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New circulating microRNAs in serum samples from malignant pleura mesothelioma affected patients as new biomarkers of this neoplasm

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ABBREVIATIONS

Abbreviations	Signification
HMC	Healthy mesothelial cells
MPM	Malignant pleura mesothelioma
µm	Micrometer
PDGF-A	Platelet-derived growth factor subunit A
SV40	Simian virus 40
agT	Large T antigen
miRNA o miR	microRNA
RNA	Ribonucleic acid
nt	Nucleotides
dsRNA	Double-stranded RNA
RISC	RNA-induced silencing complex
UTR	Un-traslated region
PPP6C	Pro-survival phosphatase PPP6C
kb	Chilobase
kDa	chiloDalton
NF-kB	Nuclear factor-kappa B
RT-qPCR	Real-time quantitative reverse-transcriptase polymerase chain reaction
RT-PCR	Real-time reverse-transcriptase polymerase chain reaction
DNA	Deoxyribonucleic acid
ng	Nanogram

µl	Microliter
°C	Celsius degrees
min	Minutes
h	Hours
mM	Millimolar
g	Gravitational coefficient
ml	Millilitro
PBS	Phosphate buffered saline
FBS	Fetal bovine serum
nM	Nanomolar
µM	Micromolar
M	Molar
fmol	Fentomole
mg	Milligrammes
FOXO	Forkhead box O
E2F	Transcription factors
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CDK	Cyclin-dependent kinase
PTEN	Phosphatase and tensin homolog
mRNA	Messenger RNA
BTG2	B-cell translocation gene 2
SV40	simian virus 40

BKV	BKPyV,BK polyomavirus
JCV	JCPyV, JC polyomavirus
KIV	KIPyV, KI polyomavirus
WUV	WUPyV,WU polyomavirus
HPyV11/STLPyV	Human polyomavirus 11
HPyV10	Human polyomavirus 10
HPyV7	Human polyomavirus 7
HPyV6	Human polyomavirus 6
HPyV9	Human polyomavirus 9
HPyV12	Human polyomavirus 12
MCPyV	Merkel cell polyomavirus
TSV	TSPyV, Trichodysplasia spinulosa-associated polyomavirus

1. INTRODUCTION

1.1 MESOTHELIUM AND MALIGNANT PLEURAL MESOTHELIOMA

1.1.1 Histo-cytological mesothelium

Mesothelium is a simple squamous tissue that covers and protects internal organs (Mutsaers 2004). It is made up of a layer of highly specialized mesothelial cells (HMC) that rest on the connective fibrous lamina, which lines the entire surface of the three serous cavities of the body: the pleural cavity in the lungs, the pericardium surrounding the heart and the peritoneal, which line the internal organs in the abdominal cavity by fixing them to the abdomen walls aiding maintenance of their natural position. (Yung, Li et al. 2006)

The embryological origin and characteristics of these cells make them a particular tissue and they are derived from mesoderm (Mutsaers 2004), not ectoderm like other tissue of coating. This peculiarity is further reflected in that mesothelial cells have both characteristics, which are typical of mesenchymal-derived cells as well as ectodermal cells. Cytokeratin rich intermediate filaments are also present and are typical epithelial markers, in addition to vimentin rich intermediate filaments, which are typical mesodermal markers. The phenotype of these mixed epithelial and mesenchymal cells promotes numerous physiological processes, such as tissue recovery following lesion. Inflammation and neoplastic transformation (Mutsaers 2004) responses are also involved in mesothelium pathophysiology. Finally, mesothelial tissue is also characterised by its response to mitogen stimulus following lesion. Indeed, mesothelium renews very slowly but if it was damaged, its mitotic index increases significantly, while its multipotent cells migrate

through the serous cavities towards the lesion site in order to regenerate tissue (Mutsaers 2004).

Mesothelial cells have an elongated polygonal shape which can vary to cuboidal or cylindrical depending on the location. Cells are connected to each other with tight junctions and adherents which are responsible for the characteristic semi-permeability of mesothelium, with both desmosomes and gap type junctions that allow intercellular communication. The apical surface of these cells is covered with numerous microvillus. In addition, the cytoplasm contains microtubules, microfilaments and scattered bundles of intermediate filaments which are rich in actin, vimentin, and cytokeratin (Jaurand and Fleury-Feith 2005). These cytological features allow mesothelial cells to play a key role in maintaining internal organ homeostasis by actively participating in fluid and cell transportation through serous cavities, antigen presentation, inflammatory response, surrounding damaged tissue restoration, coagulation and fibrinolysis and cancer cell accession (Mutsaers 2004). Previous studies have shown that compromised surface mesothelial is a preferential adhesion site for tumour cells (Cunliffe and Sugarbaker 1989). Finally, mesothelial cells are also able to endocitate foreign substances. This characteristic is the basis of their susceptibility to neoplastic transformation mediated by asbestos fibres (Liu, Ernst et al. 2000).

1.1.2 MALIGNANT PLEURAL MESOTHELIOMA

1.1.2.1 The main risks

1.1.2.1.1 Asbestos

Asbestos is a group of minerals that occur naturally as bundles of fibers. These fibers are found in soil and rocks in many parts of the world. They are made mainly of silicon and oxygen, but they also contain other elements. There are 2 main types of asbestos:

- *Chrysotile asbestos*, also known as white asbestos, is the most common type of asbestos in industrial applications. When looked at under the microscope, chrysotile asbestos fibers wrap around themselves in a spiral, which is why this form of asbestos is also called serpentine or curly asbestos.
- *Amphibole asbestos* fibers are straight and needle-like. There are several types of amphibole fibers, including amosite (brown asbestos), crocidolite (blue asbestos), tremolite, actinolite, and anthophyllite.

Both types of asbestos have been linked with cancer (http://www.cancer.org/cancer/cancercauses/othercarcinogens/pollution/asbestos?sitearea=PED).

Asbestos fibers can be useful because they are strong, resistant to heat and to many chemicals, and do not conduct electricity. As a result, asbestos has been used as an insulating material since ancient times. Since the industrial revolution, asbestos has been used to insulate factories, schools, homes, and ships, and to make automobile brake and clutch parts, roofing shingles, ceiling and floor tiles, cement, textiles, and hundreds of other products.

Asbestos was widely used during the 70's - 80's. Thus, a considerable amount of workers were exposed to asbestos fibres during this period. That notwithstanding, MPM onset occurs after a long latency period following fibre inhalation. Despite asbestos being banned in Italy in 1992, it is estimated that MPM incidence both here and throughout the world will increase strongly in the next two decades (Peto, Decarli et al. 1999, Britton 2002).

During the first half of the 20th century, growing evidence showed that breathing in asbestos caused scarring of the lungs. Exposure to asbestos dust in the workplace was not controlled at that time. Beginning in England in the 1930s, steps were taken to protect workers in the asbestos industry by installing ventilation and exhaust systems. However, in

the huge shipbuilding effort during World War II, large numbers of workers were exposed to high levels of asbestos (http://www.hse.gov.uk/research/hsl_pdf/2007/hsl0711.pdf).

As asbestos-related cancers became better recognized in the second half of the 20th century, measures were taken to reduce exposure, including establishing exposure standards and laws that banned the use of asbestos in construction materials. There has been a dramatic decrease in importing and using asbestos in the United States since the mid-1970s, and alternative insulating materials have been developed. As a result, asbestos exposure has dropped dramatically (Abelmann, Glynn et al. 2015). However, it's still used in some products, and it's still possible to be exposed to asbestos in older buildings, water pipes, and other settings. Asbestos use has been banned in the European Union since 2005, although the ban did not require removal of asbestos that was already in place. Still, heavy asbestos use continues in some countries.

Asbestos, after been recognized as a carcinogenic agent (Asbestos IARC monograph: http://www.who.int/ipcs/assessment/public_health/chrysotile_asbestos_summary.pdf?ua=1), was banned in different European Countries at various time-points between 1970-2005 (Valic 2002, Valic 2002). It was estimated that about 250,000 people will die of this tumour in Europe in the coming decades (Peto, Decarli et al. 1999, Hodgson, McElvenny et al. 2005).

Asbestos plays a critical role in tumour development as it limits and / or inhibits the immune system in controlling / eliminating transformed cells (deShazo, Nordberg et al. 1983, Jalava, Urbanucci et al. 2012). People can be exposed to asbestos in different ways:

1. *Inhaling asbestos*: Most exposures come from inhaling asbestos fibers in the air. This can occur during the mining and processing of asbestos, when making asbestos-containing products, or when installing asbestos insulation. It can also occur when older buildings are demolished or renovated, or when older asbestos-

containing materials begin to break down. In any of these situations, asbestos fibers tend to create a dust made of tiny particles that can float in the air.

2. *Swallowing asbestos*: Asbestos fibers can also be swallowed. This can happen when people consume contaminated food or liquids (such as water that flows through asbestos cement pipes). It can also occur when people cough up asbestos they have inhaled, and then swallow their saliva.

Previous studies have shown that mesothelium cells are very sensitive to asbestos fibres which, after being endocitated by the cell, interact directly with chromatin, destabilizing the structure and causing mutations primarily on tumour suppressor genes and oncogenes (Wang, Jaurand et al. 1987, Leard and Broaddus 2004).

Asbestos induces chromosomal aberrations in human cells in a non-random fashion. Indeed, frequent cases include Polysomy on chromosome 7, where the genes that encode for the growth factor platelet, derived type A (PDGF-A), receptor are located (Betsholtz, Johnsson et al. 1986, Oshimura, Hesterberg et al. 1986, Dai and Churg 2001). In addition, cases of deletion or monosomy of chromosomes 1 and 3, respectively, have been reported, including genes involved in cellular senescence and tumour suppressor genes (Flejter, Li et al. 1989, Walker, Everitt et al. 1992, Neragi-Miandoab and Sugarbaker 2009).

Its tumourigenic mechanism is linked to the activation of pro-inflammatory cytokines and NF-kB (Yang, Bocchetta et al. 2006, Sartore-Bianchi, Gasparri et al. 2007). However, the malignant transformation of mesothelial cells is required in order for Akt to be activated (Cacciotti, Barbone et al. 2005, Bertino, Marconi et al. 2007).

1.1.2.1.2 Simian Virus 40

The name of the virus SV40 is an abbreviation for Simian vacuolating virus 40 or Simian virus 40. It was assigned to the family of Papovaviridae, an acronym proposed by Melnick and obtained by fusing the names of the three representative viruses Papilloma, Polyoma,

and Vacuolating agent. More recently, SV40 has been considered a Polyomavirus, together with the human BKPyV,BK polyomavirus (BKV), JCPyV, JC polyomavirus (JCV), KIPyV, KI polyomavirus (KIV), WUPyV,WU polyomavirus (WUV), Human polyomavirus 11 (HPyV11/STLPyV), Human polyomavirus 10 (HPyV10), Human polyomavirus 7 (HPyV7), Human polyomavirus 6 (HPyV6), Human polyomavirus 9 (HPyV9), Human polyomavirus 12 (HPyV12), Merkel cell polyomavirus (MCPyV), TSPyV and Trichodysplasia spinulosa-associated polyomavirus (TSV) (Martini, Corallini et al. 2007).

SV40 was named for the effect that it produce on infected green monkey kidney cells, which developed an unusual number of vacuoles. Like other polyomaviruses, SV40 is a DNA virus that has the potential to cause tumors in animals, but most often persists as a latent infection. SV40 consists of an unenveloped icosahedral virion with a closed circular dsDNA genome of 5.2 kb (Fanning and Zhao 2009, Sowd and Fanning 2012). SV40 genome codes, at least, for six viral proteins: two early nonstructural polypeptides, the large tumor antigen (Tag) and the small tumor antigen (tag), an agnoprotein, probably involved in the assembly of viral particles and processing of late mRNA and three capsid proteins, VP1, VP2, and VP3 (Martini, Corallini et al. 2007). The early and late genes are transcribed on different DNA strands in a way that the transcription proceeds divergently from the regulatory region. This region contains the origin of DNA replication and binding sites for the transcription factors that control viral gene expression, and terminates within DNA sequences containing the polyadenylation signals. Recently, a predicted late polarity pre-microRNA (pre-miRNA) to the untranslated region 3' of the polyadenylation cleavage site in the late pre-mRNA has also been detected (Sullivan, Grundhoff et al. 2005).

SV40 was identified in the oral form of the polio vaccine produced between 1955 and 1961 by American Home Products. This is believed to be due to contamination of the substrate primary kidney cells from infected monkeys used to grow the vaccine virus during

production. Both the Sabin vaccine (oral, live virus) and the Salk vaccine (injectable, killed virus) were affected (Sangar, Pipkin et al. 1999).

It is unknown how widespread the virus was among humans before the 1950s, though one study found that 12% of a sample of German medical students in 1952 had SV40 antibodies (Martini, Corallini et al. 2007).

An analysis presented at the Vaccine Cell Substrate Conference in 2004 suggested that vaccines used in the former Soviet bloc countries, China, Japan, and Africa, could have been contaminated up to 1980, meaning that hundreds of millions more could have been exposed to the virus unknowingly. Population level studies show no evidence of any increase in cancer incidence as a result of exposure, though SV40 has been extensively studied.

SV40 sequences have been found, mainly by PCR methods, in different human cancers including mesothelioma, osteosarcoma, and non-Hodgkin's lymphoma and some different lymphoproliferative disorders, a variety of childhood brain tumors such as ependymoma and choroid plexus tumors, as well as thyroid, pituitary and parotid gland tumors (Bergsagel, Finegold et al. 1992, Martini, Dolcetti et al. 1998, Martini, Lazzarin et al. 2002, Dolcetti, Martini et al. 2003). These human tumors correspond to the neoplasms that are induced by SV40 experimental inoculation in rodents (Barbanti-Brodano, Martini et al. 1998) or by generation of transgenic mice with the SV40 early region gene directed by its own early promoter-enhancer(Brinster, Chen et al. 1984, Palmiter, Chen et al. 1985, Van Dyke, Finlay et al. 1987). SV40 sequences were detected in most cases by PCR.

Serum samples from MPM-affected patients had a prevalence of SV40 antibody that was higher than that of these cohorts of controls, that is, pregnant women and workers exposed to asbestos. The difference was statistically significant. In should be noted that the prevalence of SV40 antibodies determined in asbestos-exposed workers and pregnant women reflects the seroprevalence of the general population. Altogether, this result

remarks on the significance of the higher seroprevalence detected in the MPM patient cohort (Mazzoni, Corallini et al. 2012).

The onset and progression of MPM, as for other cancers, are associated with specific gene mutations (Bott, Brevet et al. 2011, Testa, Cheung et al. 2011). However, the agents responsible for the occurrence of mutations/chromosome alterations are poorly understood. Although asbestos is consid- ered the main cause, SV40 may be an additional candidate for causing mutations and chromosome alterations in tumors. SV40 was found to be mutagenic in human cells (Barbanti-Brodano, Sabbioni et al. 2004, Martini, Corallini et al. 2007). The viral oncoprotein Tag induces mutations and chromosomal damage characterized by numerical and structural chromosomal alter- ations such as gaps, breaks, dicentric and ring chromosomes, chromatid exchanges, deletions, duplications, and translocations (Barbanti-Brodano, Sabbioni et al. 2004). Alternatively, SV40, although able with its Tag oncogene to bind and inactivate p53- and pRB-family proteins, could only be a passenger virus, multiplying better in some transformed cells than in normal cells. Indeed, it has been proved that normal human mesothelial cells as well as other normal human cells are only semipermissive to SV40 multiplication (Bocchetta, Di Resta et al. 2000, Cacciotti, Libener et al. 2001, Morelli, Barbisan et al. 2004, Mazzoni, Rigolin et al. 2012).

Tumour induction, on the other hand, seems to be related to cooperation between the damage produced by asbestos and other transformant agents, such as the oncogenic virus, SV40 (Cacciotti, Barbone et al. 2005). Indeed, it has been observed that about 50% tissue samples derived from MPM show SV40 virus genomic sequences and oncogenic large T antigen expression (AgT) (Cacciotti, Strizzi et al. 2002). Overall, these data suggest that SV40 infection might be a cofactor in the development and progression of malignant pleural mesothelioma (Carbone, Rizzo et al. 1997). In support of this hypothesis, experiments performed on mesothelioma cells exposed to both asbestos fibres and SV40

demonstrate that cells transformed by these two co-factors are more resistant to cytotoxic drugs and chemotherapy (Tomek and Manegold 2004, Cacciotti, Barbone et al. 2005).

1.1.2.2 Mesothelioma: epidemiology, morpho-histo-cytology, clinical course and treatment.

Malignant pleural mesothelioma (MPM), like most cancers, is a somatic cell genetic disorder, which occurs as a result of several mutations that accumulate on the genome and phenotypically lead to tumour development (Dixon and Kopras 2004).

MPM is a very aggressive cancer which offers poor prognoses. MPM is derived from the neoplastic transformation of mesothelial cells and it is epidemiologically linked to exposure to asbestos fibres.

The National Register of Mesothelioma Italian includes 15,845 cases of malignant mesothelioma diagnosed from 1993 to 2008, with a gender ratio (male/female) of 2.5. 71.6% of reported cases involve men; the average age at diagnosis is 69 years. Pleural mesothelioma is the most frequent (93%) than in the peritoneal (6.4%); followed 41 cases of pericardial mesothelioma and 51 of the tunica vaginalis and testicular. The diagnosis is certain in 78% of cases, probable and possible in 11% of cases, respectively. The latency is about 45 years.

The detailed rules have been established for exposure 12,065 cases (76%), while they are in progress for 3,780 cases definition (24%).

The cases with defined exposure: (i) 69% have occupational exposure; (ii) 4% family exposure; (iii) 4% environmental exposure; (iv) 2% for non-work activity (recreation or hobby). For 20% of cases the exposure is unlikely or unknown.

The standardized rate for MPM was found in 2008 amounted to 3.55 (per 100,000 residents) in men and 1.35 in women (http://www.aimac.it/libretti-tumore/il-mesotelioma/l-epidemiologia-del-mesotelioma).

Various prognostic factors for survival in MPM have been described. The most significant prognostic factor remains histology: epithelioid mesothelioma is the sub- type with the best prognosis (Bille, Krug et al. 2016).

Asbestos was widely used during the 70's - 80's and a considerable amount of workers were exposed to asbestos fibres. MPM occurs after a long latency period following fibre inhalation. It is estimated that MPM incidence both here and throughout the world will increase strongly in the next two decades (Peto, Decarli et al. 1999, Britton 2002).

As a result of this, numerous studies have been undertaken to identify the pathogenic mechanisms that lead to disease onset. At the same time, various research studies have been performed to understand how to improve the life quality of patients who are affected by this disease and to identify new therapies (Neragi-Miandoab, Gangadharan et al. 2005, Pasello, Nicotra et al. 2011).

Histologically, there are three types of malignant pleural mesothelioma. These differ not only in terms of cell morphology but also in prognosis (Allen 2005). They are identified according to cell morphology as observed using an optical microscope and are:

1 **Epithelioid**, which is associated with less severe prognosis and represents 50% MPM cases. It is characterised only by epithelioid cells morphology.

2 **Sarcomatoid**, which is associated with less optimistic prognosis and is characterised by sarcomatoid cell morphology.

3 **Biphasic**, which is characterised by both cell types simultaneously present in varying proportions. This is the most infrequent and aggressive of the three MPM types.

The disease takes its clinical course very quickly and rarely leads to metastasis. It is characterised by tumour growth and it causes patient death due to internal organ compression, especially on the heart and lungs (Sugarbaker, Garcia et al. 1996). Pleural mesothelium can undergo different types of injuries, which can simulate mesothelioma,

making histopathological diagnoses very complex. Indeed, numerous immunohistochemistry assays with a large panel of antibodies are required.

Conventional therapies in positive MPM diagnoses include surgical resection followed by chemotherapy and radiotherapy (Cleaver, Bhamidipaty et al. 2014). However, prognosis remains dismal for this type of cancer since the effectiveness of current treatment foresees brief survival rates which are normally around 9 -12 months after diagnosis (Zellos and Christiani 2004).

1.2 MICRORNA

1.2.1 MicroRNAs: biogenesis, mechanism and function.

MicroRNA (miRNA or miR) is one approach to studying human tumours which has been most recently used in experimental oncology. MiRNAs represent a family of single-stranded, small non-coding RNAs consisting of about 21-25 nt, which are expressed in many organisms (Di Leva, Calin et al. 2006). They perform different functions, but at present, the most well-known regards the ability to regulate gene expression at transcriptional level. Indeed, negatively regulate mRNA targets are involved in many cellular processes including development, differentiation, proliferation, apoptosis and stress response (Miska 2005).

They have been divided into three classes:

1. **short interfering RNA or siRNA**: which can result from transcripts of double-stranded RNA (dsRNA) such as viral RNA, RNA hairpin, transposons, centromeres, etc.;
2. **microRNA or miRNA**: which are derived from transcripts of specific sequences;
3. **piwiRNA or piRNA**: which are often found in animals and seem to derive from precursors and single stranded small RNAs, despite limited information to date.

Micro RNA, which are derived from transcripts of specific sequences that occur as dsRNA sequences with a secondary structure (stem-loop) known as pri-miRNA, are generated using the transcription of certain genes by RNA polymerase II. Primary miRNA molecules can be as large as thousands of nucleotides and undergo polyadenylation, capping and presumably splicing introns. They are subsequently processed by a microprocessor complex formed by the RNase type III, Drosha and the DiGeorge Syndrome Critical Region 8 (DGCR8) protein which contains a domain for RNA double strand recognition. Once the pre-miRNA has been formed, it is about 70-80 nt molecules long, with hairpin shape without free ends, except for the portion 3' protruding. The pre-mirna are transported from the nucleus to the cytoplasm using the exportin 5 protein which is located on the cell membrane, where the loop is severed and then matured (Bartel 2004, Gregory, Yan et al. 2004).

Indeed, pre-miRNAs are detected and processed in the cytoplasm by the endonuclease type III, known as Dicer, leading to the formation of double-stranded molecules which are about 21-25 nt long. Dicer forms the RISC ribonucleoprotein complex (RNA-induced silencing complex) which is responsible for gene silencing using the Argonaut TRBP August protein.

Dicer and TRBP recognise the miRNA guide strand which is tied to the AGO protein, recognising and silencing the target mRNA (FIG.A) (Gregory, Chendrimada et al. 2005). As a result, the miRNA-RISC complex binds to the same target mRNA and other target mRNAs can then be identified using the miRNA-RISC complex.

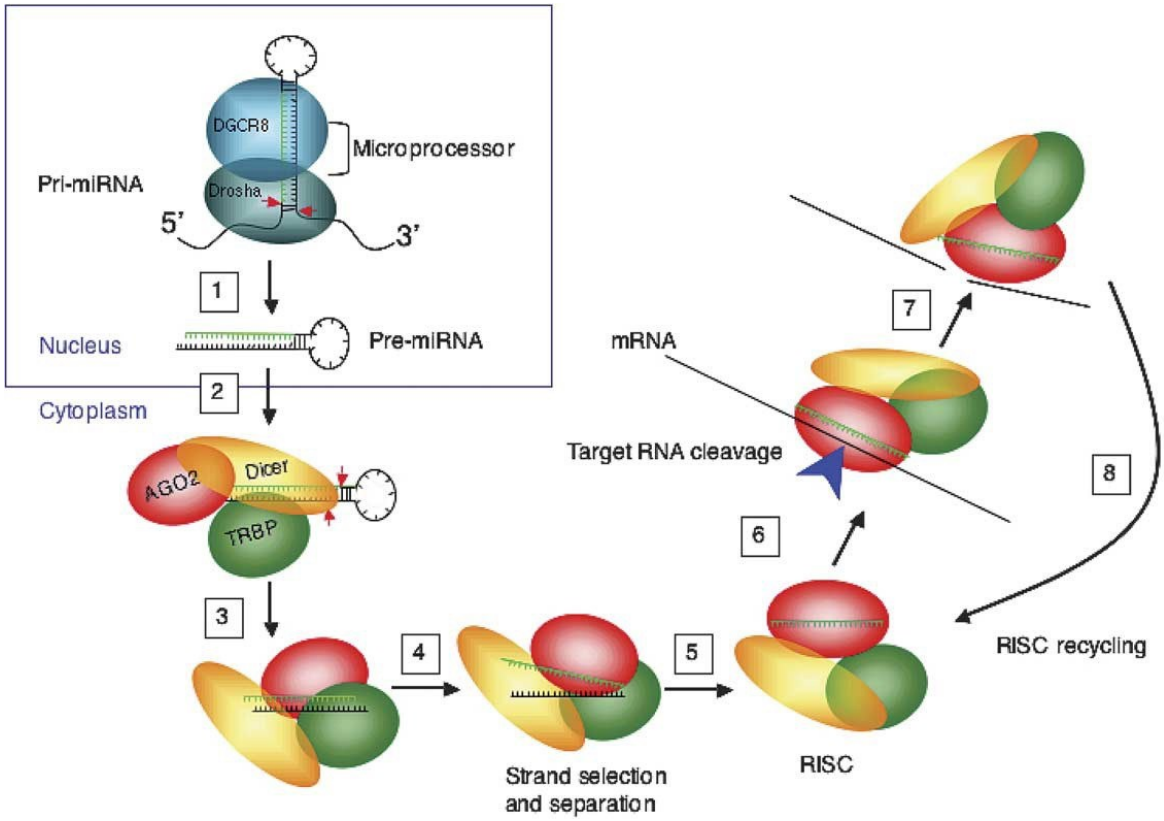

FIG. A - Model of the assembly and function of the RISC complex.

Many silencing mechanisms are operated by the RISC complex. They can be summarized as follows:

1. *miRNA cleavage or slicing*. The target mRNA is recognised and accordingly hydrolyzed. This may break the reading frame of the encoded protein and cause the complete degradation of the mRNA target by cellular exonucleases (Tolia and Joshua-Tor 2007). In order for slicing to take place the following elements are required: (i) the presence of a protein in the Argonauta catalytically active RISC complex (e.g. AGO2) and (ii) perfect miRNA and mRNA target complementarity (Liu, Carmell et al. 2004, Rivas, Tolia et al. 2005).

2. *Translational Repression*. The translation repression process causes imperfect complementarity between the miRNA region and the 3 'UTR of the mRNA target. Indeed, perfect pairing of the two strands is not required rather, only complementarity in 2-7 nt

(Bartel 2004). This process blocks mRNA translation by preventing the action of the helicases associated with the ribosomes and their catalase.

1.2.2 Circulating miRNAs

The recent discovery of circulating miRNAs has opened the possibility to study this class of biologically active agents as modes of inter-cellular information flow as well as biomarkers of disease. Here, we present an overview of the different carriers associated with extracellular miRNAs that render them stable in biological fluids, present the current level of understanding of their role in cell-to-cell communication and give an overview about the clinical utility of extracellular miRNAs as putative biomarkers (Kinet, Halkein et al. 2013).

The first accounts of extracellular miRNA biomarkers were described in serum of lymphoma patients (Lawrie, Gal et al. 2008) and in plasma and serum of prostate cancer patients (Mitchell, Parkin et al. 2008). Subsequently, it became evident that miRNAs can be exported from cells, and found in most extracellular biological fluids including plasma, serum, saliva, urine, tears, and breast milk (Chim, Shing et al. 2008, Weber, Baxter et al. 2010, Boon and Vickers 2013). Extracellular miRNAs are unexpectedly stable, and must be shielded from degradation, as naked RNA is readily targeted by exonucleases that are abundantly present in various extracellular fluids (Kamm and Smith 1972). Indeed, miRNAs are packaged in microparticles (exosomes, microvesicles, and apoptotic bodies) (Valadi, Ekstrom et al. 2007, Hunter, Ismail et al. 2008, Zernecke, Bidzhekov et al. 2009) or by their association with RNA-binding proteins including Argonaute 2 (Ago2) (Arroyo, Chevillet et al. 2011) or lipoprotein complexes such as high-density lipoprotein (HDL) (Kamm and Smith 1972, Vickers, Palmisano et al. 2011).

1. *Exosomes* are small (40–120 nm) extracellular microvesicles arising from multivesicular bodies (MVBs) and released by exocytosis of these MVBs (Heijnen,

Schiel et al. 1999). They are produced by a variety of cells including epithelial cells (Zhou, O'Hara et al. 2011), hematopoietic cells (Laulagnier, Motta et al. 2004), endothelial cells (Halkein, Tabruyn et al. 2013), and tumor cells (Mitchell, Parkin et al. 2008). Exosomes have also been identified in most circulating body fluids such as plasma, urine, milk, saliva, and sperm (Thery, Amigorena et al. 2006). The interest of exosome biology was increased following the demonstration that exosomes can serve as carriers for miRNAs (Valadi, Ekstrom et al. 2007, Gallo, Tandon et al. 2012).

2. *Microvesicles* or shedding microvesicles (SMVs) are another form of small, defined vesicles (Pant, Hilton et al. 2012) that are shed from the plasma membrane by a wide variety of cells (Heijnen, Schiel et al. 1999). They are larger (0.1–1 μm) than exosomes (Heijnen, Schiel et al. 1999) and their mechanism of production is also different. While exosomes are produced by exocytic fusion of MVBs, microvesicles are produced by budding of vesicles from the plasma membrane (Mathivanan, Ji et al. 2010). The presence of miRNAs in microvesicles were described for the first time in 2008 (Hunter, Ismail et al. 2008).
3. *Apoptotic bodies* or dying cells release membrane vesicles into the extracellular environment via bleeding of the plasma membrane (Mathivanan, Ji et al. 2010). These are larger particles (1–5 μm) with heterogeneous shape (Gyorgy, Szabo et al. 2011).
4. Apart from packaging miRNAs in cell-derived vesicles, a significant fraction of extracellular miRNAs is associated with *RNA binding proteins*, including nucleophosmin (NPM1), that provide protection from degradation (Wang, Zhang et al. 2010). It was also demonstrated that many extracellular miRNAs are bound to proteins of the Argonaute family, primarily Ago2, although additional members

such as Ago1, Ago3, and Ago4 might be also associated with miRNAs (Arroyo, Chevillet et al. 2011, Turchinovich, Weiz et al. 2012).

5. Recently study shown that extracellular miRNAs can be transported by *HDL* (Vickers, Palmisano et al. 2011, Norata, Sala et al. 2013). Whereas vesicle carriers are composed of a bilayer of phospholipids, lipoproteins have a single layer of lipids (Boon and Vickers 2013).

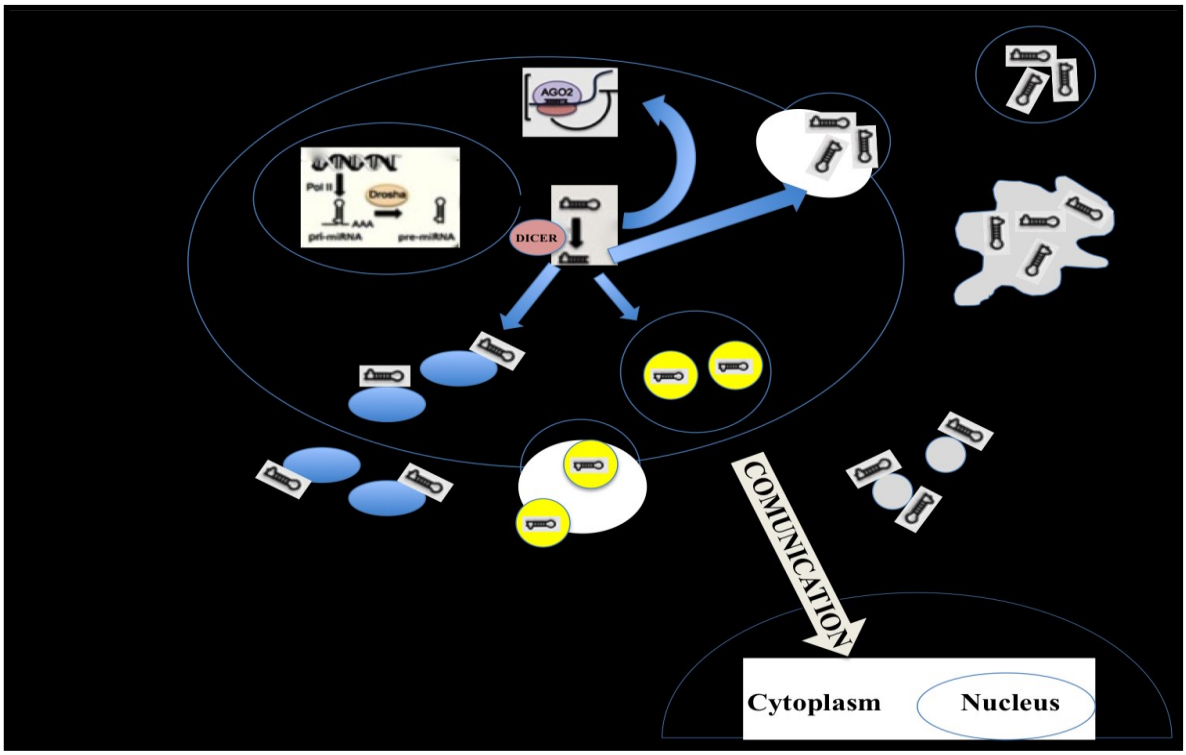

FIG. B - ***Schematic representation of cellular release (A) and inter-cellular communication (B) of miRNAs.***

(A) *In the nucleus, miRNA genes are mainly transcribed by the RNA polymerase II (Pol II) into primary miRNAs (pri-miRNAs) and processed to precursor miRNAs (pre-miRNAs) by the Drosha complex. Pre-miRNAs are exported to the cytoplasm and cleaved by Dicer to produce a double stranded miRNA duplex. The duplex is separated and a mature miRNA is incorporated into the RNA-induced silencing complex (RISC) while the other strand is likely subject to degradation. Within the RISC complex, miRNAs bind to their target messenger RNAs (mRNAs) to repress their translation or induce their degradation. In addition, miRNAs can be exported out of the cells and transported by various carriers, membrane-derived vesicles (exosomes, microvesicles, apoptotic bodies), miRNA-binding protein complexes (RBP), or high density lipoproteins (HDL).* ***(B)*** *Extracellular miRNAs can be transferred to recipient cells where they alter gene expression.*

Interestingly, extracellular miRNAs also present a newly discovered potential of intercellular communication. It is now established that transfer of genetic information in

the form of RNA exists (Valadi, Ekstrom et al. 2007) and that this form of transfer between cells is of functional relevance by exerting gene silencing in the recipient cells (Kosaka, Iguchi et al. 2010, Mittelbrunn, Gutierrez-Vazquez et al. 2011, Halkein, Tabruyn et al. 2013) (FIG. B). While the biological mechanisms driving the secretion of miRNAs are still under debate (Kosaka, Iguchi et al. 2010), this newly discovered manner of genetic exchange between cells opens a new aspect of how adjacent cells within an organ may communicate and how a miRNA can affect a cell type or a tissue where it is not produced. Since the first discovery of the extracellular miRNAs as intercellular communicators, this field of research is still growing. Increasing evidence suggests that this form of communication occurs in various physiological processes such as the regulation of the immunity (Mittelbrunn, Gutierrez-Vazquez et al. 2011) or cellular migration (Zhang, Liu et al. 2010), but also participates in pathological situations including tumor development (Yang, Chen et al. 2011).

1.2.3 MicroRNAs in human tumourigenesis.

In recent years, several researchers have identified abnormal miRNA expression in various types of cancers, suggesting their potential role as oncogenes or tumour suppressor genes (Volinia, Calin et al. 2006, Petrocca, Visone et al. 2008). Initially, particular interest was aroused in oncomir miR-17-92, which is known in the literature to be associated with tumours. In humans, this cluster, which encodes for six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1), is closely grouped within a region of 800 base pairs on chromosome 13, more precisely in the third intron of the primary transcript, at about 7 kb, called C13orf25 (Ota, Tagawa et al. 2004). In humans, the miR-17-92 cluster has two clusters paralogs: the miR-106b-25 cluster, located on chromosome 7, and the miR-106a-363 cluster, localized on chromosome X (FIG. C).

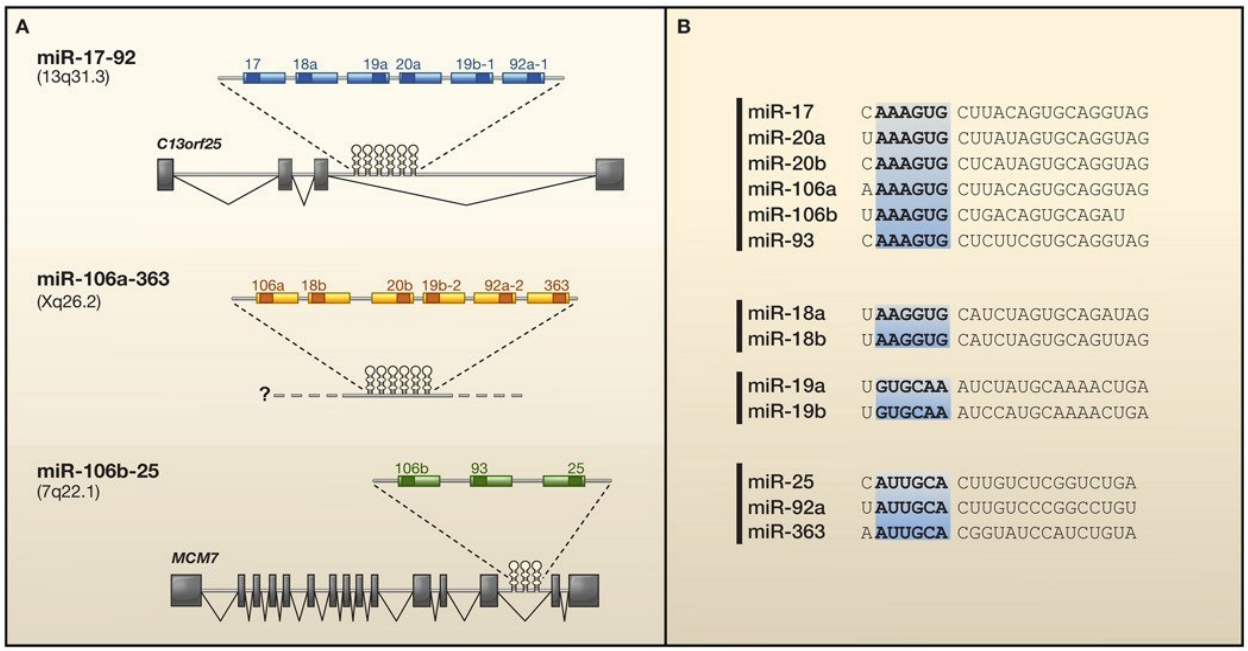

FIG. C - Cluster miR-17-92 organization and paralogs.
(A) Genomic organization and structure of miR-17-92 , miR 106b-25 and miR-106a-363 cluster primary transcript. The structure of the miR-106a-363 primary transcript cluster has not been determined; 3.5.
RKnes and oncogenes. 15-16*(B) Their sequences are shown in the blue region (2-7 nt) and are considered to be the most important recognition of the target.*

These miRNAs are involved in normal heart, lung and immune system development as well as tumourigenesis. One of the first pieces of evidence to be found regarding the miR-17-92 cluster as being functionally integrated in the central oncogenic tumourigenesis process was the discovery of its direct trans activation by c-Myc, a transcription factor which is often hyperactive in cancer cells. Subsequent studies have supported this hypothesis by highlighting the widespread overexpression of these miRNAs in various tumours including solid tumours, such as in the breast cancer, colon, lung, pancreas, stomach, prostate, as well as hematopoietic malignancies (Volinia, Calin et al. 2006, Petrocca, Visone et al. 2008).

Moreover, recent studies indicate that miRNAs, including MPM, can be used as diagnostic cancer markers (Benjamin, Lebanony et al. 2010, Gee, Koestler et al. 2010) and correlated with patient survival and clinicopathological factors (Guo, Chen et al. 2008), such as miR-29c* and miR-31.

miR-29c* has been shown to be an independent prognostic factor in MPM for both time to progression and survival after surgical cytoreduction. Indeed, high miR levels have been

found in forms of epithelial mesothelioma and associated with improved patient survival as it acts on gene methylation and other over-regulated pathways in MPM (Pass, Goparaju et al. 2010).

Thus, miR-31 tumour suppressor activity could be used for the development of potential innovative MPM therapies. It has been shown that in cell lines derived from MPM which are incapable of expressing miR-31 due to the loss of the 9p21.3 portion gene, the reintroduction of miR-31 is able to suppress the cell cycle and inhibit the expression of multiple factors involved in DNA replication and cell cycle progression, including phosphatase PPP6C, which had previously been associated with resistance to chemo- and radio-efficacy in therapy and chromosomal stability maintenance (Ivanov, Goparaju et al. 2010).

1.2.4 MicroRNAs potential biomarkers

MiRNAs are involved in several types of cancers (Miska 2005). Many studies have indicated miRNAs as diagnostic markers for cancers, including MPM (Benjamin, Lebanony et al. 2010, Gee, Koestler et al. 2010) and potential targets for new therapeutic approaches (Saito, Nakaoka et al. 2015).

In a previous study, we identified a group of 22 miRNAs which were significantly dysregulated in MPM cells compared to normal mesothelial cells. Some of these miRNAs belong to the miR-17-92 cluster, which is induced by c-Myc oncogene (Balatti, Maniero et al. 2011).

Circulating miRNAs are packaged in microparticles (exosomes, microvesicles and apoptotic bodies) or by association with RNA binding protein including Argonaute 2 (Ago2) or lipoprotein complexes such as high-density lipoprotein (HDL), with the end effect of enhancing their stability in biological fluids (Kinet, Halkein et al. 2013). It has been hypothesised that these miRNAs, which are detectable in serum samples, may allow

the inter-cellular information flow to be studied (Heneghan, Miller et al. 2010, Hu, Chen et al. 2010, Kosaka, Iguchi et al. 2010). In recent years, microRNAs (miRNAs), from cells or sera, have been proposed as new biomarkers (Busacca, Germano et al. 2010, Balatti, Maniero et al. 2011, Kirschner, Cheng et al. 2012).

Herein, we report on a comparative analysis of miRNA expression in serum samples from MPM affected patients, workers ex-exposed to asbestos fibres (WEA) and healthy subjects (HS). The aim of this investigation was to identify extracellular miRNAs as putative biomarkers for MPM and potential targets for innovative therapies.

2. AIM OF WORK

Malignant pleural mesothelioma (MPM) is a fatal cancer, with increasing incidence rates world-wide. MPM, which is resistant to conventional therapies, is diagnosed in its late stages with a median survival of 12 months.

Asbestos exposure is the main risk factor for MPM onset (van Zandwijk, Reid et al. 2012), which may occur in workers even decades after exposure to this tumourigenic mineral. However, recent data indicate that asbestos is becoming an environmental pollutant, with the result that MPM patients are not always are linked to occupational diseases (Baumann, Buck et al. 2015).

To date, there is no adequate treatment or survival prognosis for this cancer for patients after one year of diagnosis. It is of crucial importance to define new targets through greater knowledge and understanding of the molecular pathways involved in the onset / progression of this cancer in order to improve its treatment. MicroRNAs, which negatively regulate gene expression at transcriptional level, are involved in many processes including the cell cycle. Recently, abnormal miRNA expression has been identified in different tumours, both solid and hematopoietic.

The aim of this doctoral thesis was to evaluate miRNAexpression circulating in the serum of patients with MPM. To this end, differences in miRNA expression profiles circulating in the serum of patients affected from malignant pleural mesothelioma, sera from individuals formerly exposed to asbestos and the sera of healthy subjects, were investigated. The study focused on gathering new information (i) on disease onset / progression, (ii) possible new specific molecular markers and (iii) innovative therapies.

Initially, this entailed experimental analyses on microRNA expression by microarray and validation of data obtained by real-time quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR). The data obtained demonstrated that microRNAs are highly

deregulated, specifically in the serum of MPM patients. It can be posited that these newly discovered deregulated miRNAs may represent new therapeutic targets.

3. MATERIALS AND METHODS

3.1 Samples and cell cultures

The tissue biopsy used in this research was derived from healthy pleural tissues (HMC) and mesothelioma (MPM) provided by the Surgical Clinic, University Company / S. Anna Hospital of Ferrara. Some of these biopsy tissues were used to prepare primary cell cultures, defined as HMC when derived from normal mesothelial cells and MPM, if derived from mesothelioma.

The three lines of mesothelioma, that is, the three different histological types (epithelioid, sarcomatoid and biphasic) used in our immunohistochemistry analyses were provided by the Laboratory of Cytogenetics and Molecular Pathology SC Pathology A.O. S.S. Antonio and Biagio and C. Arrigo of Alessandria.

All cell lines were maintained in appropriate growth conditions in RPMI 1640, 10% fetal bovine serum (FBS) and 2 mM of L-Gluttamina.

3.2 Immunostaining

HMC and MMP cells grown on slides in multi-well plates were fixed with 4% formalin in PBS, pH 7.4 for 15 min at room temperature. After two washes in ice-cold PBS, the slides were incubated with 1% BSA for 30 min to block nonspecific binding sites for the antibodies used. Cells were then fixed with 4% paraformaldehyde, permeabilised with PBS containing 0.5% Triton X-100, and incubated with pan-cytokeratin, mouse monoclonal anti-human cytokeratin (Clone MFN1116 – DAKO m.0821) followed by secondary goat anti-mouse IgG (Fab-specific)-FITC (Sigma). Cells were then mounted with VECTASHIELD Mounting Medium with DAPI (Vector laboratories, Burlingame, CA) and observed with a confocal microscope. (NIKON ECLIPSE TE2000-E) (Figure 1).

3.3 Serum samples

Serum samples were harvested from healthy subjects (HS), workers exposed to asbestos (WEA) and patients affected by malignant pleural mesothelioma (MPM), at the Clinical Analysis Laboratory at the Ferrara University Hospital, the Occupational Medicine Faculty of the University of Ferrara and the City Hospital of Alessandria. Sera were taken from discarded laboratory analysis specimens, after routine analyses, before incineration. Anonymously collected sera were coded with indications of age, gender and pathology only. The project was approved by the County Ethical Committee, Ferrara.

Small sera aliquots were stored at −80°C until time of analysis. MicroRNAs analysis by microarray was performed on 30 serum samples (10 MPM, 10 WEA and 10 HS, with median age of 64 years), whereas RT-qPCR was extended to 49 samples (20 MPM, 15 WEA and 14 HS, with median age of 65 years).

3.4 Reverse Transcription Cel-miR-39

The six synthetic microRNAs used as spike-in went through the entire RNA isolation process and ultimately only Cel-mir-39 was assessed by qRT-PCR in the final RNA eluate, providing an internal normalisation reference (exogenous control) of technical variations between samples.

Reverse transcription reactions were performed using Taq-Man MicroRNA Reverse Transcription Kit (Life Technologies, cod. 43665979) with specific primers for Cel-mir-39 (Life Technologies, cod. 4427975, assay ID: 000200) in a 15 μL RT reaction: 7.16 μL H2O, 1.5 μL 10× reverse-transcription buffer, 0.19 μL RNase-inhibitor (20 U/μL), 0.15 μL dNTPs mix (100 mM), 1 μL Multiscribe Reverse Transcriptase enzyme (50 U/μL) and 3 μL 5x RT primers. These components were combined in order to gain the master mix.

After mixing by inversion and centrifugation, the master mix was aliquoted into 0.2 mL RNase-free tubes, followed by the addition of 2 μL input miRNAs.

A standard curve was generated using chemically synthesised RNA oligonucleotides corresponding to the known miRNA Cel-mir-39, serially diluted and added to the RT reaction mixtures at a volume of 2 μL per reactions. RT reactions were carried out on the PTC-100 thermal cycler (MJ Research) using the following conditions: 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min, and then held at 4 °C RT, products were stored at 20 °C prior to running real-time PCR.

3.5 Cel-miR-39 absolute quantification

Cel-miR-39 absolute quantification was performed using a standard curve prepared on the basis of the synthetic target miRNA. Serial dilutions (1:10, n=10) of 1 nmol/L synthetic miR-39 with an identical sequence to the mature target miRNA (UCA CCG GGU GUA AAU CAG CUU G-22 bp) were run in parallel with biological samples, starting from the reverse transcription step onwards, using the same master mix and real-time plate. Plotting the Ct values vs. the synthetic miRNA concentration in a standard curve allowed for a curve fitting that was used to determine exogenous cel-miR-39levels from the Ct values obtained using biological samples. Cel-miR-39 was expressed as pmol/L.

3.6 MicroRNA Extraction

Total RNA including microRNAs was extracted from 200 μl of serum using miRNeasy Mini Kit (QIAGEN cod. 217004) according to the manufacturer's protocol. The final amount of miRNA, extracted from serum, may be influenced either by miRNA extraction efficiency or RT-qPCR robustness (i.e. by the presence of inhibitors). Both factors can be controlled by adding synthetic non-human miRNAs to the serum sample as controls, before RNA isolation. In order to adjust these parameters specifically to 200 ul serum

volume, 2.5 µl of 5 nM synthetic miRNAs (Caenorhabditis elegans, cel-miR-39, cel-miR-54, cel-miR-238) were added to the sample (200 ul) soon after 1 ml Qiazol Reagent was added. RNA was eluted in 30 µl RNAse-free water.

3.7 MicroRNA array

MicroRNA expression profile was analysed in 30 samples using Agilent miRNA microarray (G4870A) technology. This array is capable of assessing the expression of 1,200 human miRNAs. This microarray is made from 60-mer DNA probes synthesized in situ, which represent 470 human microRNAs and human viral microRNA 64 taken from the Sanger database (release 9.1). The colorimetric analysis, hybridization and washing were performed according to the instructions provided by the company Agilent. The RNA of the same 30 samples were hybridized on an Agilent whole human genome microarray (Agilent Technologies, Palo Alto, CA) consists of 60-mer DNA probes synthesized in situ representing 41,000 human transcripts.

A constant volume of 8 µl RNA was employed for hybridisation procedures. Experiments were performed as previously described (Balatti, Maniero et al. 2011, Ferracin, Lupini et al. 2015). Microarray raw data were analysed using GeneSpring GX 13 software (Agilent Technologies). Quantile normalisation was applied before statistical analyses. A signal was detectable (i.e. above background in at least one sample) for 197 probes.

3.8 Microarray data analysis

The microarray raw data were analyzed by Gene Spring GX-software (Agilent Technologies) and pre-processed with GeneSpring plug-in for Agilent Feature Extraction software results. The data are then normalized using the average of the chip and the gene. The use of a filter that allows only the probes, which are differently expressed in at least

one sample, are taken into account in the analysis. The samples, at this point, are analyzed, pooled and compared.

MicroRNAs differentially expressed in the three groups (HS, WEA and MPM) were identified as having a 1.5 fold differential expression and an adjusted p-value < 0.1 at the ANOVA statistical analysis (GeneSpring software).

3.9 Reverse transcription of endogenous miRNAs

Reverse transcription reactions (10 μL) were performed using miRCURY LNA Universal RT microRNA PCR (EXIQON: cod EX203301). Two μL total RNA was reverse transcribed after adding 0.5 μL Synthetic RNA spike-in UniSp6 as an exogenous control. Reactions were carried out on the PTC-100 thermal cycler (MJ Research) using the following conditions: 42°C for 60 min, 95°C for 5 min, and then held at 4°C. RT products were stored at -20°C until the time of real-time PCR analysis.

3.10 Quantitative RT-qPCR analysis of endogenous miRNAs

Quantitative – RT-PCR (RT-qPCR) analysis was performed to test three differentially expressed miRNAs by microarray analysis. These miRNAs were miR-1281, miR-197-3p, and miR 32-3p. We also tested three reference miRNAs, miR-1234-3p, miR-3656 and miR-3665, which showed stable expression. This approach was employed with the aim of selecting stable endogenous controls to quantify circulating miRNAs. RT-qPCR reactions were performed using an ExiLENT SYBR® Green master mix (EXIQON) according to the manufacturer's instructions. Briefly, 1:40 diluted cDNA template was added to PCR primer mix and PCR Master mix, in 10 μL. Samples were run in triplicate (CFX96 Touch Real-Time PCR Detection System, Applied Biosystems) with denaturation at 95°C 10 min, followed by 40 cycles at 95°C 10 s, 60°C 1 min. A final melting curve analysis, from 60°C to 95°C, was carried out to test the presence of a single amplified product.

3.11 Determination of reference genes

The expression variability was assessed for each candidate reference miRNA by calculating the coefficient of variability (CV). Each candidate endogenous control miRNA was tested in triplicate in RT-qPCR and the results expressed as mean Ct. The average of the Ct value was used for the comparison in an ANOVA test, whereas the stability and rank of each candidate house-keeping miRNA genes was evaluated through the NormFinder software (http://moma.dk/normfinder-software). The NormFinder algorithm uses a mathematical model and a solid statistical framework to calculate gene expression stability. NormFinder estimates the overall expression validation of the candidate normaliser genes, as well as the intra-group and the inter-group variations.

3.12 Validation of circulating miRNAs expression

The level of three miRNAs, which resulted as differentially expressed according to microarray analysis (miR-1281, miR-197-3p and miR 32-3p), was measured by RT-qPCR. MiRNA expression level was measured using the ΔCt method, where the Ct (threshold cycle) is the fractional cycle number when the fluorescence of each sample passes a fixed threshold.

Relative quantification of each miRNAs was performed using (HKmiR), a chosen stable endogenous control miRNA, as a references gene as it shows stable expression in all sera. The results were expressed as ΔCT (CtmiR-CtHKmiR) for each subject and as a mean ± S.D. of ΔCT for each group. High miRNA ΔCT values corresponded to low miRNA expression.

The fold changes in relative miRNA expression were calculated using the equation 2-Δ(ΔCt), where

$$\Delta\Delta Ct = (Ct_{miR}\text{-}Ct_{HKmiR})_{MMP} - (Ct_{miR}\text{-}Ct_{HKmiR})_{HS}.$$

MiRNA species that were not detected in any of samples or those with a CT value >39 were excluded from the comparison. MiRNA species with at least a 1.5-fold expression change among groups were considered differentially expressed.

3.13 Statistical Analysis

The statistical analysis was performed using Prism 4.0 statistical software (GraphPad software, La Jolla, CA, USA). ANOVA test and Chi-square test were employed to evaluate age differences and the percentage of male subjects in the three groups undergoing study (HS, WEA and MPM), respectively.

Chi-square was employed to study the differences in the total number of miRNAs detected in the three groups. ANOVA test was employed to study the differences in median ΔCT of 2 dysregulated miRNA (miR-1281 and miR-197-3p) and 3 stably expressed miRNA (miR-1234-3p, miR-3656 and miR-3665) in the three groups. ANOVA test and t-test were employed to confirm the fold-change obtained from the ΔΔCt method.

MiR 32-3p, data are presented as a percentage of positive samples for the remaining dysregulated miRNA studied. 95% Confidence Intervals (CI) of the percentage of positive samples are also reported. Differences in proportions were calculated by Chi-square test for independence in the contingency tables. P value <0.05 was considered to be statistically significant.

4. RESULTS

4.1 Analysis of total miRNAs

The in first phase of my investigation, I employed as a control of the total RNA extraction procedure the Cel-mir-39. This miRNA was used as spike-in, which is an exogenous synthetic miRNA added to the solution to verify the quality and quantity of RNA extracted form our samples.

The absolute quantification of this control miRNA was performed by qRT-PCR.

Plotting the Ct values vs. the synthetic miRNA concentration, in a standard curve, I verified that amount of miRNAs extracted from the samples in analsysis was in the range of 107 - 1010 molecules. This result indicated that the procedure employed gave a good extraction of the circulating miRNAs, from serum samples, in which initially were added 1.5x1010 molecules of cel-miR-39.

4.2 Microarray analysis

In my investigation I analyzed the expression profiles of circulating miRNAs present in serum samples of (i) MPM patients, (ii) WEA and (iii) HS. The analysis was carried out by microarray technology (Agilent Technologies, Human miRNA microarray G4470A) and RT-qPCR method.

MiRNAs expression was detected in serum samples from MPM patients, compared to that WEA and HS. Form this microarray analysis it turned out that among the 1,201 miRNAs assessed, 197 miRNAs were identified, at least in 1 sample out of 30 samples analyzed.

It is worth noting that the total number of circulating microRNAs, in the three cohorts i.e. MPM, WEA and HS, was different. Indeed, differences in miRNA expression profiles were identified in MPM, WEA and HS. Specifically, more miRNAs were detected in sera

of HS compared to MPM and WEA samples. Indeed, 145 miRNAs out of 1,201 were detected in HS, whereas 119 were detected in MPM and 42 in WEA. This difference is statistically significant (HS vs MPM p = 0,0053 **, HS vs WE p < 0,0001 ***, WEA vs. MPM p < 0,0001 *** - Figure 2).

Among the 119 miRNAs expressed in MPM, 67 miRNAs were also detected in the control sample represented by HS. The remaining 52 miRNAs were found expressed only in MPM samples. In WEA cohort, 42 miRNAs were expressed. However, none of these miRNAs were exclusively present in serum samples of WEA. These 42 miRNAs were expressed, 40 of them in the three cohorts of MPM, WEA and HS, while two miRNAs miR-320c and miR-671-5p were detected in WEA and HS, but absent in MPM.

A total of 145 miRNAs were expressed in HS: 76 miRNAs were detected only in this cohort, 27 are in common with MPM, 2 are in common with WEA, while 40 miRNAs were expressed in all the three groups (Figure 3).

ANOVA test allowed to comparatively analyse the miRNAs expression in the three groups (MPM, WEA and HS). Interestingly, 37 miRNAs were found to be dysregulated: MPM vs HS 16 upregulated and 21 downregulated; MPM vs WEA 21 upregulated and 16 downregulated; WEA vs HS 4 upregulated and 33 downregulated. (Figure 4 , Table 1).

4.3 Validation of stable circulating miRNAs

Microarray analyses carried out by Agilent technology indicated that three endogenous miRNAs, miR-1234-3p, miR-3656 and miR-3665 are stable. Indeed, among 30 samples analyzed they were always present. This result suggested that these three miRNAs were stable endogenous controls for quantifying circulating miRNAs in analyzed sera of the three cohorts.

In order to verify whether these three miRNAs were stable in all 50 serum samples analyzed, the RT-qPCR technique was employed to validate the microarray data obtained in the previous analysis.

Specifically, these three candidate microRNAs, miR-1234-3p, miR-3656 and miR-3665, found with the highest expression level and with the less variability across the serum samples analyzed by microarray, were investigated. These three miRNAs were subjected to RT-qPCR to validate them as stable miRNAs, analyzing additional 20 serum samples from MPM (n=10), WEA (n=5) and HS (n=5) , together with the 30 serum samples analyzed earlier by microarray technology.

The validation analyses, to confirm the three candidates miRNAs, as stable expressed miRNAs in sera, employed thre different statistical methods. The first approach employed the average of the coefficient of variation (CV) value, across the three different groups. Then, as a second method, ANOVA test was employed to study the differences in mean ΔCT of the three candidate miRNAs in the three groups. Moreover, as a third method, the NormFinder software was used to verify the S parameter, which is the relative value indicating the stable expression.

These three statistical methods/parameters, i.e. CV, ANOVA and S, were employed to confirm the stable microRNAs among the 50 samples analyzed.

Analytical evaluations of the three parameters indicated that (i) CV values were, 0.035%, 0.023% and 0.023% for hsa-miR-1234-3p, miR-3656 and miR-3665, respectively; (ii) ANOVA test showed that miR-1234-3p has a p = 0.81, for miR-3656 a p=0.57 and miR-3665 a p=0.81; (iii) S parameter was, 0.024, 0.027, and 0.021 for miR-1234-3p, miR-3656 and miR-3665, respectively

Statistical analyses carried out on microarray and RT-qPCR data showed no different expression for these three miRNA among three cohorts.

Although RT-qPCR confirmed the stable expression for the three candidate miRNAs, the miR-3665 demonstrated the minimum CV, the maximum ANOVA value and the minimum S value, as expected for a significant data. Therefore, hsa-miR-3665 demonstrated the higher stability in our samples. This result suggested to select it as stable endogenous control for quantifying circulating miRNAs in our cohorts. This miRNA was employed as housekeeping miRNA to perform the relative quantification of miRNAs differentially expressed in the 50 serum samples. To this purpose the value of fold changes was calculated using the equation:

$$2^{-\Delta(\Delta Ct)}, \text{ where } \Delta\Delta Ct=(Ct_{miR}-Ct_{HKmiR-3665})_{MMP}-(Ct_{miR}-Ct_{HKmiR-3665})_{HS}$$

4.4 Validation of dysregulated circulating miRNAs

Microarray analysis allowed to identify three dysregulated miRNAs in the three cohort of sera from MPM, WEA and HS. In order to validate the microarray data, RT-qPCR analyses were carried out with the three miRNAs differentially expressed in the three MPM, WEA and HS cohorts.

These three miRNA, miR-197-3p, miR-1281 and miR 32-3p, were found up-regulated with both the techniques, microarray and RT-qPCR, in MPM vs. HS.

Two out of three dysregulated miRNAs, i.e. miR-197-3p and miR-1281 were detected in all 50 sera analyzed. Interestingly, these two dysregulated miRNAs allowed us to calculate the statistical values with three methods described in the MM section, i.e. the average of ΔCt by ANOVA, the t-test and the FC value. These three different values indicated that: the (i) ΔCT value of the miR-197-3p, in WEA and HS groups were similar, being 1.523 and 1.591, respectively. These two ΔCT values are different from ΔCT value of 0.175 detected in the MPM group (Figure 5).

MiR-197-3p showed a different expression in the three cohorts (ANOVA p value = 0.0093**). In particular in the comparison between MPM and HS the t-test p value was

0.0115* and FC value was 1.8; in the comparison between MPM and WEA the t-test p value was 0.0031** and the FC value was 2.5 (Table 2 and Table 3).

Conversely, for miR-1281 ΔCT value in MPM and WEA groups were similar (2.500 and 2.019), while both are statistically different from ΔCT value (4.364) of the HS group. (Figure 6).

MiR-1281 was found to be dysregulated: The statistical analysis showed that the expression of this miRNA was different in the three cohorts (ANOVA p value = 0.002**). Comparing MPM and HS sera the t-test p value was 0.0045** and the FC value was 2.5; comparing WEA and HS samples the t-test p value was 0.0047** and the FC value was 3.5 (Table 2 and Table 3).

MiR 32-3p was not detected by RT-qPCR in all 50 sera analyzed. The absence of this miRNA did not allow us to verify the statistical data by ANOVA, t-test and FC value.

In the 50 sera analyzed by RT-qPCR showed that only 6/50 (12%) samples were found positive for miR 32-3p. It is interesting to note that his miRNA was detected only in MPM samples 6/20 (30%), while it was absent both in WEA and HS sera. (Table 2 and Table 4).

4.5 FIGURES

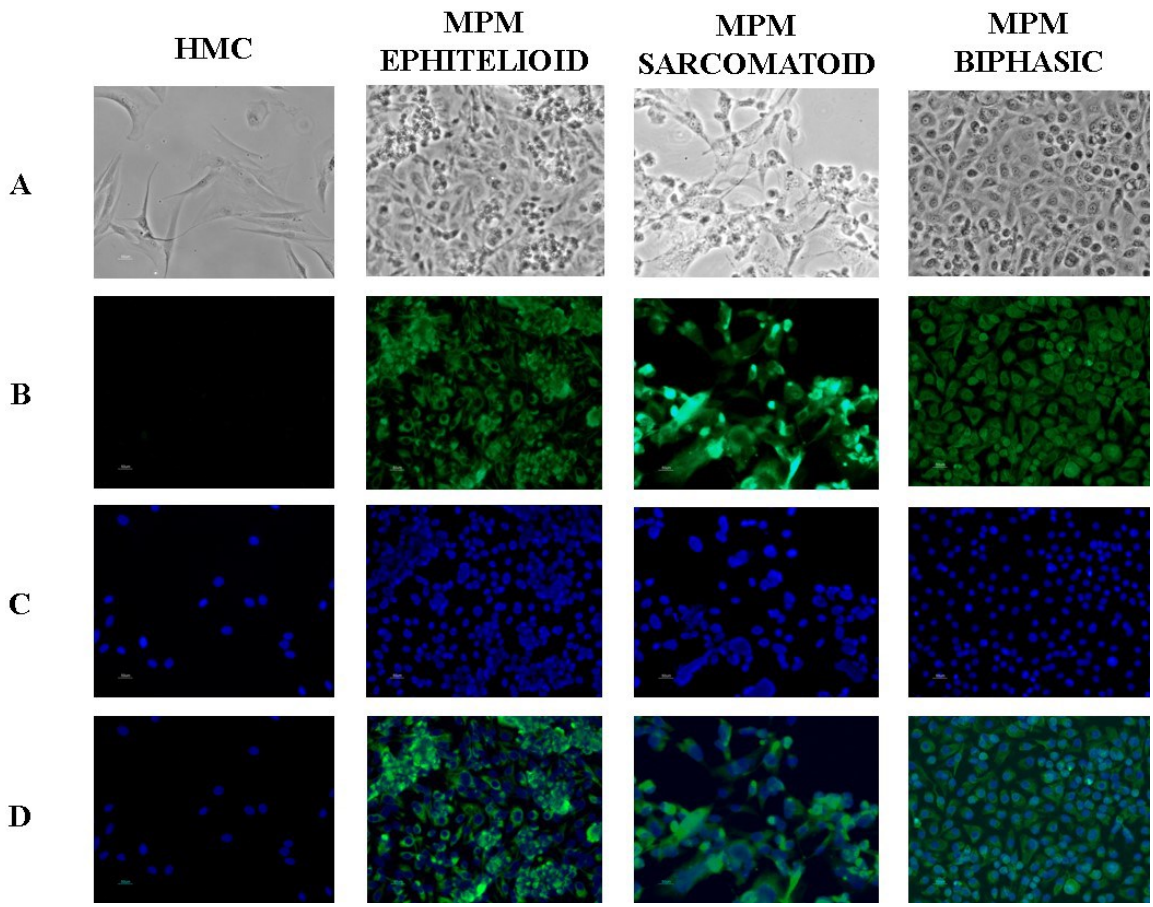

Fig.1 – Immunostaining . (A) morphology of healthy mesothelial cells (HMC) and the three histological types of malign pleural mesothelioma (MPM) epithelioid , sarcomatoid and biphasic. (B) pan-cytokeratin, mouse monoclonal anti-human cytokeratin, the HMC were negative while the MPM test positive . (C) DAPI - detection of the cell nucleus. (D) Merge – overlap pan-cytokeratin with DAPI.

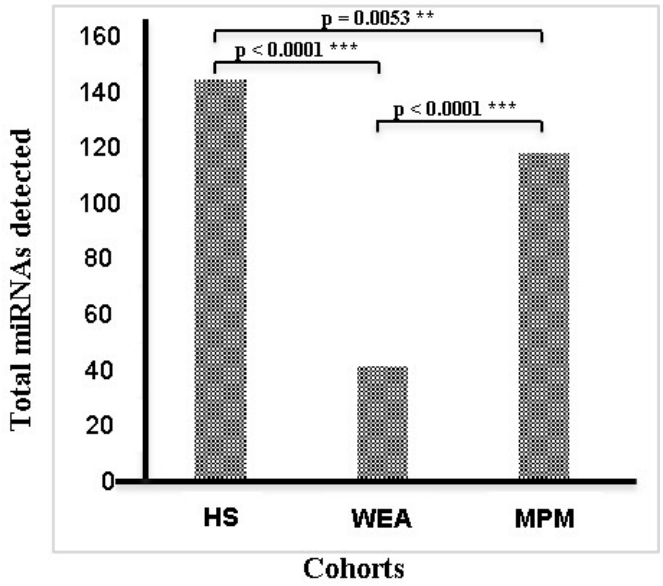

Fig.2 - Expression profiles circulating miRNAs from serum samples of MPM, WEA and HS. MiRNAs detected in serum of healthy subjects compared to that of MPM and WEA. Specifically, 145 miRNAs out of 1,201 were detected in healthy subjects, whereas they were 119 in MPM and 42 in WEA. This differences is statistically significant.

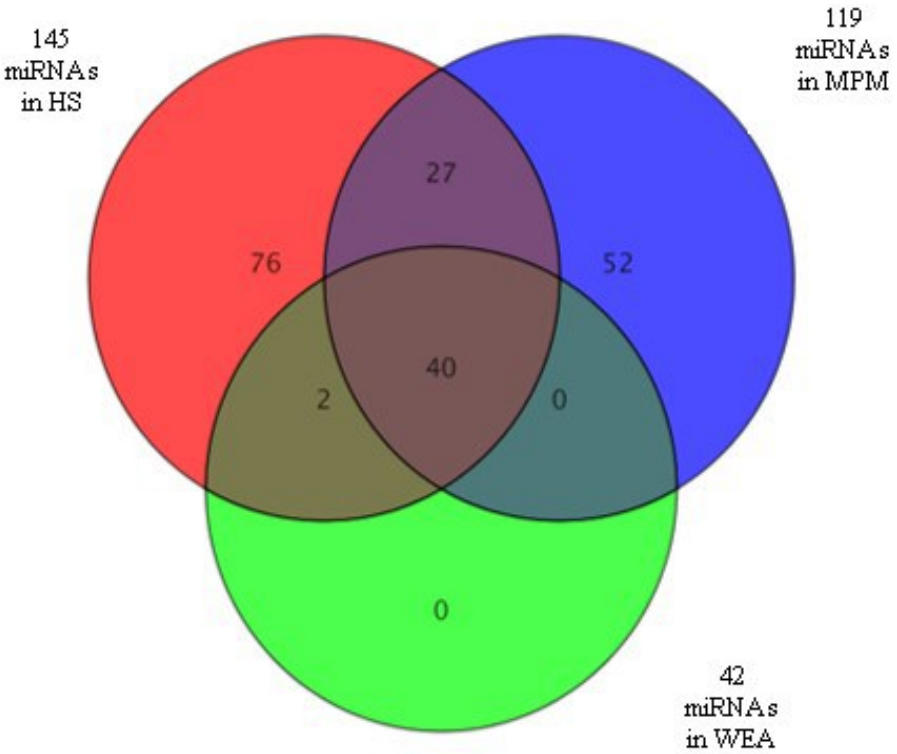

Fig.3 - Venn diagram showing miRNAs detected in sera.

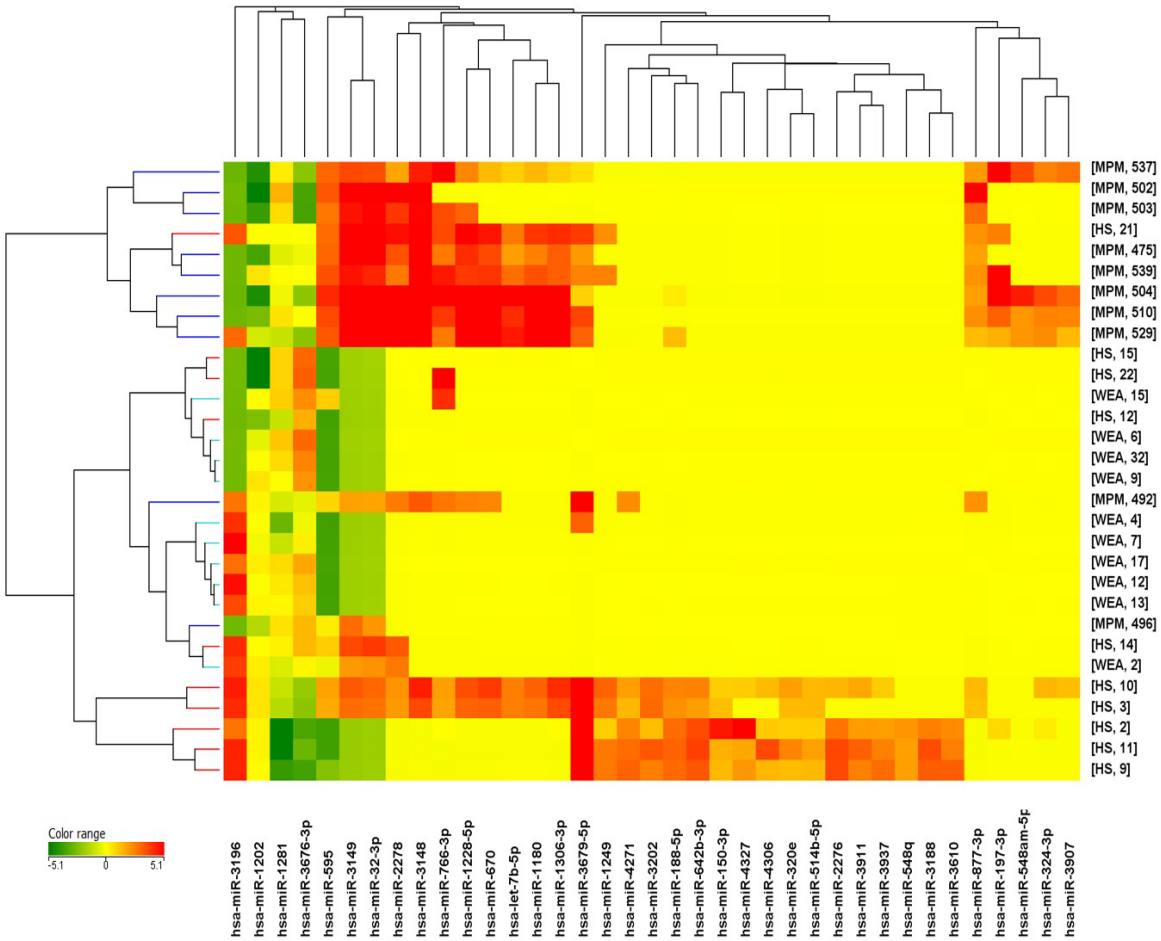

Fig.4 - Cluster analysis of microRNA expression profiles in sera from MPM patients, WEA and HS.

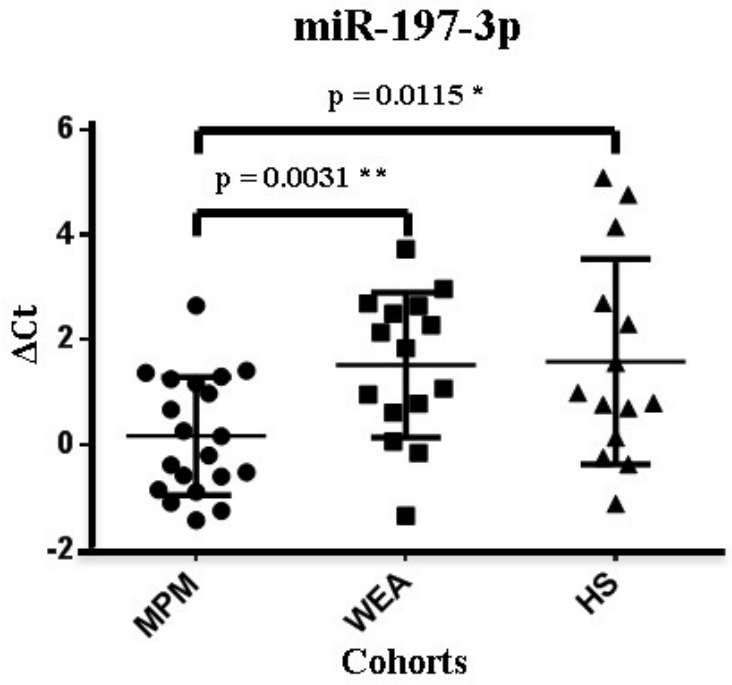

Fig.5 - MiR-197-3p expression in three cohoorts (MPM, WEA and HS)

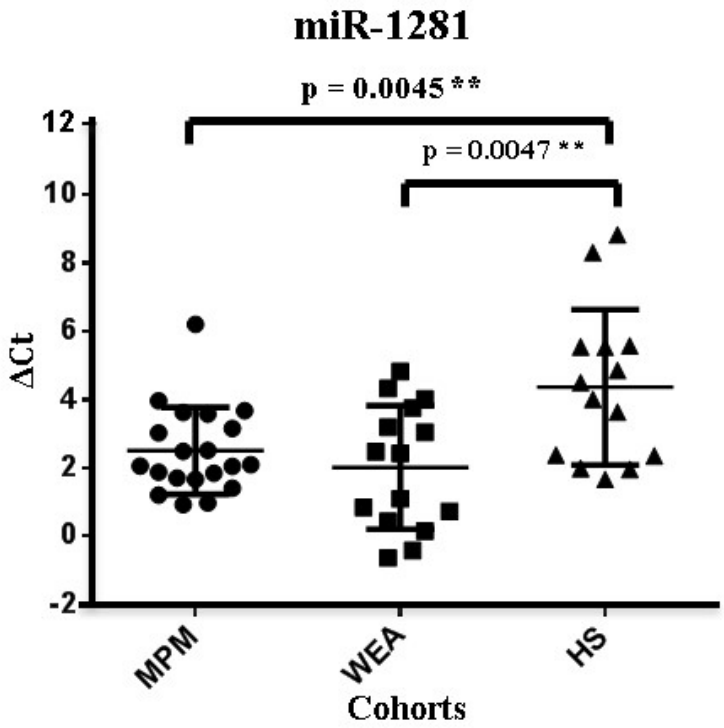

Fig.6 - MiR-1281 expression in three cohoorts (MPM, WEA and HS)

4.6 TABLES

	Normalized data			MPM vs HS		MPM vs WEA		WEA vs HS		
Systematic name	**MPM**	**WEA**	**HS**	**Regulation**	**FC**	**Regulation**	**FC**	**Regulation**	**FC**	**p**
let-7b-5p	-1,1021	-3,2156	-2,4594	*up*	2,5620	*up*	4,3271	*down*	-1,6890	0,052486
miR-1180	-0,6633	-3,2156	-2,2398	*up*	2,9824	*up*	5,8656	*down*	-1,9667	0,050773
miR-1202	1,2882	4,1715	2,5602	*down*	-2,4151	*down*	-7,3785	*up*	3,0552	0,081746
miR-1228-5p	0,3912	-3,2156	-2,0449	*up*	5,4117	*up*	12,1823	*down*	-2,2511	0,007009
miR-1249	-3,0104	-3,2156	-1,7707	*down*	-2,3615	*up*	1,1528	*down*	-2,7223	0,018978
miR-1281	3,4131	3,2472	1,5335	*up*	3,6796	*up*	1,1218	*up*	3,2800	0,097981
miR-1306-3p	-0,6182	-3,2156	-2,0092	*up*	2,6226	*up*	6,0519	*down*	-2,3076	0,071601
miR-150-3p	-3,2695	-3,2156	-2,2309	*down*	-2,0542	*down*	-1,0381	*down*	-1,9788	0,051636
miR-188-5p	-3,0793	-3,2156	-1,8494	*down*	-2,3454	*up*	1,0991	*down*	-2,5778	0,018978
miR-197-3p	-0,7610	-3,2156	-2,9142	*up*	4,4480	*up*	5,4815	*down*	-1,2323	0,047500
miR-2276	-3,2695	-3,2156	-2,0743	*down*	-2,2898	*down*	-1,0381	*down*	-2,2058	0,047500
miR-2278	0,7015	-2,9345	-1,9823	*up*	6,4258	*up*	12,4323	*down*	-1,9347	0,002154
miR-3148	1,8715	-3,2156	-1,8038	*up*	12,7759	*up*	33,9906	*down*	-2,6605	0,000109
miR-3149	3,3830	-2,8135	-0,9095	*up*	19,5963	*up*	73,3389	*down*	-3,7425	0,000092
miR-3188	-3,2695	-3,2156	-2,3036	*down*	-1,9532	*down*	-1,0381	*down*	-1,8815	0,083669
miR-3196	-2,1135	0,9510	1,5813	*down*	-12,9485	*down*	-8,3654	*down*	-1,5479	0,081746
miR-32-3p	3,1347	-2,8072	-1,0024	*up*	17,5959	*up*	61,4774	*down*	-3,4939	0,000092
miR-3202	-3,2695	-3,2156	-1,9073	*down*	-2,5708	*down*	-1,0381	*down*	-2,4765	0,014296
miR-320e	-3,2695	-3,2156	-2,4206	*down*	-1,8011	*down*	-1,0381	*down*	-1,7351	0,015185
miR-324-3p	-2,1411	-3,2156	-3,0620	*up*	1,8933	*up*	2,1059	*down*	-1,1123	0,073100
miR-3610	-3,2695	-3,2156	-2,4331	*down*	-1,7856	*down*	-1,0381	*down*	-1,7201	0,083669
miR-3676-3p	-0,9013	1,8540	-0,0857	*down*	-1,7600	*down*	-6,7518	*up*	3,8363	0,047500
miR-3679-5p	-1,3530	-2,8888	0,2802	*down*	-3,1020	*up*	2,8996	*down*	-8,9947	0,047500
miR-3907	-2,2813	-3,2156	-3,1256	*up*	1,7953	*up*	1,9109	*down*	-1,0644	0,074411
miR-3911	-3,2695	-3,2156	-2,2902	*down*	-1,9715	*down*	-1,0381	*down*	-1,8991	0,046878
miR-3937	-3,2695	-3,2156	-2,3920	*down*	-1,8371	*down*	-1,0381	*down*	-1,7697	0,047500
miR-4271	-3,0353	-3,2156	-2,0329	*down*	-2,0033	*up*	1,1331	*down*	-2,2699	0,047500
miR-4306	-3,2695	-3,2156	-2,4794	*down*	-1,7291	*down*	-1,0381	*down*	-1,6657	0,075110
miR-4327	-3,2695	-3,2156	-2,2034	*down*	-2,0936	*down*	-1,0381	*down*	-2,0169	0,096867
miR-514b-5p	-3,2695	-3,2156	-2,5183	*down*	-1,6831	*down*	-1,0381	*down*	-1,6214	0,010993
miR-548am-5p	-2,0017	-3,2156	-3,2610	*up*	2,3938	*up*	2,3196	*up*	1,0320	0,050773
miR-548q	-3,2695	-3,2156	-2,6473	*down*	-1,5391	*down*	-1,0381	*down*	-1,4827	0,081746
miR-595	3,3451	-2,4018	-0,8586	*up*	18,4264	*up*	53,7046	*down*	-2,9145	0,000096
miR-642b-3p	-3,2695	-3,2156	-1,6936	*down*	-2,9811	*down*	-1,0381	*down*	-2,8717	0,013556
miR-670	-0,1444	-3,2156	-2,0461	*up*	3,7365	*up*	8,4044	*down*	-2,2492	0,031384
miR-766-3p	0,1875	-2,7920	-1,9239	*up*	4,3209	*up*	7,8871	*down*	-1,8253	0,033535
miR-877-3p	-0,9961	-3,2156	-2,7473	*up*	3,3664	*up*	4,6572	*down*	-1,3835	0,000318

Tab.1 - MiRNAs detected dysregulated in three groups by microarray analysis.

Microarray

Systematic name	Comparison	Regolation	FC	ANOVA p
miR-197-3p	MPM vs HS	*up*	4.4	0.047*
	MPM vs WEA	*up*	5.5	0.047*
miR-1281	MPM vs HS	*up*	3.7	0.097*
	WEA vs HS	*up*	3.3	0.097*
miR-32-3p	MPM vs HS	*up*	17.6	<0.001***
	MPM vs WEA	*up*	61.5	<0.001***

Tab 2 - Microarray data of differentially expressed miRNAs miR-197-3p, miR-1281 and miR-32-3p.

qRT-PCR

Systematic name	Comparison	Regolation	FC	ANOVA p	t-tesp p
miR-197-3p	MPM vs HS	*up*	1.8	0.0093**	0.0115*
	MPM vs WEA	*up*	2.5	0.0093**	0.0031**
miR-1281	MPM vs HS	*up*	2.5	0.002**	0.0045**
	WEA vs HS	*up*	3.5	0.002**	0.0047**

Tab 3 - RT-qPCR data of differentially expressed miRNAs miR-197-3p and miR-1281.

qRT-PCR

Systematic name	Comparison	Regolation	Pos/tot (% pos)	t-tesp p
miR-32-3p	MPM vs HS	*up*	6/20 (30%) MPM 0/16 (0%) HS	0.024*
	MPM vs WEA	*up*	6/20 (30%) MPM 0/15 (0%) WEA	0.027*

Tab.4 - RT-qPCR data of differentially expressed miRNA miR-32-3p

5. DISCUSSION

My research, carried out during the three years of my PhD program, is focused on the evaluation of the expression profiles of microRNAs in the sera of patients affected by malignant pleural mesothelioma. The analysis of immunohistochemistry confirmed that date obtain from the patology department that speciemens were from patients affected by MPM. This experiment was performed to confirm that the collected sera were from MPM patients. Similarly by the same thecnical approch it was possible to verify that normal mesothelial cells were from healty subjects (HS). Once again the sera collected from this individuals rappresent sample from normal subject (Figure 1).

My experiments whith serum samples allowed to purify total RNA. Then, circulating miRNAs were isolated with commercial kit as decribe in materials and methods. The expression profile of miRNAs from sera of MPM has been poorly investigated. In my study using the microarray technology by Agilent platform, high differences in miRNA expression profiles were detcted in MPM, WEA and HS serum samples. (Figre 2 and Figure 3).

In my study, a comparative analysis of miRNA expression in serum samples from MPM affected patients, workers ex-exposed to asbestos fibers and normal subjects with the same age, was performed with the aim to reveal new MPM specific markers. These miRNAs would be predictive for the disease and targets for potential innovative therapeutic approaches.

Serum samples of 30 patients/individuals from three groups (10 MPM, 10 WEA and 10 HS) were employed to isolate total RNA. These samples were investigated using a microarray approach. Then based on the data obtainde by microarray technology, dysregulated miRNAs were validated by RT-qPCR. Togheter with the 30 initial samples,

additional 10 MPM, 5 WEA and 5 HS for a total of 50 samples were analyzed by RT-qPCR.

Significantly, 145 miRNAs were found to be expressed in the control samples than in MPM and WEA samples. This difference is statistically significant (MPM vs HS $p = 0.0053$**; WEA vs HS $p < 0.0001$***). My data are in agreement with previous reports (Lu, Getz et al. 2005). In their study these autors found that microRNAs are foud dysregulated in different human cancers, including MPM. Indeed, miRNAs in tumors were detected down-regulated compared to normal tissues. Moreover, similar results were reported in aanother study (Guled, Lahti et al. 2009) employing MPM tissues. In this investigation it is possible to distinguish the tumor profile from that of the normal tissue. A the same time this miRNA profile showed a higher miRNA expression compared to that of tumor tissues.

In my analyses deregulated miRNAs expression was detected in serum samples of WEA with a reduced number compared to those revealed in the other two cohorts (MPM and HS). It is possible that teh asbestos fibers may regulated negatively the miRNA expression, in a way similar to that detected for the immune system, which appeared down-regulated in WEA (Tulinska, Jahnova et al. 2004, Maeda, Nishimura et al. 2010, Mazzoni, Corallini et al. 2012). One may speculate that the asbestos fibers in WEA subjects induce a sort of generalized gene silencing.

On the other hand, I found that miRNAs expression is higher in MPM compared to the other two groups WEA and HS.

As shown in the results section, in my study I detected three dysregulated miRNAs, namely miR-197-3p, miR-1281 and miR-32-3p. Specificaly this three miRNAs were upregulated in MPM.

MiR-197-3p was resulted up-regulated in MPM vs HS with the fold-change of 4.4, whereas it was detected up regulated in MPM vs WEA with the fold-change of 5.5, by

microarray analysis. This result is in agreement with the RT-qPCR data, which show lower ΔCT value in MPM serum groups in comparison with both WEA and HS cohorts. Similarly, miR-197-3p was detected significantly up-regulated in sera or tissues of a variety of human cancers, such as lung, breast, pancreatic, ovarian, follicular thyroid cancers, SCLC, and NSCLC (Du, Schageman et al. 2009, Hamada, Satoh et al. 2013, Stokowy, Eszlinger et al. 2014, Dubey and Saini 2015, Mavridis, Gueugnon et al. 2015, Shaker, Maher et al. 2015, Zou, Wang et al. 2015).

The strong up-regulation of miR-197, found in MPM sera in comparison with HS and WEA cohorts, indicates that this miRNA is present at the normal level of expression in sera of HS and WEA. Subsequently, after the MPM onset the release of this miRNA may increase, allowing the detection of miR-197 up-regulated. If confirmed by other studies this result is particularly significant because miR-197 could be a new specific biomarker of this neoplasia.

The microRNA.org database indicates the *FOXO3* (Forkhead box O3) gene is one of a predictive target of miR-197. Interestingly, FOXO family is a key genes promoting the apoptosis process. Based on this data, one may speculate that mir-197 upregulated could lead to the FOXO gene down-regulation, which in turn could block the apoptosis of the cancer cells.

MiR-1281 was found up-regulated in MPM vs HS with fold-change of 3.7. Comparative analysis between WEA with HS sera showed a fold-change 3.3, by microarray technique. These results were confirmed by RT-qPCR, with a lower ΔCT values in MPM and WEA groups in comparison with HS cohort. This miRNA was investigated in human cancers by three different teams. However, our data on MPM differ from that detected in bladder cancer, where it was found down-regulated (Pignot, Cizeron-Clairac et al. 2013), in adrenocortical tumors it was not differentially expressed (Szabo, Luconi et al. 2014) and in

non-131I-avid lung metastases of papillary thyroid carcinoma is up-regulated as compared with in 131I-avid lung metastases (Qiu, Shen et al. 2015).

MiR-32-3p was detcted up-regulated in MPM vs HS group, with a the highest fold-change of 18 in microarray. Moreover, comparing MPM with WEA this miRNA was up-regulated with a fold-change of 61. These two results were confirmed by RT-qPCR. In particular, by this technique the expression of this miRNA was detected only in 6 individuals of the MPM group, while none of WEA and HS groups contained it. In another study, the expression of miR-32-3p was found up-regulated in HCC and derived cell lines. (Yan, Chen et al. 2015). It has been published that miR-32-3p down-regulates phosphatase and tensin homolog (*PTEN*) through binding to 3′-UTR of PTEN mRNA, whereas PTEN was identified as a tumor suppressor found mutated in a large number of cancers. These data indicate that the expression level of miR-32-3p could affect the proliferation, migration, and cancer cell invasion. Altogether these results suggest that miR-32-3p can be proposed as a potential target for cancer treatment (Yan, Chen et al. 2015). The expression of miR-32-3p has been shown to be upregulated in malignancies of different histotypes, including kidney (Petillo, Kort et al. 2009) and colo-rectal carcinomas (CRC) (Wu, Yang et al. 2013). Jalava et al demonstrated that has-miR-32-3p targeted B-cell translocation gene 2 (BTG2), a transcriptional cofactor with anti-proliferative properties (Jalava, Urbanucci et al. 2012). These data suggest that miR-32-3p has a fundamental role as an oncogene. Currently, there are accumulating evidences that the aberrant expression of this miRNA is linked to the development of different malignancies (Wu, Yang et al. 2013).

It has been proposed that circulating microRNAs can be used as new biomarkers. It has been suggested that these molecules could act at a distance, moving through the circulatory stream. Specific miRNAs can be incorporated into biological structures, which may protect them from degradation (Kinet, Halkein et al. 2013). It was demonstrated that exosomes contain microRNA, which can be delivered to another cell, which can be functionaly active

in new location (Valadi, Ekstrom et al. 2007, Kogure, Lin et al. 2011, Fabbri, Paone et al. 2012). One may speculate that the up or down regulation of circulating miRNAs is not linked to a variation of their synthesis within the cells, but they are probably related to the release activities into the blood stream.

In HS and WEA sera both miR-197 and miR-32-3p are expressed at low lever or even absent. In MPM sera an enhancement of expression of both these miRNAs has been shown. Considering that miR-197 down-regulates FOXO3 gene, while miR-32-3p downregulates the tumor suppressor gene PTEN and the anti-proliferative factor *BTG2*, these events may participate to the MPM onset.

In conclusion in my experiments, a stable miRNA to be used as endogenous internal control (miR-3665) togheter with circulating miRNA (miR-197-3p, miR-1281 and miR-32-3p), which were found up-regulated in MPM compared to HS sera, were detected. These three miRNAs could function as oncogenes by promoting cell cycle progression and cell mobility in MPM as shown before in other human cancers. At the same time, these miRNAs probably regulate negatively the expression of tumor suppressor mRNAs by binding to their 3'UTR. This mechanism could generate a positive feedback loop that contributes to cancerogenesis.

These up-regulated miRNAs could affect the proliferation, migration, and invasion in cancer cell and could be potential targets for innovative cancer treatments and new biomarkers for MPM.

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ABSTRACTS AND ARTICLES

A. Puozzo, S. Pietrobon, J.C. Rotondo, I. Masini, E. Mazzoni, I. Bononi, F. Martini, M. Tognon. Association between Merkel cell carcinoma and Merkel cell polyomavirus (MCPyV) in a patient affected by Von Willebrand disease and serologic similarity of MCPyV with non-human primates polyomaviruses. *Abstract XVI Congresso Nazionale AIBG* - NAPOLI 26/27 Settembre 2014

M. Tognon. F. Martini, I. Bononi, E. Mazzoni, J.C. Rotondo, S. Pietrobon, **A.Puozzo**, I.Masini. Sierologic evidence of a strong association between the glioblastoma multiforme and the DNA tumor virus SV40. *Abstract XVI Congresso Nazionale AIBG* - NAPOLI 26/27 Settembre 2014

Francesca Amoroso, Erica Salaro, Simonetta Falzoni, Paola Chiozzi, Giorgio Cavallesco, Pio Maniscalco, **Andrea Puozzo**, Ilaria Bononi, Fernanda Martini, Mauro Tognon, and Francesco Di Virgilio. P2X7 targeting inhibits growth of human mesothelioma. *Oncotarget*, 2016; submitted; pending decision.

Elisa Mazzoni, Giovanni Guerra, Maria Vittoria Casali, Silvia Pietrobon, Ilaria Bononi, **Andrea Puozzo**, Andrea Tagliapietra, Pier Francesco Nocini,4 Mauro Tognon, and Fernanda Martini. Specific antibodies against Simian Virus 40 large T antigen mimotopes in serum samples from elderly healthy subjects. Journal of Infection, 2016; submitted

Ilaria Bononi*, Manola Comar*, **Andrea Puozzo***, Massimo Bovenzi, Mariarita Stendardo, Piera Boschetto, Sara Orecchia, Roberta Libener, Roberto Guaschino, Silvia Pietrobon, Manuela Ferracin, Massimo Negrini, Fernanda Martini, Mauro Tognon.

Circulating miRNAs, found dysregulated in patients affected by malignant pleural mesothelioma, as potential new biomarkers. In preparation.