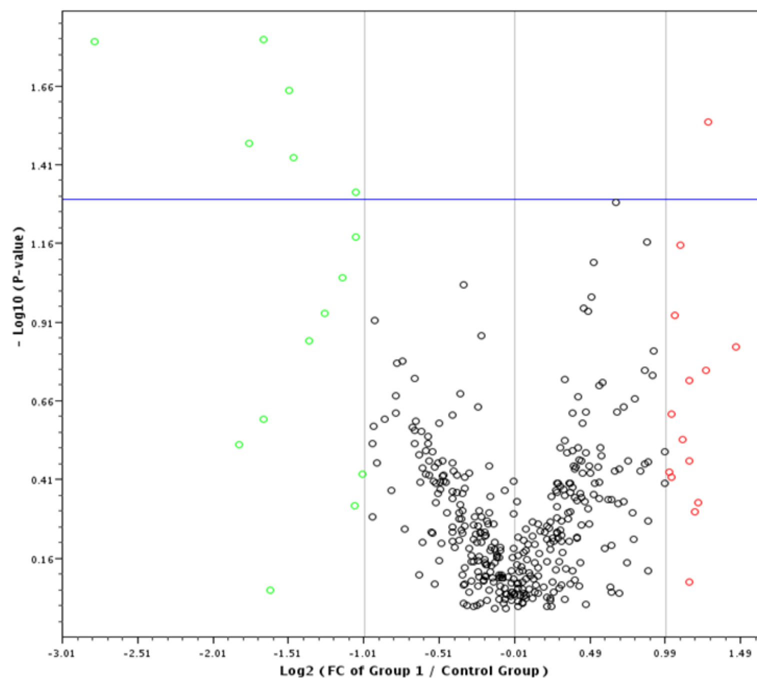


Figure 17. A volcano plot representation showing differential distribution of miRNAs based on the fold change and significance (p-value) in the group $NSCLC^{Asb}$.

Over expressed	Fold change	p-value
hsa-miR-34a-3p	2.05	0.200
hsa-miR-190a-5p	2.13	0.070
hsa-miR-205-5p	2.22	0.082
hsa-miR-485-5p	2.76	0.147
hsa-miR-495-5p	2.22	0.180
hsa-miR-520-3p	2.43	0.028
Under expressed	**Fold change**	**p-value**
hsa-miR-124-3p	-3.39	0.033
hsa-miR-302a-3p	-2.76	0.040
hsa-miR-328-3p	-3.19	0.016
hsa-miR-504-5p	-2.82	0.022
hsa-miR-765	-6.92	0.016
hsa-miR-885-3p	-2.08	0.047

Table 4. Deregulated miRNAs in the NSCLC^{Asb} group *versus* control.

The group MM shows a profile of deregulated miRNAs consisting of 20 miRNAs over-expressed and 18 miRNAs under-expressed (**Fig.18**). The miRNAs with significant differential expression are summarized in **Tab.5.**

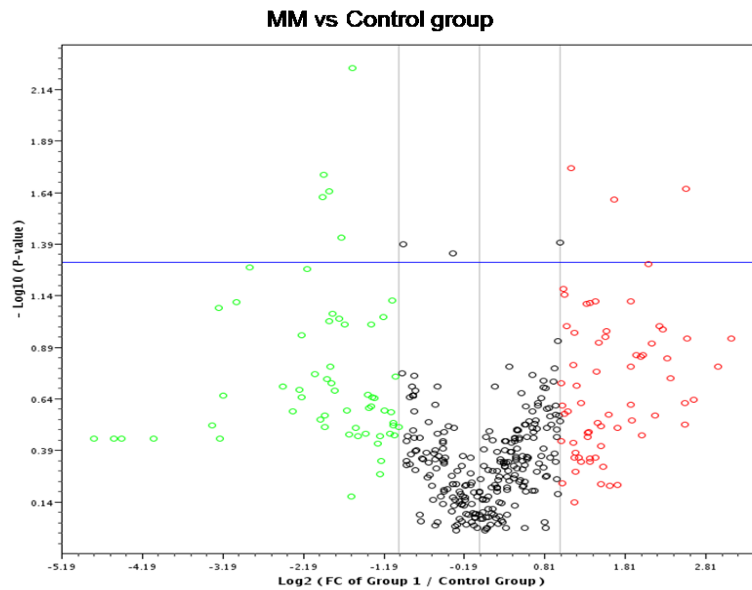


Figure 18. **Volcano plot** showing differential distribution of miRNAs based on the fold change and significance (p-value) in the group MM.

Over expressed	Fold change	p-value	Under expressed	Fold change	p-value
hsa-miR-Let-7i-3p	3.66	0.077	**hsa-miR-126-3p**	-3.63	0.022
hsa-miR-100-3p	5.87	0.022	**hsa-miR-126-5p**	-3.86	0.024
hsa-miR-100-5p	2.29	0.201	hsa-miR-139-5p	-3.20	0.099
hsa-miR-149-5p	5.00	0.146	hsa-miR-143-3p	-2.29	0.092
hsa-miR-182-5p	5.95	0.117	hsa-miR-144-5p	-8.14	0.078
hsa-miR-183-5p	4.82	0.100	hsa-miR-145-3p	-2.99	0.056
hsa-miR-193a-3p	4.27	0.050	**hsa-miR-145-5p**	-3.28	0.038
hsa-miR-193a-5p	2.49	0.078	hsa-miR-146a-5p	-3.59	0.192
hsa-miR-199a-5p	2.02	0.192	hsa-miR-185-5p	-2.12	0.076
hsa-miR-210-3p	5.20	0.180	hsa-miR-223-3p	-4.41	0.053
hsa-miR-21-3p	4.70	0.100	**hsa-miR-223-5p**	-3.82	0.019
hsa-miR-21-5p	4.40	0.120	hsa-miR-27a-5p	-2.55	0.100
hsa-miR-221-3p	2.69	0.077	hsa-miR-34b-5p	-3.62	0.160
hsa-miR-222-3p	3.20	0.025	hsa-miR-34b-3p	-3.64	0.096
hsa-miR-224-3p	2.58	0.078	hsa-miR-363-3p	-3.54	0.088
hsa-miR-224-5p	2.95	0.110	hsa-miR-451a	-7.27	0.052
hsa-miR-34a-3p	2.07	0.071	hsa-miR-486-5p	-9.43	0.083
hsa-miR-34a-5p	2.05	0.067	hsa-miR-489-3p	-2.07	0.177
hsa-miR-376b-3p	8.73	0.117	**hsa-miR-211**	-2.00	0.040
hsa-miR-708-5p	2.96	0.107			

Table 5. Deregulated miRNAs in the MM group *versus* control.

A Venn diagram of the distribution of the co-deregulated miRNAs was performed to visualise the miRNA overlapping across the groups **(Fig. 19)**.

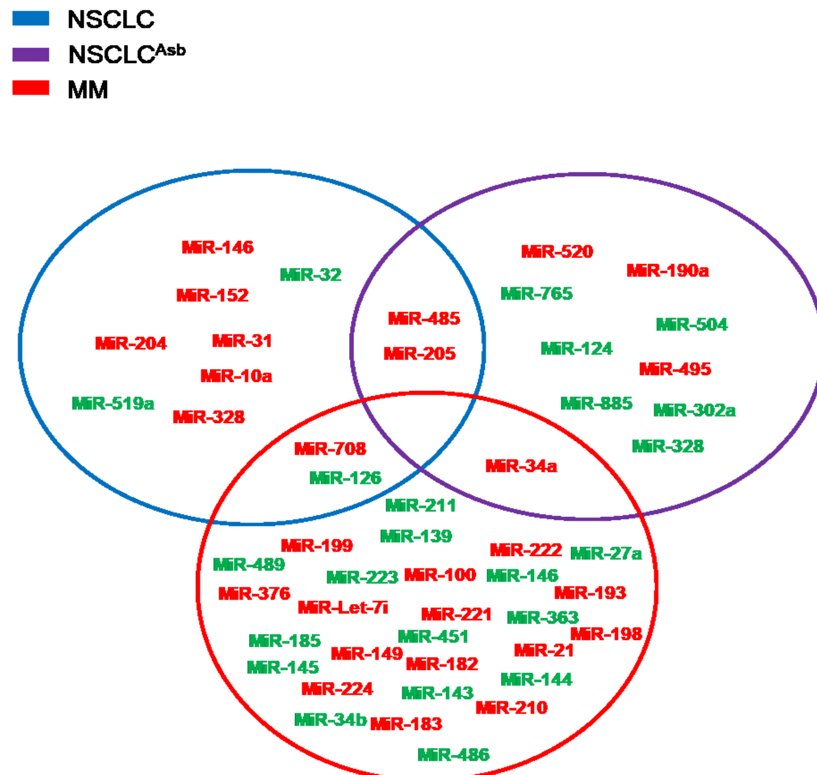


Figure 19. Venn diagram that shows differentially expressed miRNAs that were shared among three groups (NSCLC, NSCLC^{Asb} and MM).

According to the fold-change and statistical significance a panel of deregulated miRNAs within and across the groups has been identified (**Fig.20**), consisting of four miRNAs for each studied group (NSCLC, NSCLC^{Asb}, MM).

Some of them were overlapping between the different groups, such as miR-485 and miR-205, which were common for NSCLC and NSCLC^{Asb} and miR-34a, which was in common for NSCLC^{Asb} and MM group.

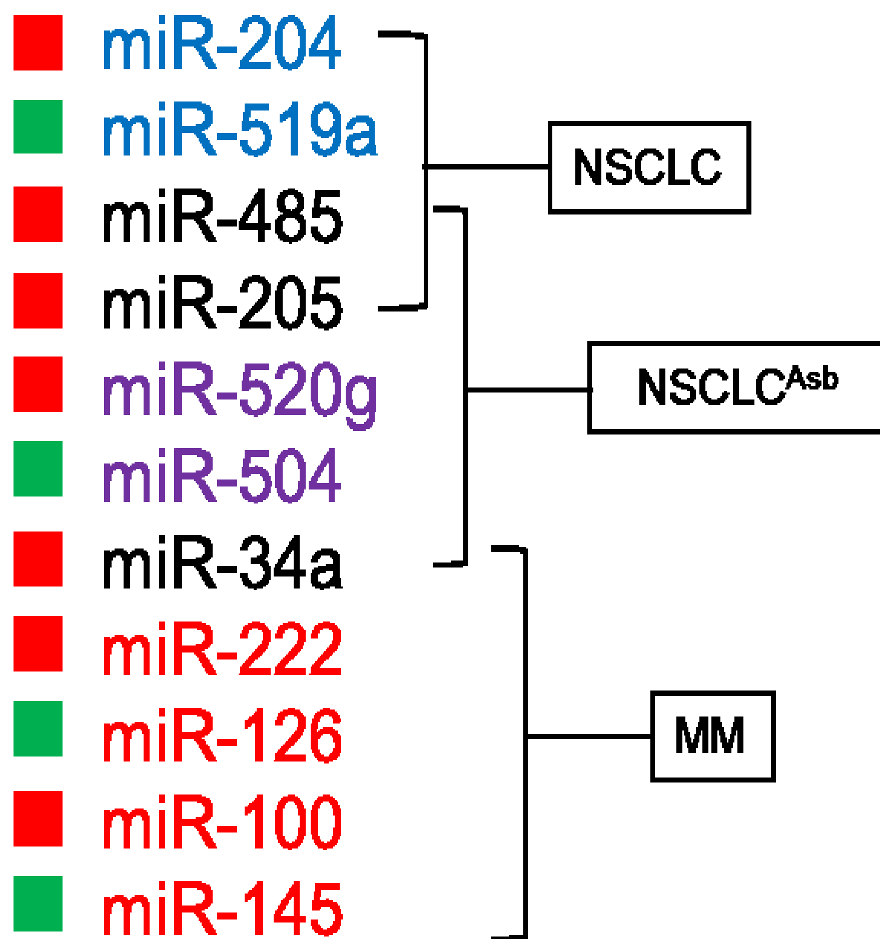


Figure 20. Schematic Representation of 11 selected miRNAs identified at the discovery phase. MiRNAs with greater and lower abundance compared to controls are shown in red and green, respectively.

6.2. Verification phase

The verification phase was performed to investigate whether the selected miRNA at the tissue levels could be detected in serum samples. In this phase the miRNA-panel, consisting of 11 miRNAs plus cel-miR-39 as exogenous control, has been evaluated in serum of patients affected by NSCLC with and without asbestos exposure, MM and compared with a population of healthy subjects as control.

Among the 11 examined miRNAs in serum samples, miR-204, miR-519a, miR-504, miR-34a, miR-145 miR-485, and miR-100 were not always detectable in the serum of the study population. Only four miRNAs (miR-126, miR-205, miR-222, miR-520g) were stably present in serum samples and were found differently expressed in NSCLC, $NSCLC^{Asb}$, MM patients respect to control group.

The miR-205 was specific for NSCLC, miR-222 was representative of asbestos-related NSCLC, miR-126 of MM and miR-520g, even though not significant, was found to be down-expressed both in $NSCLC^{Asb}$ and MM as miRNA associated with asbestos exposure (**Tab.6** and **Fig.21**).

	Group-1			Group-2			Group-3		
	NSCLC			NSCLC-Asb			MM		
miRs	**FC**	**95% CI**	***p***	**FC**	**95% CI**	***p***	**FC**	**95% CI**	***p***
3-miR-204	1.72	0.00001-3.58	0.211	2.25	0.00001-5.22	0.08	1.09	0.07-2.11	0.606
5-miR-519a	**1.72**	**0.00001-3.58**	**0.211**	**1.27**	**0.00001-2.81**	**0.315**	**1.09**	**0.07-2.11**	**0.606**
11-miR-485	**0,62**	**0.26-0.98**	**0.487**	**1.01**	**0.00001-2.20**	**0.791**	**0.61**	**0.26-0.98**	**0.05**
4-miR-205	**2.93**	**0.03-5.82**	**0.040**	**2.91**	**0.00001-6.16**	**0.170**	**0.61**	**0.00001-1.39**	**0.166**
10-miR-520g	**1.09**	**0.00001-2.76**	**0.423**	**0.63**	**0.00001-1.81**	**0.720**	**0.65**	**0.00001-1.51**	**0.128**
9-miR504	**1.72**	**0.00001-3.57**	**0.211**	**1.27**	**0.00001-2.81**	**0.315**	**1.22**	**0.00001-2.46**	**0.366**
6-miR-34a	1.72	0.00001-3.57	0.211	1.27	0.00001-2.81	0.315	1.09	0.07-2.11	0.606
2-miR-222	**1.84**	**0.12-3.57**	**0.128**	**4.14**	**0.58-7.54**	**0.011**	**0.85**	**0.23-2.16**	**0.258**
8-miR-126	**0.90**	**0.25-1.55**	**0.956**	**1.18**	**0.12-2.25**	**0.014**	**0.45**	**0.09-0.81**	**0.048**
7-miR-100	**0.16**	**0.00001-0.83**	**0.182**	**0.082**	**0.00001-0.54**	**0.461**	**2.68**	**0.14-2.27**	**0.031**
1-miR-145	1.61	0.00001-3.32	0.213	1.46	0.00001-3.33	0.156	1.02	0.09-1.96	0.615

Table 6. Fold-change (FC) in miRNA-panel expression in patients affected by NSCLC with and without asbestos exposure, MM and compared with a population of healthy subjects as control. Significance (p) and interval of confidence (95%CI) are shown.

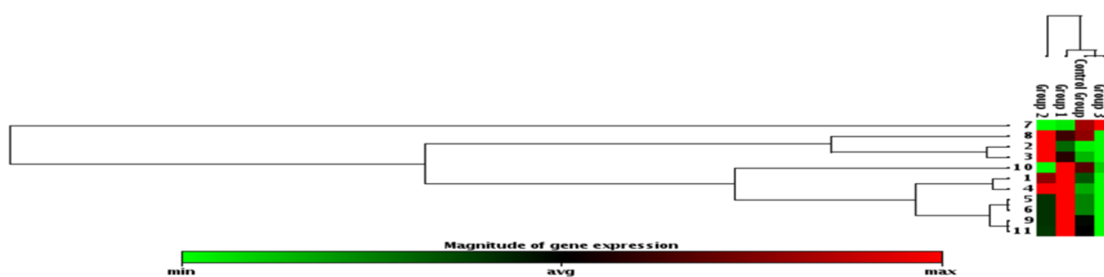


Figure 21. **Hierarchical cluster analysis of miRNAs.** MiRNA expression of patients affected by NSCLC with (Group-2) and without (Group-1) asbestos exposure, MM (Group-3) and compared with a population of healthy subjects as control. MiRNAs were considered differentially expressed if their levels were increased or decreased by more than 2-fold. Relative normalized expression for each miRNA is represented by colour intensity (green, downregulation; red, increased expression; black, miRNA not changed)

6.3. Training and validation phases

To confirm the expression of candidate miRNAs, RT-qPCR assay was used. In the training phase, miRNAs were measured in an independent cohort, which included serum samples from 24 NSCLC patients, 19 $NSCLC^{Asb}$ patients, 22 MM patients and 21 healthy controls (**Tab.7**).

The miRNAs were normalized to cell-miR-39 (exogenous control), and only miRNAs with a Fold Change (FC) > 1.5 or < 0.5 have been considered. The selected miRNAs were expressed as relative expression, or as miRNA ratio as previously reported by *Boeri et al, 2011.*

By performing binary logistic regression on the relative expression of miRNAs and miRNA ratios including age, gender and smoking as confounding variables a classifier's optimal logit (P) model was obtained able to discriminate the asbestos-related malignancies from cancer non-asbestos associated (**Tab.8**).

The miR-222 associated with age significantly detected the lung malignancies related to asbestos exposure. Next, the predictive model has been tested in another independent cohort population in the validation phase. To evaluate the prediction specificity, patients affected by sinonasal cancer (SNC) as occupational malignancy associated to wood dust and leather exposure have been included.

	CTRL	p-value	NSCLC	p-value	NSCLC^{Asb}	p-value	MM	p-value
miR-126	12.79 [0.36-260.8]		6.99 [0.09-53.36]*°	*p=0.035;°p=0.001	15.56 [1.77-129.6]		4.56 [0.02-45.81]*°	*p=0.004;°p=0.000
miR-205	1.13 [0.03-24.89]°	°p=0.001	2.50 [0.01-70.9]°	°p=0.006	3.83 [0.05-232.0]*	*p=0.000	1.29 [0.01-61.72]°	°p=0.001
miR-222	0.41 [0.01-3.03]°	°p=0.0005	0.66 [0.05-5.77]°	°p=0.000	1.19 [0.16-32.85]*	*p=0.000	0.39 [0.03-8.92]°	°p=0.000
miR-520g	0.41 [0.02-22.0]		0.52 [0.01-20.36]°	°p=0.030	1.01 [0.01-30.44]		0.60 [0.00-7.99]°	°p=0.007

Table 7. MiRNAs relative expression in healthy controls (CTRL), non-small cell lung cancer (NSCLC), asbestos-related non-small cell lung cancer (NSCLC^{Asb}), and malignant mesothelioma (MM) groups. The data are expressed as median [min-max]. Significant differences were determined using one-way ANOVA and Tukey post-hoc test. *CTRL vs NSCLC, NSCLC^{Asb}, MM; ° NSCLC^{Asb} vs CTRL, NSCLC, MM, p<0.05.

variables	**OR**	**95 % CI**	***p-value***
Age	**1.066**	**1.018-1.116**	**0.006**
miR-222	**1.28**	**1.014-1.616**	**0.038**

Table 8. Logistic regression analysis of biomarkers (miRNAs and their ratio) adjusted for age, gender, and smoking. Data was presented as OR (odds ratio), 95% CI, and p-value. Significance was determined by univariate logistic regression analysis. P-value <0.05 was considered significant.

As shown by evaluating the ROC curves reported in **Fig.22A**, the specificity and sensitivity to distinguish asbestos-related ($NSCLC^{Asb}$ and MM) and non-asbestos-related (NSCLC and SNC) malignancies and control group (CTRL) increased in the validation population respect to that of training population with an AUC=0.767 ± 0.053 and AUC=0.706 ± 0.55, respectively.

Tacking in account a Cut-off of 0.466 (80% sensitivity and 70% specificity), only 10-15% of asbestos-related malignancies ($NSCLC^{Asb}$ and MM) were depicted as not asbestos-exposed, while 40-60% of non asbestos-related NSCLC and SNC patients were detected as asbestos-related (**Fig.22B**).

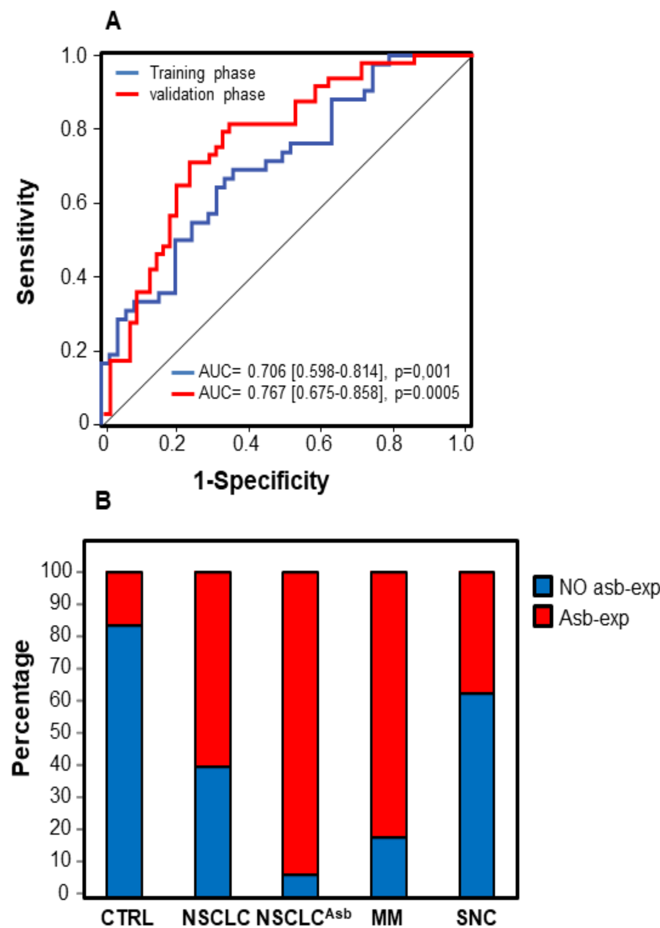
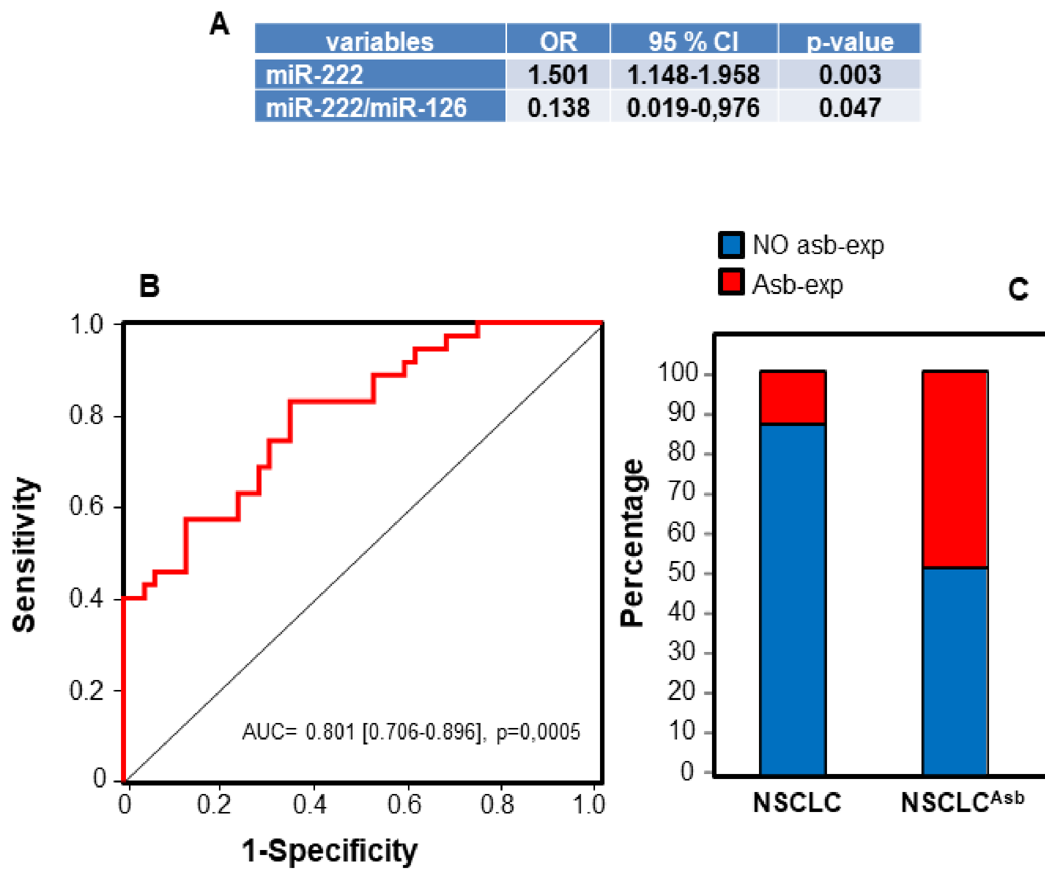


Figure 22. **Logit (P) values in study groups**. Receiver operating characteristics (ROC) curves of Logit (P) value in asbestos-related and non-asbestos-related malignancies in training and validation population. Area under the ROC curves (AUC) and 95% CI are shown (**A**). Separation of asbestos-related and non-asbestos-related malignancies using logit (P) = 0.466 as Cut-off value in the validation population (**B**).

The predictive Logit (P) model generated false positive in the non-exposed cancer group. Therefore, to best differentiate the lung cancer related to asbestos exposure, binary logistic regression was performed by considering the asbestos-related and non-asbestos related NSCLC groups for the four miRNAs and their ratio values. A predictive model was obtained, which consist of miR-222 and miR-222/miR-126 ratio (**Fig.23**).



A

variables	OR	95 % CI	p-value
miR-222	1.501	1.148-1.958	0.003
miR-222/miR-126	0.138	0.019-0,976	0.047

Figure 23. **Logistic regression analysis** and Logit (P) values in asbestos-exposed NSCLC (NSCLC^{Asb}) and non-asbestos exposed NSCLC groups. Logistic regression analysis of miRNAs and their ratio values adjusted for age, gender, and smoking. Data was presented as OR (odds ratio), 95% CI, and p-value. Significance was determined by univariate logistic regression analysis. P-value <0.05 was considered significant (**A**). Receiver operating characteristics (ROC) curve of Logit (P) value in asbestos-related and non-asbestos-related lung cancer in validation population. Area under the ROC curves (AUC) and 95% CI are shown (**B**). Separation of asbestos-related and non-asbestos-related malignancies using logit (P) = 0.316 as Cut-off value (80% sensitivity and 65% specificity) in the validation population (**C**).

The addition of the miR-222/miR-126 ratio in the predictive model best discriminated the non-exposed group (10% of false positive), but generated about 50% of false negative in the asbestos-exposed group.

The distribution of miR-222, miR-222/miR-126 ratio among the groups has been shown in **Fig.24**.

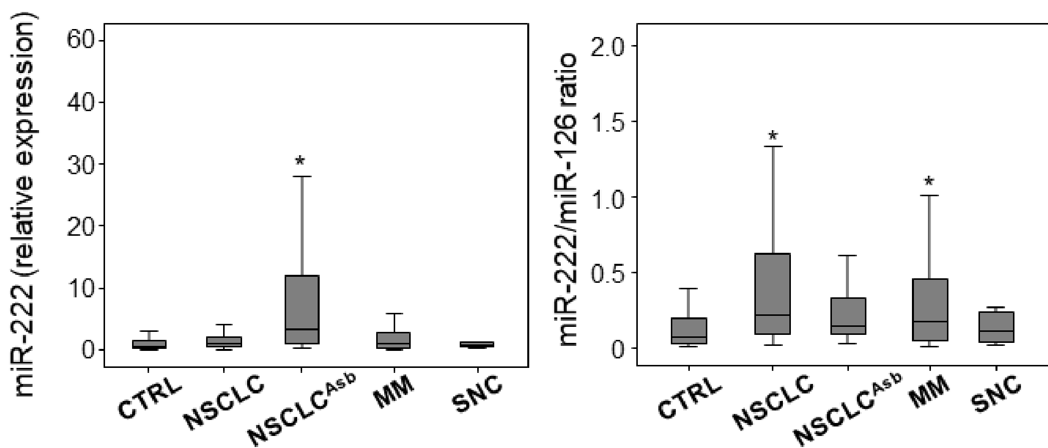


Figure 24. Tukey box plots of miR-222 (**left-panel**) and miR-222/miR-126 ratio (**right-panel**) in serum of patients with NSCLC, asbestos-related NSCLC (NSCLC^{Asb}), MM and controls (CTRL). The box delimits the 25th and 75th percentiles. Horizontal line inside the box mark the medians. Mean expression values are marched with squares. Vertical lines mark the interval between 5th and 95th percentiles. Significant differences were determined using one-way ANOVA and Tukey post-hoc test. *CTRL vs NSCLC, NSCLC^{Asb}, MM and SNC groups.

6.4. Asbetsos-related miRNAs with diagnostic value for Lung Cancer

The association of miR-222 and miR-126 with asbestos exposure was further supported by the positive correlation found between the two miRNAs and cumulative asbestos fibers (*Cf*) (**Fig.25A**).

To distinguish the NSCLC from the controls, the binary logistic regression was performed. As shown in **Fig.25B**, the association of miR-205 and miR-222 best discriminated the

patients affected by NSCLC from healthy control group. The distribution of miR-205 and miR-222 has been reported in **Fig.25C, D**.

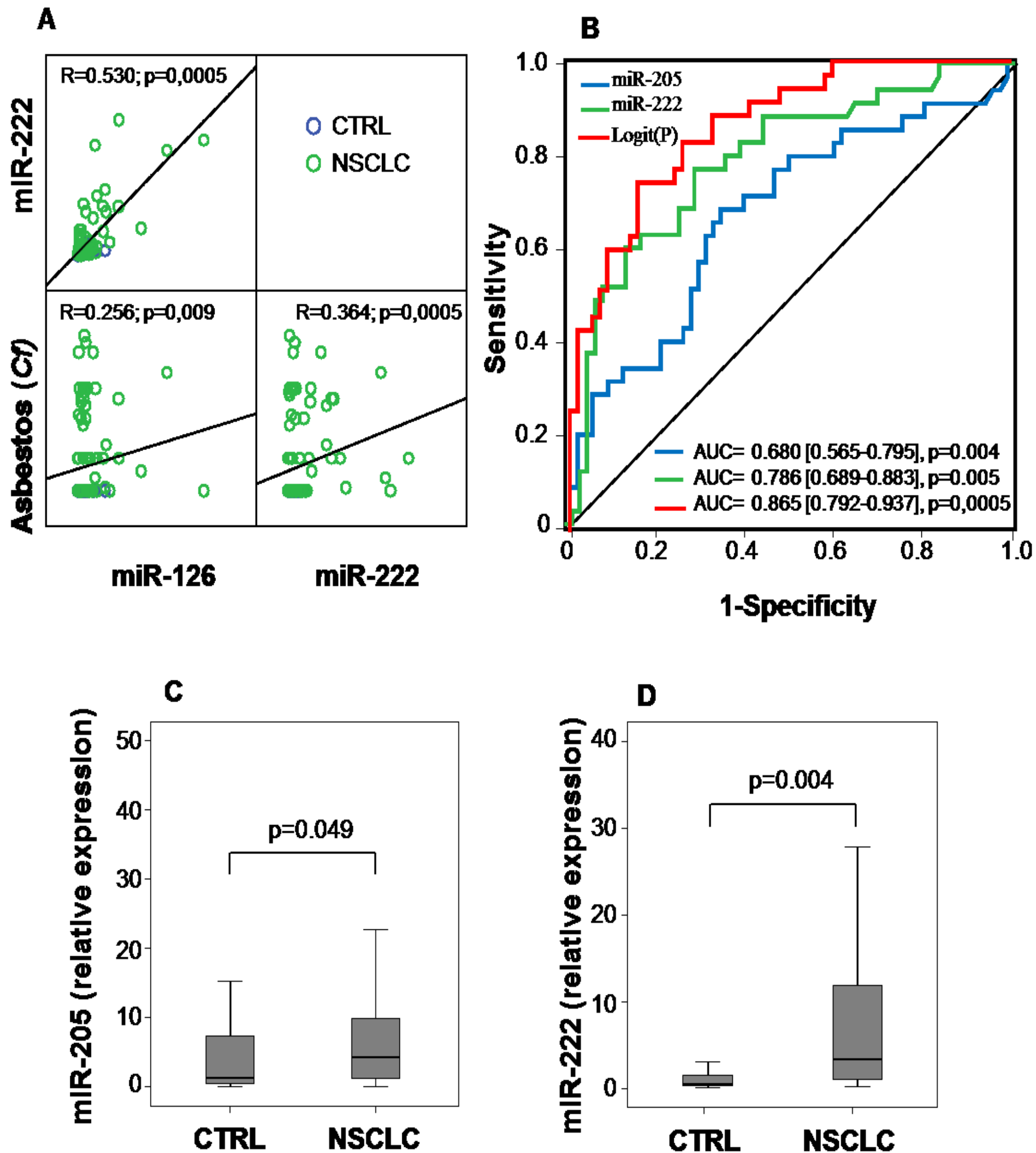


Figure 25. **Relationship between asbestos fibers and miRNAs, and their performance to discriminate NSCLC from controls (CTRL).** Correlation between cumulative asbestos fibers (*Cf*) and miRNAs. Correlation coefficient (R) was determined with the Spearman test (**A**). Receiver operating characteristics (ROC) curve of Logit (P) value in NSCLC and CTRL. Area under the ROC curves (AUC) and 95% CI are shown (**B**). Distribution of miR-205 (**C**), and miR-222 (**D**) between NSCLC and controls (CTRL). Significant differences were determined using t-Student test.

6.5. Asbestos-related miRNA with diagnostic value for Malignant Mesothelioma

As shown in **Fig.26** a positive correlation between miR-222 and miR-222/miR-126 ratio and cumulative asbestos fibers (*Cf*) has been found in MM group (**Fig.26A**). The miR-222/miR-126 ratio and miR-126 alone significantly differentiated MM from controls, with the best performance of logit (P), which include miR-126 and miR-205 in the predictive model (**Fig.26B**). The distribution of the miR-222/miR-126 ratio and miR-126 has been reported (**Fig.26C, D**).

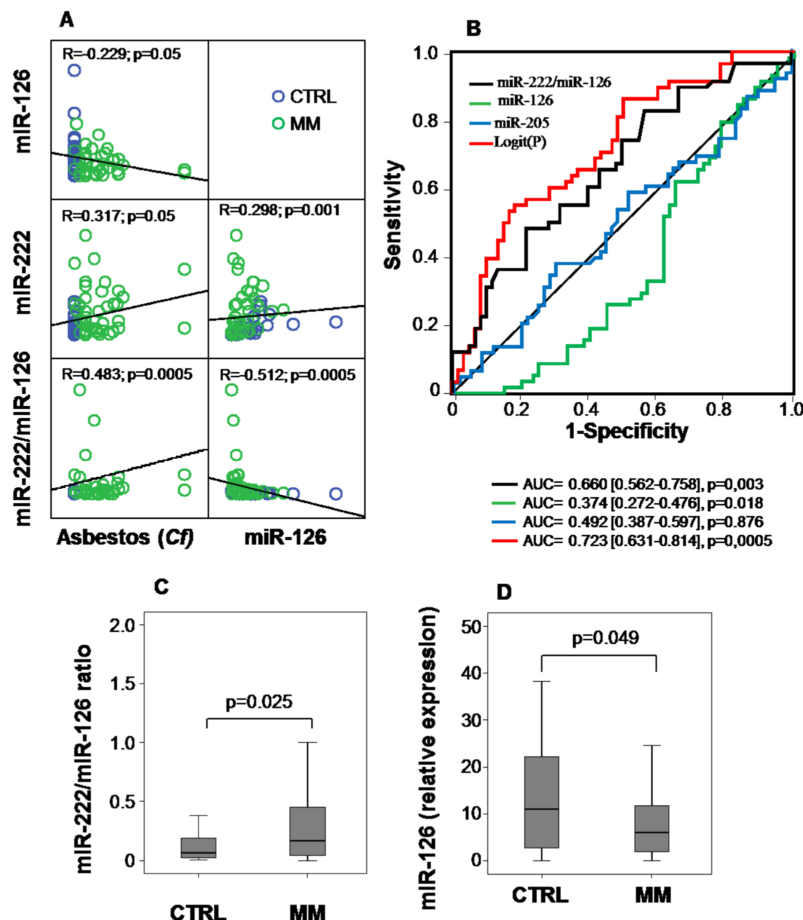


Figure 26. Relationship between asbestos fibers and miRNAs, and their performance to discriminate MM from controls (CTRL). Correlation between cumulative asbestos fibers (*Cf*) and miRNAs. Correlation coefficient (R) was determined with the Spearman test (**A**). Receiver operating characteristics (ROC) curve of miR-126, miR-222/miR-126 ratio and Logit (P) value in MM and CTRL. Area under the ROC curves (AUC) and 95% CI are shown (**B**). Distribution of miR-222/miR-126 ratio (**C**) and miR-126 (**D**) in MM and control group. Significant differences were determined using t-Student test.

6.6. Testing phase: asbestos-related miRNAs in the asbestos-exposed population

Taken together, miR-126, miR-222 and miR-205, miR-520g play important role in the asbestos-related lung malignancies. Experimental data indicates that exposure to carcinogens induces early alterations in miRNA expression. *Izzotti and Pulliero* (Izzotti and Pulliero, 2014) reported that for each carcinogen, there is a specific miRNA alteration signature. To evaluate whether the selected miRNAs are early responsive genes to asbestos exposure, the mina expression was evaluated in serum samples of a population exposed to asbestos. According to the period of asbestos-exposure, the subjects have been stratified as currently exposed, and ex-exposed with and without benign asbestos-related diseases (**Tab.8**).

	CTRL (n=20)	**EXP^{curr} (n=20)**	**EXP^{ex} (n=40)**		**EXP^{tot} (n=80)**
			No-ARDs	ARDs	
Age (yrs)	56±11	47±6	52±10	64±11	57±12
Gender (M/F %)	79/21	100/0	100/0	100/0	100/0
Smoking (%)					
No	65	25	40	25	32
Yes	10	15	17	50	33
Former	25	60	43	25	35
ARDs (%)		0	0	100	25

Table 8. **Demographic characteristics of asbestos-exposed population**. **CTRL**, healthy controls; **EXP^{curr}**, currently asbestos-exposed subjects; **EXP^{ex}**, ex asbestos-exposed subjects; **EXP^{tot}**, total asbestsos-exposed subjects; **ARDs**, asbestos-related benign diseases.

As shown in **Fig.27**, high level of miR-126 and miR-222 and their ratio with miR-205 and miR-520g were found in currently asbestos-exposed subjects. No changes in miRNA level have been observed in previously asbestos-exposed subjects (EXPex) with and without ARDs, thus supporting their role as early responsive miRNAs to the asbestos.

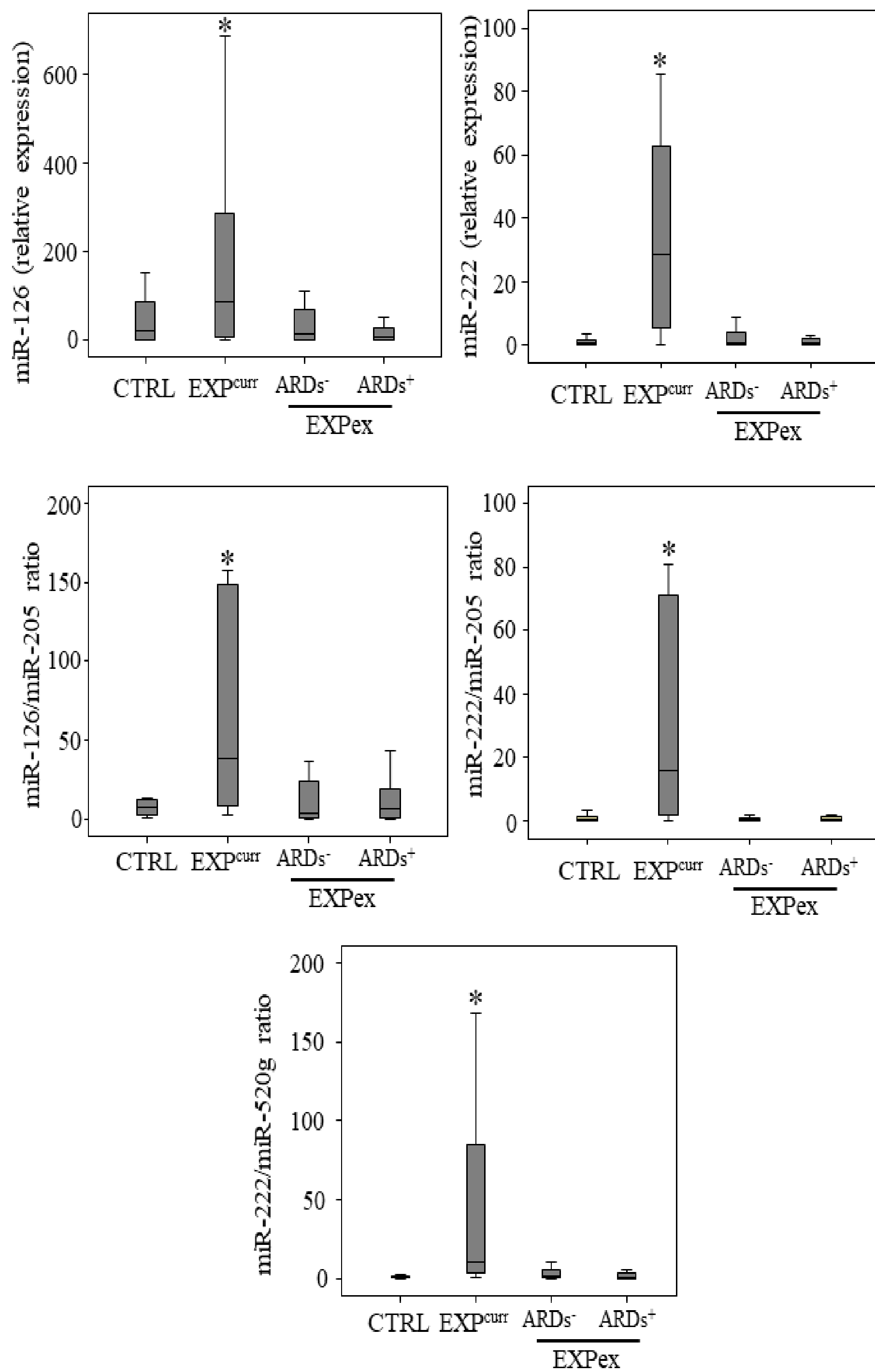


Figure 27. Tukey box plots of relative expression of miR-126 and miR-222, and miR-126/miR-205, miR-222/miR-205 and miR-222/miR-520g ratio in serum of healthy controls (**CTRL**) and asbestos-exposed subjects stratified as currently exposed (**EXP^{curr}**), ex exposed (**EXP^{ex}**) with (ARDs^{+}) and without (ARDs^{-}) benign asbestos-related diseases (ARDs). The box delimits the 25th and 75th percentiles. Horizontal line inside the box marks the medians. Mean expression values are marched with squares. Vertical lines mark the interval between 5th and 95th percentiles. Significant differences were determined using one-way ANOVA and Tukey-Kramer post-hoc test. *CTRL vs asbestos-exposed groups.

The first evidence that miRNA expression is early altered by exposure to environmental chemical carcinogens was shown for cigarette smoke (Izzotti et al., 2009). It was reported that miRNA alterations induced by occupational/environmental exposure to carcinogens are predictive of the feature appearance of cancer only when these miRNAs alterations are irreversible (Izzotti et al., 2011). Conversely, reversible miRNA changes represent adaptive rather than pathogenic mechanisms. The irreversibility of miRNA alterations is reflected in the inability of the cells to restore the physiological miRNA expression level despite the cessation of exposure to the carcinogen. Accordingly, the alterated level of miR-126, miR-222, miR-205 and miR-520g observed in currently asbestos-exposed subjects was not found in long-term asbestos-exposed subjects not affected by cancer (ex-exposed subjects), but was found alterated in long-term asbestos-exposed subjects affected by lung malignancies (lung cancer and malignant mesothelioma). Alterations of miRNA expression indicate their relevance to cancer development and progression. Such alterations should be cause of cancer onset rather than a consequence of cancer.

6.7. Asbestos-related miRNAs in carcinogen-induced pre-cancerous cells

To evaluate the asbestos specificity of identified miRNAs and the mechanism by which the asbestos exposure specifically altered their expression, an *in vitro* model was performed. Three different carcinogens involved in the lung cancer onset, Arsenic (As) Crocidolite (Asb), and hexavalent Chrome (Cr) were used to induce carcinogenicity in immortalized human alveolar epithelial BEAS-2B cells (He et al., 2014; Wang et al., 2016; Pratheeshkumar et al., 2016; Pratheeshkumar et al., 2016). After one month of exposure to

the carcinogens the survived cells were collected, and the transformed cell were selected in soft-agar system. As illustrated in **Fig. 28** the cells with acquired pre-cancerous phenotype (initiated cells) grew in agar suspension culture by forming colonies.

Figure 28. Selection of carcinogens-induced cell transformation. BEAS-2B cells were exposed to sodium arsenate ($NaAsO_2$, 1µM), crocidolite fibers (5µg/cm^2) and potassium dichromate ($K_2Cr_2O_7$, 0.5µM) for one month, and soft-agar colony formation evaluated.

The formed masses were collected, the pre-cancerous cells isolated and characterized for their proliferation rate, endogenous reactive oxygen species (ROS) level, and metabolism. Among the carcinogens tested, only chrome-induced pre-cancerous cells (BEAS-2B^{Cr}) showed increased cell proliferation, while high level of ROS, including ROS from mitochondria (mtROS), was found in Arsenic-induced pre-cancerous cells (BEAS-2B^{As}). No changes both in cell proliferation and in ROS level were found in Asbestos-induced pre-cancerous cells (BEAS-2B^{Asb}) (**Fig.29**).

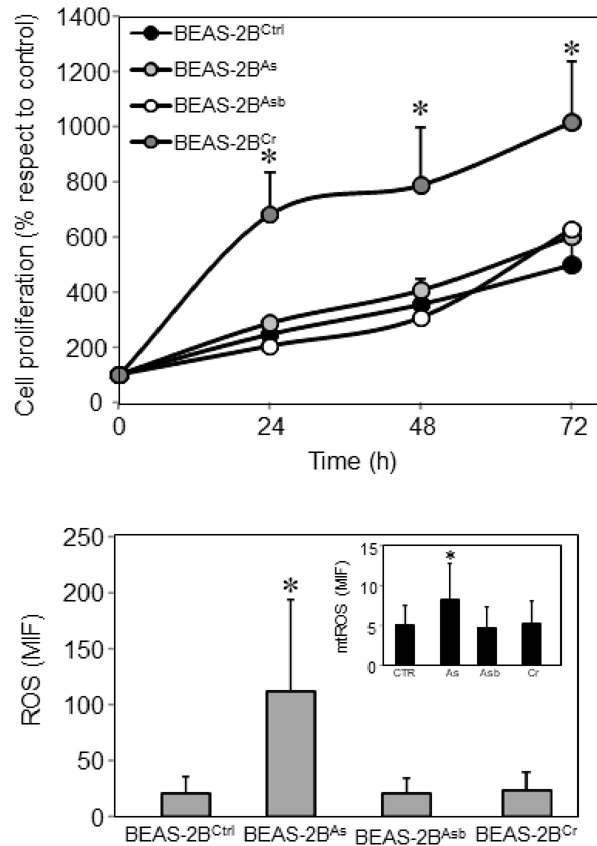


Figure 29. Cell proliferation and endogenous ROS level in pre-cancerous cells. Carcinogens-induced pre-cancerous cells (BEAS-2B^{As}, BEAS-2B^{Asb}, BEAS-2B^{Cr}) were growth at 24-48-72 hours and cell viability evaluate by MTT assay. Cell viability was represented as percentage respect to control (non-exposed cells). ROS generation was assessed using DCF (an indicator of hydrogen peroxide) or DHE (an indicator of superoxide, mtROS) probes, and the results expressed as Mean Intensity Fluorescence (MIF). Data shown are mean values ± SD of at least three independent experiments performed in triplicate. The symbol '*' indicates statistically significant differences between pre-cancerous cells and wild-type BEAS-2B (BEAS-2B^{Ctrl}) cells with $P < 0.05$.

Conversely, Asbestos-induced pre-cancerous cell (BEAS-2B^{Asb}) showed higher mitochondrial redox activity respect to wild-type BEAS-2B^{Ctrl}, and carcinogen-induced initiated cells (BEAS-2B^{As} and BEAS-2B^{Cr}). The increased mitochondrial activity was associated with increased mitochondrial membrane potential in all the carcinogen-induced initiated cells (**Fig.30**).

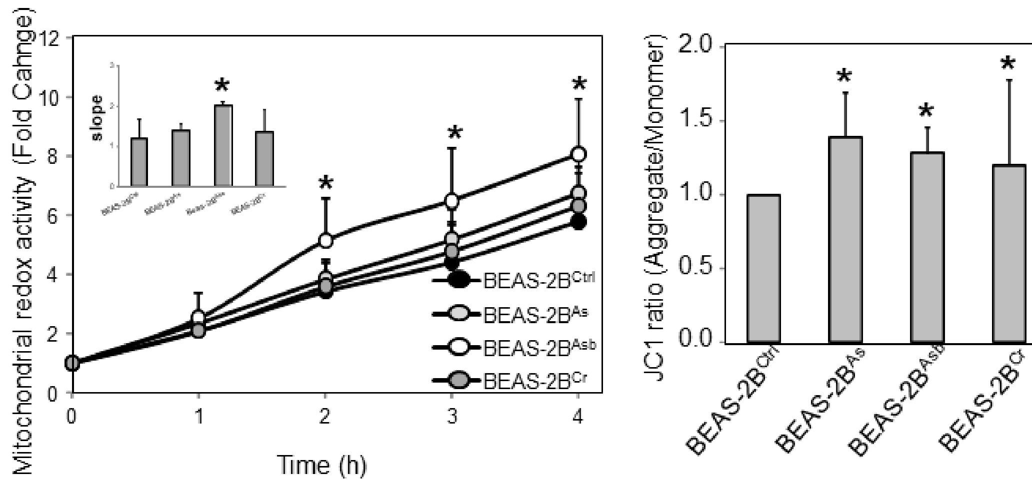


Figure 30. Mitochondrial activity in carcinogens-induced pre-cancerous cells. Carcinogens-induced pre-cancerous cells (BEAS-2B^{As}, BEAS-2B^{Asb} and BEAS-2B^{Cr}) and wild-type BEAS-2B (BEAS-2B^{Ctrl}) were evaluated for mitochondrial redox activity (MRA) by resuzarin assay. Results were normalized for protein content and were expressed as fold-change respect to basal (**left-panel**). The mitochondrial membrane potential was evaluated by JC1 probe and the $\Delta\Psi_m$ change was detected by flow cytometer and the results expressed as aggregates/monomer ratio (**right-panel**). The results are mean values ± S.D. of three individual experiments performed in triplicate. Comparisons among groups were determined by one-way ANOVA with Tukey post-hoc analysis, the symbol '*' indicates significant differences compared to control with $p<0.05$.

These pre-cancerous cells showed increased glucose up-take associated with a decrease of its intracellular level (**Fig.31A, B**). Either the inhibition of the mitochondrial oxidative phosphorylation (OXPHOS) at the complex I by rotenone (Rot), or the inhibition of the glycolysis by the inhibitor 2-deoxyglucose (2DG) slightly increased the intracellular glucose level respect to the wild-type BEAS-2B. The ATP level did not change among the pre-cancerous cells and wild-type BEAS-2B cells. The inhibition of OXPHOS or glycolysis did not affect ATP, which was reduced when rotenone and 2DG were combined, indicating a compensatory mechanism. Conversely, an increase in lactate was observed in BEAS-2B^{Asb}, and the inhibition of OXPHOS further increased its levels. Taken together, the increased mitochondrial redox activity found in Asbestos-induced pre-cancerous cells resulted in low intracellular glucose steady-state, thus stimulating its up-take. The high glucose rate consumption rather than reflecting in ATP production, resulted

in lactate formation, thus supporting the glycolytic metabolic change occurred in these cells (**Fig.31 C, D**).

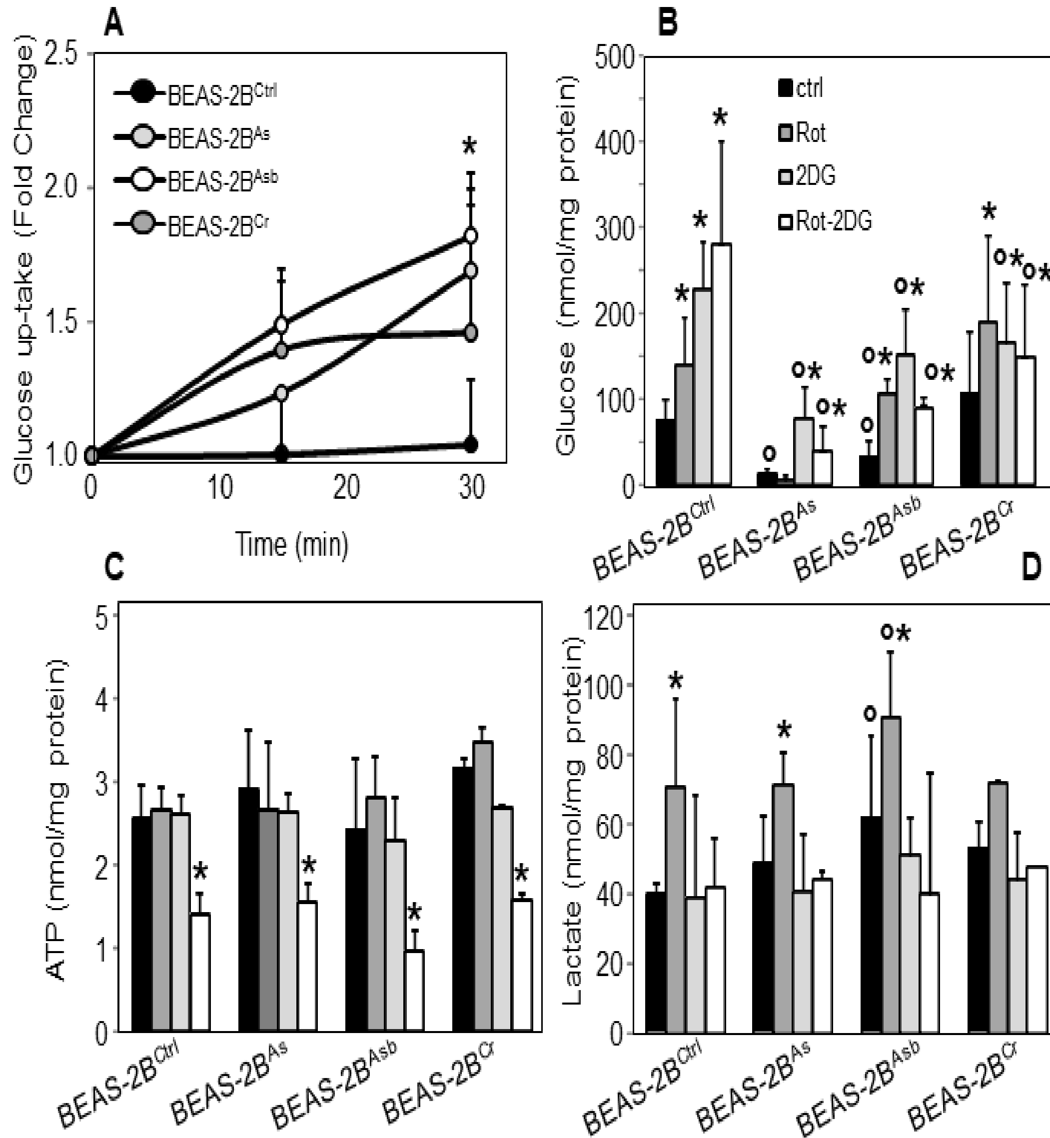


Figure 31. **Metabolic changes in carcinogens-induced pre-cancerous cells**. (**A**)The glucose up-take was evaluated with 2-nitrobenzodeoxyglucose (2-NBDG) probe (50µM) in low-glucose DMEM. The level of fluorescent glucose analogue in the cells was evaluated at 550/590 nm using a fluorescence plate reader. The cells were evaluated for intracellular glucose (**B**), ATP (**C**), and lactate (**D**) in the presence of Rotenone (Rot, 20 µM, 5 h) or 2-deoxyglucose (2DG, 5 mM, 5 h), or both (Rot+2DG), and the results expressed as nmol/mg protein. The results are mean values ± S.D. of three individual experiments performed in triplicate. Comparisons among groups were determined by one-way ANOVA with Tukey post-hoc analysis, the symbol '*' indicates significant differences compared to treatments, symbol '°' indicates significance compared to control with $p<0.05$.

6.8. Signal transduction pathway modulates the asbestos-related miRNA expression: role of stroma environmental

It was reported that asbestos fibers induced protracted phosphorylation of the mitogen-activated protein (MAP) kinases and extracellular signal-regulated kinases (ERK) 1 and 2, and increased kinase activity of ERK2 (Zanella CL et al, 1996).

To explore whether asbestos causes receptor tyrosine kinase (RTK) activation, the carcinogen-induced pre-cancerous cells were evaluated for signal transduction pathway in absence and presence of the macrophages-induced proinflammatory cytokines in a co-culture system (*see Materials and Methods*).

As reported in **Fig.32**, Cr-induced, and in major extent Asbestos-induced pre-cancerous cells (Beas-2B^{Asb}) caused a persistent phosphorylation of EGFR resulting in downstream activation of the AKT and MAPK signalling pathways. The presence of inflammatory cytokines attenuated the signalling response.

Previous studies have shown that aberrant activation of cell signalling cascades, like those associated with epidermal growth factor receptor (EGFR), may be a critical factor in mineral fibres associated carcinogenic responses (Carbonari et al., 2011; Taylor et al., 2013).

To investigate the involvement of EGFR-pathway in asbestos-related miRNAs, the selected miRNAs (miR-126, miR-205, miR-222, and miR-520g) were evaluated in carcinogen-induced pre-cancerous cells in absence and presence on the EGFR inhibitor AG1478. Excepted for miR-520g, all miRNAs were upregulated in the pre-cancerous cells. Higher expression of miR-126, miR-205, miR-222, and down-regulation of miR-520g were found in BEAS-2B^{Asb} respect to BEAS-2B^{As} and BEAS-2B^{Cr}.

The inhibition of EGFR-pathway markedly increased miR-126 and miR-520g and significantly reduced miR-222 expression to the basal level. The incubation with macrophages-induced cytokines markedly boosts the results (**Fig.33 A, B**).

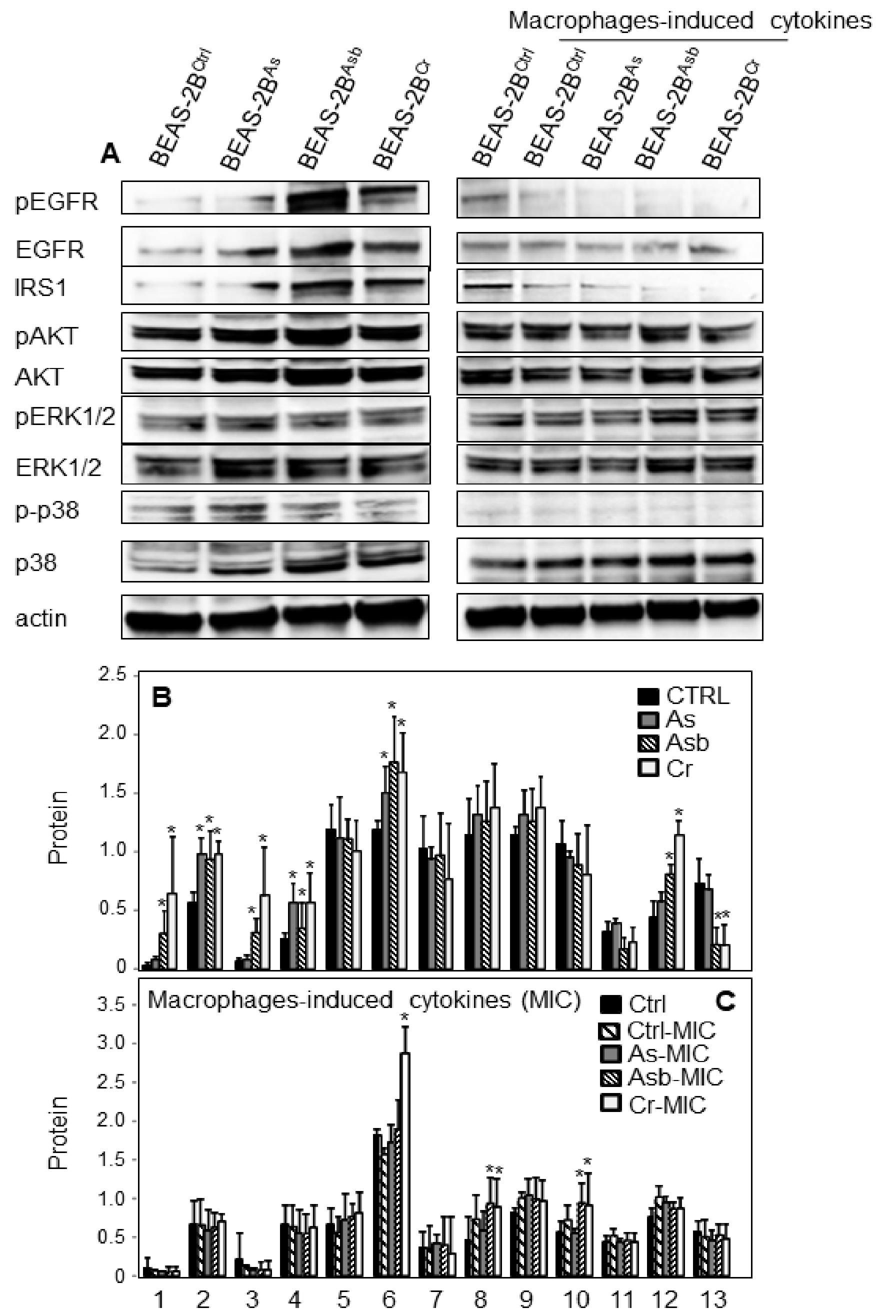


Figure 32. Immunoblotting evaluation of expression and phosphorylation of signalling intermediates including pEGFR (1), EGFR (2), pEGFR/EGFR ratio (3), IRS1 (4), pAKT (5) AKT (6), pAKT/AKT ratio (7), pERK1/2 (8), ERK1/2 (9), pERK1/2/ERK1/2 ratio (10), p-p38 (11), p38 (12), p-p38/p38 ratio (13) in

BEAS-2B^{As}, BEAS-2B^{Asb}, BEAS-2B^{Cr} and BEAS-2B^{Ctrl} in absence and presence of the macrophages-induced cytokines (MIC) system. β-Actin staining is a loading control (**A**) and the densitometry analysis in absence (**B**) and presence of the macrophages-induced cytokines (MIC) system (**C**) has been shown. The data shown are mean values ± S.D. of three independent experiments. Comparisons among groups were determined by one-way ANOVA with Tukey post hoc analysis, the symbol '*' indicates significantly different values compared to wild-type BEAS-2B (BEAS-2B^{Ctrl}).

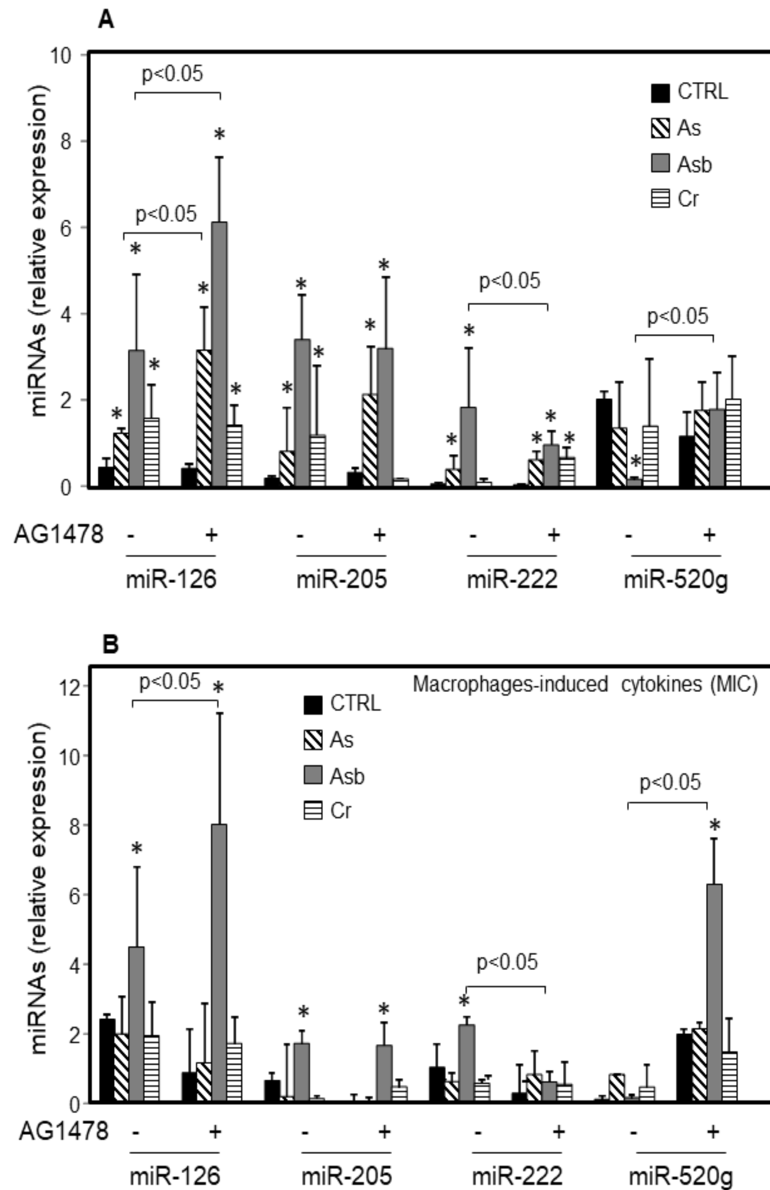


Figure 33. MiRNA expression in carcinogens-induced pre-cancerous cells. BEAS-2B^{Ctrl}, BEAS-2B^{As}, BEAS-2B^{Asb}, BEAS-2B^{Cr} have been evaluated for miRNA-panel consisting of miR-126, miR-205, miR-222, and miR-520g, with and without EGFR inhibitor (AG1478) in absence (**A**) and presence (**B**) of macrophages-induced cytokines. The data shown are mean values ± S.D. of three independent experiments. Comparisons among groups were determined by one-way ANOVA with Tukey post hoc analysis, the symbol '*' indicates significantly different values compared to wild-type BEAS-2B (BEAS-2B^{Ctrl}).

Carcinogenesis involves active cross talk between cancer cells and stromal cells to develop supportive microenvironment. In this context, asbestos-related miRNAs were evaluated in *a vitro* model mimicking tumour stroma by coculturing carcinogen-induced pre-cancerous cells with fibroblasts (IMR-90) and human umbilical vessel endothelial cells (HUVEC). As showed in **Fig.34,** the interaction of BEAS-2B^{Ctrl}, BEAS-2B^{As}, BEAS-2B^{Asb}, BEAS-2B^{Cr} with the stroma cells (IMR-90 and HUVEC) confers upregulation of all asbestos-related miRNAs (miR-126, miR-205, miR-222, miR-520g) in the pre-cancerous cells.

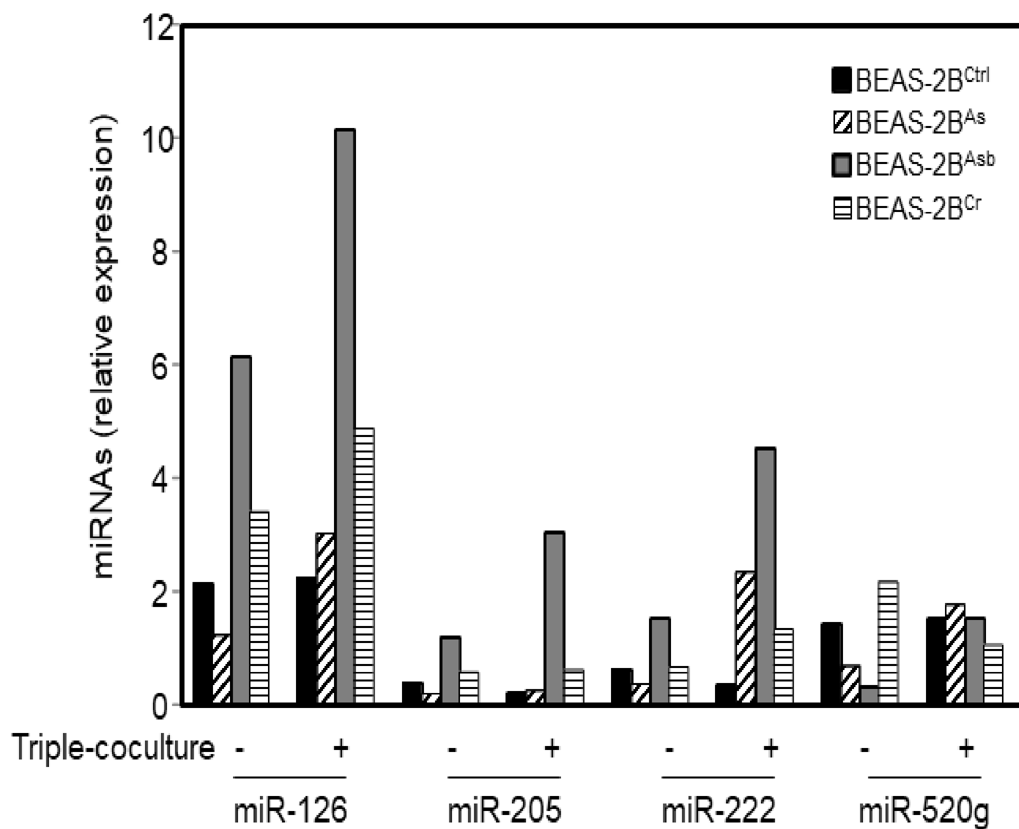


Figure 34. Asbestos-related miRNAs in triple-coculture model. MiR-126, miR-205, miR-222, miR-520g expression was evaluated in BEAS-2B^{Ctrl}, BEAS-2B^{As}, BEAS-2B^{Asb}, BEAS-2B^{Cr} alone or cocultured with fibroblasts (IMR-90) and human umbilical endothelial cells (HUVEC).

7. Discussion

The most common asbestos-associated diseases are benign pleural disease, asbestosis, lung carcinoma (small cell, squamous, and adenocarcinoma) and malignant mesothelioma. Asbestos causes more lung cancer deaths than malignant mesothelioma of the pleura and no safe threshold for asbestos exposure has been established (Hodgson and Darton, 2000). Asbestos-related diseases persist, because millions of workers have had prior exposure and many industrializing countries continue to use asbestos. Globally, an estimated 107,000 people die annually from lung cancer, malignant mesothelioma, and asbestosis due to occupational and environmental asbestos exposure. In combination with smoking, asbestos exposure increases the risk of lung cancer in a synergistic manner (Ngamwong et al., 2015), which has made difficult to elucidate those alterations which are related specifically to asbestos exposure.

Multiple epigenetics play an important role in gene expression whose abnormality might be reflected in the alteration of the expression of genes. It is known that epigenetic mechanisms are involved in the regulation of miRNAs, a class of non-coding RNAs. MiRNA can be targeted by epigenetic modification; as well, miRNAs can target regulators of epigenic pathway. In recent years, the analysis of microRNA profile has been established in diagnosis for their high specificity and sensitivity in detecting many cancers (Pal et al., 2015; von Brandenstein et al., 2012).

MiRNA expression profile can be used to distinguish normal from malignant tissues, to identify the tissue origin in poorly differentiated tumors or tumors of unknown origin and to distinguish the different subtypes of the same tumor.

Furthermore, miRNA expression is early altered by exposure to environmental carcinogens (Izzotti et al., 2014). Accordingly, altered miRNA levels can be proposed as biomarker signature to characterise the carcinogen-induced cell transformation.

In the present study, consequential study phases have been performed to identify miRNAs associated to the development of asbestos-induced malignancies. These phases included, a first discovery phase, where asbestos-associated miRNAs have been identified, which was followed by a verification phase to determine whether specific tissue miRNAs are detectable in serum samples. MiRNAs deregulated in tissue specimens were rarely detected in serum samples. Here, four asbestos-related miRNAs consisting of miR-126, miR-205, miR-222 and miR-520g involved in malignant diseases were detected in serum. Because the normalization of miRNA data in serum samples is still a controversial issue, (Boeri et al., 2011; Fortunato et al., 2014) the ratios between the expression values of all miRNAs consistently expressed in serum were also computed. Each value of a single miRNA as relative expression and their ratio values were used for class prediction of asbestos-related diseases in the training and validation sets.

Class comparison analysis was initially performed in the training set to identify a group of miRNAs showing a predictive model to discriminate asbestos-related malignancies ($NSCLC^{Asb}$ and MM) from non-asbestos-related lung cancer (NSCLC), and disease-free patients (CTRL). The asbestos signature obtained was then used to calculate specificity and sensitivity in an independent validation set.

In the predictive model, both miR-222 and age best depicted the asbestos-related malignancies (***cf Fig.21)***.The association of miR-222 with miR-222/miR-126 best characterised the non-asbestos related NSCLC group. We found that miR-126 and miR-222 were strongly associated with asbestos exposure and both miRNAs were involved in

major pathways linked to cancer. MiR-126 is classified as “angio-miR” for its involvement in regulating angiogenesis and vascular integrity through the expression in endothelial cells. The regulation of angiogenesis is carried out through the restriction of VEGF-induced signals. MiR-126 targets SPRED1(Sprouty-related EVH1 domain-containing protein 1) and PI3KR1 (subunit p85β di PI3K), these proteins inhibit receptor kinase-induced signals via the MAPK and PI3K pathways (Fish et al., 2008).

Then, low levels of miR-126 were detected in many cancers (Zhou et al., 2013; Yanaihara et al., 2006; Tavazoie et al., 2008; Guo et al., 2008; Li et al., 2014; Luan et al., 2015; Santarelli et al., 2011; Tomasetti et al., 2012; Grimolizzi et al., 2017).

Abnormal hypermethylation of the Egfl-7 gene (coding for miR-126) observed in many tumor cells may explain the low levels of miR-126 found in cancers (Saito et al., 2009; Sasahira et al., 2012). Rather than the direct methylation of Egfl-7 gene in cancer cells, the cross talk between tumor cells and CAFs in the stroma induces miR-126 down-regulation in endothelial cells causing an increase of tube formation. The concomitance up-regulation of pro-angiogenic factors, such as andromedullin (ADM) and vascular endothelial growth factor (VEGF) induces tumor growth and invasion by facilitating angiogenesis (Huang and Chu, 2014).

The expression of miR-126 decreased significantly in cancer, particularly in highly metastatic cell lines (Tavazoie et al., 2008; Du et al., 2014; Png et al., 2011).

Insulin-like growth factor binding protein 2 (IGFBP2) secreted by metastatic cells recruits endothelia by modulating insulin growth factor (IGF1)-mediated activation of the IGF type-I receptor on endothelial cells; whereas c-Mer tyrosine kinase (MERTK) receptor cleaved from metastatic cells promotes endothelial recruitment by competitively antagonizing the binding of its ligand Growth arrest-specific 6 (GAS6) to endothelial

MERTK receptors. Co-injection of endothelial cells with breast cancer cells rescues their miR-126-induced metastatic defect, revealing a novel and important role for endothelial interactions in metastatic initiation (Png et al., 2011). MiR-126, independently suppress the sequential recruitment of mesenchymal stem cells and inflammatory monocytes into the tumor stroma to inhibit lung metastasis by breast tumor cells in a mouse xenograft model (Zhang et al., 2013).

In addition, miR-222 is associated with lung cancer aggressiveness (Mao et al., 2014) and AKT signalling is the major pathway influenced by miR-222 (Wong et al., 2010). Although, miR-222 was upregulated in NSCLC samples and associated with advanced clinical stage and lymphnode metastases as previously reported (Mao et al., 2014), the association of miR-205 in the diagnostic predictive model resulted in the best disease prediction (***cf Fig.24***).

Indeed miR-205 was revealed as a novel diagnostic and prognostic biomarker for lung cancer (Li et al., 2017; Nymark et al., 2011). It is located in a lung cancer-associated genomic amplification region at 1q32.2, participates in tumorigenesis of lung cancer, especially in the occurrence, development and prognosis of NSCLC. It markedly overexpresses in the tissue of lung cancer and serves as a prospective diagnostic biomarker for pulmonary diseases.

Overexpression of miR-205 promoted NSCLC cell invasion and metastasis through regulating an epithelial phenotype with increased E-cadherin and reduced fibronectin. MiR-205 was further developed to identify squamous cell lung carcinoma (SCC) and adenocarcinoma (ADC) subtypes of NSCLC.

Indeed, *Jiang et al.* (Jiang et al., 2013) showed that miR-205 expression was significantly higher in NSCLC tissues and serum, which was a good diagnostic biomarker

for NSCLC. Then, over-expression of miR-205 was also detected in the normal samples from asbestos-exposed patients with SCC compared with those from nonexposed patients. It is tempting to hypothesize that asbestos is able to induce the expression of miR-205 at an earlier stage of carcinogenesis in exposed individuals as compared with nonexposed subjects. Indeed, *Nymark et al.* (Nymark et al., 2011) observed that although this miRNA is not specific for asbestos exposure in lung cancer, it might be an asbestos-specific early event in SCC.

The over-expression of miR-205 in the asbestos-related samples correlated with the down-regulation of distinct target genes; e.g., TXNRD1, which is involved in oxidative stress; SMAD1, which shows reduced protein levels in asbestos-treated mouse lungs and the histone demethylation involved gene KDM4B at 19p13.3 (Myllärniemi et al., 2008).

As previously reported (Santarelli et al., 2011; Tomasetti et al., 2012), miR-126 and in a major extent miR-222/miR-126 ratio showed significant diagnostic value for malignant mesothelioma, with higher ROC curve when miR-126 was combined with miR-205 in the predictive model (***cf Fig***.***25***).

To elucidate the role of mediation effect of these miRNAs between asbestos-exposure and the tumour development, a disease-free population exposed to asbestos, stratified as currently exposed and ex-exposed with and without benign asbestos-related diseases (ARDs), was evaluated for the selected miRNAs. Notably, increased expression of miR-126 and miR-222 including miR-126/miR-205, miR-222/miR-205, and miR-222/miR-520g ratios were found only in currently exposed subjects (***cf Fig.26***) and not in ex-exposed. This suggests that increased expression of miR-126 and miR-222 may represent an adaptation response to the stimulus of the carcinogen, as shown in currently exposed subjects.

Increased expression of miR-222 was observed in air pollution exposure and metal-rich particulate (Vriens A et al., 2016; Vrijens et al., 2015; Bollati et al., 2010). MiRNA changes may be sensitive indicators of the biological effects of acute and chronic environmental exposure. Results from animal studies suggest that miRNA expression changes in response to environmental carcinogens exposure are transient and revert to normal levels after recovery from exposure (Rager et al., 2013; Rager et al., 2014). Accordingly, ex-exposed subjects to asbestos did not show any changes in miRNA expression (***cf Fig.26***).

Based on these evidences, we can postulate that irreversible alterations of miRNA expression can result in carcinogenesis when accompanied by other molecular damages. The molecular mechanisms behind the altered expression of miRNA in cancer have been investigated (Riedmann et al., 2015). Altered miRNA expression is coupled to changes in chromatin structure effecting differential transcriptional and splicing patterns of genes, which can affect downstream signalling pathways. Increasing body of evidences suggest that receptor tyrosine kinase (RTK) activation participates in the oncogenic progression from non-neoplastic mesothelial progenitor cells to mesothelioma (Menges et al, 2010; Perrone et al, 2010). The RTKs epidermal growth factor receptor (EGFR), MET, AXL, and EPHB4, are upregulated and activated in some mesotheliomas (Ou et al, 2011), resulting in downstream activation of the AKT and MAPK signalling pathways (Zanella et al, 1996; Pache et al, 1998). Increased expression of EGFR was found in the Asbestos-initiated cells, causing activation of the downstream effector AKT and p38 MAPK signalling (***cf Fig.31***). Asbestos-mediated activation of EGFR-AKT pathway resulted in miR-126 and miR-222 upregulation associated with miR-520g downregulation. Both miR-222 and miR-520g were reversed by inhibiting EGFR (AG1478), suggesting its involvement in asbestos-

induced miRNA regulation (***cf Fig.32***). Moreover, the upregulation of miR-126 and miR-222 was enhanced when Asbestos-induced pre-cancerous cells were cocultured with tumour stromal cells, such as fibroblasts and endothelial cells. Thus, highlighting the role of the cancer stroma cross talk in the expression of miR-126 and miR-222 to facilitate angiogenesis and invasion growth of lung malignancies (***cf Fig.33***).

The EGFR upregulation of this pathway has been found in several tumours, including NSCLC, head and neck carcinoma, gliomas, and colorectal carcinoma (Chung et al., 2006). The EGFR transactivation stimulates a network of cytoplasmic transduction molecules, leading to a transcriptional activation and consequent modulation of a wide variety of cellular functions, including cell proliferation, migration, adhesion, and differentiation. The activation of the EGFR pathway is also responsible for the transcriptional activation of specific microRNAs, including miR-222 (Teixeira et al., 2012; Garofalo et al., 2011). The upregulation of miR-222 has been described in several human cancers including glioblastoma, melanoma, hepatocellular carcinoma, kidney and bladder cancers, gastric cancer, pancreatic cancer, ovarian cancer and prostate cancer (Negrini et al., 2009; Coppola et al., 2010). This upregulation was also observed in circulation, as free miRNAs, in renal cell carcinoma patients compared with healthy individuals (Teixeira et al., 2012).

In addition, miR-222 is involved in the metastatic process. Its expression levels are correlated with the repression of transcriptional factors, such as the zinc finger transcription factor Trps1 (TRPS1). The repression of this factor causes an increase in the levels of the zinc finger E-box-binding homeobox 2 protein (ZEB2), which promotes a crucial step in the epithelial-to-mesenchymal transition (EMT), essential for the development of metastasis (Shah and Calin, 2011).

MiR-222 seems to have the ability to modulate cell cycle progression. *Miller and co-workers* demonstrated that miR-222 could modulate cell cycle progression, by repressing cell cycle inhibitor proteins p27/Kip1 and p57, facilitating cell proliferation and self-renewal (Miller et al., 2008).

In addition to downstream modulators of the EGFR-RAS-RAF-MEK pathways, the high-mobility group A1 (HMGA1) in NSCLC samples regulates miR-222. Chromatin immunoprecipitation (CHIP) assay revealed that HMGA1 directly bound to the proximal promoter of miR-222 in NSCLC cells. HMGA1 silencing reduced miR-222 transcriptional activity, whereas forced HMGA1 expression increased it, indicating that miR-222 was directly regulated by HMGA1. In addition, the overexpression of miR-222 in NSCLC could lead to repression of phosphatase 2A subunit B (PPP2R2A) expression and activation of AKT signalling (Zhang et al., 2011).

In conclusion, this study uncovers miRNAs that are potentially involved in asbestos-related malignancies and their expression outline mechanisms whereby miRNAs may be involved in Asbestos-induced pathogenesis.

8. Conclusions.

Asbestos-related lung diseases remain a significant challenge to health care providers as well as to investigators studying the basic mechanisms that underlie asbestos-induced pulmonary toxicity. Given the long latency between asbestos exposure and disease as well as the direct relationship between asbestos consumption and mortality from asbestos-related lung diseases, a total worldwide asbestos ban is strongly supported. It is believed that the morbidity and mortality caused by asbestos exposure will peak in the next decade (Sen, 2015). Minimally invasive monitoring approaches are thus urgently needed, both to extend patient lifespan and to preserve their quality of life. Increasing evidence supports the key importance of miRNAs in asbestos-related malignancies diagnosis, prognosis and treatment. Computed tomography and chest x-rays remain the more sensitive approaches to evaluate objective clinical parameters of exposure (Yusa et al., 2015; Elshazley et al., 2011; Terra-Filho et al., 2015). The identification of circulating miRNAs is one of the major scientific breakthroughts in recent years and it has revolutionized cell biology and medical science. Blood-based miRNA profiling is not as reliable as tissue-based miRNA-profiling, but offers the potential for early, non-invasive, sensitive and specific asbestos-related cancer detection and screening. Recently, several circulating miRNAs have been identified as potential serum biomarkers in different cancer types (Allegra et al., 2012). These serum miRNAs may be effective as predictive biomarkers in cancer. It has been suggested that these molecules could act at a distance, moving through the circulatory stream. In summary, this study identified a panel of four serum miRNAs (miR-126, miR-205, miR-222 and miR-520g) as a signature for lung asbestos-related malignancies.These miRNAs are useful in discriminating asbestos-related malignancies ($NSCLC^{Asb}$ and MM) from non-asbestos-related lung cancer (NSCLC), and disease-free patients (CTRL). These

finding may provide a foundation for development of a novel noninvasive test to predict lung asbestos-related malignancies, determination of innovative therapeutic strategies and for their possible and future applicability in follow-up of ex- and currently asbestos-exposed workers.

Thus, this study will serve as a basis for further large-scale validation of circulating non-coding RNAs to enable their introduction into the clinical setting. However, furher studies are needed to validate those serum miRNA signature in an independent cohort study.

It is foreseeable that the molecular analysis of liquid biopsies (blood, urine, etc.) will become the gold standard for developing noninvasive diagnostic and prognostic tests. Such tests will not require hospitalization and could be offered in peripheral health centers, therefore potentially augmenting the accrual of high-risk individuals to cancer screening programs. In addition, the possibility to collect multiple samples from the same patients will definitely help clinicians to better monitor therapy response and decide for an optimal treatment.

In conclusion, even if further validation studies are needed, here we demonstrate that asbestos-related microRNAs deserve attention as potential non-invasive biomarkers for asbestos-related lung malignancies in the diagnostic setting.

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