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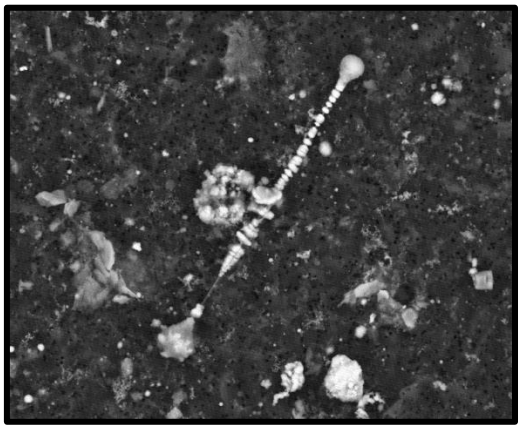
Asbestos exposure and malignant mesothelioma: the role of inorganic fiber burden and disruption of iron homeostasis in lung microenvironment. A postmortem study on human lungs.



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ABSTRACT

Asbestos-related diseases still represents a major public health problem all over the world. Among them, malignant mesothelioma (MM) is a highly aggressive, poor-prognosis cancer, arising from the serosal lining of pleura, pericardium and peritoneum, triggered by asbestos exposure. Asbestos is the collective name of six kinds of naturally occurring minerals, namely chrysotile (the only serpentine asbestos), and the amphiboles crocidolite, amosite, tremolite asbestos, actinolite asbestos, anthophyllite asbestos. The response of human lungs to asbestos inhalation and the molecular mechanisms which lead to MM development several decades after exposure are still largely unknown. One of the most debated issues is the formation of asbestos bodies, that are asbestos fibers covered by an iron-rich amorphous substance. Literature data suggest the key role of iron metabolism in the coating process leading to the formation of asbestos bodies, that has been regarded as both protective and harmful. This study aims to understand more about the reaction of the human organisms to asbestos inhalation and the individual susceptibility to MM. First, the lung inorganic fiber burden has been characterized in lungs of individuals who were previously exposed to asbestos (occupationally or environmentally) using electron scanning microscopy with energy dispersive spectroscopy (SEM-EDS). All the subjects used to work at an asbestos-cement industry, which was active between 1932 and 1993 in Broni, a small town in Lombardy. Unexpectedly, a significantly lower concentration of asbestos fibers has been found in MM compared to asbestosis patients. Chrysotile was not detected at all in any of the examined samples, despite it was largely used at the plant, suggesting a complete clearance of this type of asbestos from lungs. Crocidolite was the most represented asbestos, followed by amosite, tremolite/actinolite asbestos and anthophyllite asbestos, consistently with the data about the industry production. The ratio between asbestos fibers and asbestos bodies was widely different from subject to subject.

Based on the well-known role of iron in asbestos-induced pulmonary toxicity, the second part of the study investigated the frequency of a group of single nucleotide

polymorphisms (SNPs) in genes involved in iron homeostasis in individuals who died from MM compared to controls. Despite the successful DNA extraction from formalin-fixed paraffin-embedded samples (FFPE), we failed to identify any genotype associated with a protective or predisposing effect in relation to MM development as a consequence of asbestos exposure. Finally, on the basis of the established role of BAP1 in MM pathogenesis and its association with ferroptosis impairment observed in various kinds of cancers, the expression of BAP1, transferrin receptor 1 (TRF1), ferritin heavy chain 1 (FTH1) and ferroportin (FPN) has been investigated using immunohistochemistry and rtPCR, finding that asbestos affects the expression of the mentioned proteins in lungs differently in MM patients compared to subjects exposed to asbestos but died of other causes. These findings suggest that a different biological response to asbestos inhalation and to the consequent iron overload in lungs may play an important role in cancer initiation. The formation of asbestos bodies appears to be a key mechanism in the formation of a pro-neoplastic microenvironment, as well as ferroptosis impairment. These results are important from a prevention point of view, as iron metabolism, as well as the consequent oxidative stress, chronic inflammation and cancerogenic stimuli might be targets for therapeutic strategies aiming to delay or prevent MM onset in individuals previously exposed to asbestos. Moreover, knowing the mechanism that can make an individual vulnerable to asbestos can be of crucial importance for prevention.

ABBREVIATIONS

ABs= asbestos bodies
ACM=asbestos-containing materials
ARDs= asbestos-related diseases
EXP-C= exposed controls
Ff/Gdw= fibers per gram of dry weight
FFPE= formalin-fixed-paraffin embedded
FTH1= ferritin heavy chain 1
GWAS= genome wide association study
HC= healthy controls [used in SNP study]
HFE= hephaestin
MM= malignant mesothelioma
NON-EXP= non-exposed controls [used in IHC study]
ROS= reactive oxygen species
SEM-EDS= scanning electron microscope equipped with energy dispersive spectometry.
SNP= single nucleotide polymorphism
TF= transferrin
TFR1= transferrin receptor 1

1. INTRODUCTION

1.1 Asbestos and human diseases

The link between asbestos exposure and Asbestos Related Diseases (ARDs), first of all pleural mesothelioma, has been hypothesized for the first time in 1960 [1], and since then it was largely investigated.

Asbestos has been established to cause two main group of pathologies: the first group is represented by the non-neoplastic diseases, such as the benign manifestation known as “pleural plaques” and, on the other hand, asbestosis, the form of pneumoconiosis (that is defined as a pulmonary fibrosis due to the presence of mineral dust in the lung parenchyma) related to excessive amounts of asbestos fibers [2]. The second group includes the malignant neoplastic diseases: lung cancer and malignant mesothelioma (MM), the neoplasm arising from the serosal linings of the pleural, pericardial or peritoneal cavities.

Among ARDs, MM has to be considered of particular importance, even though its incidence is extremely low in the general population, causing, on a global scale, about the 1% of deaths due to tumors [2]. The extreme relevance of this disease, and consequently the great need of research in this field, is owed to its known relationship with a well-defined trigger (asbestos) and its exceptionally poor prognosis. Indeed, although there is significant variation with important prognostic variables such as age, stage at presentation and histologic subtype, median survivals for patients with mesothelioma generally range from 6 to 14 months [3]. The worldwide incidence and mortality of MM are increasing, despite the ban of asbestos in the early 90s in most European countries and the strict regulations introduced in the US. In particular, the mortality rate increased by 5,37% per year worldwide [4]. Nowadays, even though several important advances have been made recently in the care of patients, currently there are no effective therapies available for MM treatment.

Asbestos (from the Greek word meaning “inextinguishable”) is the term for a family of naturally occurring fibrous minerals widespread in all the world. The word asbestos defines mineral species that occur as bundles of fibers and that can be separated into thin threads. They are classified as asbestos by WHO when they occur in a respirable size with certain dimensions (length > 5 μm, width < 3 μm, aspect ratio greater than or equal to 3:1 [5]). Six different minerals belong to the asbestos group: asbestos actinolite, asbestos tremolite, asbestos anthophyllite, asbestos grunerite (also called amosite from its commercial name, that is the acronym for Asbestos Mines of South Africa), crocidolite and chrysotile (the first five are amphiboles, whereas chrysotile is a serpentine) according to the international nomenclature.

The start of the modern asbestos industrial age began in the second half of the 19th century; due to their distinct physical and chemical properties (such as thermal and

chemical resistance, flexibility, and tensile strength, etc...) chrysotile, crocidolite, amosite and, to a lesser extent, anthophyllite asbestos, have been exploited to create asbestos-containing materials (ACM) used in a large number (at its peak, >3000 products were registered) of practical and industrial application (i.e. building materials, heat and noise insulators, car brakes and gaskets, even toys, jewelry, and cigarette filters). Tremolite asbestos and actinolite asbestos were not used commercially in ACM to any significant extent but they do occur abundantly in several kinds of rocks worldwide mined for ornamental stones and railway ballast. They are particularly abundant either in serpentinites where they can be found as the main mineral in rock veins or as accessory minerals along with other commodities that are mined, such as chrysotile, vermiculite, and talc.

Even though the use of asbestos has been banned in European countries (in Italy, with law n. 257 enacted on 12th march 1992), and strictly regulated in the US, the widespread production and use of asbestos have caused unprecedented human suffering and still represents a major public health problem all over the world. Nowadays, in many countries the mining and/or use of asbestos is still allowed. The highest consumption of asbestos in the period 1995-2003, on a global scale, occurred in Russia, China, Thailand, Brazil, India, Kazakhstan, Iran and Ukraine [4]. In addition, it must be underlined that, even though only six types of natural fibers were defined “asbestos” and therefore regulated, there are more than 400 kinds of naturally occurring mineral fibers that are demonstrated to be carcinogenic, among those erionite (present in Cappadocia - Turkey, Mexico, North Dakota, Nevada), asbestiform antigorite (revealed in New Caledonia) [4], and fibers with fluoro-edenitic composition (Biancavilla, Sicily, Italy) [6].

The latent onset of disease, that occurs after thirty to fifty years since the first exposure [4], has led to a catastrophic epidemic and a continuing onslaught as a result of exposures ended several decades ago. The reason for such a long latency is still unclear; it has been suggested that this is the time required for asbestos fibers to migrate from the lung to the pleural/peritoneal tissue [7].

Despite the open, intense debate still ongoing about the different hazardousness and cancerogenic potential of the various type of asbestos [8–11], according to the World Health Organization (WHO) and the International Agency for Research on Cancer (IARC), all types of asbestos are classified as class I carcinogens [12]. Chrysotile has been demonstrated to be a potent carcinogen in animals [13].

However, the current scientific evidence suggests that the carcinogenic potency of chrysotile asbestos is lower compared to amphiboles in humans [8, 11, 14], due to its more rapid clearance from lungs. The dynamics of lungs clearance of asbestos have been largely investigated on animal models, but few studies address this issue with data on humans. In 1987 Churg and De Paoli observed autoptic lung samples of two groups of asbestos workers whose last exposure occurred, respectively, 2 and 12 years before death; they found that the ratio between chrysotile and tremolite did not show any variation with time, suggesting that chrysotile clearance in lungs occurs shortly after

exposure [15]. These results were confirmed by more recent reviews, considering also experimental data on animals [16, 17].

The same author, on the basis of lung content analysis, suggested that the dose of chrysotile required to cause MM is higher than the dose of amphiboles and, therefore, amphiboles are more potent carcinogens compared to chrysotile, and that tremolite (that can be found in certain types of rocks that are mined to extract chrysotile) is the real cause of chrysotile-induce MM [18]. However, the limitations of human lung content studies in assessing the carcinogenic potential of chrysotile compared to other asbestos types were pointed out by Smith and Wright [10]: this method often excludes fibers shorter than 5 microns, and therefore the role of short fibers may be underestimated. Secondly, chrysotile fibers are often too thin to be detected at SEM. Moreover, the rapid lung clearance of chrysotile leads to underestimate the real exposure to this type of asbestos; the strong presence of tremolite, being a minor component of the chrysotile mineral ore, provides proof of a massive exposure to chrysotile. On this basis, and after reviewing a large amount of epidemiological data and experimental studies on animals, they conclude that chrysotile is, indeed, the main cause of pleural MM [10].

Conversely, an extensive review published in 2000, based on epidemiological data, concluded that the chrysotile, amosite and crocidolite contributed to the specific risk of MM respectively in the ratio 1:100:500 [8]. The same paper stated a less marked difference between the three commercial types of asbestos in relation to the risk of lung cancer, with a ratio chrysotile/amosite of 1:10 and chrysotile/crocidolite of 1:50.

A more recent review, based on in-vitro studies on biodurability of different kind of asbestos fibers, as well as on animal models, human lung burden studies and epidemiological data, concludes that low exposures to chrysotile do not present a detectable risk to health. Moreover, the authors suggest that even high exposures, if of short duration, imply low risks for health [19]. However, the chrysotile rapid clearance, widely accepted, was questioned by Feder at al., who performed the only longitudinal study on fiber lung content, comparing biopsies, broncho-alveolar lavage and postmortem data of the same 12 patients [20]. They demonstrated that asbestos concentrations are stable across intervals of 4-21 years between two samplings and also chrysotile fibers can be detected several years after the cessation of exposure. Anyway, these results indicate that the well-known chrysotile clearance had occurred very early after the end of exposure (this is in line with previous results, as the minimum time elapsed since the cessation of exposure was 3 years in this study).

Interestingly, not all cases of MM are related to a documented exposure to asbestos, occupational or environmental. It is reported that about 20-25% of MM patients have never been exposed to asbestos [21]. Often the subject can be unaware of the exposure, because of the wide diffusion of asbestos in the environment. Consistently with this statement, Krayne et al. found, among 516 cases of MM for which an electron microscopy study had been performed, 83 MM with lung contents comparable to the preference population [22]. Overall, the literature data agree in indicating that a very

small dose is sufficient to cause MM in predisposed individuals and there is no evidence of a threshold below which there is no risk of developing MM [23].

1.2 Malignant mesothelioma and asbestos fiber burden in lungs

In literature, there are few studies that assess the inorganic lung content using analytical electron microscopy. This technique can be performed on a small portion of lung parenchyma, after acid digestion, using a transmission or a scanning electron microscopy equipped with a microprobe for energy-dispersive spectroscopic elemental analysis. This method represents the only way to quantify the inorganic fibers in lungs, as well as asbestos bodies (ABs), and, at the same time, to classify the fibers, according to their elemental composition, into mineralogical types. Analytical electron microscopy represents, therefore, an irreplaceable tool for the assessment of asbestos exposure, and despite the costs and the time required, that can be several days for each sample, it is of crucial importance in the study of asbestos effect on human organisms.

The lack of studies in literature is due to the scarce availability of suitable samples. In fact, for the optimal execution of this technique, abundant samples of formalin-fixed normal lung parenchyma (free from neoplastic invasion and fibrosis) are required. The samples must be still in formalin (not paraffin embedded). Moreover, the sampling site must always be the same, namely the inferior lobe of the right lung. Obviously, the availability of such samples (and of suitable controls) is limited.

The interest of the scientific community for asbestos-related diseases started in the 1960s, and since then the fiber quantification and classification in lungs was addressed by some scientists, who tried to understand if a different quantity or quality of fibers in lungs drives differently the risk of developing MM. Wagner and coll. were among the first to undertake this research; in 1982 they studied, on postmortem samples, the lung content of former employees of an asbestos textile factory using transmission microscopy [24]. Interestingly, they found similar concentrations of asbestos, and in particular of crocidolite and chrysotile, in MM and controls, suggesting that MM is not related to any peculiar kind of asbestos. In 1984, Churg et al. analyzed the lung content of 6 long-term chrysotile miners and millers with pleural MM and controls (miners and millers without asbestos-related diseases) finding similar lung burdens and similar dimensional characteristics of fibers in cases and controls, but MM patients presented more components of chrysotile ore (chrysotile and tremolite asbestos) [25]. Mc Donald et al. conducted electron microscopy observations on lung samples of Quebec miners, revealing a similar amount of chrysotile in MM and controls and attributed most MM cases to amphiboles [26]. Another important lung content study was carried by Roggli et al. in 1993, on lung samples of insulators or shipyard workers, finding that amosite was the most frequent kind of asbestos, followed by non-commercial amphiboles, chrysotile and crocidolite. The authors concluded that tremolite probably accounted for a much higher exposure to chrysotile than that observed on autoptic samples and questioned the role of crocidolite as the most carcinogenic kind of asbestos [27]. In the same years, two lung-content studies were conducted in Japan. Morinaga et al. found asbestos in 19 of the 23 examined MM cases [28]. Amphiboles were detected in 13

cases, while in five cases only chrysotile was found. Five out of the 17 controls' lungs contained asbestos fibers. This study indicates that chrysotile has, indeed, a role in MM causation. Sakai et al. found a significantly higher concentration of asbestos fibers in MM compared to controls [29].

Rogers et al. studied a large series of Australian samples, finding a significant dose-response relation between fiber concentration in lungs and the risk of MM for all fiber types; moreover, longer fibers were related to a higher risk [30].

Another electron microscopic study on lung content conducted on 126 autoptic samples (divided into MM, lung cancers, asbestosis and normal lungs) concluded that the concentration of chrysotile was similar among the groups, whereas the amphibole concentration shows higher levels in MM and asbestosis compared to normal lungs and lung cancer patients. ABs were found mainly in asbestosis group [31]. Likewise, a 1994 study on autoptic lung samples of shipyard and insulation workers (exposed to chrysotile and amosite) evidenced significantly higher levels of amosite in asbestosis patients compared to subjects without asbestos-related diseases, but failed to identify a correlation between asbestos concentration in lungs (and concentration of each kind of asbestos) and MM [32]. The same study excluded any correlation between fibers of different lengths and specific diseases. De Klerk et al. performed TEM lung content analysis on lung samples of Australian crocidolite workers (Wittenoom industry, Gorge) and compared the results with the data about airborne concentration of asbestos [33]. They found a significant association between asbestos fiber amounts in lungs and both duration and intensity of exposure, as well as a negative correlation between asbestos in lungs and time since the end of exposure. This work did not investigate the relation between asbestos in lungs and diseases.

Magnani et al. studied lung samples taken during 48 consecutive autopsies conducted in Casale Monferrato, where an important asbestos-cement factory used to be active, revealing a similar concentration of both asbestos fibers and ABs in subjects with and without occupational exposure to asbestos, indicating that environmental exposure can be as heavy as occupational [34]. More recent studies investigated the lung content of MM patients. Barbieri et al. analyzed eight cases of MM and 13 controls, finding a significant difference in asbestos concentration was detected only between MM with occupational exposure and the control group. Notably, in the latter group, asbestos was found in 6 subjects [35]. The concentration of asbestos in environmentally exposed MM and controls were not statistically significant.

In 2013, Gilham et al. examined at TEM 133 lung samples taken at autopsy form MM patients and 262 from individuals with lung cancer [36]. Interestingly, they found a relation between the risk of developing both MM and lung cancer and the fiber burden measured in lungs. They found mainly amosite and crocidolite, whereas concentrations of chrysotile, tremolite, anthophyllite and tremolite were much lower. Anyway, it is not clear what the reference population was, as they did not examine any individual without asbestos-related diseases.

On the whole, this brief review of the literature about lung content analysis revealed very inconsistent conclusions about the link between the concentration of asbestos in lungs and the risk of developing MM. The studies above summarized, most of which are quite dated, were conducted using different methods of sample collection and preparation and different electron microscopy techniques (TEM, SEM, FEG SEM) and instrumentations. On this basis, more research on large series of patients and controls is necessary in this field.

1.3 The genetic substrate of mesothelioma predisposition

Although mesothelioma has been considered for many years the paradigm of environmentally determined cancers, much evidence, reported in literature, suggests a potential role of a genetic component in the etiology of this disease. Currently, MM is believed to follow the gene x environment model [37]. This hypothesis is supported by several findings:

- Only a minority of asbestos-exposed subjects develop MM (5–17% of heavily exposed individuals)[38].

- Some subjects develop MM following very low doses of asbestos exposure, whereas others, exposed to higher quantities, do not suffer from this disease [39].

- The frequent reports of MPM familial clustering [40–44].

To date, BRCA1-associated protein 1 (BAP1) is the only gene whose role in mesothelioma predisposition is recognized and accepted.

BAP1 is a nuclear deubiquitinating enzyme with several functions, such as regulation of cell cycle and replication, gene transcription, DNA damage response, apoptosis and cell differentiation [45]. Studies on families with high incidence of MM led to the discovery that they were carriers of germline mutations in BAP1 [43]. The role of BAP1 mutations in the susceptibility to mesothelioma has been extensively investigated and confirmed by many studies [46–48]. Subsequently, BAP1 syndrome has been identified as a defined nosological entity, associated with an increased risk of developing rare neoplasms, including, in addition to MM, cutaneous and uveal melanoma, atypical Spitz tumors, clear cell renal carcinoma [49–52].

Even though the incidence of germline mutations of BAP1 is low (1-2% at most), the loss of expression of BAP1 is observed in around 60% of MM [4]. Immunostaining for that protein has been recently validated as a useful diagnostic tool in order to distinguish MM, especially those very poorly differentiated, from other metastatic malignancies [53–55]. Immunohistochemistry (IHC) represents a cost-effective way to investigate the biallelic BAP1 loss. In fact, most BAP1 mutations generate a truncated protein,

which is degraded. Thus, the vast majority of biallelic BAP1 mutations are associated with negative IHC staining [56].

BAP1 gene is a tumor suppressor whose loss of function drives carcinogenesis in various tissues. As other tumor suppressors, BAP1 follows the "two-hit" model [57]. The first hit is represented by the deletion of 3p21, (which is inherited in the familial forms of the above-mentioned cancers). A subsequent mutation causes the inactivation of the remaining allele and the alteration of the protein function or homeostasis [57].

Despite the identification of some frequent somatic mutations in MM, germline alterations in genes other than BAP1 that might be responsible for MM susceptibility have still to be found. Indeed, the familial form of MM, provoked by BAP1 germline mutations, represents a small proportion of MM.

Two genome-wide association studies (GWASs) on MM patients' blood were carried out [58, 59]. Cadby et al. reported an increased frequency of SNPs in three genes (CRTAM, SDK1 and RASGRF2), never reported to be associated with MM before, whose functions are related to cell adhesion and/or cell migration and therefore are likely to play a role in the response to inhaled asbestos. However, this study failed to detect any association of MM with mutations in GSTM1, XRCC1, XRCC3, SOD2, and EPHX, previously identified in candidate-gene studies [60–62]. Equally important, mutations were not found in p16, NF2 and BAP1, major drivers of MM carcinogenesis [59]. Such findings suggested that BAP1 mutations are not a common predisposing factor for MM, but are typical of a peculiar familial form of MM.

Matullo et al. conducted a similar GWAS on 407 MM cases and 389 controls, identifying a number of SNP associated with the risk of developing MM, located, respectively, on genes involved in lung development, respiratory tube development , respiratory system development, metalloendopeptidase activity, and metallopeptidase activity [58]. None of the SNPs identified in this study were the same found in the australian study conducted by Cadby.

These studies found significant associations with several genes analyzing a big cohort, but none of them managed to identify a single gene or even a signature of genes associated with a defined predisposition to MM. Indeed, since 2013, this approach did not lead to any significant continuation.

1.4 The mechanism of asbestos-induced carcinogenesis

After the inhalation of long and thin fibers and their penetration into the pleural space, the interaction of the fibers with mesothelial and inflammatory cells causes a prolonged progress of tissue damage, repair and inflammation that finally lead to carcinogenesis. In a 2013 review, four mechanisms have been suggested to explain the asbestos-induced carcinogenesis [63]:

- Reactive oxygen species are produced by mesothelial cells and inflammatory cells.
- The asbestos fibers interact with the mitotic spindle (creating a physical disruption) and consequently they increase the risk of chromosomal abnormalities.
- The accumulation of hazardous molecules on the fibers.
- Asbestos-exposed mesothelial cells and macrophages release a variety of cytokines and growth factors, which induce inflammation and tumor promotion. Those include tumor necrosis factor-α, interleukin-1β, transforming growth factor-β and platelet-derived growth factor. Thus, the aberrantly activated signaling network among mesothelial cells, inflammatory cells, fibroblasts and other stromal cells may create a pool of mesothelial cells, which harbor aneuploidy and DNA damage, potentially developing into cancer cells and together forming a tumor microenvironment that supports and nourishes them.

Another extensive review focusing on the molecular basis of asbestos-induced lung disease identifies, as key mechanisms, alveolar cell apoptosis (both mitochondria and p53 related pathways), mitochondrial DNA damage and repair mechanisms (especially involving 8-oxoguanine DNA glycosylase and mitochondrial aconitase, a mtDNA repairing system that reacts to redox state), and the asbestos-induced production of reactive oxygen species [64]. This review highlights five factors determining the asbestos fiber toxicity: dose, dimension, biodurability, surface reactivity, genetic background of the exposed individual.

It must be highlighted that the mentioned review focuses on asbestosis rather than mesothelioma or lung cancer, on the (questionable) basis that in nearly all patients with asbestos-related cancers asbestosis is present. As discussed later in this thesis, our results are in contrast with this assumption, as most of subjects who died of MM did not present asbestosis, and most subjects with asbestosis (because they were occupationally exposed to high doses asbestos during life) did not develop MM. However, certainly asbestosis represents a model of heavy exposure to asbestos and studying this disease can provide interesting insights about the biological response of the human lung to high doses of asbestos. Yet, we should remember that MM does not require high doses of asbestos to develop.

A recent review [37] attributes the asbestos-induced carcinogenesis to the chronic inflammation induced by the pro-inflammatory environment determined by the ROS. Mesothelial cells, if exposed to asbestos, die via programmed cell necrosis, that is characterized by the releasing of HGMB1. This protein, together with reactive species of oxygen (ROS), promotes the assembly of NLRP3 inflammosome, which induces the release of IL1, IL1alfa, TNFalfa. In this microenvironment, the surviving mesothelial cells proliferate, accumulating mutations.

MM genome sequencing studies revealed that inactivating mutations occur randomly and are rarely shared among MM biopsies, with the exception of BAP1, and to a lesser

extent NF2, CDKN2A1, and possibly CUL12 [65]. These frequent mutations occurring in MM reveal the most likely carcinogenic alterations. The potent oncosuppressor function of BAP1 is mainly due to its role in both DNA repair and cell death by apoptosis and ferroptosis, a non-apoptotic, iron-mediated cell-death [4, 66]. BAP1 is linked to ferroptosis by decreasing the expression of SLC7A11, a subunit of the Xc- transporter system that internalizes cystin inside the cell [66]. Cystin is essential for glutathione synthesis, that prevents the lipid peroxidation, the main trigger of ferroptosis. Therefore, one of the physiological functions of BAP1 is increasing the sensitivity of mesothelial cells to reactive species of oxygen and facilitating the ferroptosis in response to oxidative stress. Another key role of BAP1 in carcinogenesis is represented by the metabolic shift, induced by low cytoplasmic levels of this protein, from oxidative phosphorylation to anaerobic glycolysis (Warburg effect), that facilitates the neoplastic transformation and is typical of tumoral cells [67].

The other most frequently mutated genes in MM are the following: CDKN2A, whose sequence generates a tumor suppressing gene called *ARF* by alternative splicing that works as an inhibitor of TP53 specific ubiquitin ligase [68]; NF2, a component of Hippo pathway, whose inactivation leads to nuclear accumulation of YAP and TAZ, promoting multiple oncogenes expression ; and CUL1, a core component of SCF E3 ubiquitin-protein-ligase complexes that mediate the ubiquitination of proteins involved in cell-cycle progression, signal transduction and transcription [48].

Many issues about the molecular pathogenesis of mesothelioma have still to be clarified, especially concerning the precise physio pathological mechanism that finally leads, after decades since the first exposure (and often after several years since the end of exposure), to the development of the rapidly lethal disease. The role of fiber type and dimension (length and width) and the importance of exposure dose still remain unsolved.

The asbestos-induced carcinogenesis and, in general, the disruption of the lung microenvironmental after asbestos exposure have been associated with iron overload [13, 69] for many reasons. Iron has a central role in the generation of reactive oxygen species by asbestos interacting with normal products of metabolism, such as H_2O_2, in the biological microenvironment. The surface of asbestos fibers generates highly reactive oxygen species through the chemical reaction below. The presence of iron enhances greatly the production of ROS.

O_2-+H_2O_2 ---------IRON -> HO^- + $HO^{\cdot}$ + O_2

Fe^{2+} + ROOM -> Fe^{3+} + $RO^{\cdot}$ + HO^-

Asbestos-induced radicals, very soon after exposure, damage DNA in several ways, resulting in apoptosis, gene mutation, chromosomal aberrations, and finally cell transformation [64].

Despite its exact origin is still controversial and not fully understood, iron has undoubtedly a central role in the first tool of "defense" of the human organism against asbestos: the formation of asbestos bodies (ABs).

ABs consist of an inhaled fiber coated with iron and organic matter (mainly proteins, mostly ascribable to ferritin). They have a wide range of shapes and dimensions [**FIGURE 1**] and the distribution of the coating is never homogeneous [70].

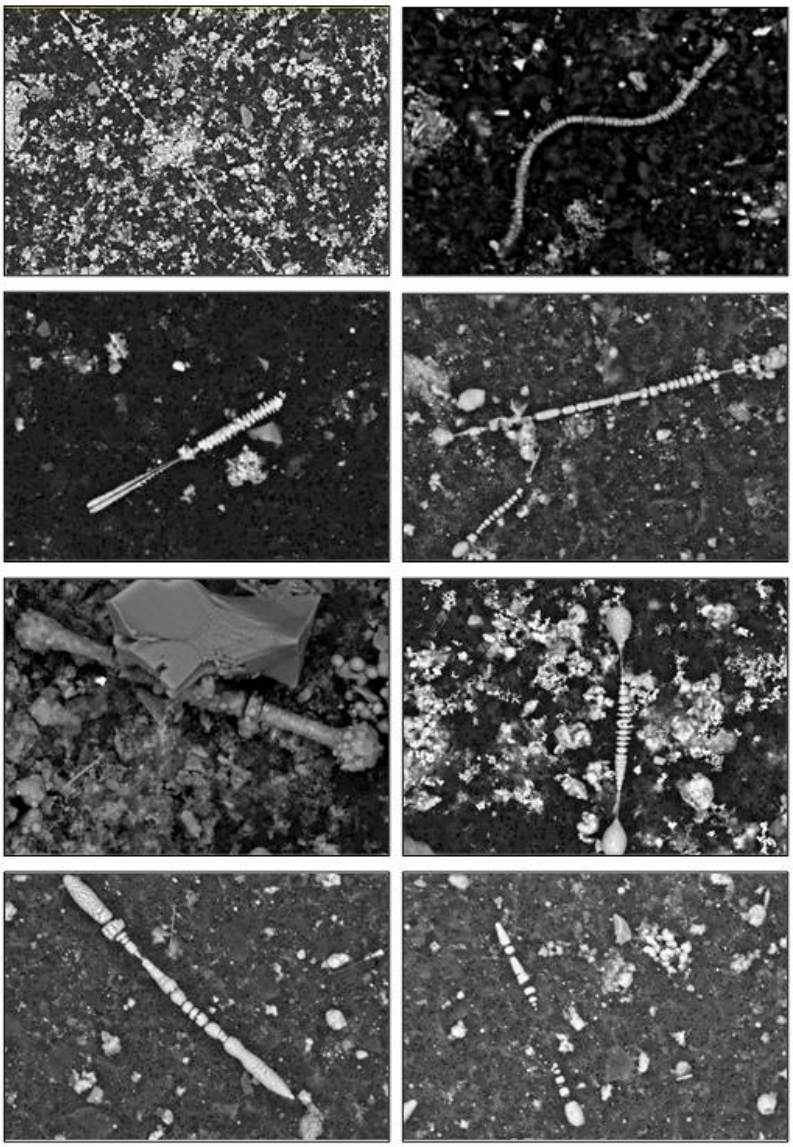


FIGURE 1: SEM images of asbestos bodies.

What is known about the mechanism of formation can be summarized as follows: when an asbestos fiber is introduced into the respiratory tract, macrophages try to phagocytose it. If a fiber is longer than about 20 μm, a single cell is not able to ingest it entirely, consequently the "frustrated phagocytosis" triggers a series of inflammatory mechanisms that promote the accumulation of iron in the cells. Iron micelles appear in macrophages cytoplasm in proximity to the fiber and by accumulation of this ferruginous micelles, together with homogeneous matrix material, the coating is formed around the fiber [2]. Moreover, asbestos fibers have the intrinsic capacity to complex iron from the surrounding environment [69, 71]. Indeed, a vicious cycle is established: the more iron the fiber attracts from the tissue, the more inflammation is triggered and, consequently, more iron is accumulated in close proximity to the fiber.

Then, the Fe^{3+} on the fiber surface is reduced to Fe^{2+} by reductants available in the lung parenchyma (such as superoxide) in order to be internalized in the cells by divalent metallic ion transporter (DMT). Reduced iron has the ability to produce oxidative stress. Once internalized into cells, iron must be safely accumulated. To be stored in ferritin (the principal and safer way of storage) iron must be oxidized again to Fe3+: such reaction happens directly inside the ferritin, that is able to catalyze it. Another way of iron storage consists of hemosiderin, a product of partial degradation of ferritin with a higher iron-to-protein ratio. Indeed, the asbestos bodies are composed mainly of ferritin and hemosiderin, with a very low presence of hematite and metallic iron, found to be well below 5% [72].

It is, therefore, evident that asbestos bodies have two faces: on the one hand, they can be regarded as an attempt of protection against asbestos, separating the cytotoxic fibers from the biological tissues; on the other hand, the formation of asbestos bodies around fibers implies an intrinsic cytotoxic effect, given the generation of reactive oxygen species [71, 73]. Interestingly, in a study conducted using XRF spectroscopy, iron was found to be more concentrated in the inner part of the AB. This could be explained by the release of iron, and consequent Fe overload, originating from the degradation of the fiber [72]. Not all the asbestos bodies are composed mainly of iron, as the finding of asbestos fibers coated by calcium oxalate crystals or calcium phosphate spherules have also been reported [74, 75][75]. Anyway, the iron homeostasis is clearly altered by asbestos, as suggested by increased levels of ferritin, transferrin, transferrin receptors, lactoferrin [71].

The presence of iron on asbestos fibers (but, more importantly, their tendency to bind it in a biological environment) is established to be crucial in determining the cytotoxicity and carcinogenic effect of asbestos fibers [64, 69, 76, 77]. Crocidolite and amosite, compared to chrysotile, are recognized to be more carcinogenic [78]: such effect has been attributed, by some authors, to the higher iron content [71]. Conversely, other experts stated that it is very unlikely that the iron contained in the fiber crystal lattice contributes directly to the cytotoxicity, as it is very strongly bound and, above all, inaccessible to reductants [71]. Moreover, even though chrysotile is recognized to be less dangerous, due to the low iron content, the same cannot be said about tremolite, containing almost no iron. Moreover, experiments carried out on mesothelial cultured

cells demonstrated a high carcinogenic potential of chrysotile, related to the local overload of iron as a consequence of exposure to this kind of asbestos [13]. Moreover, chrysotile induces MM as well as amphiboles in experimentally injected animals [13]. Therefore, the current, shared opinion is that the accumulation of catalytically active iron, responsible for the production of free oxygen radicals, comes from the biological microenvironment that surrounds the fiber after inhalation, rather than from the asbestos fiber itself.

Crovella et al. [79] found an association between variants in some single nucleotide polymorphisms (SNPs) in genes involved in iron metabolism and the risk of developing mesothelioma after asbestos exposure [79]. In particular, they found a significant relation between FTH1 *rs76059597*, TF *rs2715631* and HEPH *rs3747359*, located, respectively, on ferritin heavy chain 1 (FTH1), transferrin (TF) and haephestin genes (HEPH). The same results have been confirmed in another study published in 2019 by the same authors, showing, again, a protective role of HEPH *rs3747359* and TF *rs2715631* [80].

Other studies investigated proteins involved in iron trafficking and metabolism in relation to carcinogenesis. Transferrin receptor 1 (TFR1), an essential protein for iron uptake and cell growth, and therefore expressed in almost all the human cells, is a transmembrane glycoprotein homodimer, that binds diferric transferrin. Its expression levels are regulated post-transcriptionally, in an iron-dependent manner, by iron regulatory proteins (IRPs). TFR1 levels are reported to be increased in several cancers, such as breast, lung, renal cancer [81].

Ferroportin (FPN) is the only known iron efflux protein in mammals, with the essential function of exporting excess iron from the cytosol. [81]. In senescent cells, FPN changes its localization from outer membrane to intracellular compartments, indicating that this protein does not participate actively in iron efflux in this particular setting [82]. In many cancer types the expression of FPN is significantly decreased and lower levels of this protein are associated with a poorer prognosis. FPN expression is regulated by iron regulatory proteins (similarly to TFR1), hypoxia (that upregulates its expression), as well as by hepcidin, that increases iron levels in the cells promoting internalization and degradation of FPN. Conversely, an increase of FPN expression has been observed shortly after experimental asbestos exposure in mice, with a return to baseline after one month since the exposure [69]. The same experiment provided evidence of a persistent increased expression of ferritin in every cell of the lower respiratory tract (and especially macrophages and airway epithelium). The same authors found significantly higher levels of iron, lactoferrin, ferritin, and transferrin receptor in bronchoalveolar lavage in individuals exposed to asbestos (regardless the presence of pathologies) compared to unexposed controls.

1.5 The Fibronit factory

The subjects of this study used to work at Fibronit or to live nearby the plant. The factory, located in Broni, a small town in Lombardy region, was an important asbestos-

cement plant that had been active from 1932 until 1993, producing asbestos-cement artifacts, using mixtures of commercial types of asbestos, including chrysotile, crocidolite, and amosite (the latter used in small quantities, as an additive). Processes gave origin to diffuse emissions from building openings and local exhausts were not installed until the end of the 1970s. The records of the plant reported a total number of 3455 workers employed between 1932 and 1993.

Inhabitants of the area of Broni (and asbestos workers) have been subjected to several epidemiological studies demonstrating an increased mortality from mesothelioma [83–88]. Yet, the analysis of asbestos lung content has never been performed on this population.

2. AIMS

The present project aims to investigate a large series of deaths occurred in individuals previously exposed to asbestos, in order to clarify its role in causing human diseases, first of all MM. The main goal is to understand more about the reaction of the human organisms to asbestos inhalation and possible differences from subject to subject, maybe genetically determined.

The first part of the study consists in the analysis of the lung inorganic fiber burden by electron scanning microscopy with energy dispersive spectroscopy (SEM-EDS). The aim is to evaluate if the amount of inorganic fibers in human lungs, and especially asbestos, their mineralogic characteristics and dimensions, as well as the concentration of ABs, drives differently the occurrence of MM.

The second part of the study aims to compare the frequency of a group of SNPs in genes involved in iron homeostasis (previously found to be related to protection/predisposition to MM development after asbestos exposure) in individuals who died from mesothelioma, in exposed and in non-exposed controls, in order to identify genotypes that could have a protective role or may, on the contrary, increase the susceptibility to developing MM as a consequence of asbestos exposure.

Based on the pivotal role of iron in asbestos-induced carcinogenesis, the third part of the study aims to investigate the expression of four proteins involved in iron trafficking and ferroptosis, in order to understand if asbestos inhalation alter the expression of such proteins in lungs and if those alterations are different in lungs of patients who died of MM compared to subjects exposed to asbestos but died of other causes. In particular, beside BAP1, related to ferroptosis, the expression of transferrin receptor 1 (TRF1), ferritin heavy chain 1 (FTH1) and ferroportin (FPN) is investigated in order to address the effect of asbestos on the crucial points of iron homeostasis in lung cells: iron import, storage and export.

3. MATERIALS AND METHODS

3.1 Scanning electron microscopy and Energy Dispersive X-ray Spectrometry (SEM-EDS)

3.1.1 Subjects and study design

People who died for asbestos related diseases (ARDs) confirmed by autopsy and histological examination were the target population. The eligible were all those died for MM and asbestosis living in a small town in Northern Italy (Broni, Pavia province) - where an important factory manufacturing asbestos-cement was operating from 1932 to 1993 – and included in the records of Forensic Medicine Department of the University of Pavia from 2000 to 2018. Forensic autopsies were ordered by the Prosecutor in the context of a penal trial for manslaughter related to violation of regulations concerning safety at the workplace. A total of 188 subjects were initially enrolled in the study. At the time of forensic autopsy, in each case, the whole lungs had been collected, formalin-fixed and stored for further examination at the Section of Legal Medicine and Forensic Sciences of University of Pavia. For each case, the report of the Pathologist, that includes anamnesis, clinical data, autoptic and histopathologic findings is available. The dataset has been built with respect to the kind of exposure to asbestos (occupational, environmental or both of them), the cause of death (MM or not), the histological classification of mesothelioma if present, the survival time since the diagnosis, the latency time (the time interval between the first exposure and diagnosis), the time elapsed between the end of exposure and death. A retrospective cohort design was used. A subsample of 72 subjects (59 MM and 13 exposed controls) was randomly selected from the cohort of eligible subjects.

3.1.2 Endpoints

The co-primary endpoints were the concentrations of total inorganic fibers, asbestos fibers and ABs, as well as the concentrations of each type of asbestos (chrysotile, crocidolite, amosite, tremolite/actinolite asbestos and anthophyllite asbestos) in lung samples of the selected subjects. To compute these concentrations, the inorganic fibers and ABs contained in 0,25 g of right wet lung (inferior lobe) were counted, measured and analyzed using SEM-EDS, according to the protocol described by Belluso et al.[89].

The method consists in chemical digestion (using 13% sodium hypochlorite) of 0,25 g of formalin fixed lung parenchyma (to disregard organic materials), filtration of the suspension through a polycarbonate membrane (Millipore, Darmstadt, Germany) with a diameter of 25 mm and a pore size of 0.45 μm. Then, the filter, dehydrated and pasted on a pin-stub using a carbon tape, is examined by SEM. The observation is performed on an area of 2 mm^2 of filter at 2000 M using backscattered electrons.

According to fiber definition [5], only particles with length-to-width ratio >3, length > 5 μm, and width < 3 μm have been considered. ABs were also counted.

The fiber chemical composition was analyzed using EDS Oxford Inca Energy 200 equipped with INCA X-act SDD detector (Oxford Instruments NanoAnalysis, Bucks,UK).

The number of detected inorganic fibers and ABs were normalized to 1 g of dry tissue as indicated by international guidelines [90, 91] reporting the concentration in terms of burden of inorganic fibers, asbestos and ABs per gram of dry lung tissue weight: ff/gdw.

To identify the different types of inorganic fibers, we compared the EDS spectra with a database internal to laboratory.

Since the technique here used does not allow univocal identification of certain minerals having similar chemical composition and analogous morphology, it is not possible to distinguish chrysotile from asbestiform antigorite and tremolite asbestos from actinolite asbestos. Therefore, we used respectively the following mineral group names: chrysotile/asbestiform antigorite and tremolite/actinolite asbestos.

3.1.3 Control of bias

The investigation on inorganic fiber lung burden has been carried out in two laboratories by two independent observers. In order to minimize the possible bias due to different instruments and microscopists, a detailed protocol including the sample preparation and the data collection parameters was defined before the data collection started and according to the procedure described by Belluso et al. [89]. In addition, periodic inter-laboratory control has been made, before starting the collection of data and when the number of 10, 20, 40, 60 and 72 samples was reached. Besides, five samples were analyzed by both observers, revealing homogeneous measurements.

3.1.4 Variables

Anthropogenic environmental and occupational exposure information, cause of death (investigated through a complete autopsy with histopathologic examination), as well as socio-demographic characteristics, were extracted from the archives of the Forensic Medicine Department. Regarding the type of exposure, we adopted the term “anthropogenic environmental exposure” referred to people who lived in an area with air dispersed asbestos from the asbestos-cement plant [92, 93]. The term “occupational exposure” refers to people who worked in the asbestos-cement industry [94].

3.1.5 Statistical methods

Quantitative variables were summarized as mean with standard deviation, if the normality was respected, and with median and 25th and 75th if not. To verify the normality, the Shapiro-Wilk test was used. Three groups with different types of

exposure were computed: occupational alone, anthropogenic environmental alone, both exposures. To evaluate differences in quantitative variables across groups of exposure to asbestos and histological type of MM the analogous non-parametric test of analysis of variance (Kruskal-Wallis's test) was applied, followed by the appropriate post-hoc test if significant. Bonferroni's correction for multiple comparisons tests was applied. The evaluation of differences between subjects who died from MM and those who died from other causes was performed using the non-parametric unpaired t test (Mann-Whitney test). A p-value less than 0.05 was considered significant, apart from the post-hoc test, in which, taking into account the correction for multiple comparison tests, the significance threshold was 0.0167 (p/k, assuming k=3 contrast), but also in this case the p-value was reported in the same scale and multiply again for k. All analyses were performed using STATA 15®.

3.2 Single Nucleotide Polimorphisms chiaracterization

3.2.1 Subjects and study design

The participants are deceased subjects who underwent a forensic autopsy, followed by a complete histopathological examination, at the Section of Legal Medicine and Forensic Sciences of University of Pavia. This part of the study was conducted on the following three groups of subjects.

1) CASES OF MM WITH KNOWN ASBESTOS EXPOSURE (MM): 50 males with a documented asbestos exposure (assessed through anamnestic information retrieved from the clinical and judicial records) deceased from malignant mesothelioma. The study group was selected among the 188 cases of mesothelioma retrieved retrospectively at the Section of Legal Medicine and Forensic Sciences of the University of Pavia. MM cases are the same examined in the SEM-EDS part of the study, except for the females, who were not considered here.

2) SUBJECT EXPOSED TO ASBESTOS WHO NEVER DEVELOPED MESOTHELIOMA (EXP-C): 50 males with a documented asbestos exposure, either occupational or environmental (assessed through anamnestic information retrieved from the records) who did not develop mesothelioma, but died of other causes (not neoplastic). 35 of them suffered from asbestosis. A time interval of at least 40 years between exposure and death was requested for inclusion. Similarly to the mesothelioma cases, they used to live in Broni or adjacent towns [83] and/or work at the asbestos-cement plant located in Broni. The 50 subjects of this group include the 13 individuals with asbestosis examined with SEM-EDS.

3) HEALTHY CONTROLS WITHOUT ASBESTOS EXPOSURE (HC): 76 unrelated subjects without known exposure to asbestos (general population), randomly selected among the cases handled for forensic purposes in the Forensic Genetic Laboratory of the Section of Legal Medicine and Forensic Sciences. Broni and neighboring towns inhabitants were excluded.

For the DNA extraction, for each case, two 10 μm-thick sections were cut from a formalin-fixed paraffin-embedded (FFPE) block of cardiac tissue retrieved from the archive.

3.2.2 DNA extraction from FFPE samples

The following procedure was performed in order to extract the DNA from the paraffin embedded tissues (note that it is an extraction, not a purification, the latter being unfeasible due to the chemical and physical properties of paraffin).

First, the excess of paraffin around the sample was cut out. Then, the tissue section was put into a non-ionic buffer (300-500 nl) composed as follows:

- 50 mM kCl;
- 10 mM Tris/HCl pH 8.3 (in order to induce lysis of the cells);
- 2.5 mM MgCl2 (salt);
- 0.1 mg/ml gelatin;
- Non-ionic surfactant detergents;
- 0.45% NP40 (Ottifenolethylene condensed oxide);
- 0.45% tween20 (polyoxymethylene sorbitan monolaurate);

Then, 20 μl proteinase K (10 mg/ml) is added and the buffer, containing the tissue, is incubated overnight at 58°C.

The following day, the proteinase k is inactivated by 10 min boiling.

The, the solution in centrifuged at 12000 rpm for 10 min and transferred into a new test-tube.

10-15 μl of the DNA suspension so obtained is then amplified in 50 μl (35 cycles).

3.2.3 SNPs analysis

SNP selection: on the basis of previous literature [79][58][95] the following six SNPs, located respectively in the indicated genes, were selected.

- *rs224575* and *rs224589* gene SCL11A2 (divalent metal iron transporter 1).
- *rs3747359* gene HEPH (hephaestin).
- *rs2715631* gene TF (transferrin).
- *rs76059597* gene FTH1 (ferritin heavy polypeptide 1).
- *rs243865* gene MMP2 (metalloprotease 2).

Quantification by rtPCR

The quantification of the total amount of amplifiable human DNA and human male DNA in the samples obtained from extraction was performed using the Quantifiler® Duo DNA Quantification Kit.

PCR-multiplex amplification

The PCR multiplex reaction was carried out in a total volume of 25 µl using 5 µl of Multiplex PCR Master Mix (Qiagen), 3-6 µl (depending on quantification results) DNA extract (DNA template), 2,5 µl of 10X premixed primers, 2 µl dNTPs, 1,5 µl MgCl2, 0.2 µl Taq Polymerase (plus h2o2). Primer sequences and concentrations in the reaction mix are shown in **Table 1**.

Table 1: sequences of the extension primes used to target the selected SNPs.

PRIMERS				
Gene	**direction**	**sequence**	**primer length**	**segment length**
FTH1	Forward	tgcacagaaaacttacagccag	22 bp	102 bp
	Reverse	atacaggagcagggaggaga	20 bp	
TF	Forward	gattttctctgctgagtgtgcc	22 bp	100 bp
	Reverse	tccttgaagcagcctttcca	20 bp	
HEPH	Forward	cacaaattctggcctggtgg	20 bp	103 bp
	Reverse	gcaggctttccagccagata	20 bp	
MMP2	Forward	tccagtgcctcttgctgttt	20 bp	113 bp
	Reverse	tgagctgagacctgaagagc	20 bp	
DMT1 rs224575	Forward	aaccaatctacccacaattactga	24 bp	104 bp
	Reverse	gccccgcttcaacaaaagac	20 bp	
DMT1 rs224589	Forward	ccaacatgcagggtggagaa	20 bp	100 bp
	Reverse	ctggagcagtggctggattt	20 bp	

Multiplex PCR thermal cycling conditions were: 95°C pre-incubation step for 2 min, then 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 60 s, with a 5 min final extension at 72°C. The size of the PCR products was checked on agarose gels by bromophenol blue loading solution. Positive and negative controls were included.

SNaPshot minisequencing reaction

After PCR amplification, 1 µl Exo + 1 ul SAP (USB, Cleveland, OH) was added to 2-4 µl (depending on the gel results) of the PCR products, in order to remove primers and unincorporated deoxynucleotides, was incubated for 10 min at 37°C followed by 10 min at 80°C for enzyme inactivation.

Multiplex primer extension reactions were carried out in a total volume of 7 µl, containing 2 µl of SNaPshot Ready Reaction mix, 1,5-3 µl of purified PCR product and 0.7 µl of probe mix 10X (+ h2o2). Positive control (control primer as probe for control), as well as negative control, were included [**Table 2**].

Table 2: probes data.

PROBES			
Gene	**SEQUENCE**	**SIZE (BP)**	**added ddNTPs**
FTH1	ttttttgagagggcgctggagtactgaccc	30 b (24 b + 6 poliT)	T/C
TF	agtgtgcctggctgatcttt	20 b	T/G
HEPH	tgcagggctggtgccttgggtgca	24 b	G/C
MMP2	ttttgagacctgaagagctaaagaggt	27 b (24 b + 3 poliT)	G/A
DMT1 rs224575	tttcgctccctgaagtcggttaggtta	27 b (24 b + 3 poliT)	C/T
DMT1 rs224589	ttttttttttcccctgtccttttaagcacataatac	36 b (26 b + 10 poliT)	A/C

Thermal cycling conditions for single base extension reactions were performed according to the SNaPshot multiplex kit user's manual: 24 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s and extension at 60°C for 30 s. The extension primers were designed using Primer3 software and HPLC purified to remove incomplete synthesis products (Invitrogen, Carlsbad, CA). Resulting minisequencing product sizes are around 100 bp.

After the SNaPshot reaction, the excess fluorescence labelled dideoxynucleotides (ddNTPs) were inactivated by adding 1 U of shrimp alkaline phosphatase (SAP) (USB) to 3 µl of the minisequencing reaction. Incubation was performed at 37°C for 60 min followed by 15 min at 80°C for enzyme inactivation.

The minisequencing SAP treated products (1 µl) were mixed with 10 µl of Hi-Di formamide (in order to increase the melting temperature) and 0.1 µl of LIZ- 120 internal size standard (AB). The samples were separated by capillary electrophoresis on an ABI PRISM 310 Genetic Analyser (AB) after denaturation. Data were analyzed using the GeneScan ver. 3.1 software (AB).

3.2.4 Statistical methods

Sample size estimation: allelic and genotypic frequencies were reported in a previous study for three of the polymorphisms of interest [79] for a population living in the area of Trieste, both in cases of MM and healthy controls. Assuming a power of at least 80%, the sample size was computed for each one of the three polymorphisms and the highest estimated was considered. Thus, the minimum sample size is 86 (43 cases and 43 controls).

Statistical analysis: The statistical analyzes were conducted using the software PLINK (v1.07), an open-source C/C++ WGAS tool set developed by Shaun Purcell [96],

The Hardy-Weinberg equilibrium was preliminarily assessed for the genetic data obtained from the three study groups, using a significance p-value <0.001. Hardy Weinberg's law establishes that the genotype frequencies of a population follow a predictable binomial distribution model, that can be calculated from allelic frequencies, if the following equilibrium conditions are satisfied in the target population: random

assortment of gametes, no mutation, no migration, Mendelian segregation of alleles and absence of selection for one of the genotypes [97, 98].

In order to compare the genetic data obtained from the analysis of the three groups of samples, the Pearson Chi-Square ($\chi 2$) test was used for both genotypic and allelic genetic models. The latter can be considered an explorative association analysis; a p-value of less than 0.05 has been defined for significance. In cases where the expected values were lower than 5, the non-parametric Fisher's exact test, was used as an alternative. For the values that were found to be significant, a further study of the models was carried out, including the dominant and recessive models [99].

3.3 Immunohistochemistry and rtPCR

3.3.1 Subjects

This part of the study was conducted on lung samples taken from the same 50 MM and 50 EXP-C mentioned in the SNPs section. As healthy controls, we selected from the archive 20 males over the age of 60 without any asbestos exposure and respiratory diseases who died for traumatic causes (NON-EXP).

3.3.2 Tissue preparation and immunohistochemistry

Serial 5-µm-thick sections were cut from FFPE lung tissue specimens. In MM cases, lung samples free from neoplastic invasion were selected. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 12 min. Then, heat induced antigen retrieval was performed with a Lab Vision™ PT Module (Thermo Fisher Scientific, Fremon, CA). The following primary monoclonal antibodies were used: mouse anti-BAP-1 (1:200; sc-28383, Santa Cruz Biotechnology), rabbit anti-ferritin heavy chain (FTH1) (1:2500; polyclonal, Invitrogen), mouse anti-Transferrin receptor (TFR) (1:1000; H68.4, Invitrogen), and rabbit anti-ferroportin (FPN) (1:2500; Invitrogen). The following secondary antibodies were used: biotinylated anti-rabbit Ig (H+L; made in horse) (Vector Laboratories); biotinylated anti-mouse IgG (H+L, made in goat) (Vector Laboratories). After overnight incubation with the primary antibody at 4 °C, immunoreactions were performed with a standard avidin-biotin complex kit (Vectastain ABC Kit, Vector Labs's) and visualized with diaminobenzidine (Sigma-Aldrich, Milan, IT).

Semiquantitive analysis of immunopositivity

Immunoreactivity was graded semi-quantitatively using an optical Nikon NI-U microscope (Tokio, Japan). BAP1 and FPN were graded as positive or negative, given that we did not identify any different grades or patterns of expression in case of positivity. FTH1 and TFR were scored as negative (0), positive (1) and strongly positive (2) depending on more or less that 50% of the tissue in each 20x field is characterized by a positive immunohistochemical reaction.

Statistical methods

Antigen findings were scored as quantitative discrete variables: overall comparisons among the groups (MM, EXP-C, NON-EXP) were performed with non-parametric Kruskal-Wallis tests; pairwise group comparisons were then evaluated with Mann-Whitney U tests. A p-value of 0.05 was considered statistically significant; a Bonferroni corrected statistical significance of $p < 0.00125$ was considered for post hoc comparisons.

3.3.3 rtPCR

RNAextraction

The RNA extraction was performed using The RecoverAll™ Total Nucleic Acid Isolation Kit (Thermofisher), that is a specific kit designed to extract total nucleic acids (RNA, miRNA, and DNA) from FFPE tissues.

First, four 15-µm thick slides were cut from each paraffin embedded lung block and deparaffinated by in xylene incubation at 50°C for 3 minutes. After centrifugation for 2 minutes at maximum speed, the xylene is removed, and the tissue has been washed in alcohol twice. After that, the tissue pellets have been air dried to remove the residual ethanol. Then, the protease digestion has been performed, adding the digestion buffer and protease and incubating the samples at 50°C for 15 min and then at 80°C for 15 minutes. The next step is the nucleic acid isolation: isolation mixture has been added to the tissue and the mixture is passed through a filter cartridge, followed by wash solutions. Then, DNAse were added to each filter cartridge and they are incubated for 30 minutes, followed by another wash cycle and centrifugation. Finally, the cartridge is eluted with 50 µl of the elution solution provided in the kit.

cDNA synthesis and qPCR

cDNAs were prepared from 200 ng of total RNA using iScript™ cDNA Synthesis Kit (Bio-Rad). qPCR was performed according to manufacturer's instructions with iQ™ SYBR® Green Supermix (Bio-Rad). Primers were designed with Primer3Plus software [100]. Genes were quantified in triplicates, GAPDH was used as housekeeping gene. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

4. RESULTS

4.1 SEM-EDS analysis of fiber burden in lungs

The SEM-EDS analysis was completed in 72 cases, 86.1% males. 36,1% of the entire study group had only anthropogenic environmental exposure to asbestos, 27,8% only occupational, and 36,1 % were exposed in both ways.

In subjects with occupational exposure, the duration of asbestos exposure ranged from 6 to 480 months (median=264, IQR 108-360 months), whereas in anthropogenic environmental exposed individuals the exposure was found to last between 36 and 720 months (median= 414, IQR 258-576). The latency (calculated only in MM cases), defined as the time elapsed between the beginning of exposure and the diagnosis, ranged between 16 and 60 years considering occupational exposure (median= 41 years, IQR 33-48) and 19-80 years (median=53 years, IQR 42-65). Note that some individuals used to live in Broni, nearby the plant, for their entire life.

The time elapsed since the end of exposure ranged between 8 and 44 years (median=21 years, IQR 18-26).

In 81,9% of the analyzed cases, the cause of death, revealed by a forensic autopsy followed by histopathological examination and immunohistochemistry, was pleural MM. In particular, 65.5% % of the deceased had epithelial MM, 10,3% sarcomatoid MM and 24,1% biphasic MM.

The survival time since the diagnosis ranged between 1 and 379 months (median= 15 months, IQR 9,5-28,5). The characteristics above illustrated are summarized in **Table 3**.

Table 3: demographic and anamnestic data of the 72 subjects of SEM-EDS analysis

	N=72
sex	
male	62 (86.1%)
female	10 (13.9%)
cause of death	
no MM	13 (18.1%)
MM	59 (81.9%)
hystological type of MM	
epithelial	38 (65.5%)
sarcomatoid	6 (10.3%)
biphasic	14 (24.1%)
type of exposure	
Both exposures	26 (36.1%)
Environmental exposure	26 (36.1%)
Occupational exposure	20 (27.8%)
latency (occupational exposure), years	median=41.0 (IQR 33.0-48.0)
latency (environmental exposure), years	median=53.0 (IQR 42.0-65.0)
exposure duration (occupational), months	median=264.0 (IQR 108.0-360.0)
exposure duration (environmental), months	median=414.0 (IQR 258.0-576.0)
survival time since diagnosis of MM, months	median=15.0 (IQR 9.5-28.5)
Time since end of exposure, years	median=21.0 (IQR 18.0-26.0)

4.1.1 Concentration of inorganic fibers, asbestos and ABs and type of exposure

Overall, the concentration of total inorganic fibers ranged from 0 ff/gdw to 6679195 ff/gdw (median= 62928.8, IQR 13801,8-253703,5); the concentration of asbestos ranged from 0 ff/gdw to 5689685 ff/gdw (median= 24199,7, IQR 0,0 – 167984,5), and the concentration of ABs ranged from 0 ff/gdw to 3003538 ff/gdw (median= 6292,0, IQR 0-62459,3).

In most samples (77,8 %) the concentration of uncoated fibers **[Figure 2a**] was higher compared to ABs **[Figure 2b]**, with a fibers/ABs ratio ranging from 0.48 to 0.92).

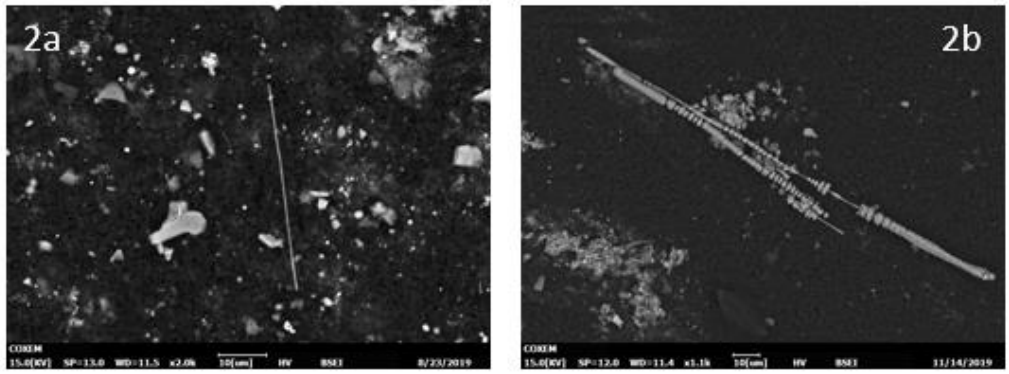


Figure 2: an example of an uncoated asbestos fiber (amphibole) [2a] and of an AB [2b].

Generally, a significant correlation between the amount of asbestos fibers and ABs was detected (Spearman's rho = 0.471; p-value= <0.0001). Yet, the ratio between asbestos fibers and ABs is extremely variable, ranging from 0.0085 to 157.

In 22,2% of the examined lung samples, only uncoated asbestos fibers (without any ABs) were detected. On the contrary, in only 4 samples (5,5%) zero fibers and a number of ABs were detected.

In 19,4 % of the examined lung samples neither fibers nor ABs were observed; 8 of them had only environmental exposure, 3 occupational exposure and 3 of them had both kinds of exposure.

Not only asbestos fibers were detected, but also a considerable amount of other inorganic fibers. The proportion of inorganic fibers classified as not asbestos, compared to asbestos, was not significantly different according to the kind of exposure (p= 0.987). In individuals exposed only occupationally, considering the median, asbestos was 50% of the total inorganic fibers. In subjects exposed only environmentally asbestos was 50% of the total inorganic fibers and in both exposures the percentage of asbestos was the highest (59% of total fibers) **[Figure 3].**

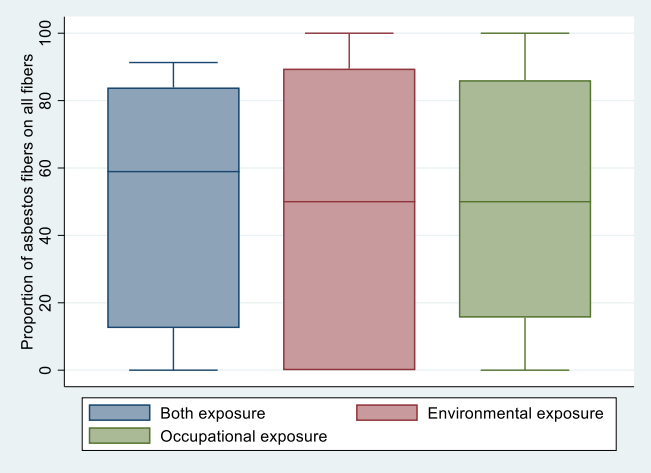


Figure 3: the proportion of asbestos fibers and other inorganic fibers (not asbestos) in the three categories of asbestos exposure, represented as median proportion.

We searched for differences in the amount of asbestos and ABs per ff/gdw among the three types of exposure (**Table 4).**

Table 4: Asbestos fibers and ABs, and length of asbestos: comparison between subjects with occupational exposure alone, anthropogenic environmental exposure alone and both exposures.

	Occupational exposure alone (n=20)	**Anthropogenic environmental exposure alone (n=26)**	**Both exposures (n=26)**	**Test and p-value**
Asbestos per ff/gdw, median (iqr)	22530.5 (IQR 4426.2-267890.8)	20336.9 (IQR 0.0-65623.0)	24199.7 (IQR 0.0-297895.0)	KW= 3,30 0,192
ABs per ff/gdw, median (iqr)	6292.0 (IQR 0.0-364735.6)	0.0 (IQR 0.0-19985.9)	34002.4 (IQR 3369.0-353750.0)	KW = 9,85 **0.007**
Mean length of asbestos, median (iqr)	23.8 (IQR 15.8-40.3)	20.3 (IQR 18.1-31.4)	20.6 (IQR 13.9-26.2)	KW= 0,53 0,764
Length/width of asbestos, median (iqr)	47.8 (IQR 18.0-66.3)	29.0 (IQR 21.0-51.3)	32.9 (IQR 24.8-43.7)	KW= 0,4 0,818

The only statistically significant result concerning this point regarded the concentration of ABs, which was different according to the kind of exposure. In particular, the amount of asbestos bodies was significantly higher in individuals with both exposures compared to those exposed only environmentally. Unexpectedly, the amount of asbestos fibers

was not significantly different across the three groups of exposure, in other words individuals who worked in contact with asbestos had similar concentrations of asbestos in their lungs to subjects who lived nearby the plant.

The median length, width and length/width ratio of asbestos fibers was not significantly different among three types of exposure [Table 4]. However, the higher median length was observed in occupationally exposed subjects (47,8 μm), compared to 32.9 μm in environmental exposure and 29 μm in subjects with both exposures.

4.1.2 Concentration of each kind of asbestos by type of exposure

The asbestos fibers were classified, on the basis of their shape, dimensional characteristics and EDS spectrum, as follows, according to the international guidelines: 1) chrysotile/asbestiform antigorite; 2) crocidolite 3) amosite 4) anthophyllite asbestos 4) tremolite/actinolite asbestos [**Figure 4**]. Tremolite and actinolite were classified together, like chrysotile and asbestiform antigorite, because they cannot be differentiated based on the EDS spectrum, as they have a similar chemical composition. Tremolite and actinolite are both non-commercial asbestos; whereas the chrysotile/asbestiform antigorite group includes chrysotile (classified as asbestos, widely used to produce asbestos maufacts) and antigorite (not belonging to the asbestos family).

Chrysotile/asbestiform antigorite was detected (in extremely low quantity) in only one of the examined samples. Overall, crocidolite is the most represented asbestos (51 % of the totality of asbestos in all the samples) [**Figure 4a**], followed by amosite (46%) [**Figure 4b]**, tremolite/actinolite asbestos (3,3%) [**Figure 4c**] and anthophyllite asbestos (0.9%) [**Figure 4d**].

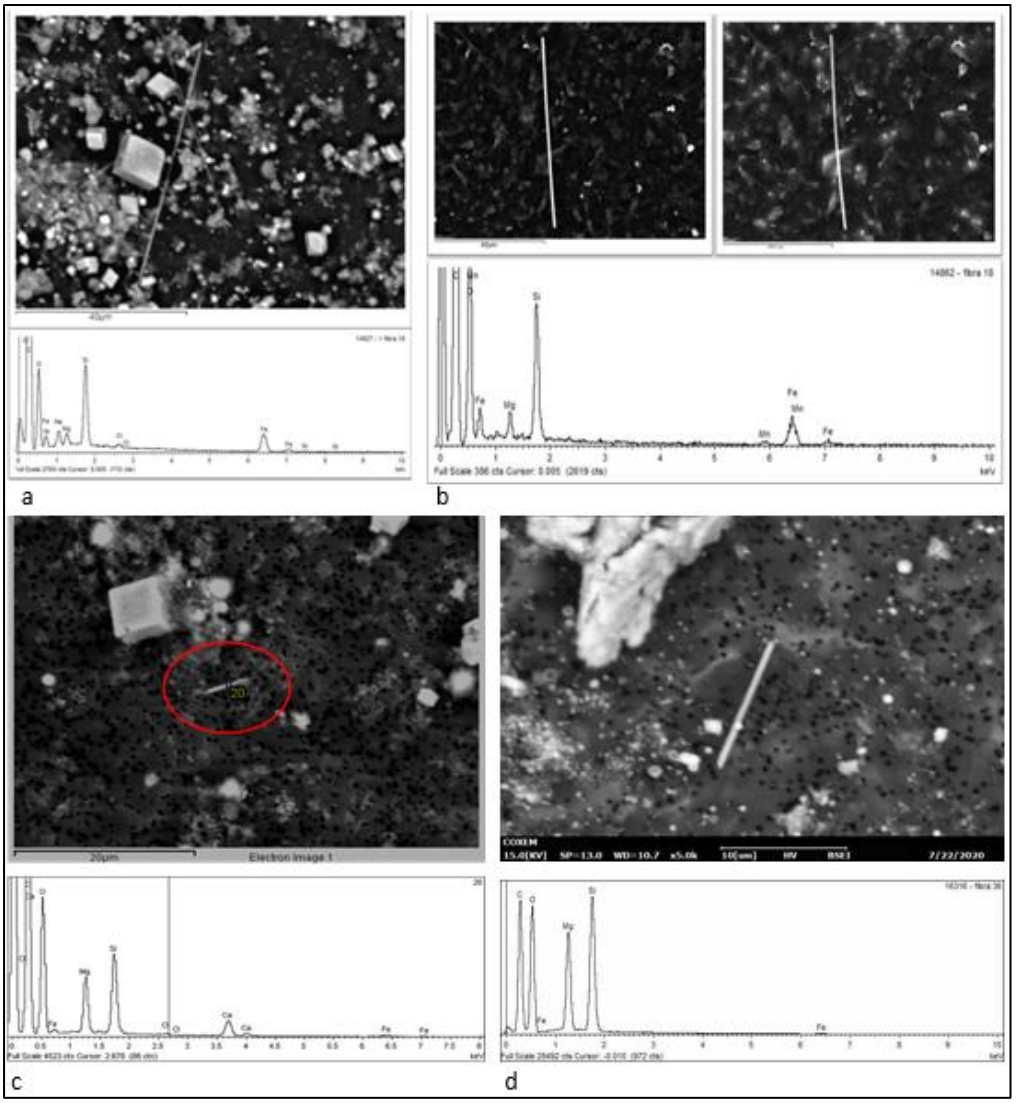


Figure 4: some examples of images and EDS spectra of each detected kind of asbestos. 4a: Backscattered electron SEM image and EDS spectrum of crocidolite. 4b. Backscattered (left) and secondary (right) electron SEM image, and EDS spectrum of amosite. Figure 4c. Backscattered electron SEM image and EDS spectrum of tremolite/actinolite asbestos. The cubic particles are NaCl residuals. 4d Backscattered electron SEM image and EDS spectrum of anthophyllite asbestos.

In the lung samples of subjects with occupational exposure, the median concentration of crocidolite and amosite are similar, with prevalence of the former [**Figure 5].**

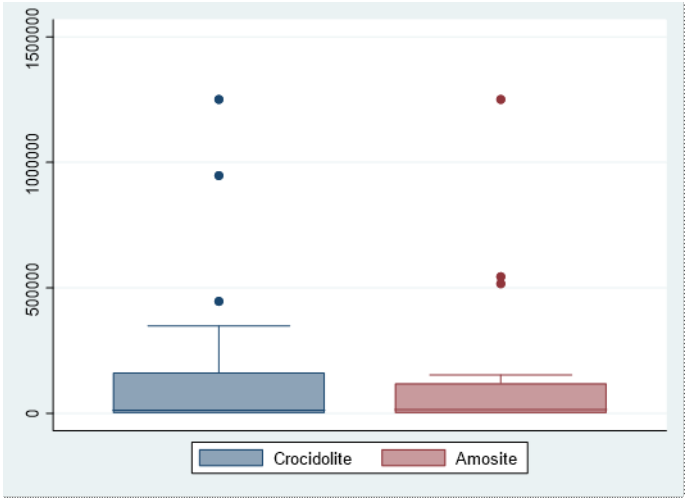


Figure 5: summary of crocidolite and amosite concentration in lungs of occupationally exposed subjects.

We did not find any significant differences in the amount of the fibers belonging to the five asbestos species comparing the lung content of individuals with the three types of exposure. In the three exposure groups, the definitely prevalent species were crocidolite and amosite. Smaller quantities of tremolite/actinolite asbestos were also found, as well as anthophyllite.

4.1.3 Concentration of asbestos, other inorganic fibers and ABs and cause of the death

The totality of subjects without any ABs nor asbestos in lung samples (19,4%) died of MM.

As previously specified, the subjects of the present study underwent a forensic autopsy because they died with a disease related to asbestos exposure. The subjects who died of MM were 59, whereas 13 out of 72 suffered from asbestosis and died of its complications, such as cardiac-respiratory failure, pneumonia or other natural causes related to their interstitial lung disease.

Most of asbestosis patients were elderly affected by multiple diseases. Yet, the most important aspect is that they underwent a documented heavy asbestos exposure, but they did not develop MM. In consideration of their old age and the time elapsed since the beginning of asbestos exposure, it is possible to assume that they would never have developed MM if they had survived more.

Subjects who died of MM showed a significantly lower median amount of asbestos fibers per ff/gdw as compared to subjects who died of other causes ($p<0,001$) [**Table 5**].

Table 5: amount of asbestos fibers, ABs and of each asbestos type: comparison between subjects died of MM and of asbestosis.

	No mesothelioma (n=13)	**Mesothelioma (n=59)**	Test and p-value
Asbestos fibers per ff/gdw, median (iqr)	297895.0 (IQR 30321.4-881567.5)	11320.0 (IQR 0.0-92282.6)	MW= 3,71 **P<0,001**
ABs per ff/gdw, median (iqr)	452800.0 (IQR 0.0-664502.8)	4579.3 (IQR 0.0-50535.7)	MW=1,97 0,048
Chrysotile/asbestiform antigorite median (iqr)	0 (0-0)	0 (0-0)	MW=2,13 0.033
Crocidolite median (iqr)	141450.0 (IQR 70750.0-348134.9)	0.0 (IQR 0.0-28605.1)	MW=4,23 **<0,001**
Amosite median (iqr)	178736.8 (IQR 15160.7-516587.3)	0.0 (IQR 0.0-28605.1)	MW=3,50 **<0,001**
Anthophyllite asbestos median (iqr)	0.0 (IQR 0.0-0.0)	0 (0-0)	MW=0,21 0,828
Tremolite/actinolite asbestos median (iqr)	0.0 (IQR 0.0-5660.0)	0.0 (IQR 0.0-9158.6)	MW=1,70 0,087
Mean length of asbestos fibers median (iqr)	24.2 (IQR 19.7-36.7)	19.3 (IQR 15.1-32.2)	MW=1,40 p=0.159
Length/width of asbestos fibers, median (iqr)	43.7 (IQR 30.2-50.4)	29.0 (IQR 20.3-58.0)	MW=1,70 p=0,087

Similarly, the amount of ABs per ff/gdw was significantly smaller in MM patients (p=0,048).

Moreover, no relevant difference was found in mean length (nor in length/width ratio) of asbestos between subjects who died of MM and those who died from other causes.

4.1.4 Concentration of asbestos types by cause of death and histological characteristics of the neoplasm

Concerning the cause of death, the median concentrations of each type of asbestos showed the tendency to be lower in MM patients compared to subjects without MM, consistently with the significantly lower total amount of asbestos observed in the former group. This difference was statistically significant for crocidolite and amosite. On the contrary, the concentration of anthophyllite asbestos and tremolite/actinolite asbestos did not show significant differences in relation to the cause of the death nor to the type of exposure.

Finally, subjects who died from MM were divided into three groups according to the histological characteristics of the neoplasm (epithelial, sarcomatoid and biphasic). No statistically significant differences were pointed out in the total amount of asbestos, as well as the distribution of each type of asbestos, in relation to the histologic classification of MM **[Table 5].**

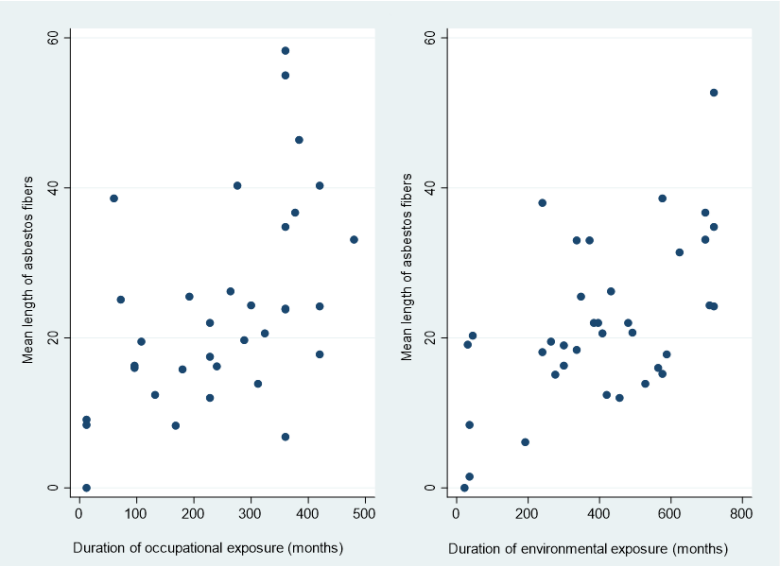
4.1.5 Correlation of asbestos fibers and ABs with duration of exposure, latency, survival, time since end of exposure

Unexpectedly, the amount of both asbestos fibers and ABs, as well as the amount of the single species of asbestos, did not show any significant correlation with the duration of exposure, regardless the type of exposure considered, occupational or anthropogenic environmental. Similarly, no correlation was found between the amount of asbestos and ABs and the latency period (the time between the first exposure and the diagnosis of MM, obviously considering only MM patients), nor the survival time (period of time between diagnosis of MM and death).

Interestingly, even though the time elapsed since the end of exposure varied greatly across the 72 individuals, ranging from 8 and 44 years (median=21 years, IQR 18-26), the amount of asbestos and ABs did not show any correlation with this period of time .

4.1.6 Length and width of asbestos fibers

The mean length of asbestos fibers and the ratio between length and width increase with the duration of the exposure to asbestos (regardless the type of exposure, occupational or anthropogenic environmental), as it is represented in Figure 6.



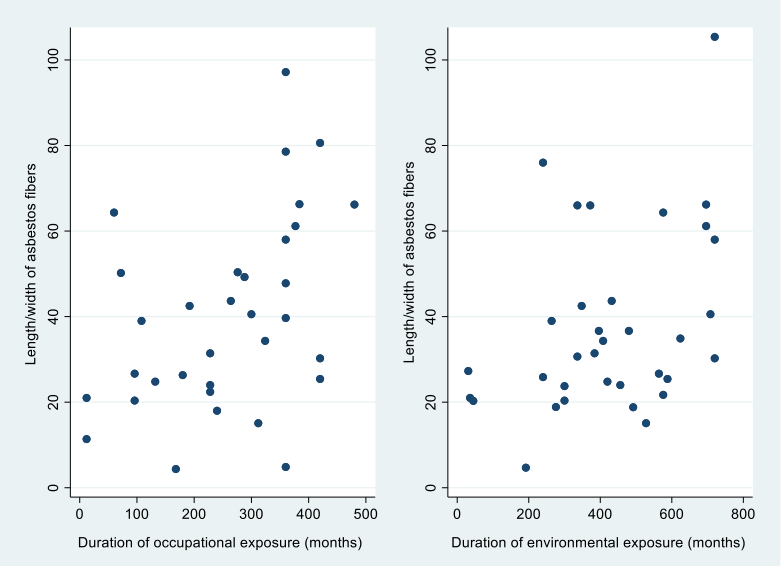


Figure 6: graphs representing correlations between duration of occupational exposure and mean length of asbestos fibers (rho=0.530, p=0,001); duration of environmental exposure and mean length of asbestos fibers (rho=0.510, p=0,001); duration of occupational exposure and length/width ratio of asbestos fibers (rho=0,448, p=0,008); duration of environmental exposure and length/width ratio of asbestos fibers (rho=0,346, p=0,044).

4.2 Single nucleotide polymorphisms (SNPs) in genes involved in iron metabolism

4.2.1 Subjects characteristics

In total, we included 50 patients exposed to asbestos who died from malignant mesothelioma (MM), 50 subjects with ascertained asbestos exposure who died from other causes (not neoplastic) (EXP-C) and 76 healthy males (HC) as a control group.

Demographic and clinical characteristics of MM and EXP-C are summarized in **Tables 6 and 7**. All the subjects were selected among males in order to avoid a potential confounding factor in this phase of the study (so, the 10 women examined in the previous section were not considered here). There were no significant differences between cases and controls regarding age.

Table 6: anamnestic and demographic data of MM patients.

Median age		71
Histological type of MM	**epitelioid**	33 (66%)
	sarcomatoid	3 (6%)
	bifasic	11 (22%)
	unknown	3 (6%)
Asbestos exposure	**occupational**	34 (68%)
	Anthropogenic environmental	16 (32%)
smoking	**Yes**	25 (50%)
	No	11 (22%)
	unknown	14 (28%)
Median survival since diagnosis		12 months
Median latency since first exposure		46 years
Median postmortem interval		66 hours

Table 7: anamnestic and demographic data of EXP-C.

Median age		79,5
Cause of death	**traumatic**	13 (26%)
	natural death	37 (74%)
Asbestosis	**yes**	35 (70%)
	no	15 (30%)
Asbestos exposure	**occupational**	35 (75%)
	environmental	15 (30%)
smoking	**Yes**	26 (52%)
	No	7 (14%)
	unknown	15 (15%)

4.2.2 DNA quantification

After extraction (as described in M&M section), the DNA deriving from the samples belonging to MM and EC groups was quantified using rtPCR. The quantification targets are, for the kit, a human autosomal probe (HA) and a male human DNA probe (HY). For both groups of subjects exposed to asbestos, the arithmetic mean and median were calculated for each of the two targets, in order to determine which of the two is the best reference to normalize the amount of DNA to be introduced in the PCR reaction mix.

This evaluation would not be necessary in case of intact DNA, as the two probes would provide almost overlapping values of quantification. Conversely, DNA from FFPE samples (the only available material for this study) is very likely to be damaged, due to formalin fixation. In case of damaged DNA, the small probe (in the present case the probe Y) amplifies preferentially. The mean, standard deviation and median of the quantification values of the autosomal (HA) and Y (HY) probes are reported below.

MM:

HA: M_a = 0,261. M_e = 0,186. σ = 0,297.

HY: M_a = 0,468. M_e = 0,341. σ = 0,490.

EXP-C:

HA: M_a = 0,17311075. M_e = 0,066525. σ = 0,309.

HY: M_a = 0,28781042. M_e = 0,10003. σ = 0,450.

The values of the quantifications provided by the Y probe are almost double compared to the autosomal probe, with a greater dispersion of the data for the Y probe in one group and, vice versa, for the autosomal probe in the other.

Therefore, in order to normalize the DNA quantification data for the subsequent amplification reactions of the selected SNPs markers, the Y probe was considered more reliable in the presence of damaged DNA.

The quantification identified 4 unsuitable samples (2 MM and 2 EC), that were excluded due to excessive degradation of DNA.

For both groups, a possible correlation between the time elapsed since the sample preparation and the quantity of DNA extracted from them was investigated. The quantification data were plotted in relation to the time and the regression coefficients obtained were not significant.

The quantification of the DNA extracted from the biological samples of the group of healthy controls was carried out by fluorimetry, with the use of the Quantus ™ fluorometer (Promega). The DNA extracted from these samples (generally blood or salivary samples) is abundant and of high quality.

Then, the six selected markers were amplified in a single PCR reaction, starting from a quantity of DNA varying between 500 pg and 1 ng and, after that, the correct molecular weight of the amplified fragments was evaluated.

Since the DNA extracted from paraffin-embedded samples can be damaged in its primary structure due to the formalin fixation, primers capable of amplifying small fragments (around 100 bp) were selected.

In order to verify the efficiency and specificity of the individual amplification reactions, a high molecular weight control DNA sample (DNA from the 2800M cell line) was initially used. The successful amplification and the correct molecular weight of the amplicons were verified, for each marker, on an agarose minigel.

4.2.3 SNPs frequency and cause of death

Subsequently, the simultaneous amplification of all six SNPs markers was carried out in a single PCR reaction (multiplex PCR). Overall, 48 MM, 48 EXP-C and 76 HC were analyzed. The amplification of the markers was verified on an agarose minigel as documented in **Figure 7**.

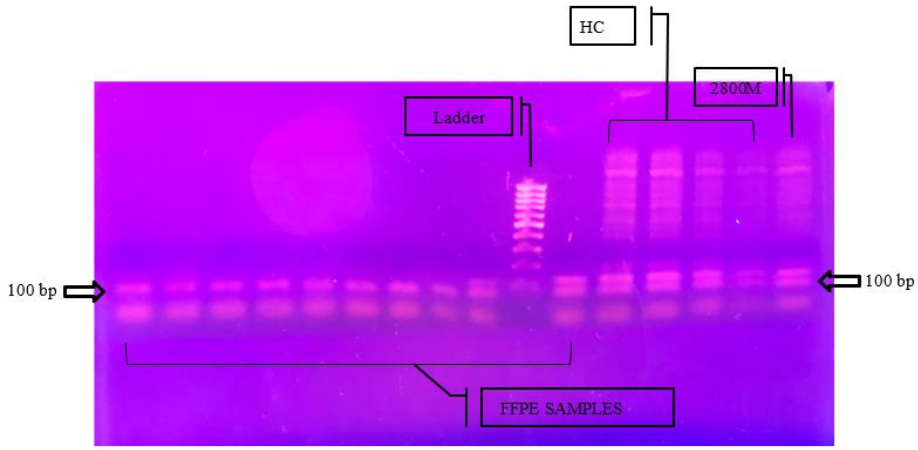


Figure 7: 2% agarose minigel in 0.5X TAE buffer. On the right, indicated by the white arrow, amplicons obtained from high molecular weight DNA samples of the group of healthy controls and cell line 2800M; in the center there is a molecular weight marker (100 bp ladder) and, on the left, amplicons from paraffin embedded tissues. The weak band present below the specific amplicons indicated by the white arrow is attributable to an amplification artifact (primers dimerization).

The genotypic characterization assay of the samples was then set up using the minisquencing or SNaPshot method [101, 102]. This approach was designed to provide an alternative to the complex and expensive sequencing of the entire amplicon. In fact, the scope is to define genetically only the position of interest (the SNP).

The method is based on the elongation of a primer-probe, positioned exactly one base before the SNP of interest. The DNA polymerase will then incorporate the di-deoxynucleotide complementary to the base on the DNA template and stops there. Each di-deoxynucleotide is labeled with a different fluorochrome; in this way it is possible to identify the incorporated base and consequently the genotype of the sample.

The six SNP markers were initially characterized individually in the SNaPshot reaction, in order to verify the effective incorporation of a di-deoxynucleotide in the primer-probe and the corresponding electrophoretic mobility of the extended fragment, as shown in Figures 8-13.

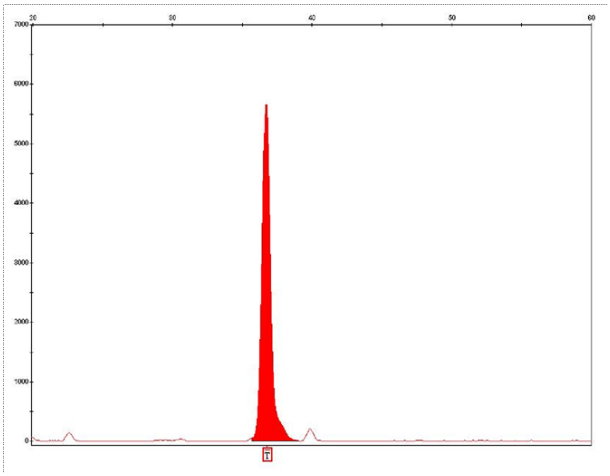


Figure 8: Electropherogram of the SNP characterization of the Ferritin gene rs76059597. The 37 bp red peak corresponds to the incorporation of the T base; the corresponding genotype is consequently TT homozygous.

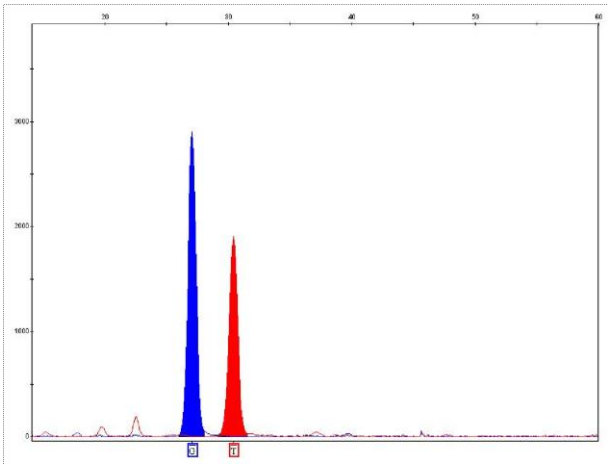


Figure 9: Electropherogram of SNP characterization of the transferrin gene rs2715631. The first blue peak of 27 bp corresponds to the incorporation of the base G, while the second red peak of 31 bp corresponds to the base T; the corresponding genotype is consequently GT heterozygous.

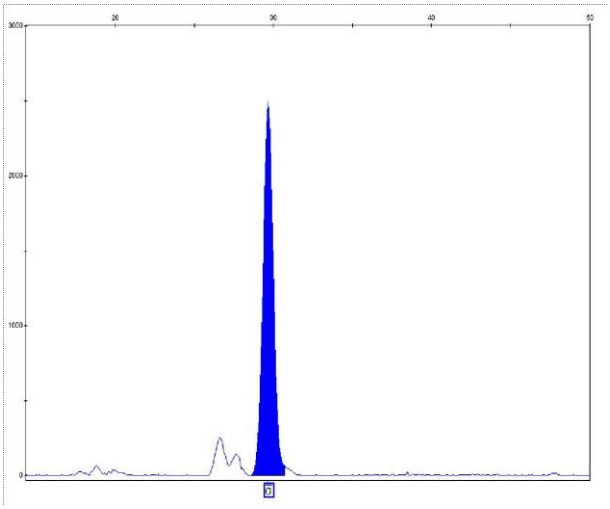


Figure 10: Electropherogram of SNP characterization of the Hephaestine gene rs3747359. The 29 bp blue peak corresponds to the incorporation of the G base; the corresponding genotype is consequently a homozygous GG genotype.

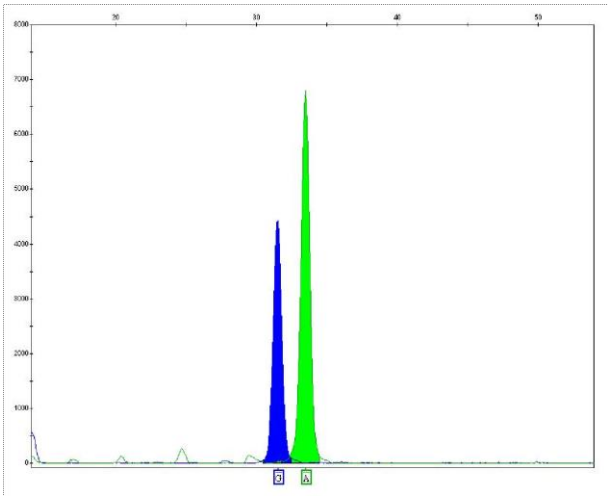


Figure 11: Electropherogram of the SNP characterization of the MMP2 rs243865 gene. The first blue peak of 31 bp size corresponds to the incorporated base G, while the second green peak of 33 bp corresponds to the base A; the corresponding genotype is consequently heterozygous GA.

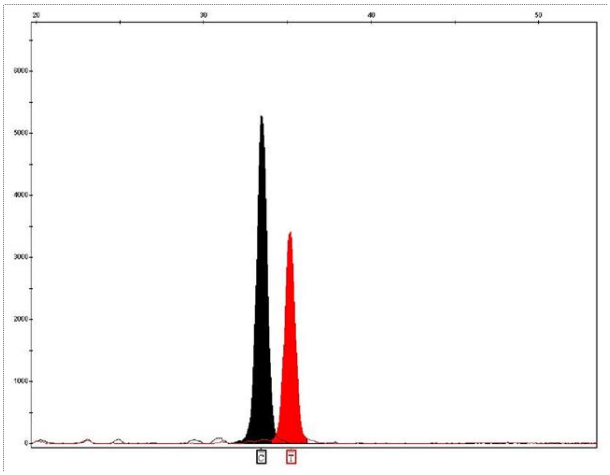


Figure 12: Electropherogram of the SNP characterization of the DMT1 rs224575 gene. The first black peak of 33 bp size corresponds to the incorporation of the base C, while the second red peak of 35 bp corresponds to the base T; the corresponding genotype is consequently heterozygous CT.

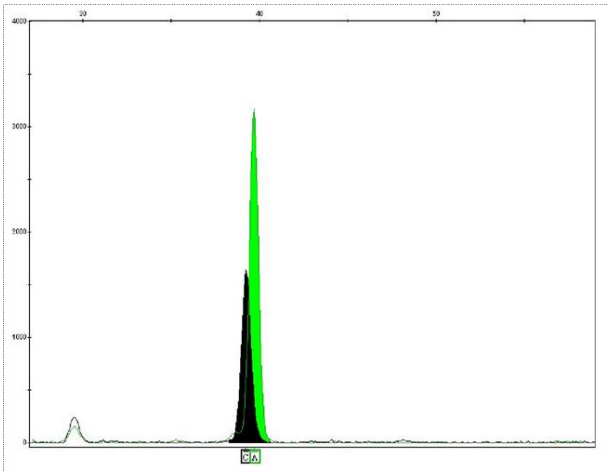


Figure 13: Electropherogram of the SNP characterization of the DMT1 rs224589 gene. The first black peak of 39 bp size corresponds to the incorporation of the base C, while the second green peak of 40 bp corresponds to the base A; the corresponding genotype is consequently CA heterozygous.

Once the correct extension of the primer-probe has been verified for all six markers, a mini-sequencing reaction was set up for the simultaneous extension, in a single reaction, of the six polymorphic markers considered. This multiplex mini-sequencing reaction was initially tested starting with a control sample represented by the 2800M cell line DNA. Based on the experiments conducted, the suitable amount of DNA for amplification and subsequent SNaPshot sequencing reaction was identified in 10 ng.

The following figure [**Figure 14**] shows the electropherogram of the genotype markers obtained for the six SNPs markers relative to the DNA of the 2800M cell line.

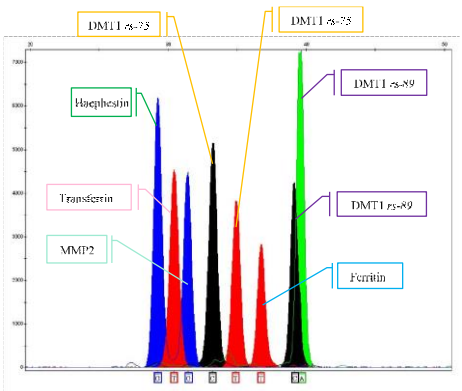


Figure 14: Electropherogram representing the characterization of the 6 SNPs in DNA taken from the cell line 2800M (10 ng). The top labels identify the SNP and the bottom labels identify the embedded base. The characterization of this DNA therefore provided the following genotypes: Hephaestine GG, Transferrin TT, MMP2 GG, DMT1 rs-75 CT, Ferritin TT and DMT1 rs-89 CA.

Once the separation of the electrophoretic peaks corresponding to the alleles of the different markers was verified (excluding overlapping of identical fluorochromes), the mini-sequencing reaction was carried out. The amount of DNA added to the mini-sequencing reaction was estimated comparing empirically the intensity of the DNA amplification products with the 2800M cell line. **Figures 15-17** represent electrophoretic patterns obtained from some of the paraffin embedded samples.

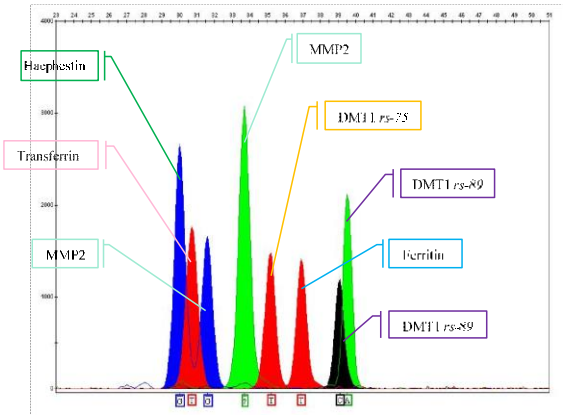


Figure 15: Electropherogram of sample 17543 (MM). The top labels identify the SNP and the bottom labels identify the embedded base. The characterization of this DNA

provided the following genotypes: Hephaestine GG, Transferrin TT, MMP2 GA, DMT1 rs-75 TT, Ferritin TT and DMT1 rs-89 CA.

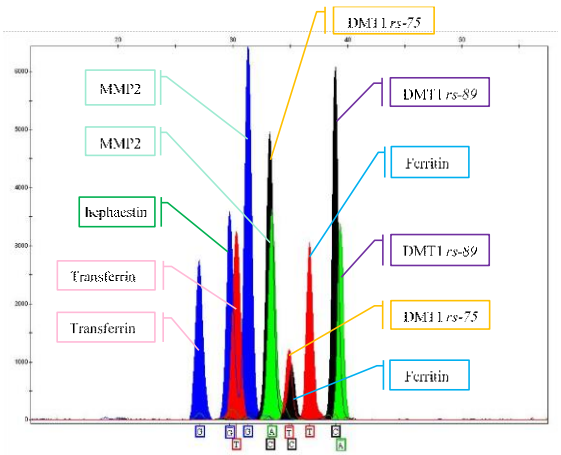


Figure 16: Electropherogram of sample 16761 heterozygous for all markers except Hephaestine (ec). The top labels identify the SNP and the bottom labels identify the embedded base. The characterization of this DNA therefore provided the following genotypes:

Hephaestine GG, Transferrin GT, MMP2 GA, DMT1 rs-75 CT, Ferritin CT and DMT1 rs-89 CA.

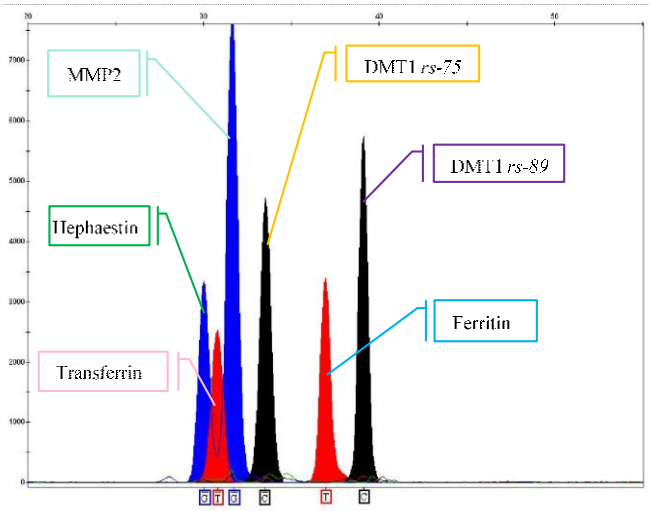


Figure 17: Electropherogram of sample 17042, homozygous for all markers (MM). The top labels identify the SNP and the bottom labels identify the embedded base. The characterization of this DNA has therefore provided the following genotypes:

Hephaestine GG, Transferrin TT, MMP2 GG, DMT1 rs-75 CC, Ferritin TT and DMT1 rs-89 CC.

Based on the results obtained from the analysis of the genotypes of each of the six selected SNPs markers, the allele and genotype frequencies were calculated for the three groups of subjects, as reported in the following **Tables 8 and 9**. The allele and genotype frequencies in the general database of 1000 Genomes and in its Italian subgroup (consisting of 107 individuals from Tuscany) are also reported.

Table 8: genotype frequencies of the samples here studied and of the 1000 Genomes database.

Genotypes frequencies					
Gene	**Alleles**	**MM**	**EXP-C**	**HC**	**1000 Genomes**
		n = 48	n = 48	n = 76	**Italians (TSI)** n = 107
Ferritin rs76059597	TT	44 (91,67%)	46 (95,83%)	70 (92,11%)	100 (93,46%)
	TC	4 (8,33%)	2 (4,17%)	6 (7,89%)	7 (6,54%)
	CC	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Transferrin rs2715631	TT	26 (54,17%)	22 (45,83%)	41 (53,95%)	66 (61,68%)
	TG	13 (27,08%)	24 (50%)	28 (36,84%)	33 (30,84%)
	GG	9 (18,75%)	2 (4,17%)	7 (9,21%)	8 (7,47%)
Hephaestin* rs3747359	G (C) GG (CC)	48 (100%)	48 (100%)	52 (100%)	107 (100%)
	GC (CG)	-	-	-	0 (0%)
	C (G) CC (GG)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
MMP2 rs243865	GG (CC)	27 (56,25%)	24 (50%)	47 (61,84%)	58 (54,20%)
	GA (CT)	17 (35,42%)	21 (43,75%)	27 (35,53%)	42 (38,25%)
	AA (TT)	4 (8,33%)	3 (6,25%)	2 (2,63%)	7 (6,54%)
DMT1 rs224575	CC	17 (35,42%)	19 (39,58%)	25 (32,89%)	37 (34,57%)
	CT	25 (52,08%)	22 (45,83%)	35 (46,05%)	51 (47,66%)
	TT	6 (12,50%)	7 (14,58%)	16 (21,05%)	19 (17,75%)
DMT1 rs224589	CC (GG)	27 (56,25%)	28 (58,33%)	37 (48,68%)	52 (48,59%)
	CA (GT)	21 (43,75%)	18 (37,50%)	33 (43,42%)	48 (44,85%)
	AA (TT)	0 (0%)	2 (4,17%)	6 (7,89%)	7 (6,54%)

Table 9: allele frequencies of the samples here studied and of the 1000 Genomes database.

ALLELES FREQUENCIES						
Gene	**Alleles**	**MM**	**EXP-C**	**HC**	**1000 Genomi**	
		n_{AL} = 96	n_{AL} = 96	n_{AL} = 152	**General** n_{AL} = 10016	**Italians (TSI)** n_{AL} = 214
Ferritin rs76059597	T	92 (95,83%)	94 (97,92%)	146 (96,05%)	9906 (98,9%)	207 (96,73%)
	C	4 (4,17%)	2 (2,08%)	6 (3,95%)	110 (1,1%)	7 (3,27%)
Transferrin rs2715631	T	65 (67,71%)	68 (70,83%)	110 (72,37%)	8534 (85,2%)	165 (77,1%)
	G	31 (32,29%)	28 (29,17%)	42 (27,63%)	1482 (14,8%)	49 (22,9%)
Hephaestin* rs3747359	G (C)	48 (100%)	48 (100%)	76 (100%)	N/A	161 (100%)
	C (G)	0 (0%)	0 (0%)	0 (0%)	N/A	0 (0%)
MMP2 rs243865	G (C)	71 (73,96%)	69 (71,88%)	121 (79,61%)	8644 (86,3%)	158 (73,83%)
	A (T)	25 (26,04%)	27 (28,13%)	31 (20,39%)	1372 (13,7%)	56 (26,17%)
DMT1 rs224575	C	59 (61,46%)	60 (62,50%)	85 (55,92%)	4387 (43,8%)	125 (58,41%)
	T	37 (38,54%)	36 (37,50%)	67 (44,08%)	5629 (56,2%)	89 (41,59%)
DMT1 rs224589	C (G)	75 (78,13%)	74 (77,08%)	107 (70,39%)	7362 (73,5%)	152 (71,03%)
	A (T)	21 (21,88%)	22 (22,92%)	45 (29,61%)	2654 (26,5%)	62 (28,97%)

The three groups of subjects were then compared with each other using the PLINK software (v.1.07) [96], in order to find any statistically significant differences.

First, it is worth noting that all the groups under study are in Hardy-Weinberg equilibrium (significance value $p < 0.001$), thus suitable to be compared with each other. Subsequently, using the Pearson $\chi 2$ test (significance value $p < 0.05$) the genotypic and allelic models were evaluated to verify if the genotypes of each marker were significantly different between the three groups. For groups whose expected results were less than 5 subjects, Fisher's exact test was used.

First, the allelic and genotypic frequencies in MM subjects were compared to EXP-C. **Table 10** shows the statistically significant results of these comparisons. The marker of Hephaestine, rs3747359, on the X chromosome, was not included in the statistical analysis as it is non-polymorphic in our sample, being characterized by the same allele in all samples.

Table 10: comparison of genotypes and alleles frequencies between MM and EXP-C. Statistically significant results are highlighted in bold.

COMPARISON OF GENOTYPIC AND ALLELIC MODELS BETWEEN MM AND EXP-C				
Gene	**Model**	**Test**	χ^2	***p-value***
Ferritin rs76059597	Genotypic	Fisher	-	0,677
	Allelic	Fisher	-	0,755
Transferrin rs2715631	**Genotypic**	**Fisher**	**-**	**0,022**
	Allelic	χ^2	0,69	0,407
MMP2 rs243865	Genotypic	Fisher	-	0,893
	Allelic	χ^2	0,24	0,624
DMT1 rs224575	Genotypic	χ^2	0,38	0,827
	Allelic	χ^2	0,03	0,863
DMT1 rs224589	Genotypic	Fisher	-	0,442
	Allelic	χ^2	0,02	0,882

Considering the statistical significance found for the transferrin SNP rs2715631 (p = 0.012), the dominant and recessive models were evaluated. The odds-ratio (OR) was also calculated, and the confidence interval (CI) was indicated. The values obtained for these two models are highlighted in Table 14. The distribution of the two models between MM and EC did not show a significant difference.

Table 11: comparison of dominant and recessive genotypes for the Transferrin SNP rs2715631 between mesothelioma patients and exposed subjects.

COMPARISON OF GENOTYPIC AND ALLELIC MODELS BETWEEN MM AND EXP-C						
Gene	**Model**	**Test**	χ^2	***p-value***	**OR**	**CI (95%)**
Transferrin rs2715631	Dominant	Fisher	-	0,541	-	-
	Recessive	Fisher	-	0,051	-	-

The genotypic and allelic models were compared between MM and non-exposed controls (**Table 12**) and between MM and the Italian samples (TSI) from the database of 1000 genomes (**Table 13**).

Table 12: comparison of genotypic and allelic models between MM and HC.

COMPARISON OF GENOTYPIC AND ALLELIC MODELS BETWEEN MM AND HC				
Gene	**Model**	**Test**	χ^2	***p-value***
Ferritin rs76059597	Genotypic	Fisher	-	1,000
	Allelic	Fisher	-	1,000
Transferrin rs2715631	Genotypic	χ^2	2,92	0,232
	Allelic	χ^2	0,615	0,433
MMP2 rs243865	Genotypic	Fisher	-	0,516
	Allelic	χ^2	0,72	0,395
DMT1 rs224575	Genotypic	χ^2	1,49	0,475
	Allelic	χ^2	0,74	0,389
DMT1 rs224589	Genotypic	Fisher	-	0,135
	Allelic	χ^2	1,80	0,180

Table 13: comparison of genotypic and allelic models between MM and 1000Genomes data.

COMPARISON OF GENOTYPIC AND ALLELIC MODELS BETWEEN MM AND 1000 GENOMES (TSI)				
Gene	**Model**	**Test**	χ^2	***p-value***
Ferritin rs76059597	Genotypic	Fisher	-	0,739
	Allelic	Fisher	-	0,743
Transferrin rs2715631	Genotypic	χ^2	4,31	0,116
	Allelic	χ^2	3,06	0,081
MMP2 rs243865	Genotypic	Fisher	-	0,858
	Allelic	χ^2	$5 \cdot 10^{-4}$	0,981
DMT1 rs224575	Genotypic	χ^2	0,71	0,702
	Allelic	χ^2	0,26	0,614
DMT1 rs224589	Genotypic	Fisher	-	0,185
	Allelic	χ^2	1,70	0,192

As shown in the tables, comparison of the allelic and genotype frequencies between MM, HC and the data about the Italian subgroup of 1000Genomes (TSI) did not provide any statistically significant results.

The allelic and genotypic frequencies of these samples are therefore homogeneous.

Subsequently, the group of EXP-C were compared to the HC (healthy subjects) and to the Italians in the 1000 Genomes database (TSI) (**Tables 14 and 15**, respectively).

Table 14: comparison of genotypic and allelic models between exposed controls and non-exposed healthy subjects.

COMPARISON OF GENOTYPIC AND ALLELIC MODELS BETWEEN EXP-C AND HC				
Gene	**Model**	**Test**	χ^2	***p-value***
Ferritin rs76059597	Genotypic	Fisher	-	0,483
	Allelic	Fisher	-	0,490
Transferrin rs2715631	Genotypic	Fisher	-	0,299
	Allelic	χ^2	0,07	0,794
MMP2 rs243865	Genotypic	Fisher	-	0,322
	Allelic	χ^2	1,96	0,161
DMT1 rs224575	Genotypic	χ^2	1,04	0,596
	Allelic	χ^2	1,05	0,306
DMT1 rs224589	Genotypic	Fisher	-	0,533
	Allelic	χ^2	1,34	0,248

Table 15: comparison of genotypic and allelic models between exposed controls and data from 1000genomes.

COMPARISON OF GENOTYPIC AND ALLELIC MODELS BETWEEN EXP-C AND 1000 GENOMES (TSI)				
Gene	**Model**	**Test**	χ^2	***p-value***
Ferritin rs76059597	Genotypic	Fisher	-	0,722
	Allelic	Fisher	-	0,726
Transferrin rs2715631	Genotypic	Fisher	-	0,082
	Allelic	χ^2	1,40	0,238
MMP2 rs243865	Genotypic	Fisher	-	0,922
	Allelic	χ^2	0,13	0,719
DMT1 rs224575	Genotypic	χ^2	0,45	0,798
	Allelic	χ^2	0,46	0,497
DMT1 rs224589	Genotypic	Fisher	-	0,560
	Allelic	χ^2	1,23	0,268

No statistically significant differences in the distribution of allelic and genotypic frequencies were pointed out between the sample of exposed controls and the non-exposed healthy subjects, nor between exposed controls and data from 1000genomes database.

Finally, to verify that our choice of HC is not biased, we compared the frequencies of genotypes and alleles in this group with those from 1000genomes database. No significant differences were pointed out.

Table 16 comparison of genotypic and allelic models between non-exposed healthy controls and data from 1000genomes.

COMPARISON OF GENOTYPIC AND ALLELIC MODELS BETWEEN HC AND 1000 GENOMES (TSI)				
Gene	**Model**	**Test**	χ^2	*p-value*
Ferritin rs76059597	Genotypic	Fisher	-	0,775
	Allelic	Fisher	-	0,779
Transferrin rs2715631	Genotypic	Fisher	-	0,606
	Allelic	χ^2	1,07	0,302
MMP2 rs243865	Genotypic	Fisher	-	0,399
	Allelic	χ^2	1,64	0,201
DMT1 rs224575	Genotypic	χ^2	0,31	0,855
	Allelic	χ^2	0,23	0,635
DMT1 rs224589	Genotypic	Fisher	-	0,945
	Allelic	χ^2	0,02	0,896

Finally, in the subjects for which the lung content analysis was available, the relation between SNPs frequency and amount of asbestos fibers, ABs, ratio between asbestos fibers and Abs and the amounts of each kind of asbestos was investigated. None of the five genotypes showed any significant difference according to the amount of asbestos fibers nor of asbestos bodies. We also tested the frequencies of each genetic variant in relation to the ratio between asbestos fibers and asbestos bodies, in order to investigate a possible relation between the SNPs and a different "attitude" to coat asbestos fibers, without any significant result.

Finally, limited to MM cases, we analyzed the data in order to find out if the SNPs in the five genes here investigated are related to a different latency (time between the first exposure to asbestos and the diagnosis of mesothelioma) and/or a longer/shorter survival time (between diagnosis and death). Again, no significant results were obtained.

4.3 BAP1 expression and iron trafficking in lungs of asbestos-exposed individuals with and without MM: immunohistochemical and rtPCR findings

First, BAP1 IHC was performed on MM neoplastic tissue, resulting negative in all the cases but two [**Figure 18**].

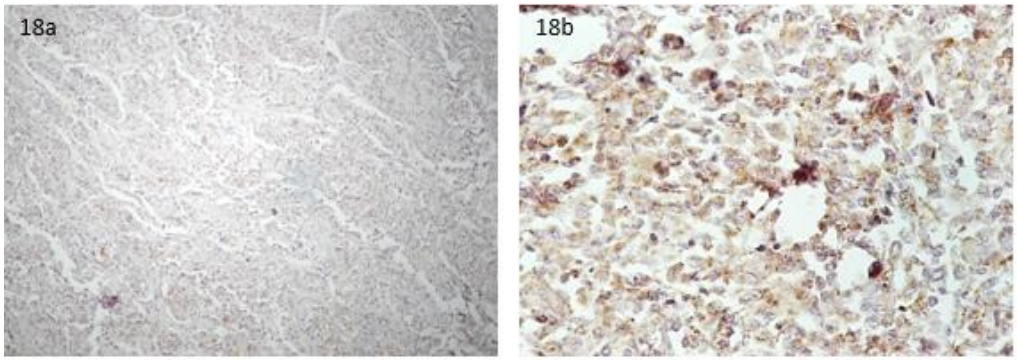


Figure 18: examples of negative (a) and positive (b) immunostaining for BAP1 in MM tissue. (magnification: 18a: 100x; 18b:200x)

The immunohistochemical staining for BAP1, TFR1, FTH1 and FPN on lung tissue provided significant results in regard to the comparison between the MM patients, exposed controls (EXP-C) and non-exposed subjects dies of traumatic causes (NON-EXP).

BAP-1 staining in normal lung tissue was positive in the totality of the HC, in 86,5% of EC, and in only 59% of MM [**Figure 19, 20**]. The statistical analysis revealed a significant difference among the three groups and, in particular, MM positivity score was significantly lower compared to EXP-C (p=0,044) and NON-EXP.

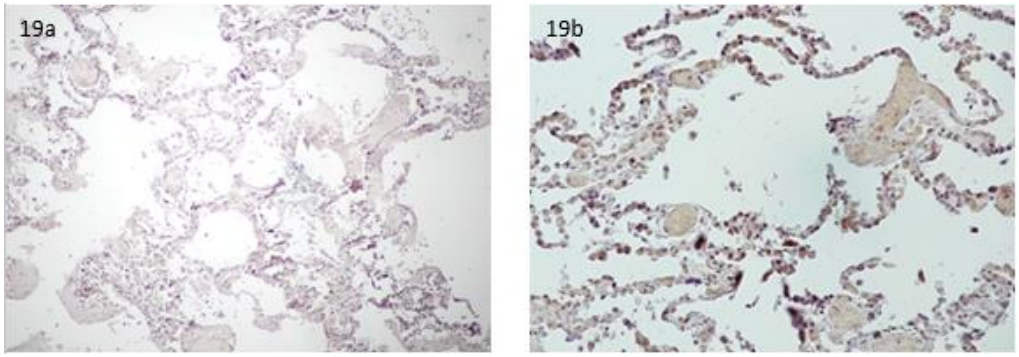


Figure 19: two examples of BAP1 staining in normal lung tissue. a (100x): negative staining in a MM. b (200x): positive staining in one NON-EXP.

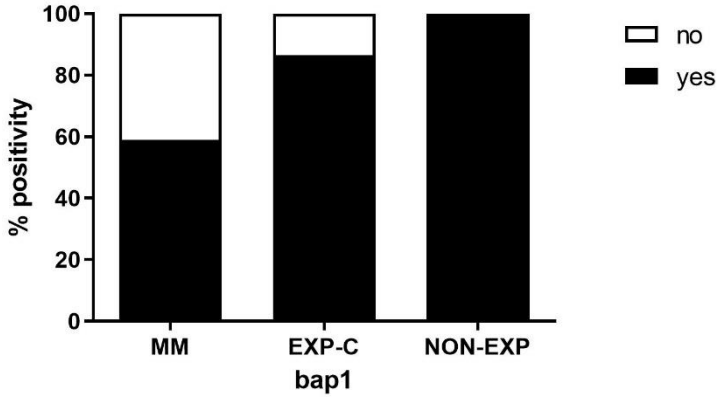


Figure 20: distribution of BAP1 IHC positivity in the three groups.

TRF1 staining was negative in the totality of NON-EXP, whereas it was mildly positive in 66,7% of MM and strongly positive in 10,3% of them. Among EXP-C, TFR1 staining was negative in only one case, mildly positive in 25% of them and strongly positive in 71,9% [**Figure 21, 24**]. The statistical analysis revealed a significant difference among the three groups ($p<0,00001$), and, in particular, the TFR1 staining score was significantly higher in MM compared to EXP-C ($p<0,00001$) and in MM compared to NON-EXP ($p<0,00001$), whereas no significant difference was observed between EXP-C and NON-EXP.

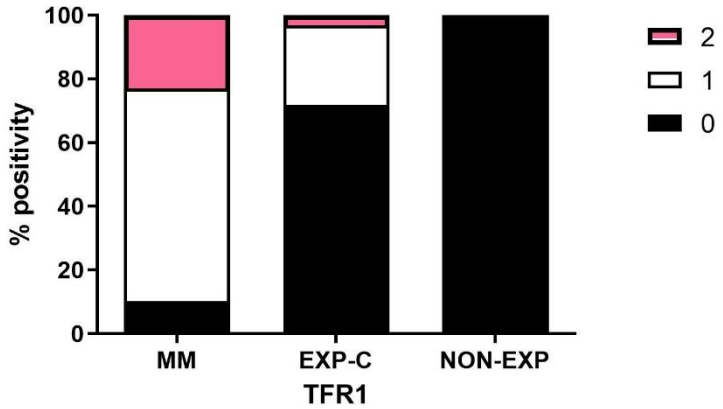


Figure 21: distribution of TFR1 IHC positivity in the three groups.

FTH1 staining was negative in 52,6% of NON-EXP (except for sporadic positive macrophages), mildly positive 36,8% of them and strongly positive in 10,5%. Regarding MM, the reaction was negative in 10,8% of them, mildly positive in 56,7% and strongly positive in 32,4% [**Figure 22, 24**]. The statistical assessment revealed a

significant difference among the three groups (p=0.00139) and, in particular, the staining score resulted higher in MM compared to NON-EXP (P=0.00374) and in EXP-C compared to NON-EXP (P=0.00046), whereas no significant difference was observed between MM and EXP-C.

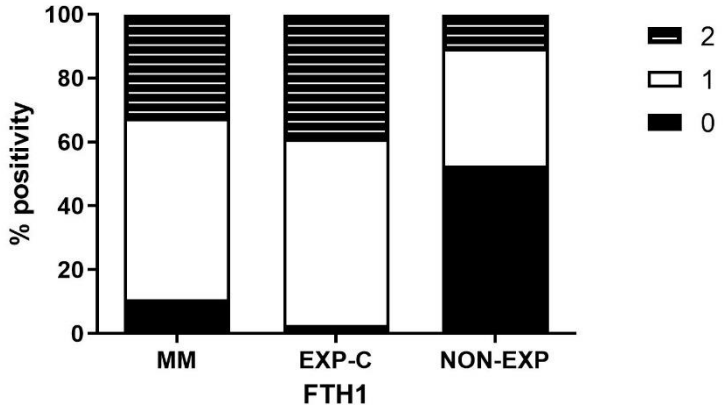


Figure 22: distribution of FTH1 IHC positivity in the three groups.

FPN staining resulted negative in 100% of NON-EXP, in 53,8% of MM and in 78,4% of NON-EXP [**Figure 23, 24**] A significant difference was observed among the three groups (P=0.01229). In particular, the staining positivity resulted more represented in MM in respect of HC (p=0.0048), whereas the difference between MM and EC did not reach the statistical significance(p=0.0601), even though a tendency to a stronger positivity is appreciable in MM compared to EC (P=0.06).

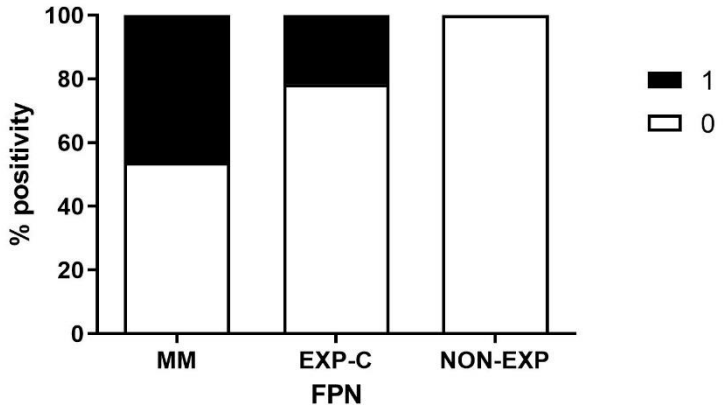


Figure 23: distribution of FPN IHC positivity in the three groups.

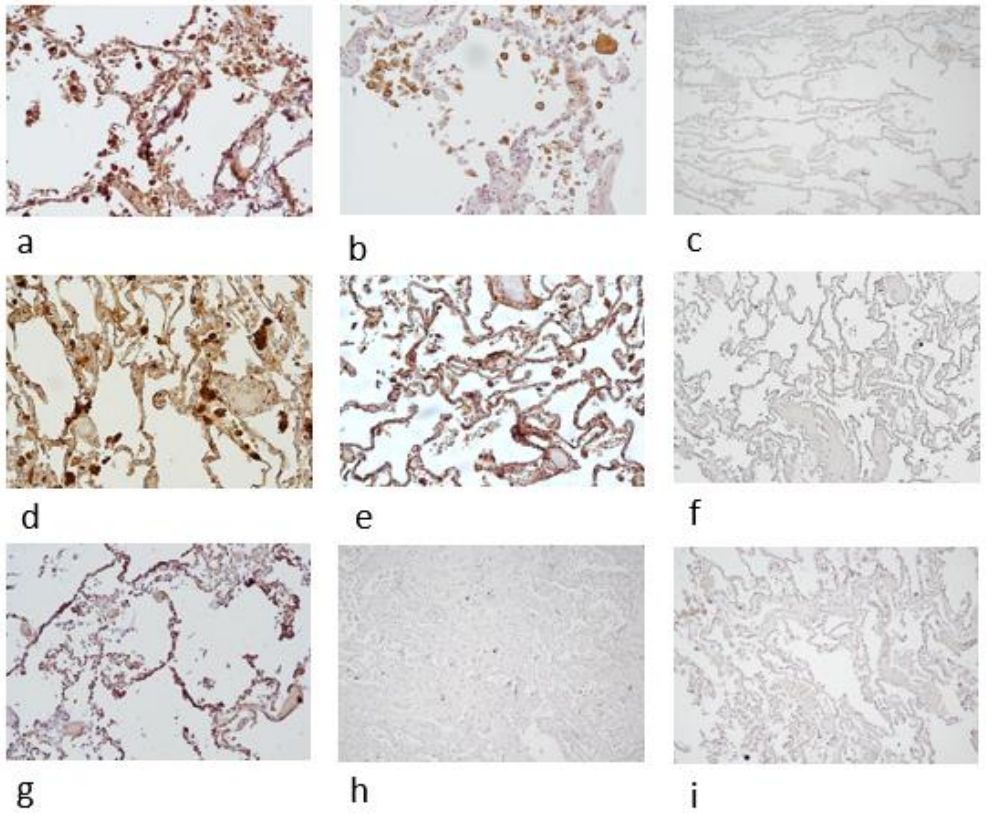


Figure 24: a) MM, antiTFR1 (200x). b) EXP-C, anti TFR1. (200x) c) NON-EXP, antiTFR1 (100). d) MM, antiFTH1 (200x). e) EXP-C, antiFTH1 (200x). f) NON-EXP, antiFTH1 (200x). g) MM, antiFPN (200x). h) EXP-C, antiFPN (100x). i) NON-EXP, antiFPN (200x).

Analysis of expression of the mRNA was possible in only 12 subjects per group, due to technical limitations related to bad RNA quality (as expected in FFPE samples). The experiment was performed in samples for which the expression provided a satisfying quantification of RNA and an appreciable expression of housekeeping gene GADPH. Even after this selection, rtPCR did not provide convincing results. Indeed, the expression of the housekeeping gene showed a marked variability among the 36 samples, revealing a considerable degradation of mRNA. A preliminary statistical analysis of the obtained measures did not reveal any statistically significant result. This is obviously due, besides the RNA quality, to the low number of examined samples and to the high standard deviation in each group.

Despite such limitations, some considerations can be made about the expression of the genes of interest. rtPCR for BAP1 mRNA did not show any appreciable tendency among the three groups. This is partly due to an outlier in the MM group, that raises the mean value of expression in this group [**Figure 24a**]. The same subject shows a positive

IHC for BAP1. The analysis of expression of TFR1 gene showed a tendency to a higher expression in HC compared to MM and EC (even if not statistically significant) [**Figure 24b**]. This result is in contrast with the literature data and with the IHC staining, that show exactly the opposite. FTH1 mRNA expression did not reveal any remarkable difference among the three groups, showing only a slight tendency to a lower level in EC compared to both MM and HC [**Figure 24c**]. This is in contrast with IHC data, that show a marked lower positivity for this protein in HC compared to MM and EXP-C. Finally, FPN mRNA expression analysis revealed results that are consistent with IHC staining for FPN protein, showing a higher expression level in MM compared to EXP-C and NON-EXP [**Figure 24d**].

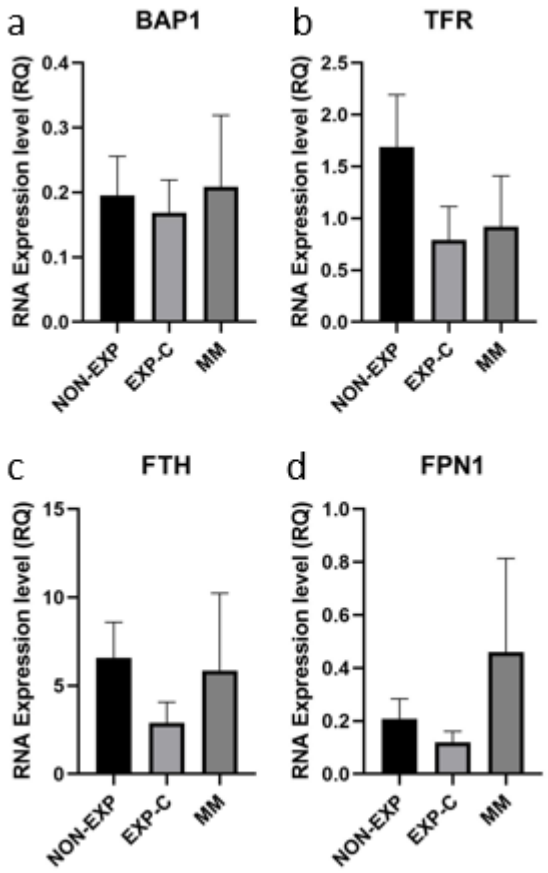


Figure 24: quantitative assessment of the expression of the four genes (rtPCR).

The IHC findings were then assessed in respect of the results of lung content analysis performed using SEM-EDS, available for 72 subjects (all belonging to the MM and EC group, as asbestos exposure for HC was assumed to be absent). The level of positivity of each marker was compared to the amount of asbestos fibers, asbestos bodies, and of

each asbestos species. Also possible relations between IHC staining levels and the dimensional characteristics of the detected asbestos fibers were evaluated.

BAP1 IHC did not seem to be related to none of the above-mentioned variables. The statistical analysis performed revealed p values far from significance for all the parameters considered.

TRF1 IHC showed significant differences in relation to the amount of asbestos fibers, of asbestos bodies and the amount of crocidolite. In particular, the staining was significantly stronger in subjects with lower amounts of asbestos fibers (p=0,032).

A significant difference was observed also for the amount of ABs. Subjects with a strong staining for TFR1 (2) have less ABs. Curiously, the group with mild positivity showed the lowest amount of ABs and those with negative staining showed the highest amount of ABs. This tendency is even stronger for crocidolite amounts: subjects with strong TFR1 positivity showed significantly lower amounts of crocidolite compared to subjects with negative staining (p>0.001) and subjects with mild positivity have significantly lower levels of crocidolite in their lungs compared to those with negative staining for TFR1.

Regarding FTH1 IHC, the statistical analysis showed that subjects with a strong FTH1 staining have higher levels of ABs in their lungs (p=0,028). In particular, subjects with strong FTH1 staining (graded as 2) have significantly higher amounts of ABs compared to those with a negative staining (p=0.014). Conversely, FTH1 staining did not show any difference in respect of the amount of uncoated fibers, nor with any of the other evaluated parameters, namely the amount of each type of asbestos and dimensional characteristics of fibers.

The positivity rate of FPN IHC did not show any significant difference in relation to the amounts of asbestos fibers not ABs, but, interestingly, in subjects with positive staining for FPN, asbestos fibers were significantly shorter (p=0,044).

Then, the existence of a relation between the positivity rate of each marker and the time since the end of exposure and the duration of exposure, without pointing out any significant difference.

Finally, only for MM patients, the positivity rate of each of the investigated markers was assessed in relation to the latency time (period of time between the first exposure to asbestos and diagnosis of MM) and survival time (time passed between diagnosis and death), without revealing any significant difference nor appreciable tendency.

5. DISCUSSION

5.1 SEM-EDS analysis of fiber burden in lungs

Interestingly, in the majority of samples taken from MM patients here analyzed, the concentration of asbestos was lower than expected. In 45 out of 59 MM cases (76,3 %) the amount of asbestos fibers is well below the threshold value considered demonstrative for exposure to asbestos, that is more than 0,1 million of amphibole asbestos longer than 5 μm per 1 gdw or more than 10 x 105 amphibole asbestos longer than 1 μm per 1 gdw as measured by electron microscopy in a qualified laboratory [103]. It is also notable that, in 14 of the 59 subjects who died from MM, asbestos was not detected at all, as well as ABs. This does not mean that the subjects' lungs are totally free from asbestos, but that we did not find any in our investigation, that involves a limited amount of lung parenchyma. Anyway, this result indicates that asbestos fibers, if present in lungs, are certainly very few.

In this connection, it is worth to remind that a low, "background" exposure to asbestos is widely diffused. Previously, Capella et al. performed the SEM -EDS analysis of lung samples taken from people who resided in Torino all their life, without any occupational exposure to asbestos (and without anthropogenic environmental exposure, given that in the area of Torino, in that period, there were no plants using asbestos), died from causes not related to asbestos exposure. In most of these subjects, a low amount of asbestos, belonging to tremolite/actinolite asbestos and chrysotile/asbestiform antigorite group, were detected [93]. Likewise, as reported in another paper, not negligible amounts of asbestos have been found also in the general population of Milan [104]. Such data suggest that the general population is potentially exposed to a low amount of asbestos. Yet, evidently, such "background" exposure is not sufficient, in most cases, by itself, to cause MM. The finding of a lower amount of asbestos in lungs of people who died of MM compared to exposed people deceased from other causes corroborates this concept, suggesting that the quantity of asbestos is not decisive in determining MM. In fact, on the other hand, we detected high amounts of asbestos (as well as ABs) in occupationally exposed subjects who never develop MM.

Of course, the absence of asbestos in 14 of the 72 subjects here analyzed, who were certainly exposed to asbestos during their life, may be related to the lung clearance of fibers. In all the 72 subjects of this study, the asbestos exposure had ceased at least 8 years before death (median=21 years). As it is further discussed below, lung clearance involves mainly chrysotile, that represents the less bio-persistent asbestos type [17, 19, 105]. Therefore, the possible explanation for the low amount or even the absence of asbestos, despite the certain exposure during life, may be that in those subjects the inorganic content of lungs consisted mainly of chrysotile, which was cleared from the lung microenvironment. This hypothesis is also corroborated by the absence of chrysotile in our samples, as discussed below. However, despite the controversies in

this field, chrysotile has been considered much less potent in causing MM compared to amphibole asbestos, with a ratio of 500:100:1 between crocidolite, amosite and chrysotile [78]. Therefore, it would be quite unlikely that in our cases of MM without any detected asbestos fiber in lungs the responsible for the development of the neoplasm was exclusively the chrysotile which had been previously cleared from the lungs. Anyway, the finding of no asbestos, and especially of no amphiboles, in MM subjects is unexpected and not consistent with previous studies, investigating the inorganic fibers lung content, which found an increased amount of amphibole asbestos in subjects who died of MM [35, 106]. However, such studies not always include controls (defined as exposed subjects without MM). On the other hand, consistently with our findings, it was previously reported that not in all MM cases asbestos are detectable in lungs using SEM-EDS [22].

It is noteworthy not only that many MM patients had few fibers in their lungs (below the threshold for exposure), but the amount of asbestos in MM was significantly lower as compared to subjects who died from causes related to asbestosis (and therefore heavily exposed) but never developed MM.

Such findings are consistent with previous studies about lung content, in which the concentration of amphibole asbestos in MM patients showed no statistically significant differences compared to controls [24]. Other studies found that the MM risk is proportional to fiber burden [36] or, however, an increased asbestos concentration in mesothelioma patients [35, 106][29, 30, 35, 106].

Our findings suggest that MM risk is not related to the dose of asbestos in lungs. In other words, it means that an extremely small quantity of inhaled asbestos is sufficient to cause MM.

Indeed, the carcinogenic potential of asbestos also at very low doses was underlined for the first time by Selikoff's pioneer study [107]; the author also hypothesized for the first time the possible role of an individual susceptibility, perhaps genetically mediated. The present results confirm the absence of a threshold level of asbestos exposure below which there is no risk for MM, as already stated before [8, 107, 108] .

In order to better understand the link between asbestos dose of exposure and MM risk, the electron microscopy analysis of lung content is necessary. In fact, epidemiological studies often failed to characterize the exposure, as demonstrated by subsequent SEM-EDS studies on the same cohorts [109]. Indeed, despite epidemiological studies suggesting a dose-response relation in the risk of developing MM (e.g.,[36, 110]), there is no evidence of this relation deriving from lung inorganic fiber burden studies, giving objective information about the actual inorganic fibers content of lungs, supporting this hypothesis. As demonstrated by electron microscopy, MM often occurs in patients with asbestos burden comparable to the general population (the so-called “background” or “spontaneous mesothelioma”)[106].

Interestingly, there is a great variability in the amount of ABs, that, even though statistically correlated with the number of fibers, is extremely variable and often very different from the amount of fibers. In three cases, we observed a concentration of fibers above the threshold of 0,1 million per gdw, but no ABs. On the other hand, in 6 cases we detected zero asbestos fibers, but a number of ABs. This result is in line with previous reports, that pointed out a considerable variability in the coating process from subject to subject [111].

In subjects who died with asbestosis, compared to MM patients, a higher concentration of ABs was observed. Even though this can be related simply to the higher concentration of asbestos fibers in those subjects, resulting in ABs formation (consistently with the significant correlation between asbestos fibers and ABs) this finding suggests the hypothesis that the formation of ABs, one of the most controversial and unclear points in the cellular reaction to asbestos, might be the expression of a different biological response to asbestos, leading to a stronger capability to isolate and neutralize asbestos [37, 79, 112]. In other words, this finding may reflect a protective role of fiber coating in the lung microenvironment.

Moreover, the present study pointed out that the total amount of asbestos, as well as ABs, was not significantly different between subjects with only occupational exposure compared to those with anthropogenic environmental exposure. The anamnestic data, that include a detailed residential history, showed that most subjects with anthropogenic environmental exposure used to live very close to the asbestos-cement plant (500 m or closer). In six cases the environmental exposure was both residential and household. The relevance of non-occupational exposure in the determination of the inorganic fiber burden in lungs is in conformity with previous epidemiological studies [88, 92, 110, 113–115], as well as with previous electron microscope investigations[35]. This finding is in line with the concept that asbestos concentrations in lungs due to anthropogenic environmental exposure can be as high as those provoked by occupational exposure, as stated, as well, by the above cited papers (mainly based on epidemiological data without any morphologic validation). It means that the environmental exposure to asbestos fibers, nowadays still present worldwide due to the diffusion of asbestos manufacts in many countries, is as effective as occupational one in determining the fiber burden in lungs.

In addition, a significant difference was found in ABs amount between subjects exposed in both manners and those with only anthropogenic environmental exposure. The concentration of uncoated fibers shows a similar trend, even though it did not reach statistical significance. Anyway, ABs concentration, even though not always proportional to the number of uncoated fibers, is an established marker of asbestos exposure [103, 112]. The presented data suggest that the additional effect of the two exposures, when occurring in the same subject, increases significantly the entity of the accumulated fibers in lungs (that, to a certain extent, are coated and detectable as ABs).

The present study also pointed out remarkable findings about the presence of various types of asbestos in lungs. Occupationally exposed subjects were employed at the

asbestos-cement factory, where large amounts of chrysotile and crocidolite were used. Amosite was used in smaller quantities as an additive [84]. Note that also the anthropogenic environmental exposure was related to production of the same factory, involving people that used to live nearby the plant or whose husband or son used to work at the industry. Notwithstanding, as already stated above, we found almost no fibers attributable with certainty to chrysotile (belonging to the category of chrysotile/asbestiform antigorite). Two possible explanations can be proposed: the first one is obviously related to the well-established rapid clearance of this mineral in lungs [15, 16, 105, 116]. Despite the intense debate, still ongoing in the literature, the actual biopersistence and the carcinogenic effect of chrysotile is still an unanswered question.

From the mineralogical point of view, chrysotile is very different from amphiboles asbestos in regard to the chemical composition and structure [11, 19]. It is well known that the retention of chrysotile in human lungs is much lower compared to amphiboles, due to its rapid clearance, rather than a lower rate of deposition[17]. The mechanism of chrysotile clearance, even though not fully understood, is related to the fragmentation of the fibers in the lung microenvironment and the subsequent phagocytosis by macrophages (airway macrophages, alveolar macrophages, interstitial macrophages, intravascular macrophages, pleural macrophages) [116].

The chemical instability of chrysotile is due to the dissociation of magnesium from the crystalline structure in the acid lung microenvironment; as a consequence, the structure of chrysotile becomes friable and fragments into very short fibrils that can be phagocytized and removed from the alveoli [19]. Recent experimental studies on rats and baboons confirmed a very rapid clearance of chrysotile from lungs (with very few fibers after 90 days since the end of exposure, compared to high concentration of amphiboles after the same period of time) [117, 118]. Yet, other studies showed findings that implies opposite deductions.

For instance, a recent paper by Feder and coll. showed that asbestos, and in particular, chrysotile, is stable in human lungs up to 37 years [20], and chrysotile is the main fiber they observed in human lung samples using a high-resolution type of electron microscope, a FEG-SEM. Similarly, previous studies on animals, as well as on humans, pointed out the presence of chrysotile as late as 60 years after exposure [106].

Churg and De Paoli, in 1988 [15], measuring the fiber burden in lungs of subjects with different time intervals since exposure cessation, concluded that inhaled chrysotile may end up as two populations: one is cleared quickly from the air spaces and the other, composed of fibers which managed to reach the interstitium, remain for longer. Such observations suggested that the degradation of chrysotile in human lungs, leading to its clearance, must occur at a very early time since inhalation and after that the remaining chrysotile, not degraded in a short time, is not significantly cleared in the following years.

The present data, instead, suggest a complete degradation and removal of chrysotile in all the investigated subjects but one (that had, anyway, very few chrysotile fibers in his

lungs). In our series, the time intervals since the cessation of the exposure (occupational or anthropogenic environmental) were extremely inhomogeneous (range: 8-44 years), but we did not notice any difference in chrysotile presence, suggesting that the clearance of this mineral occurs relatively rapidly, consistently with what was previously known [16, 17].

Another possible explanation should be considered: chrysotile fibers might be too thin to be detected using the technique above described (SEM-EDS observation using the mentioned instrumentation), due to the resolution power limited to 0.2 μm. Indeed, in the study By Feder et al. [20] a FEG-SEM was used, with a higher resolution: at such conditions the chrysotile has been detected. Nevertheless, studies about fiber burden in lungs measured by TEM found very few chrysotile fibers compared to amphibole asbestos [34, 36], even in subjects known to be exposed to chrysotile. Such results, in line with the data here presented, support the hypothesis of the preponderant role of the pulmonary degradation of chrysotile.

It was also reported that chrysotile short and thin fibers are the most common type of asbestos in the parietal pleura, but not in random samples of lung [119]. Yet, the same authors did not find any chrysotile fiber in pleura.

The predominance of crocidolite, followed by amosite, in lungs of almost all the subjects is consistent with the production at the Fibronit plant [84, 120] and the durability of this kind of asbestos.

Moreover, in subjects with anthropogenic environmental exposure in Broni, compared to those who were occupationally exposed, a higher proportion of tremolite/actinolite asbestos was observed (even not statistically significant). Tremolite/actinolite asbestos was never used commercially, but it is dispersed from natural source from the outcropping rocks containing them (serpentinite rocks in Western Alps) and also from talc containing materials, in case the talc contains these types of asbestos as natural contaminants [93], and sometimes also anthophyllite asbestos [121]. Therefore, their air dispersion, inside the firm, could be due to the use of talc or talc containing products, given the large industrial use of talc [122].

The different carcinogenic potential of chrysotile and tremolite/actinolite asbestos is still questioned. It was suggested that chrysotile-induced MM in miners (exposed to the mine dust) is, indeed, related to the presence of tremolite/actinolite asbestos in the mineral ore before milling and therefore separate chrysotile from tremolite/actinolite asbestos[18, 78]. Our observations suggest a high carcinogenic potential of tremolite/actinolite asbestos, even though its association with chrysotile is not clear.

When exposed both occupationally and environmentally, subjects showed a prevalence of amosite, whereas in the only occupational exposure crocidolite and amosite were similarly distributed. Interestingly, subjects undergone to anthropogenic environmental exposure showed a different distribution compared to occupational exposure: tremolite/actinolite asbestos were similarly represented to amosite and there was a

presence of anthophyllite asbestos, that was not used at the plant (and, in fact, not detected on the lungs of occupationally exposed subjects). This may suggest other sources of exposure (maybe utensils or other products containing asbestos that were widely used at the time).

Concerning the differences in the various types of asbestos according to the cause of death (MM or not), the present investigations pointed out that subjects who died from MM have not only a lower amount of total asbestos, but specifically less crocidolite and amosite, considered the most noxious kind of asbestos (according to their different biopersistence)[11, 14, 123]. This might be related to the fact that all subjects who were heavily exposed to asbestos and died from causes other than MM were employed at the asbestos-cement factory, where amosite and crocidolite were used. In fact, they had high concentrations of such asbestos types in their lungs. However, crocidolite and amosite have been detected in most MM (even though in low amounts). On the contrary, previous studies reported the absence of these kind of asbestos in 32 the general population[93, 104]. This suggests that MM onset is likely to be related to crocidolite and amosite, even though in small quantities. Yet, given that even more crocidolite and amosite were detected in lungs of subjects exposed to asbestos but not died of MM, it seems obvious that the presence of such type of asbestos in lungs is not sufficient, even in large amounts, to cause MM.

Regarding the dimensional characteristics of asbestos fibers, for this study we considered only fibers longer than 5 μm with length/width ratio >3, according to the widely accepted definition of fiber [5]. Indeed, even though a relevant role in causing MM of ultra-short fibers had been proposed by some authors [119, 124–126], this theory was firmly contested [108]. The current opinion, widely shared in the scientific community, and supported by a recent review [127] and a metanalysis [14] is that fibers longer than 10 μm play the most important role in provoking mesothelioma and lung cancer, whereas shorter fibers are less potent (even though their hazardousness cannot be denied, especially in relation to their capability to cause inflammation, fibrosis and pleural reactions).

Our analysis showed a tendency to a greater length/width ratio in subjects with occupational exposure as compared to those with anthropogenic environmental exposure or both exposures, even though the statistical significance was not reached. Anyway, given that we did not consider in this analysis fibers shorter than 5 μm, the different length of fibers do not seem to play a crucial role in determining MM, as no relevant difference was found in mean length (nor in length/width ratio) of asbestos fibers between subjects who died of MM and those who died from other causes. Notwithstanding, we have to consider that the smallest fibers are cleared more easily and therefore the time elapsed since the end of exposure may have reduced the amount of the shortest and thinner fibers.

Moreover, we observed an interesting correlation that was never reported in literature: the length of asbestos fibers, as well as the length/width ratio, increases with the duration of exposure, but not with the time which elapsed since the end of exposure.

We must specify that such evaluations are limited by the fact that we do not know the real concentration of fibers to which the subjects had been exposed, but we can only evaluate the concentration of fibers in lungs at death (that occurred after at least 8 years after the end of the exposure). Anyway, we can hypothesize that a longer exposure, and therefore a continuous deposition of "fresh" fibers for a long period of time, impair the efficiency of fragmentation, and therefore of lung clearance, according to the "overload" mechanism [128], that implies a dose dependent depression of the alveolar macrophage activity, resulting in an increased rate of particle accumulation in the lung interstitium.

Lung clearance does not regard only chrysotile, but was experimentally demonstrated for amphiboles by Rendall and Du Toit, who estimated a half-life of about 50 months for crocidolite and 18 months for amosite [118]. These results correlate well with observations conducted on humans by the same authors [129]. As opposite as expected, and also inconsistently with previous observations [130], we did not observe any negative association between the fiber burden and the time since the end of exposure. Indeed, Du Toit conducted his study on a smaller number of subjects (36 patients) using light microscopy, so the present finding can be considered more accurate. Probably, the exposure to asbestos of the subjects here analyzed was sufficiently high to exceed the clearance rate of amphiboles, resulting in a fiber overload and in an impaired macrophages function [128]. Moreover, particles in the interstitium are "sequestered" from clearance and are inevitably retained. Anyway, overload is not even necessary when the particles are highly cytotoxic, like asbestos, as they are able to depress phagocytosis and clearance also at lower doses, as they alter the chemotactic stimuli and slow the cell extravasation and random migration. Those mechanisms explain why the lung burden does not decrease with the passing of time after the end of exposure.

Morgan et al hypothesize that the ratio between uncoated and coated fibers decreases with the time elapsed since the end of exposure, due to the ongoing removal of uncoated fibers and not of the ABs, as well as the prosecution of fiber coating [33, 111]. In our series the amount of ABs did not show any correlation with time since the end of exposure. This result might indicate that the coating process does not involve all the fibers and it is probably different from one individual to another in terms of efficiency. Anyway, the coating process does not depend only on time.

It was stated that the risk of MM increases with the duration of exposure and its intensity (and thus the amount of inhaled fibers) [108]. Indeed, such reports erroneously assumed that a longer exposure means a higher fiber burden in lungs: our findings did not show any significant correlation between duration of exposure and asbestos fibers and ABS amount. So, once again, what was taken for granted based on epidemiological data is questioned by the analysis of lung content. The data here presented suggest, instead, that also the accumulation of fibers reaches a limit after a period of time, and do not continue if the exposure proceeds.

Similarly, the analysis of inorganic lung content leads to reject the statement of some authors, according to whom a reduction in asbestos dose lengthens the latency [108] .

In fact, we did not observe any correlation between the amount of asbestos fibers and ABs and the duration of latency period.

On the contrary, other authors, who performed studies about lung content, found a clear correlation between the fiber concentration in lungs and the duration and intensity of exposure, as well as with the time since the end of exposure [33].

5.2 Single nucleotide polymorphisms (SNPs) in genes involved in iron metabolism

FFPE samples have always been traditionally regarded as not suitable for molecular and genetic analysis, as nucleic acids are inevitably damaged by formalin fixation. Notwithstanding, recently the methods for extraction and analysis of DNA, RNA and proteins considerably improved, allowing molecular genetics studies using FFPE [131, 132]. Even molecular diagnostic studies and retrospective GWAS association studies have been conducted using FFPE samples, providing valuable results [133].

Of course, DNA extracted from FFPE samples produces lower quality profiles than those from fresh tissue [134]. The main cause of alteration in the DNA structure is represented by the fixation in formaldehyde, that is the active component of the formalin, which cross-links the DNA and the surrounding proteins [135] and causes the breaks in the sequences nucleotides [136, 137]. Furthermore, both the pH and the duration of fixation with formalin affect the quality of the nucleic acid [138].

Beside formalin fixation, we must consider that we are dealing with autoptic samples, and therefore with the postmortem degradation of nucleic acids occurred before formalin fixation. Following cell death, endogenous nucleases initiate the DNA degradation [139]. The rate at which DNA is degraded by endogenous nucleases depends on various factors, such as the tissue, the expression of enzymes, temperature and pH [140].

At the same time, the non-enzymatic modification of the primary DNA structure also occurs [139]. Among the most susceptible areas of the DNA molecule are the N-glycosidic bonds between the nitrogenous bases. Other mechanisms of modification of the structure of the molecule can be oxidations, deaminations and methylations; the absence of a nitrogen base can also cause cross-links between DNA and proteins or between the two DNA strands.

Despite the above illustrated difficulties, FFPE samples often represent the only available source of information about human deceased patients and therefore retrospective, observational studies on archived samples are of essential importance, especially when we are dealing with rare tumors like MM. Pathology Departments usually conserve the FFPE blocks indefinitely, whereas blood samples and other biological materials (stored at -20°C or -80°C) are periodically discarded, given the economic and storing issues.

Consistently with the results of the above cited studies, the extraction of DNA from FFPE samples provided sufficient quantities of DNA in all the subjects except four. Even though degraded, as proved by the difference between the results provided by the two quantification probes, the DNA extracted from our heart samples was of sufficient quality for SNPs analysis.

Similarly, the approach of molecular characterization of the six SNPs markers associated with iron metabolism, using the SNaPshot mini-sequencing method and subsequent separation in capillary electrophoresis, has produced electropherograms of clear interpretation and, consequently, reliable genetic typing of the samples in the study.

Interestingly, the time elapsed since the fixation process did not appear to influence the DNA quality, contrary with previous literature [137]. This result suggests that the damage induced by post-mortem degradation and fixation is not significantly worsened by the subsequent storing time of the FFPE.

Given the significant results found by Crovella et al. [79], showing a relationship between some genotypes and the risk to develop mesothelioma, we decided to look for the polymorphisms which resulted related to MM in our case series. Beside the genes studied in Crovella's paper, we decided to include MMP2 because of the evidence that metallic trivalent ions, such as Al and Fe, can bind MMP2 with inhibiting effects and consequent deprivation of tumor cells' metastatic potential [141]. Moreover, polymorphisms in MMP2 have already been proven to be related to a decreased risk to develop mesothelioma [95].

The statistical analysis performed to compare the incidence of each SNP among the three groups, as well as with the data provided by 1000Genomes database, did not provide significant results, as the frequencies of the different alleles and genotypes showed no differences between the three groups of subjects. In particular, the alleles and genotype frequencies were similar in MM, exposed controls, healthy controls and in the Italian subgroup of 1000 genomes database.

The only significant difference was pointed out for SNP rs 2715631 in Transferrin gene between MM and exposed controls, but this result was not confirmed by the further analysis. *rs2715631* is an intronic variant of gene TF, located on chromosome 3:133764045, encoding for transferrin, a glycoprotein involved in iron transport into the bloodstream. Each transferrin molecule has the ability to carry two iron ions in the ferric form (Fe3+). Transferrin has a key role in iron trafficking: under physiological conditions, almost all the circulating iron is bound to transferrin [142]. The main way to uptake iron for cells consists in internalizing Tf-bound iron by its binding to transferrin receptor 1 (TFR1) after receptor-mediated endocytosis. Ferric iron is released from TF within the endosome after its acidification and is then reduced by an endosomal ferrireductase. This ferric reduction is followed by transport of the resulting ferrous iron across the endosomal membrane by DMT1 or ZIP14 [142].

Our results are in contrast with Crovella's paper [79], according to which GG genotype of this SNP confers a protection against MM. However, it is necessary to point out that exposed controls group in Crovella's work do not appear to be in the Hardy-Weinberg equilibrium and, consequently, cannot be compared with other data. Anyway, the statistical analysis of association of dominant and recessive models with the risk of mesothelioma, even though suggestive for an increased risk of developing mesothelioma for carriers of recessive model GG, did not reach the statistical significance, not allowing any conclusion in this regard.

Rs224575 and *Rs224589* are both intronic variants of gene SCL11A2, located on chromosome 12 and coding for the Divalent Metallic Iron Transporter 1, a solute carrier (family 11 member 2). Even though in Crovella's work the differences in this SNPs frequencies did not reach statistical significance, based on the observed tendency we decided to include them anyway because of the central role of this protein in transporting Fe2+ (the reduced form of iron that produces the oxidative injury) from the extracellular environment to the cytoplasm, where iron will be safely stored. Until now, the distribution of the different alleles and genotypes of this gene was very similar in MM, EXP-C and HC, as well as in Italian subgroup of 1000Genomes database.

rs3747359 is a coding SNP of the gene HEPH, located in Chromosome X, encoding hephaestin, a protein belonging to the multi-copper oxidase family that is known to be involved in cellular iron ion homeostasis, iron transport through the epithelial layer of bowel, as well as in oxidation-reduction processes [143]. The SNP results in a non-synonymous aminoacidic substitution (Asp/his) at position 601 of HEPH. The presence of the C allele was found to be associated with protection against development of mesothelioma [79]. In the same study, also the C/G and C/C genotype in the HEPH gene were associated with an increased protection against malignant mesothelioma, following a dominant genetic model. However, some doubts arose comparing Crovella's conclusions to our observations: first, as the HEPH gene is located on chromosome X, it is hard to understand how it was possible to find two alleles in males. Secondly, as the allele with C is extremely rare, with a minimum allele frequency (MAF) <0.01, as reported in the 1000 genomes data of the gene HEPH, it is, at least, surprising to find such a higher frequency of the C allele in the 48 subjects investigated, as well as such a strong deviation from Hardy Weinberg equilibrium. In fact, in all the 168 samples here analyzed, we found the allele G.

rs76059597 is an intronic variant of a FTH1 gene (ferritin heavy polypeptide 1), located on Chromosome 11:61965877, encoding for H-subunit of ferritin. Ferritin is a multimeric protein composed of 24 subunits capable of storing ~ 4,500 iron atoms forming a hollow sphere[142]. In order to be stored in ferritin, Fe2+ must be oxidized to Fe3+ by the ferroxidase activity of H-ferritin in an oxygen-dependent manner[142]. This enclosure and sequestration of iron as ferrihydrite is vital, as it allows to avoid toxic redox reactions. We hypothesize, according to previous findings [79, 80], that C allele of FTH1 might play a protective role against asbestos-induced cell damage. Conversely, our observations showed a similar, low frequency of the C allele of this SNP in MM (4%) and exposed controls (2%), as well as in HC (4%). Also, the

genotypes distributions did not suggest any defined tendency, being TT largely predominant in MM (92%), EXP-C (96%) and HC (92%).

rs243865 is a promoter polymorphism, known to be associated with lip and oral cavity cancer [144]. It might change the transcription of the MMP2 gene (matrix metalloproteinase 2), located on Chromosome 16:55477894. Matrix metalloproteinases (MMPs) are calcium-dependent, zinc-containing endopeptidases that play an important role as modulators of the tumor microenvironment. They are involved in tissue remodeling by interfering with the cell-cell and cell-extracellular matrix interactions. MMPs, particularly MMP-2 and MMP-9, play an important role in tumor angiogenesis, invasion and metastasis[145]. Interestingly, as already explained above, metallic trivalent ions, such as Al and Fe, can bind MMP2 with inhibiting effects and consequent deprivation of tumor cells' metastatic potential[141]. Overall, polymorphisms in matrix metalloproteinase have been found to confer protection against lung cancer in studies conducted on Asian population[145, 146]. In a previous research, carriers of MMP2 rs243865 CT or CT/ TT genotypes appeared to have significantly decreased risk for developing malignant mesothelioma, especially individuals exposed to asbestos, in comparison with CC homozygous genotype[95]. In contrast, our findings showed very similar alleles and genotypes frequencies in cases and exposed controls.

In summary, none of the analyzed polymorphisms showed any significant difference between cases and controls in the distribution of both alleles and genotypes. The results here presented are, anyway, relevant because they highlight the importance of being cautious in presenting results of SNPs studies carried out on limited numbers of subjects. It is worth noting that the alleged protective role of HEPH coding SNP, as well as the two non-coding SNPs, respectively in FTH1 and TF, claimed by Crovella et al, in 2016 [79], was confirmed by a further study published by the same authors in 2019, conducted on a new cohort of lung cancer subjects from the same geographic area of subjects with mesothelioma [80]. Therefore, this approach, even though very appealing given the central role of iron trafficking molecules in the response to asbestos, does not seem to be promising in addressing the complex problem of genetic susceptibility to MM.

5.3 BAP1 expression and iron trafficking in lungs of asbestos-exposed individuals with and without malignant mesothelioma

In literature, studies similar to the present work are very rare due to the lack of suitable human samples. As opposite to studies about MM, often conducted on biopsies, the peculiarity of the series of deceased subjects here studied is that they underwent a forensic autopsy and, in this circumstance, it was possible to collect abundant samples of lung tissue free from neoplasm, suitable both for fiber burden analysis and for assessing the biological response of the lung to asbestos inhalation. Moreover, we were able to perform all the experiments above illustrated not only on individuals who died of MM, but also on people who certainly were exposed to asbestos but did not develop the neoplasm and on suitable healthy controls.

Despite the substantially negative results of SNPs assessment, we decided to keep exploring the iron homeostasis and its possible disruption as a consequence of asbestos exposure.

Prior to the investigation on iron intake (TFR1), storing (FTH1) and efflux (FPN) BAP1 staining was performed, as this protein is strictly connected with iron metabolism and ferroptosis [66] and because the loss of expression of BAP1 gene is almost constantly observed in MM neoplastic tissue, to such an extent that IHC for BAP1 is used to differentiate MM from other tumors [53–55]. Moreover, it is well known that BAP1 germline mutations are responsible for an increased susceptibility to MM after an even minimal asbestos exposure [43, 46, 47]. In the present series, BAP1 staining in MM tissue was negative in almost the totality of cases. This finding, in line with what previously known, confirms that the loss of expression of BAP1 is a key step in mesothelial carcinogenesis.

In brief, what we know is that BAP1 germ-line mutations predispose to MM and, on the other hand, in MM neoplastic tissue BAP1 is very often not expressed. Similarly to the other tumor-suppressor genes, BAP1 follows the two-hit model, as the first hit usually is the loss of heterozygosity consequent to a 3p21 deletion (pre-existent on a familial basis or sporadic) and the second mutation, occurring in the remaining allele, affects the protein function or homeostasis [57]. What it has still to be clarified is when and how the "second hit" happens. The loss of function of BAP1 implies pro-neoplastic consequences because it promotes the switch of cells towards a glycolytic metabolism ("Warburg effect"), leading to acidification of microenvironment and to a immunosuppressive phenotype of macrophages (M2) [67]. On this basis, it is clear that BAP1 inactivation can be a cause (and not a consequence) of carcinogenesis. In this study we found that BAP-1 IHC positivity is less represented in lung tissue (not invaded by the neoplasm) of individuals who died of MM compared to both EXP-C and NON-EXP. This observation might suggest that asbestos exposure can impair the expression of BAP1 in lung tissue, outside the process of mesothelial carcinogenesis. Iron overload [13, 147–149] induced by asbestos inhalation and the consequent oxidative stress and chronic inflammation [37], both well-established mechanisms of MM pathogenesis, might affect BAP1 expression, facilitating cell proliferation and neoplastic transformation and preventing cell death (in particular, ferroptosis). Similarly, S. Toyokuni proposed that iron overload induces deletions in CDKN2A/2B gene, another gene that is mutated in a large proportion of mesotheliomas [68]. These findings were not observed by rtPCR, maybe because the hypothetical mechanism of asbestos induced BAP1 inactivation might lie in post-transcriptional or post-translational alterations, that might happen preferentially in individuals who develop MM after asbestos exposure.

As above mentioned, BAP1 inactivation impairs ferroptosis, a preferential regulated cell death pathway for mesothelial cells. Consequently, escaping ferroptosis is likely to be a key factor in mesothelioma development [150]. One way to circumvent the iron-induced cell death is to reduce iron bioavailability by increasing iron-storage in the cell. This situation has been observed in cellular senescence models, where high levels of transferrin receptor (TFR1), Ferritin (increased 10-fold), and ferroportin (FPN) have

been demonstrated [82]. Iron storage, mainly exerted by ferritin, is a crucial point in carcinogenesis in many kinds of tumors. Ferritin removes excess iron from the cytoplasm, preventing the iron-mediated generation of free radicals and, at the same time, it allows the conservation of iron inside the cell.

As suggested by previous studies both on animals and on humans [13, 69, 71], the present findings suggest an asbestos-induced alteration of iron intake, storage and efflux in lung cells. Starting from iron intake, we found that TFR1 IHC positivity is more represented in MM patient's lungs compared to EC and HC, but similar between EXP-C and NON-EXP. This might mean that individuals who develop MM after asbestos exposure, as opposed to exposed individuals who do not, are more prone to accumulate iron inside cells, not being able to regulate the expression of TFR1 as a response to iron overload in the lung microenvironment. This excessive iron intake might worsen the oxidative stress and the consequent chronic inflammation, leading to a pro-carcinogenic shift. This hypothesis must be verified on mesothelial normal cells (that are likely to be more sensitive to asbestos effects). Indeed, TFR1 is overexpressed in many types of cancer cells [151].

Assessing the TFR1 positivity in relation to asbestos fibers and ABs, we found that TRF1 staining was significantly stronger in subjects with less asbestos fibers (and specifically less crocidolite) and less ABs. This can be linked to the fact that subjects with MM have significantly lower amounts of fibers and ABs (as extensively illustrated in the paragraphs dedicated to SEM-EDS) and must be verified on a larger number of EXP-C. Anyway, this result corroborates the fact that MM patients overexpress TFR1 in response to asbestos exposure.

Regarding the asbestos effect on iron storage, we found out that FTH1 positivity is increased in MM patients' lungs and in EXP-C compared to NON-EXP, whereas similar between MM and EXP-C. This suggests that, as it is reasonably expectable, asbestos exposure increases the expression of ferritin, given the need of safely storing excess iron inside the cells. The increased ferritin presence in lungs is not significantly different between MM and EXP-C, suggesting that iron storage is similarly affected by asbestos in individuals who develop MM compared to people who do not.

Consistently with the present results, a study conducted in-vitro pointed out an increased expression of FTH as a consequence of asbestos exposure and also an anti-apoptotic role of this protein in human mesothelial cells and in mesothelioma cells [152]. Evading apoptosis is a well-established hallmark of cancer.

Predictably, we found that a stronger FTH1 staining is significantly related to higher levels of ABs in lungs but not to higher amounts of uncoated fibers (regardless the type). This result suggests that the ABs are more important than uncoated fibers in determining iron overload in the lung microenvironment and, in the light of the anti-apoptotic role of FTH1, in promoting cell survival (and, therefore, proliferation of damaged cells). Moreover, it seems that iron contained in the ABs is not a "sequestered cluster", but it actively affects the levels of iron in cells of airways epithelia and

macrophages. Therefore, the ABs should not be regarded as an inert iron storage system, but as an active source of oxygen radicals [73].

When a cell is facing an iron excess, there is an increased expression of FTH1 and FPN, in order to reduce the labile iron pool and prevent ROS formation [151]. Iron metabolism is a semi-closed system, due to the extreme importance of this metal for life [151, 153]. Therefore, during evolution, mammals did not develop efficient ways to eliminate iron and are not prepared to face iron overload. FPN-mediated efflux is the only way for the cell to dismiss excess iron once it is internalized. Similarly, the human organism has very limited capacity to eliminate iron once it is absorbed through intestinal mucosa via DMT1. FPN is normally expressed in lungs (airway epithelial and macrophages) and iron exposure induces an increased expression of this protein [154]. We observed a more represented FPN IHC positivity (both in airway and alveolar epithelia and macrophages) in MM compared to HC, whereas the difference between MM and EXP-C did not reach the statistical significance (even though there was a defined tendency to an higher positivity in MM compared to EC). FPN is the only marker for which rtPCR confirmed IHC results, corroborating this finding. The overexpression of FPN in response to asbestos exposure (and therefore iron overload) is quite expectable. Besides, trying to explain the tendency to be more strongly expressed in MM compared to EXP-C, we can hypothesize that, even though the increased expression of this protein in airways epithelia leads to a more efficient iron detoxification, in macrophages it can lead to the transportation of larger quantities of iron in the extracellular matrix and into lymphatic vessels and, finally, towards the pleura, facilitating oxidative stress and carcinogenesis in this tissue. In fact, as long as iron is safely stored in intracellular ferritin, its hazardousness, in terms of oxidative stress and inflammation, is reduced. Maybe higher levels of FPN expression can explain a stronger tendency to pour iron in the interstitium.

Overall, we found a scarce correspondence between rtPCR and IHC findings. Beside the above-described limitations, essentially due to RNA degradation, this might be related to post-transcriptional regulatory mechanisms that play a role in the control of the expression of the examined proteins. Such mechanisms have been already pointed out for TFR1, FTH1 and FPN [151], whose translation can be blocked or promoted by IRP (iron regulatory proteins) that binds mRNA preventing the recruitment of ribosome.

Moreover, we are well aware that we are looking at the lungs of individuals whose asbestos exposure had ceased several years before their death. Therefore, it is clear that only long-term and stable alterations can be pointed out in these samples. Asbestos amphibole fibers are evidently still present in lungs of the examined subjects (even though their amount is extremely variable from subject to subject, as illustrated in the SEM-EDS section). Reasonably, their effect can still be observed. Indeed, given the exceptionally long latency of asbestos-induced cancers, especially MM, evaluating what happens in the lung after several years since exposure (and when MM onset has occurred) is of paramount importance. However, the time since the end of exposure (quite variable among our series) and the duration of exposure are not related to the

positivity of the above-described markers. Likewise, the latency time and survival time (in MM cases) did not show any relation with the IHC positivity of none of the examined proteins.

6. CONCLUSIONS

The present thesis is the results of a multidisciplinary work involving different scientific disciplines (legal medicine, pathology, occupational medicine, environmental mineralogy), and have relevant implications from a prevention point of view. SEM-EDS analysis of 72 lung samples provided interesting results, corroborating the hypothesis that asbestos exposure, by itself, is not sufficient to provoke MM. In fact, we found high quantities of asbestos in heavily exposed subjects who never developed MM. On the other hand, numerous subjects who died of MM showed an asbestos lung content comparable to the general population and, in a not-negligible proportion of them, no asbestos was detected. These results confirm that the risk of MM is not related to asbestos dose. Moreover, this first part of the study suggested a complete clearance of chrysotile asbestos, whereas crocidolite, amosite, tremolite/actinolite asbestos anthophyllite asbestos are clearly biopersistant. Equally important, our findings pointed out individual differences in the biological response to asbestos inhalation. In particular, there was a wide inter-individual variability in terms of fiber coating and ABs formation. As iron homeostasis plays a pivotal role in the response to asbestos inhalation, as well as in AB formation, we decided to investigate the frequency of a group of SNPs in genes involved in iron metabolism to point out potential genotype differences in relation to the susceptibility to develop MM as a consequence of asbestos exposure. This part of the research did not provide any convincing results. On the contrary, the immunostaining (coupled with rtPCR) for BAP1 and for the key proteins involved in iron trafficking (TFR1, FTH and FPN) in normal lung tissue gave interesting findings, suggesting that a different biological response to asbestos and to the consequent iron overload in lungs may play an important role in cancer initiation. This is of paramount importance from a prevention point of view, as MM is still a highly lethal tumor without any effective therapies. The prevention of its onset, by intervening in the iron homeostasis alteration and the ROS production, may represent a valuable approach in order to reduce the impact of this neoplasm, that still represent a major health issue all over the world, given the extremely diffuse presence of asbestos at a global scale. The results here presented, obtained through observational studies on deceased humans, need further investigation, in particular in-vitro studies to examine in depth the biological response of mesothelial cells to asbestos and to better understand the role of ferroptosis and its impairment in mesothelioma onset and development. Also the structure of ABs and the coating process still have to be fully understood. In conclusion, the importance of lung content examination using SEM-EDS should be underlined, as it represents a fundamental and irreplaceable tool to obtain objective information about the inorganic fiber burden in lungs (and, therefore, about the subject's exposure to asbestos during life). Indeed, evaluations based only on epidemiological and anamnestic data cannot provide such information and have been reported to produce misleading results.

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