CHRONIC INFLAMMATION IN MALIGNANT MESOTHELIOMA
PATHOGENESIS: FOCUS ON HMGB1 ISOFORMS AND GERMLINE
BAP1 MUTATIONS

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Malignant mesothelioma (MM) is an aggressive cancer arising from the mesothelial cells forming the lining of the pleural, pericardial, and peritoneal cavities. Prolonged exposure to carcinogenic mineral fibers, such as asbestos, is the most important risk factor for MM development. Mechanistically, asbestos-induced chronic inflammation is considered a crucial event in MM pathogenesis.

High Mobility Group Box 1 (HMGB1) is a pro-inflammatory molecule previously identified as mediator of both asbestos-induced carcinogenesis and MM progression. Here, we show that total HMGB1 levels can reliably distinguish asbestos-exposed individuals and MM patients from healthy controls. Moreover, a specific HMGB1 isoform—hyper-acetylated HMGB1—is significantly increased in MM patients compared to asbestos-exposed individuals and healthy controls. Finally, total and hyper-acetylated HMGB1 can also help differentiating MM patients from patients with pleural effusions due to other causes. Our results suggest that hyper-acetylated HMGB1 might represent a promising novel biomarker for early diagnosis of MM.

Continuing our effort to refine the identification of individuals at risk of developing MM, we sought to investigate the role of asbestos exposure in presence of germline mutations in the gene encoding BRCA1 associated protein-1 (BAP1), which have been recently reported as predisposing events to MM and several other cancers. However, in individuals with germline BAP1 mutations that developed MM, occupational exposure to asbestos is not usually reported. Here, using a genetically engineered murine model, we discovered
that minimal exposure to asbestos causes a deregulated chronic inflammatory response and increased risk of MM in presence of germline \textit{BAP1} heterozygosity, suggesting that doses of asbestos considered not harmful for the general population might cause the disease in genetically predisposed individuals.
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LIST OF ABBREVIATIONS AND SYMBOLS

8-HdG  8-hydroxy-2’-deoxyguanosine
AIF  Apoptosis-Inducing Factor
BAP1  BRCA1 Associated Protein-1
DAMPs  Damage-Associated Molecular Patterns
ELISA  Enzyme-Linked Immunosorbent Assay
ERM  Ezrin/Radixin/Moesin
GPI  Glycosylphosphatidylinositol
HCFC1  Host Cell Factor C1
Het  Heterozygous
HM  Human Mesothelial cells
HMGB1  High Mobility Group Protein 1
IL  Interleukin
LATS2  Large Tumor Suppressor homolog 2
LPS  Lipopolysaccharide
MBAITs  Melanocytic BAP1-mutated Atypical Intradermal Tumors
MFI  Mean Fluorescence Intensity
MM  Malignant Mesothelioma
MPF  Megakaryocyte Potentiating Factor
mTOR  mammalian Target of Rapamycin
NAD  Nicotinamide Adenine Dinucleotide
NF2  Neurofibromatosis 2
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>NF-κB</td>
<td>Nuclear Factor κ B</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>PAR</td>
<td>Poly(ADP-Ribose)</td>
</tr>
<tr>
<td>PARP</td>
<td>PAR Polymerase</td>
</tr>
<tr>
<td>PTMs</td>
<td>Post-Translational Modifications</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RNS</td>
<td>Radical of Nitrogen Species</td>
</tr>
<tr>
<td>ROS</td>
<td>Radical of Oxygen Species</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for Advanced Glycation Endproducts</td>
</tr>
<tr>
<td>SMRP</td>
<td>Soluble Mesothelin-Related Peptides</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian Virus 40</td>
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<tr>
<td>TIM-3</td>
<td>T-cell immunoglobulin domain and mucin domain 3</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll Like Receptor</td>
</tr>
<tr>
<td>TAMs</td>
<td>Tumor Associated Macrophages</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TPS</td>
<td>Tumor Predisposition Syndrome</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor Protein p53</td>
</tr>
<tr>
<td>UCH</td>
<td>Ubiquitin Carboxyl-terminal Hydrolase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-Type</td>
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<tr>
<td>YAP</td>
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CHAPTER 1
INTRODUCTION

1.1 Malignant Mesothelioma: Brief Overview

Malignant mesothelioma (MM) is a very aggressive cancer that derives from the malignant transformation of the mesothelium. The mesothelium is a membrane consisting of a single layer of simple cuboidal cells with cobblestone-like appearance (Figure 1.1).

Figure 1.1: Microscopic appearance of mesothelial cells: cobblestone-like pattern.

Mesothelial cells—whose embryological origin is the mesodermal layer—form the lining of the pleural, peritoneal, and pericardial cavities, and the tunica vaginalis testis [1] (Figure 1.2). The main function of the mesothelium is to provide organs with non-adherent surfaces to decrease attrition during cycles of organ expansion and contraction [2]. This is achieved via physiological secretion of small amounts of fluid, which in case of pathological conditions can
lead to the accumulation of conspicuous amounts of liquid effusions in body cavities [2]. Despite their mesodermal origin, mesothelial cells share a number of biological and biochemical characteristics with epithelial cells, such as expression of surface microvilli, epithelial cytokeratins, and tight junctions [2,3]. However, compared to classical epithelial cells, mesothelial cells have more differentiation plasticity—possibly to the level of pluripotent stem cells [2,3]. This dual nature of mesothelial cells is also reflected in the histological presentations of MM. In most cases (50–60%), MM has histological characteristics similar to carcinomas, with epithelioid-like cells; this subtype is known as “epithelioid MM”. Less frequently (10–20%), MM presents features more reminiscent of its true mesodermal origin, with elongated spindle-like cells; this subtype is known as “sarcomatoid MM”. When epithelioid and sarcomatoid cells coexist within the same tumor, MM is defined as “biphasic” (25–35%) [4]. More rarely, MM can be primarily characterized by poorly differentiated cells [4] or cells with unusual differentiations, like osteoclast-like giant cells [5] or cells with osteoid morphology [6]. In MM, recognition of the histological subtype is important, since it has an important prognostic value—see later.

Overall, MM is a rare cancer, with ~1 case every 100,000 individuals per year, for a total of ~3,200 diagnoses every year in the U.S. [7]. About 70–80% of all cases of MM are pleural in origin [1], consistent with the finding that prolonged occupational exposure to airborne carcinogenic mineral fibers—such as the six fibers widely used commercially in the past decades with the name of “asbestos”—is the first and most important risk factor for MM development [1,8]. Epidemiological studies have indeed shown that ~5% of asbestos miners have
Figure 1.2: **Anatomical locations of mesothelium and potential sites of MM development.** Adapted from http://www.cancer.gov
been and still are diagnosed with MM [1]. In these cases, MM is usually diagnosed around or after the age of 70 [1], as the latency time between exposure to fibers and MM development is 40–60 years [1,9], and is more frequent in males than females (≥5:1 ratio) [1,10].

Most Western countries have banned asbestos production and commercial use, but surprisingly the incidence of MM, which in the U.S. peaked around the early ’90s, has not dramatically decreased since, remaining stable around 1 case per 100,000 individuals per year [1].

The most likely explanation of this phenomenon is that decreased occupational exposure to asbestos has been paralleled in U.S. and other Western countries by an increase in non-occupational exposure to environmental sources of asbestos or asbestos-like minerals released in the air: 1) following industrialization of rural areas with natural deposits [11–14]; 2) from old mines and industry plants, or asbestos present in old buildings [14–16]. Notably, in rapidly industrializing countries such as China, India, and Brazil, the use of asbestos is still unrestricted and a peak in MM cases is expected in the next decades [17].

Other risk factors that have been associated to MM development include exposure to non-regulated asbestos-like carcinogenic mineral fibers [10]—e.g., erionite [11] and antigorite [18]—and genetic factors [19–25]. Familial clustering of MM has indeed been reported by several groups in the last decades, both in absence of known exposure to carcinogenic fibers, and in presence of such exposure, as in the case of the Turkish families heavily exposed to erionite in the Cappadocia region [26,27]. In the last years, reports of MM in patients with hereditary cancer predisposition syndromes due to germline mutations in the tumor suppres-
sor genes \textit{NF2}, \textit{TP53}, and \textit{MLH1} have accumulated [28–32], but in none of these syndromes is MM part of the classical clinical presentation. In 2011, germline mutations in BRCA1 associated protein-1 (\textit{BAP1}) were shown to cause the first hereditary cancer predisposition syndrome with significantly increased risk of MM, and several other cancers [33,34].

Notably, MM cases not associated with occupational exposure to asbestos fibers—i.e., where exposure to environmental carcinogens starts early in life and/or genetics play a major role—are usually diagnosed at a younger age compared to cases associated to occupational exposure, are more frequently diagnosed in the peritoneum, and have a male/female ratio close to 1.0 [10,35,36].

Exposure to ionizing radiation—usually iatrogenic exposure to X-rays as part of treatment protocols for prior malignancies—has also been linked to an increased risk of MM [37–40]. Finally, controversial epidemiological, pathological, and experimental evidences coexist on the role of Simian Virus 40 (SV40) infection in MM pathogenesis in humans [41,42].

Clinically, MM symptoms vary based on its localization. Patients with pleural MM usually present with shortness of breath—mostly due to the presence of pleural effusions—and localized pain in the side of the chest. Aspecific symptoms of chronic diseases such as fatigue, weight loss, and fever are also commonly found [43]. Patients with peritoneal MM usually present with abdominal pain and swelling due to presence of ascites. Also in this case, aspecific signs often prevail, causing delays in the correct diagnosis [43]. MM has a very dismal prognosis with a median survival in the absence of any treatment of $\sim 1$ year [1]. Factors associated to improved prognosis are: peritoneal localization [44], early stage
(III) at diagnosis, female sex, age <50 years old at diagnosis, epithelioid subtype, treatment with curative intention, and lower levels of platelets and neutrophils [45, 46]. Interestingly, we recently reported that MM arising in individuals with germline BAP1 mutations have an exceptionally long survival [35].

Standard therapeutic options in MM are surgery, systemic chemotherapy (cisplatin + pemetrexed) and/or localized hyperthermic intraperitoneal chemotherapy for peritoneal MM, and radiotherapy. Only rarely, however, are these therapies curative, possibly because of intrinsic chemo-resistance of MM [47,48] and its polyclonal origin [49]. Targeted therapies in MM will likely soon be part of the standard therapy: bevacizumab, a monoclonal antibody that acts as an inhibitor of vascular endothelial growth factor (VEGF), already proven to be effective in increasing overall survival in a phase III study, and several other molecules are currently being tested in phase II and phase III trials [50]. In particular, immunotherapy appears to be also effective in a conspicuous subgroup of MM patients, opening novel horizons on MM therapy [50].
1.2 Chronic Inflammation and Pathogenesis of Asbestos-induced Mesothelioma

1.2.1 Inflammation, Immunity, and Cancer

Inflammation is a complex physiological response to the presence of endogenous or exogenous harmful stimuli—physical and chemical irritants, damaged cells, pathogens—whose five clinical cardinal signs are dolor (pain), calor (heat), rubor (redness), tumor (swelling), and functio læsa (loss of function) [51]. The inflammatory response must be closely regulated by the body, since an insufficient reaction—as in the case of individuals suffering from immunodepression—might not result in the successful elimination of the damaging agent, while on the contrary an excessive inflammatory response might ultimately lead to tissue damage. An adequate inflammatory response results in elimination of the harmful stimulus and in subsequent healing with only very limited tissue damage. However, especially when protracted over a long period of time, the inflammatory response can significantly alter the biological, morphological, and functional characteristics of tissues—as in the case of liver cirrhosis from chronic hepatitis and pulmonary fibrosis from chronic exposure to airborne pollutants [51, 52].

The most important cells responsible of the inflammatory process are the immune cells—also known as leukocytes or white blood cells. Leukocytes can be divided into five major classes: lymphocytes; monocytes and their mature differentiated counterpart, macrophages (Mφ); neutrophils; basophils; eosinophils. Each of these cell types has a variety of functions and
participates in different measure to inflammatory responses due to specific causes; for example lymphocytes are the key players in the physiological response against viral infections, neutrophils and macrophages are more important in bacterial infections, and eosinophils for parasitic infections and allergies [52]. In addition to their role in the inflammatory response, leukocytes are also pivotal cells in the prevention and control of clinically overt cancers, a process called immune-surveillance [53]. Indeed, spontaneous malignant transformation happens continuously in our bodies, with a rate proportional to the number of random genetic and epigenetic mutations—affected by inherited and environmental factors—and to the number of cell divisions—a factor that changes extensively in different tissues [54]. It is therefore not surprising that individuals with severe conditions of immunodepression, for example patients with advanced acquired immune deficiency syndrome, have a significantly delayed wound healing, and increased risk of both opportunistic infections and cancer [55–57]. If escape from immune-surveillance is in fact a necessary step for a malignant cell to survive and eventually continue cancer development, the presence of leukocytes within solid tumors—an observation that dates back to 1863, by Dr. Rudolf Virchow—may seem counterintuitive. This apparent paradox has been extensively studied in the last decades, and based on several independent lines of evidence, chronic inflammation is now considered a crucial event in all the stages of tumor development [52]. In the initiation phase, a chronic inflammatory response can stimulate cancer development both directly, promoting the transformation of normal cells, and indirectly, acting on the immune cells [52]. It is indeed now clear that the same stimuli associated with benign
forms of tissue damage such as cirrhosis and fibrosis—namely growth factors, cytokines, chemokines, prostaglandins, reactive oxygen and nitrogen species—also directly predispose cells to malignant transformation [52]. Moreover, chronic inflammation is physiologically characterized by the presence of immunosuppressive cells—e.g., regulatory T cells or myeloid-derived immunosuppressive cells—whose biological role is to dampen the excessive damage caused by the acute reaction and promote wound healing. These very same cells ultimately contribute to the creation of a microenvironment that favors cancer cell escape from immune-surveillance [52,53] (Figure 1.3).

Figure 1.3: The complex interactions between immune cells and cancers. Adapted from [58]

From an epidemiological perspective, chronic inflammation may indeed be considered the driving force behind the vast majority of human cancers with strong etiological factors of en-
vironmental origin, such as smoking-associated cancers, hepatitis- and/or alcohol-associated liver cancers, and *H. pylori*-associated gastric cancers [51, 52]. Similarly, colitis-associated colorectal cancers have a clear association to chronic inflammation, and also obesity and metabolic syndrome might promote tumorigenesis in the pancreas and liver at least partially through activation of a sub-clinical chronic inflammatory response [52].

The contribution of chronic inflammation to cancer progression is even clearer. Virtually all malignancies, even those arising in absence of clear exogenous carcinogenic stimuli—e.g., breast cancer—trigger an intrinsic pro-tumorigenic chronic inflammatory response [52]. The immune cells most frequently found within the tumor microenvironment are tumor-associated macrophages (TAMs) and T cells. TAMs mostly promote tumor growth, releasing growth factors and pro-angiogenic cytokines [58], and their presence is usually correlated to worse prognosis in several cancers [59], including MM [60]. TAMs phenotypically resemble alternatively-polarized “M2” macrophages, a subpopulation of macrophages physiologically important in the late stages of inflammation to reduce the tissue damage and promote wound healing [61–63]. Therefore, it has been speculated that M2-like macrophages could be involved in the early step of inflammation-associated cancers [61, 62]. On the other hand, classically-polarized “M1” macrophages have been known to be responsible of pathogen clearance during the acute inflammatory response and also show anti-tumoral characteristics [61–63] (Figure 1.4).

In conclusion, immune cells are the important players that, due to their functional plasticity, can act both as anti-tumoral and pro-tumoral cells. The cross-talk between cancer cells
Figure 1.4: Macrophage plasticity is associated to different functions. Adapted from [69]
and immune cells is so important that cancer-associated chronic low-grade inflammation has been recognized as one of the novel hallmarks of cancer [64].

1.2.2 Asbestos and Asbestos-like Fibers

“Asbestos” is the collective name given to six mineral fibers—all now known as human carcinogens—that were commercially used in the past due to their characteristics of electric and thermic insulators. The name “asbestos” itself derives from the ancient Greek and translates into “inextinguishable”. Indeed, the basic characteristics of these mineral fibers were already known in ancient times, as demonstrated by the descriptions present in Roman author Pliny the Elder’s Natural History [65]. Most of the modern asbestos-containing products included: retardant coatings; concrete; bricks, pipes and fireplace cement; heat, fire, and acid resistant gaskets; pipe insulation; ceiling insulation; fireproof drywall; flooring; roofing; lawn furniture.

On the basis of their chemical composition—i.e., amount of iron, calcium, magnesium, and silicon—and crystalline structure, asbestos fibers are classified into two groups: 1) amphiboles (which includes amosite, crocidolite, tremolite, anthophyllite, and actinolite); 2) serpentines, with the only member being chrysotile. Amphibole class fibers are needle-like, while chrysotile fibers are curly [1,66](Figure 1.5).

The carcinogenic potential of asbestos fibers depends on several factors: 1) fiber bio-persistence; 2) geometry and size; 3) chemical composition. Amphibole fibers are characterized by prolonged bio-persistence, with fiber concentration in tissues proportional to the length of expo-
Figure 1.5: Scanning Electron Microscope Images of Crocidolite and Chrysotile Fibers. USGS Denver Microbeam Laboratory
sure, while chrysotile fibers have a significantly shorter half-life and are more easily cleared from the body [67]. Not surprisingly, the association to MM is much stronger for amphiboles than chrysotile [68], even though continuous exposure to the latter results in the same cellular alterations observed after exposure to amphiboles [66], and has been epidemiologically associated with increased risk of MM [69]. In regard to geometry and size, fibers with length >4 m and a needle-like shape lead to an increased risk of pleural inflammation and MM, possibly because they cause a prolonged frustrated phagocytosis by resident mesothelial cells and macrophages [70]. Finally, fibers with increased iron content have been proposed to be more carcinogenic, due to the action of the metal as catalyst in the production of oxygen and nitrogen radical species [71, 72].

Importantly, almost another 390 fibrous asbestos-like materials not subject to any regulation are present in nature [10], and several of these might be carcinogenic—possibly even more so than asbestos itself, as in the case of erionite [73] or other fibrous non-asbestos amphiboles [74]. Increasing concern is also directed at man-made fibers, such as carbon nanotubes. Several in vitro and in vivo studies indeed showed that multi-walled carbon nanotubes share characteristics similar to classical asbestos fibers [75–77]. In this manuscript, we will mostly refer to studies with crocidolite asbestos fibers, since they have been more extensively investigated, but the mechanisms described here for asbestos exposure might hold true also in the case of exposure to natural and artificial “asbestos-like” fibers.
1.2.3 Pathogenesis of Asbestos-induced Mesothelioma

In humans, most of the asbestos fibers that are inhaled deposit along the airways, and only a small percentage—mostly constituted by thin and long fibers—reaches the alveoli and the pleural mesothelial cells [78]. When these events were first mimicked in vitro, asbestos fibers were surprisingly found to be cytotoxic for human mesothelial cells [79,80]. This unexpected finding suggested that a more complex model of MM pathogenesis was required, which would overcome the former simplistic idea that direct genotoxic effects of asbestos would be sufficient to cause MM. Asbestos fibers per se are in fact weak mutagens, especially when compared to other known cancer-inducing agents, for example cigarette smoke [81]. The observation that asbestos deposition in tissues is associated with a substantial inflammatory response [82,83] suggested that leukocytes might be important in MM pathogenesis. Recent evidences indeed suggest that the chronic inflammatory response activated by damaged and/or dying mesothelial cells represents the missing piece in the puzzle. In fact, these cells release a plethora of molecules—collectively referred to as “alarmins” or DAMPs (damage-associated molecular patter molecules)—in the microenvironment to signal their condition of distress [84,85]. These alarmins in turn are responsible of activating resident inflammatory cells and recruiting from the bloodstream other leukocytes, particularly neutrophils and macrophages [84,85]. Moreover, leukocytes can also be directly activated by asbestos fibers, during the process of frustrated phagocytosis [86,87]. To complete this vicious cycle, activated inflammatory cells release radical species of oxygen (ROS) and nitrogen (RNS), chemokines, and cytokines—the most important ones being tumor necrosis
factor (TNF)-α and interleukin (IL)-1β—which on the one hand promote DNA damage in mesothelial cells, while on the other they provide them with strong survival stimuli [88, 89]. The complete identification of the molecules directly or indirectly responsible for promoting the survival of mesothelial cells carrying DNA damage might result in potential novel targeted therapies designed to modulate asbestos-induced inflammation in order to reduce MM incidence [89, 90].

Among the alarmins that trigger asbestos-induced inflammation, High Mobility Group Protein 1 (HMGB1) has recently emerged as a potential diagnostic biomarker of MM and a novel therapeutic target [88, 91, 92]. Paragraph 1.3 will be dedicated to review the biological functions of HMGB1; in chapter 2, we will present results on specific HMGB1 isoforms as biomarkers of asbestos exposure and MM.

To further understand the pathogenesis of asbestos-induced MM, it has to be considered that only only 2–10% of individuals professionally exposed to asbestos fibers develop MM, while radiological and histological signs of asbestos-induced chronic inflammation—fibrosis of the lung parenchyma or thickening of the pleural lining—can be found in the vast majority of these individuals [93, 94]. It is therefore likely that individual genetic characteristics might modulate the intensity, length, and type of asbestos-induced inflammation. Indeed, association studies based on single nucleotide polymorphisms have suggested that genes involved in DNA repair, inflammation, and xenobiotic and oxidative metabolism might modulate the risk of asbestos-induced pathologies, including MM [95–98].

In this regard, we recently discovered that germline mutations in the BAP1 gene cause a
novel autosomal dominant hereditary tumor predisposition syndrome characterized by high incidence of MM and other cancers [33, 34]. Whether germline $BAP1$ mutations could predispose to MM in the absence of or after only very limited exposure to asbestos remained a major question to be tackled in the field. In paragraph 1.4 we will review $BAP1$ biological functions, particularly focusing on somatic and germline $BAP1$ mutations. In chapter 3, we will present the results of our studies on how germline $BAP1$ mutations might modulate asbestos-induced chronic inflammation and increase the risk of MM even after minimal exposure to asbestos fibers.

In conclusion, the current model of MM pathogenesis suggests that individual genetic factors determine susceptibility to asbestos carcinogenicity, which is mediated from a molecular point of view by alarmins, inflammatory cytokines, and ROS/RNS released by damaged mesothelial cells and activated inflammatory cells, which globally result in the survival and expansion of several clones of mesothelial cells carrying DNA damage, eventually resulting in the development of a clinically overt polyclonal MM (Figure 1.6).
Figure 1.6: Working hypothesis for MM carcinogenesis. (HMC, human mesothelial cells). Reproduced from [94]
1.3 Biomarkers of Asbestos Exposure and Malignant Mesothelioma

1.3.1 Definitions of Biomarker

Biomarkers are defined as measurable biologic indicator used to distinguish precisely, reproducibly, and objectively two or more biological or clinical states [99]. In oncology, biomarkers are used for numerous purposes: to quantify exposure to known carcinogens (exposure biomarkers); to aid in the diagnose of a disease (diagnostic biomarkers); to monitor disease progression (monitoring biomarkers); to predict survival (prognostic biomarkers); to assess drug safety and evaluate target engagement (pharmacodynamics biomarkers); to predict patients who are more likely to benefit from a treatment (predictive biomarkers); to anticipate clinical endpoints after a specific therapy (surrogate biomarkers).

In general, biomarkers can be represented by both discrete variables—e.g., gene polymorphisms giving rise to different alleles—and continue variables—e.g., specific molecules whose levels define the risk of disease. In either case, there are several and diverse biological sources of potential biomarkers. For clinical implementation, sources that do not require invasive procedures to be collected—e.g., urine, saliva—should be preferred, whenever possible, to sources requiring invasive collection. Among the latters, blood sampling should be preferred over other sources—e.g., pleural fluid, cerebrospinal fluid—that require more expensive and potentially hazardous procedures.

Given its association to known environmental carcinogens—i.e. asbestos and asbestos-like
mineral fibers—the long latency time from exposure to cancer development and diagnosis, and its dismal prognosis, MM represents an ideal disease whose natural history could be significantly altered from the development of reliable biomarkers. In the last decades, research efforts in this direction have been important, and numerous candidate biomarkers have been proposed.

### 1.3.2 Biomarkers of Asbestos Exposure

Asbestos workers have a significantly higher risk compared to the general population of malignant conditions, particularly mesothelioma and lung cancer [100], but also of laryngeal cancer [101], cancers of the digestive tract [102], ovarian cancer [103], and possibly others. Moreover, they also often present with nonmalignant asbestos-related diseases—e.g., pleural plaques and diffuse pleural fibrosis [104]. The identification, among potentially exposed individuals, of subjects with early-stage asbestos-related clinical conditions is an unmet medical need.

The pathogenesis of both malignant and nonmalignant asbestos-related diseases is strongly connected to the tissue damaging and inflammatory properties of the asbestos fibers. It is therefore not surprising that most proposed biomarkers of asbestos exposure are molecules involved in inflammation or DNA damage. Compared to unexposed controls, asbestos-exposed individuals showed indeed significantly deregulated blood levels of several inflammatory cytokines and chemokines [105, 106]. One of the consequences of asbestos-induced inflammation is the increase in radical of oxygen species. Clinically, this translates into in-
creased levels of the marker of DNA oxidative damage 8-hydroxy-2'-deoxyguanosine (8-HdG) in circulating leukocytes of exposed individuals [107–109]. Similarly, levels of anti-oxidant enzymes peroxiredoxins were also found increased in asbestos-exposed individuals compared to controls, together with the cytoskeletal protein tropomyosin 4 [110]. Moreover, levels of known growth factors associated to both wound healing and cancer progression, such as interleukine-6, platelet-derived growth factor ββ, hepatocyte growth factor, basic fibroblast growth factor, and vascular endothelial growth factor β, were also increased in asbestos-exposed individuals [107, 108].

None of these biomarkers has however moved to clinical testing, mostly due too their poor specificity: for example, 8-HdG levels can be increased by exposure to other cancer-causing agents, such as tobacco smoke, heavy metals, and polycyclic aromatic hydrocarbons [111], and alteration in blood levels of cytokines and chemokines are common also in other inflammatory and autoimmune diseases [112].

1.3.3 Biomarkers of Malignant Mesothelioma

Soluble mesothelin related peptides (SMRPs)

The mesothelin gene (MSLN) encodes a 69-kDa precursor protein, which is cleaved into a 40-kDa membrane-bound C-terminal fraction known as mesothelin or C-ERC/mesothelin, and a 31-kDa soluble N-terminal fraction, known as megakaryocyte potentiating factor (MPF) or N-ERC/mesothelin [113]. Mesothelin is expressed at very low levels in normal tissues compared to MM (limited to the epithelioid tumor cells), pancreatic cancer, ovarian cancer,
and non-small cell lung cancer, and represent therefore a potential target of cancer immunotherapy [114]. The biological functions of mesothelin are not well-understood, however mesothelin seems to be involved in cell adhesion processes, via its interaction with cancer antigen-125 (CA-125, also known as mucin 16 or MUC16) [115]. Mesothelin has three presumed isoforms: variant 1 is the most expressed in cancer cell and is attached to the cell surface via a glycosylphosphatidylinositol (GPI) anchor. Compared to variant 1, mesothelin variant 2 and 3 have respectively a 24– and 82–base pair insertions. Variant 2 retains the GPI anchor and is membrane-bound, while variant 3 is soluble as a result of a frameshift mutation. Both variant 1 and 2 can however enter the circulation through protease-dependent shedding at the GPI anchor; in fact soluble variant 1 is the most abundant isoforms found in cancer patients [116–118]. Here, we will use “soluble mesothelin” to indicate all mesothelin variants measurable in the blood, and “soluble mesothelin-related peptides” (SMRPs) to indicate both soluble mesothelin and MPF.

Soluble mesothelin is as of today the only approved diagnostic biomarker for MM. In 2003, the first enzyme-linked immunosorbent assay (ELISA) capable to measure soluble mesothelin variants 1 and 3 was reported, with soluble mesothelin proposed as a diagnostic and prognostic biomarker [119]. This ELISA assay was later developed and commercialized by Fujirebio Diagnostics (Malvern, PA) with the name of Mesomark®. In January 2007, the U.S. Food and Drug Administration (FDA) approved the Mesomark® ELISA under the Humanitarian Device Exemption programs, which is exempt from the effectiveness requirements of a pre-market approval, to aid in the monitoring of patients with epithelioid and
biphasic mesothelioma. For this reason, soluble mesothelin has been the most extensively studied biomarker for MM so far.

A first meta-analysis conducted in 2012 and considering all the studies available using the Mesomark® ELISA for diagnosis of MM patients showed that at a common diagnostic threshold of 2.00 nmol/l, the sensitivities and specificities of mesothelin in the different studies ranged widely from 0.19 to 0.68 and 0.88 to 1.00, respectively. The authors concluded that in patients suspected of having MM, a positive blood test for mesothelin at a high-specificity threshold is a strong incentive to urge further diagnostic steps. However, the poor sensitivity of mesothelin limits its added value to early diagnosis [120].

A second more recent meta-analysis analyzed the diagnostic accuracy of SMRPs in MM diagnosis across 28 studies. In total, data from 1562 patients with pleural MM were compared to those from 5988 individuals without MM. The sensitivity ranged from 0.33 to 0.95 (pooled 0.61, 95% CI 0.58-0.63) while specificity ranged from 0.60 to 1.00 (pooled 0.87, 95% CI 0.86 to 0.88). The specificity of serum SMRPs was highest when comparing MM patients to healthy controls, and lower when comparing to asbestos-exposed individuals or patients with other cancers. Among the SMRPs, MPF showed better sensitivity and specificity than soluble mesothelin. Notably, the 28 studies showed significant heterogeneity, whose sources (e.g. differences in study population, sample collection and storage, and technical factors) have to be identified and addressed in the future to improve the clinical applicability of SMRPs in MM diagnosis [121]. It is important to consider that clinical covariates, such as body-mass-index, glomerular filtration rate, and tumor stage, can significantly affect the
performance of SMRPs [122,123].

The role of soluble mesothelin as prognostic or predictive biomarker has also been recently evaluated in MM patients. Mesothelin levels provide information about patient prognosis, but do not predict treatment response [124]. However, a decrease in mesothelin levels has been observed in MM patients with radiological responses following chemotherapy, suggesting that mesothelin could be a good biomarker to monitor treatment efficacy and possibly recurrence of disease [125, 126]. These results were recently confirmed in a multicenter prospective observational study, which showed that soluble mesothelin is a useful early treatment response biomarker when measured serially during chemotherapy [127].

In conclusion, wide-spectrum clinical application of SMRPs as diagnostic biomarkers is still questionable. The poor sensitivity of SMRPs, especially when comparing MM patients to high-risk asbestos-exposed individuals, significantly limits its use in the early diagnosis of MM. If novel platforms, like the recently proposed ELISA system for the detection of MPF [128], will overcome the current poor sensitivity, SMRPs role in MM diagnosis might become clinically relevant and will have to be revisited. However, SMRPs (especially soluble mesothelin), hold significant potential as monitoring biomarkers in patients with measurable levels at diagnosis.

**Osteopontin**

Osteopontin (OPN) is prevalently an extracellular matrix glycoprotein expressed by a variety of cell types, including fibroblasts, osteoblasts, endothelial cells and inflammatory cells, often overexpressed in lung, breast, colorectal, and other cancers [129]. Besides extracellular OPN,
also intracellular OPN has been described, and recently several studies supported a role in regulation of immunity and inflammation by this variant [130,131]. Secreted OPN can exist in three differentially spliced isoforms (A-C), which can exert tissue specific functions [132]. In MM, OPN plays an important role in adhesion, proliferation, and migration [133]. This is likely mediated by the selective up-regulation of OPN isoform A in MM compared to healthy pleural cells, which possess all three isoforms [134]. Moreover, OPN expression has been associated to increased resistance of MM cells to anti-cancer drugs vinorelbine, etoposide, and gemcitabine [135], but also to increased sensitivity to the anti-folate drug pemetrexed [136].

In 2005, OPN was for the first time reported as a sensitive and specific biomarker capable of distinguishing individuals exposed to asbestos fibers without MM from those with MM [137]. After this report, several studies by different groups were performed to validate these initial findings. A recent meta-analysis of eight studies using OPN assay for the diagnosis of MM concluded that the pooled sensitivity was 0.57 (95% CI 0.52-0.61), specificity was 0.81 (95% CI 0.79-0.84) [138]. As in the case of SMRPs, also for OPN there was significant heterogeneity among studies. A potential source of heterogeneity is the test specimen, with some studies testing OPN in plasma and others in serum [138]. On this relevant issue, several groups reported that plasma OPN has a superior diagnostic accuracy and more stability compared to serum OPN [139]. As more studies will be conducted, the clinical potential of OPN to diagnose MM will become more clear.

Besides its possible role in MM diagnosis, other potential applications of OPN in MM have
been studied: indeed, baseline levels of OPN in MM have been proposed as prognostic biomarker [140], whereas OPN (contrary to SMRPs) does not seem a valid biomarker to monitor treatment efficacy, since its levels were not associated to shrinkage of tumor volume after therapy [126,140].

**Fibulin-3**

Fibulin-3 is the product of the epidermal growth factor containing fibulin-like extracellular matrix protein 1 (*EFEMP1*) gene. It is expressed at low levels in normal tissues [141]. Little is known about the molecular functions of fibulin-3; some evidences suggest that, just like the previously proposed MM biomarkers, also fibulin-3 might have a role in the regulation of cell adhesion and migration [142].

Recently, fibulin-3 was reported to be overexpressed in MM [143], and plasma levels of fibulin-3 measured with a commercially available ELISA kit were shown to be have high sensitivity and specificity (96% and 95% respectively at a 52 ng/mL threshold) in distinguishing MM patients from asbestos-exposed individuals without tumor [141]. These findings were independently reproduced in two small studies, which reported sensitivity and specificity values around or more than 80% [144,145]. However, a larger study with a head-to-head comparison between fibulin-3 and mesothelin found the former to have much lower sensitivity (22% with 95% specificity) at the previously reported threshold in distinguishing MM patients from asbestos-exposed individuals without MM [146].

Besides its diagnostic value, potential prognostic and predictive roles of fibulin-3 are being also explored. Fibulin-3 levels in pleural effusions might have a prognostic value, which was
however not observed in the case of plasma fibulin-3 levels [141]. Finally, plasma fibulin-3 levels were also not predictive of response to chemotherapy [127].

The relative inconsistency of fibulin-3 performance among different studies is, at least in part, likely due to sample preparation and storage. Fibulin-3 levels are more stable in plasma rather than serum, possibly because of the presence in the fibulin-3 molecule of two thrombin cleavage sites [147], and incorrect preparation of the samples significantly affects the results [141]. Another important factor to be considered when studying biomarkers that could affect the reproducibility of the results is the selection of appropriate control groups, whose samples should be prepared exactly as for the MM cases, ideally in the same institution.
1.4 HMGB1: a Pivotal Alarmin in Mesothelioma Development

1.4.1 HMGB1 Functions Depend on Localization and Redox Status

HMGB1 is a highly conserved and ubiquitous protein, mostly but not exclusively localized in the nucleus, where it represents the most abundant non-histone protein [148]. HMGB1 presents high homology with other members of the HMGB family of proteins (>80%), which are however expressed only in specific tissues—lymphoid tissues and testis for HMGB2—and/or specific developmental stages—embryos or hematopoietic stem cells for HMGB3 [148, 149]. The crucial role of HMGB1 in mammalian biology is confirmed by the observation that HMGB1 knock out mice die shortly after birth because of hypoglycemia [150].

The first described function of HMGB1 was to participate in the organization, together with histones, of nucleosomes and chromatin structure [151, 152]. HMGB1 can in fact bend the DNA, and it interacts with many transcription factors and other chromatin remodeling proteins to regulate gene transcription [153, 154] and DNA damage response [155–157].

Structurally, HMGB1 is characterized by the presence of two conserved homologous domains, the HMG boxes Box A (amino acids 1–79) and Box B (amino acids 89–163), and by an acidic—i.e., rich in glutamic and aspartic acids—N-terminal tail [148]. Box A and B bind the DNA, whereas the acidic domain interacts with basic residues abundant on histone proteins. HMGB1 only contains three cysteine residues (amino acids 23, 45, and 106), whose
redox status is extremely important for its function. In fact, HMGB1 can exist in three main different redox isoforms: fully reduced HMGB1, disulfide HMGB1—with a disulfide bond between Cys23 and Cys45 and reduced Cys106—and terminally oxidized HMGB1—when Cys106 in the sulfonic state -SO$_3$H [148,158,159]. Finally, HMGB1 presents two functional nuclear localization signals (NLS1, amino acids 28–44, and NLS2, amino acids 179–185), which are rich in lysine residues and are important for its intracellular localization [148] (Figure 1.7).

Figure 1.7: Structure of HMGB1 (C: cysteine; K: lysine; NLS: nuclear localization signal).

The localization of HMGB1 within the cell has major functional consequences. In the cytoplasm, HMGB1 promotes autophagy via its interactions with beclin-1 and p53 [160–162]. On restricted cell types, such as activated platelets, HMGB1 can also be present as a membrane-associated protein [163]. Finally, HMGB1 can be actively secreted in the extracellular space by several cell types, including mononuclear phagocytic cells [164,165], endothelial cells [166], and cancer cells [91,167], or be passively released by cells severely damaged or necrotic, following loss of plasma membrane integrity [168]. Apoptotic cells release substantially less
HMGB1, even though activation of mononuclear cells by apoptotic remnants can itself stimulate HMGB1 active release [169]. Extracellular HMGB1 acts as an alarmin and stimulates the inflammatory response. In fact, in its fully reduced form, HMGB1 promotes leukocyte migration, whereas in its disulfide form, it induces the production of pro-inflammatory cytokines by inflammatory cells [158, 159, 169].

Importantly, post-translational modifications (PTMs) regulate the localization of HMGB1 to specific cell compartments. So far, the most studied PTMs that regulated HMGB1 localization are serine phosphorylation and especially the acetylation of key residues in the NLSs. Specifically, in activated immune cells, HMGB1 can be phosphorylated on serine residues within the NSLs, and this phosphorylation prevents HMGB1 from being shuttled to the nucleus and re-directs it toward active secretion [170, 171]. Acetylation of HMGB1 was described for the first time in 1979 [172], and it is now clear that acetylation of specific lysine residues within the NLSs determines in inflammatory cells re-localization of HMGB1 to the cytoplasm and primes it for active secretion [164, 165]. The interplay between all these PTMs in determining the fate of HMGB1 is currently the subject of research. As mentioned, the functions of extracellular HMGB1 vary according to its redox status. Fully reduced HMGB1 binds the chemokine CXCL12 and stimulates chemotaxis via its cognate receptor CXCR4 [173]. Disulfide HMGB1, on the contrary, binds primarily to the Toll Like Receptor (TLR) 4 and its co-receptors CD-14 and MD-2, which act as the main receptors for bacterial lipopolysaccharide (LPS) [174, 175]. HMGB1 can also bind other receptors, such as the Receptor for Advanced Glycation Endproducts (RAGE) [176, 177], the receptor T-cell
immunoglobulin domain and mucin domain 3 (TIM-3) [178], and other TLR, specifically TLR2 and TLR9, especially when HMGB1 is bound to nucleic acids [174,179,180]. Activation of TLRs by HMGB1 results in nuclear translocation of Nuclear Factor κ B (NF-κB) and transcription and translation of pro-inflammatory molecules, including tumor necrosis factor TNF-α, IL-6, and IL-1β [181]. Terminally oxidized HMGB1 loses both its chemoattractant and cytokine-inducing properties, and might be involved in the resolution of the inflammatory process [169,182] (Figure 1.8). In vivo, the redox status of HMGB1 is dynamically regulated, and the presence of ROS/RNS in the microenvironment controls which isoform prevails [169].

![Figure 1.8: Different functions of HMGB1 according to its redox status. Adapted from [139]](image)
1.4.2 HMGB1 in Human Diseases

As one of the most important mediators of inflammation, HMGB1 has been shown to be involved in a great number of different pathologies, which can be divided into four main categories: 1) infectious diseases; 2) autoimmune/chronic inflammatory diseases; 3) other diseases associated to extensive tissue damage; 4) cancer. Here, we will briefly describe the established contributions of HMGB1 in these conditions.

In infective diseases, HMGB1 has been extensively studied in the context of LPS-induced septic shock [183, 184]. In macrophages, LPS induces HMGB1 hyper-acetylation and secretion via activation of the JAK/STAT1 pathway [165], as well as via activation of poly(ADP-ribose) (PAR) polymerase (PARP)-1 and inhibition of the deacetylase SIRT1 [185, 186]. HMGB1 is considered a late mediator of endotoxemia, and inhibition of its secretion or function results in significant decreased inflammatory response with consequent increased survival after LPS challenge \textit{in vivo} [168, 184, 187].

The contribution of HMGB1 in autoimmune and chronic inflammatory diseases has also been recently evaluated [188, 189]. In the synovia of patients with rheumatoid arthritis (RA), as well as in animal models of this disease, HMGB1 is increased and blockade of HMGB1 expression attenuates disease in animal models [190–192]. In particular, in synovial fibroblasts HMGB1 regulates angiogenesis [193] and prostaglandin synthesis [194]. In myositis, expression of HMGB1 is enhanced in inflamed muscle and indicates muscle dysfunction [195,196]. In systemic lupus erythematosus, HMGB1 enhances macrophages inflammatory response [197] and could represent a biomarker of disease [198].
Other benign diseases associated with increased levels of HMGB1 include acute conditions with extensive tissue damage such as: physical trauma [199]; severe burns [200]; ischemia and/or ischemia-reperfusion injury [201]; acetaminophen-induced hepatotoxicity [202].

Finally, HMGB1 has been involved in the pathogenesis and progression of several malignancies besides MM, most notably hepatocellular carcinoma [203], gastric cancer [204], and colorectal cancer [205]. In all these cases, HMGB1 expression is associated with disease progression and worse prognosis.

HMGB1 might also have a crucial role in determining the efficacy of chemotherapy and radiotherapy, through its interaction with dendritic cells: mature dendritic cells interact with HMGB1 via TLR4, resulting in stimulation of tumor antigen processing and presentation [206, 207], whereas tumor-infiltrating dendritic cells bind HMGB1/nucleic acids complexes with the TIM-3 receptor, thus diminishing the immunogenicity of nucleic acids released from dying tumor cells [178]. Whether different HMGB1 isoforms contribute more to tumor progression or to the response to anti-tumoral therapy is still an object of research.

1.4.3 HMGB1 in Mesothelioma Initiation and Progression

As discussed in previous paragraphs, chronic inflammation is believed to have a crucial role in MM pathogenesis. The identification of key molecules starting and propagating such response is therefore considered an important step to refine our understanding of MM pathogenesis. In this regard, our group has shown in the last years that HMGB1 is one such molecule. Human mesothelial cells exposed to asbestos activate a programmed necrosis pathway called
“parthanatos” [88]—i.e., cell death morphologically sharing characteristics of both apoptosis and necrosis caused by PARP over-activation [208]. PARP catalyzes the synthesis of the PAR polymer, which physiologically represents an important post-translational modification [208]. Utilization of ribonucleotides in this process is accompanied by depletion of the cofactor NAD$^+$ and the energy storage molecule ATP [208]. Simultaneously, excessive PAR polymer triggers the release of AIF (apoptosis-inducing factor) from the mitochondria, its translocation to the cell nucleus and cleavage of DNA [208]. During parthanatos, HMGB1 is modified by PARP in several amino-acids, especially in its acidic tail. This modification causes HMGB1 to lose affinity for nuclear histone proteins and is associated with HMGB1 translocation to the cytoplasm [209]. Following loss of cell membrane continuity, HMGB1 is then passively released in the extracellular space, where it can bind its cognate receptors and activate immune cells. Cytokines produced by activated macrophages, e.g., TNF-α, in turn promote survival of mesothelial cells [83]. Indeed, immunohistochemical staining of tissues from asbestos-exposed mice clearly showed co-expression of HMGB1 and TNF-α [88]. Also, we reported that circulating levels of HMGB1 in asbestos-exposed individuals were significantly higher when compared to healthy controls or heavy smokers [88].

HMGB1 has also been associated with MM progression [91]. Indeed, MM cell lines actively secrete HMGB1 that acts as an autocrine and paracrine pro-tumoral mediator. HMGB1 inhibition results in reduced cell migration, invasion, and colony formation in vitro, as well as slower tumor growth in vivo [91]. In this setting, the RAGE receptor appears to be the most important mediator of HMGB1 effects [91]. HMGB1 levels are higher in MM
patients, suggesting that HMGB1 could represent a novel biomarker of diagnosis [91, 210] and/or prognosis in MM [211]. Finally, aspirin and its metabolite salicylic acid were recently shown to act as novel HMGB1 inhibitors with significant activity in a MM model [92]. These evidences support HMGB1 as a potential biomarker and therapeutic target for MM [90].
1.5 BAP1 in Cancer and Mesothelioma

1.5.1 Landscape of Somatic Mutations in Mesothelioma

MM is a tumor characterized by relatively few recurrent somatic mutations and by frequent structural alterations with large copy number variations. Chromosomal losses are more common in MM than gains: regardless of the MM subtype, the most commonly lost regions are at 1p, 3p, 6q, 9p, 13q, 14q, 15q and 22q; the most commonly gained regions are 5p, 7p, 7q, 8q and 17q [212]. Many of the chromosomal losses observed correspond to second hits after mutations of important tumor suppressor genes. In fact, recent high-throughput sequencing studies have confirmed that MM tumors rarely harbor activating mutations in oncogenes, while frequently present inactivating mutations in the tumor suppressors CDKN2A, NF2, and BAP1 [213,214].

A frequent cytogenetic abnormality in MM (50-80% of samples) is deletion at 9p21, the locus of CDKN2A (encoding p16\textsuperscript{INK4A} and p14\textsuperscript{ARF}) and CDKN2B (p15\textsuperscript{INK4B}) [215, 216]. p16\textsuperscript{INK4A} and p15\textsuperscript{INK4B} control the cell cycle preventing the activation of the complex of cyclin-dependent kinase 4/6 with cyclin D, which is upstream of the retinoblastoma protein pathway. On the other hand, p14\textsuperscript{ARF} regulates p53 through inactivation of the human ortholog of mouse double minute 2, which is an upstream regulator of p53. Thus, the homozygous deletion of this locus indicates the inactivation of two major tumor suppressing pathways in the cell [217]. Murine models of asbestos-induced carcinogenesis have confirmed that both p16\textsuperscript{INK4A} and p14\textsuperscript{ARF} independently contribute to the suppression of asbestos carcinogenicity [218]. Although mutations in TP53 (the gene encoding p53) are the most
common genetic alterations found in human malignancies, they are actually only rarely found in human MM [219]. Despite that, p53 can be involved in MM pathogenesis through functional inactivation—e.g., by deletion of p14\textsuperscript{ARF} or through interaction with SV40 large T antigen [220, 221]. To support the functional contribution of the p53 pathway to MM pathogenesis, there are several cases of MM in patients affected by Li-Fraumeni syndrome, the autosomal dominant cancer syndrome due to germline TP53 mutations [29–31], as well as murine models of MM in p53 heterozygous mice showing accelerated carcinogenesis after exposure to asbestos fibers [222,223] or long multiwalled carbon nanotubes [224].

Another tumor suppressor gene commonly lost (~40-50% of all cases) in MM is Merlin (neurofibromin 2), the product of the gene NF\textsubscript{2}, mapped to 22q12.2 [225]. Germline mutations of NF\textsubscript{2} are causative of type 2 neurofibromatosis, an autosomal dominant hereditary disease characterized by tumors of the nervous system [226]. As expected, the risk of MM development after asbestos exposure is increased in patients affected by type 2 neurofibromatosis [227]. Merlin is a member of the Ezrin/Radixin/Moesin (ERM) protein family and its biological function is not well understood. Sporadic schwannomas, meningiomas, and MM are often characterized by bi-allelic inactivation of NF\textsubscript{2} [225, 228]. Wild-type merlin mediates contact-dependent inhibition of cell proliferation in normal cells, primarily through inhibition of the mammalian target of rapamycin (mTOR). Without merlin, mTOR activity is aberrantly up-regulated, which leads to increased cell proliferation; in MM tumors and cell lines phospho-mTOR expression and NF\textsubscript{2} expression are inversely related, confirming the link between the two [229]. Merlin also regulates multiple cell signaling cascades, and
is thought to inhibit cell signaling by negatively regulating cell surface receptors, including epidermal growth factor receptor, EGFR [230]. Specifically, one of the major downstream effectors of Merlin is the Hippo signaling pathway, which regulates cell differentiation and division, and promotes apoptosis. Alterations of components in this cascade were identified in MM cells, including constitutive activation of Yes-associated protein (YAP) and inactivation of large tumor suppressor homolog 2 (LATS2) [231]. YAP expression was observed in >70% of primary MM tissues, with most positive cases showing increased nuclear YAP staining when compared to cytoplasmic staining [232]. It is clear that deregulation of the mFerlin-Hippo signaling cascade is one of the frequent and key events of MM cell development and/or progression. Classical ERM proteins consist of an N-terminal domain, a coiled-coil domain, and a C-terminal actin-binding motif, and readily switch between an open dormant conformation and a closed active conformation. Merlin shares much sequence homology with the ERM proteins, but lacks an actin-binding motif and suppresses tumorigenesis in the closed form [233]. Recent studies showed that the closed, growth-inhibitory form of Merlin accumulates in the nucleus, binds to the E3 ubiquitin ligase CRL4(DCAF1), and suppresses its ubiquitination activity [234]. Li et al. [234] proposed that Merlin suppresses tumorigenesis following its translocation to the nucleus, where it binds and inhibits CRL4(DCAF1), causing changes in the expression of several hundred genes by ubiquitinating transcription factors, histones, or chromatin-remodeling enzymes. Importantly, Merlin has been recently proposed as a novel regulator of p53, suggesting an additional pathway through which p53 could be functionally inhibited.
in MM [235]. Two different mice with heterozygous NF2 mutations have been used as MM models: the NF2(KO3/+), created by insertion of a selection marker in NF2 exon 3 [236–238] and the NF2(KO2-3/+) created by replacing with a selection marker from the 3’ part of exon 2 up to the 5’ part of intron 3 [239,240].

Following repeated intraperitoneal injections of crocidolite asbestos, NF2(+/-) mice show markedly accelerated MM tumor formation compared to asbestos-treated wild-type (WT) littermates. Constant biallelic inactivation of Nf2 was found in MM from NF2(+/-) compared to only 50% of WT mice similar to the incidence documented in human MM. MM from asbestos-treated NF2(+/-) frequently exhibited deletions of p16\(^{\text{INK4A}}\), p19\(^{\text{ARF}}\) and p15\(^{\text{INK4B}}\), with p53 expression lost in 44% of MM [237–240].

### 1.5.2 Somatic Mutations of BAP1 in Mesothelioma and Other Cancers

The BAP1 gene is found on chromosome 3p21. It encodes a 729 amino-acid long prevalently nuclear C-terminal deubiquitinase. Structurally, BAP1 has a UCH (Ubiquitin Carboxy-terminal Hydrolase) domain, an HCFC1 binding domain (H), and a complex N-terminal domain (NTD)—with a functional NLS—which is also responsible of several of the protein-protein interaction of BAP1 (Figure 1.9).

Somatic inactivating mutations of BAP1 have been consistently described in \(\sim 20\text{--}25\%\) of MM sequenced [33,213,214,241]. Immunohistochemical absence of the protein has however revealed in \(\sim 60\text{--}65\%\) of all MM cases. This large difference has been found to be due to
the fact that a large number of MM carries large deletions—several exons or whole gene—or complex splicing alterations which can be frequently missed by genomic DNA sequencing technologies [242].

Figure 1.9: Schematic representation of BAP1 protein domains.

Somatic BAP1 mutations are differentially present according to MM histology: ∼70–80% of epithelioid MMs is BAP1 negative, while ∼0–5% of sarcomatoid MMs shows loss of BAP1 [33, 213, 214, 241, 243]; biphasic MMs are BAP1 negative in ∼50% of the cases, and importantly demonstrated loss of staining in both the epithelioid and sarcomatoid components [243], therefore suggesting that BAP1 expression in spindled mesothelium can help the discrimination of reactive and malignant cells, thus providing a more objective means of distinguishing epithelioid from biphasic morphology compared to histology alone [243, 244]. From a prognostic point of view, in MM—contrary to what happens in other cancers—BAP1 loss is associated with improved prognosis. Whether this beneficial effect is true or just due to the association between BAP1 loss and the epithelioid subtype—which has per se better prognosis—is still a matter of debate [33, 213, 214, 241, 243, 245].

Besides MM, recurrent somatic BAP1 mutations have been described in numerous cancer types. Inactivating mutations have been identified in ∼85% of metastasizing uveal melanomas [246, 247], and loss of BAP1 and monosomy of chromosome 3 are associated
with significantly worse prognosis [246,247]. In clear cell renal cell carcinoma, loss of BAP1 is observed in \( \sim 10\text{–}15\% \) of all cases, it is mutually exclusive with mutations in \textit{PBRM1}—a gene encoding for the epigenetic regulator BAF180—and is also associated with worse prognosis [248–251].

Other tumor types with recurrent BAP1 mutations are intrahepatic cholangiocarcinoma (\( \sim 10\text{–}15\% \) of all cases) [252–254], thymic carcinoma (\( \sim 10\text{–}15\% \) of all cases) [255,256], bladder cancer (\( \sim 15\% \) of all cases) [257], and esophageal squamous cell carcinoma (\( \sim 2\text{–}5\% \) of all cases) [258,259]. For these tumor types, limited sample sizes have not yet enabled studies on the prognostic role of BAP1.

**1.5.3 Germline Mutations of \textit{BAP1} and BAP1 Tumor Predisposition Syndrome**

We recently reported for the first time germline \textit{BAP1} mutations in two families with high incidence of MM. In addition to MM, individuals in these two families also developed uveal melanoma [33]. We later also reported the presence of benign cutaneous lesions called MBAlTs (melanocytic BAP1-mutated atypical intradermal tumors) [34] and proposed the existence of an hereditary BAP1 tumor predisposition syndrome (TPS) (MIM: 614327). We also found germline \textit{BAP1} mutations in two other unrelated individuals with MM and uveal melanoma, suggesting that alterations of BAP1 could be the genetic cause for all these malignancies [33]. All the tumors displayed loss of heterozygosity, consistent with a tumor suppressor role of BAP1. The presence of asbestos fibers was documented in the homes
in which affected individuals of these families lived, raising the possibility that minimal asbestos exposure may be necessary to develop MM in the presence of germline \textit{BAP1} mutations [33]. Other investigators independently confirmed our findings and reported other cancer types frequently associated with heterozygous germline \textit{BAP1} mutations, such as cutaneous melanoma, renal cell carcinoma, and other cancers [260–262].

To date, more than 60 families carrying germline \textit{BAP1} mutations have been described worldwide, allowing a better characterization of the BAP1-TPS. The four malignancies more commonly found in the BAP1-TPS are MM (~25% of all carriers), uveal (~30%) and cutaneous (~15%) melanoma, and renal cell carcinoma (~10%). Other cancers frequently reported in these families with frequencies <10% are: basal cell skin carcinoma, intrahepatic cholangiocarcinoma, meningioma, and soft-tissue sarcoma. The penetrance of the BAP1-TPS is extremely high (>90%), and cases of multiple tumors are not exceptional. Importantly, while sporadic MM has a very dismal prognosis, individuals with germline \textit{BAP1} mutations that develop MM—especially those with other associated malignancies—have a very favorable outcome, with an overall survival up to 7 times better than sporadic cases [35].

For most of these malignancies, the age of cancer onset in genetically predisposed individuals is lower compared to sporadic cases, and biallelic loss of \textit{BAP1} is observed [263]. The germline \textit{BAP1} mutations associated with the BAP1-TPS are spread throughout the gene, as expected for a tumor suppressor gene, and there is no apparent genotype-phenotype correlation, suggesting that environmental factors and/or other genetic variations modulate the clinical phenotype in presence of \textit{BAP1} mutations (Figure 1.10).
1.5.4 Molecular Biology of the Tumor Suppressor BAP1

BAP1 is a deubiquitinating enzyme that has been implicated in numerous cellular processes such as cell proliferation, DNA damage response and repair, and control of gene expression through chromatin modifications [260]. The tumor suppressive function of BAP1 was first hypothesized in 1998 when this deubiquitinase was identified as a partner of BRCA1 [264]. Mutations affecting either the ubiquitin hydrolase activity of BAP1 or its nuclear localization abolish BAP1 tumor-suppressor activity [265]. Importantly, BAP1 catalytic activity itself contributes to determine its nuclear localization, via auto-deubiquitination of its NLS that protects BAP1 from cytoplasmic sequestration mediated by the ubiquitin ligase UBE2O [266].

Originally, it was hypothesized that most of BAP1 tumor suppressive activities relied mostly on its capability to disrupt the BRCA1/BARD1 heterodimer [267]. While the regulation of BRCA1 is an important activity of BAP1 in several models, including BCR-ABL induced
chronic myeloid leukemia [268], it is now clear that BAP1 has other important BRCA1-independent tumor suppressive functions.

Indeed, BAP1 is part of high-molecular weight multi-protein complexes which include the HCFC1 transcriptional scaffolding protein [269,270], O-linked N-acetylglucosamine transferase (OGT) [271,272], human orthologs of additional sex combs (ASXL1/ASXL2), and forkhead transcription factors (FOXK1/FOXK2) [269–274]. Importantly, BAP1 can act as an epigenetic regulator, interacting with ASXL1 to form the Polycomb repressive deubiquitinase (PR-DUB) complex [275]. This complex mediates deubiquitination of monoubiquitinated histone 2A at lysine 119 (H2AK119), a critical mechanism in the control of transcription initiation and elongation, transcription silencing, and DNA repair. Confirming the contribution of this activity in tumor suppression, cancer-associated ASXL1 mutations may act as a gain-of-function of the PR-DUB complex [276]. Recently, also the BAP1/ASXL2 complex has been shown to be an H2AK119 deubiquitinase complex which regulates cell proliferation and senescence and is often disrupted in cancer [277]. BAP1-mediated regulation of gene transcription can also happen via formation of a ternary complex between BAP1, HCFC1 and the transcription factor YY1 [278]. A direct regulation of cell-cycle associated proteins has been also proposed, based on the regulation of HCFC1 levels by BAP1 [269].

One of the main recently discovered functions of BAP1 is the promotion of DNA damage repair after double strand breaks. BAP1 loss is associated with increased persistence of DNA damage and chromosomal abnormalities [279,280]. Also, BAP1 is phosphorylated
following DNA damage, and this post-translational modification affects its binding to the chromatin, further supporting a role of BAP1 in the transcriptional response to double strand breaks [281].

Moreover, BAP1 seems capable of regulating centrosome stability and mitotic spindle fiber formation via interaction respectively with MCRS1, a component of the centrosome that plays a critical role in spindle assembly [282], and γ-tubulin, a cytoskeletal protein forming the mitotic spindle [283]. BAP1 is also capable of deubiquitinating other substrates, such as PGC-1α—possibly modulating cell bioenergy [272]—and INO80—contributing to the regulation of DNA replication [284].

Considering its multiple functions, it will be challenging to identify which alterations of these BAP1-mediated functions are most important in order for MM and other tumors to develop. In fact, cell lines from different tumors associated with the BAP1 syndrome have different, often paradoxical, phenotypes after BAP1 silencing: BAP1 silencing in MM cells for example is associated with a significant alteration of Polycomb target genes, consistent with BAP1’s function as an epigenetic regulator. However, BAP1 silencing is also associated with a concomitant reduction of cell growth, unusual when silencing a tumor suppressor gene [241].

In a murine model, complete loss of BAP1 is embryonically lethal, while its post-natal inactivation is associated with rapid myeloid transformation [271]. In the absence of external stimuli, BAP1 heterozygous mice are viable and don’t display any tumor predisposition, suggesting that interaction with environment and other genes might contribute to what is observed in humans. In conclusion, BAP1 is a complex tumor suppressor that exerts impor-
tant roles in the control of gene transcription, DNA repair, mitotic spindle formation, and cellular metabolism.

Ongoing studies are investigating the contributions of these different functions of BAP1 in the transformation of different tissues.
2.1 Background and Aim

We reported that HMGB1 contributes to asbestos carcinogenesis and MM progression [66, 88, 91]. As described, HMGB1 localization and secretion is regulated by acetylation of lysine residues in its two NLSs. Non-acetylated HMGB1 is mostly localized in the nucleus, whereas hyper-acetylated HMGB1 is sequestered into the cytoplasm and can be actively secreted into the extra-cellular space.

Therefore, we hypothesized that 1) the HMGB1 secreted by MM cells was hyper-acetylated and that this isoform would be found in the sera of MM patients; 2) the HMGB1 released when human mesothelial cells (HM) die following asbestos exposure was the passively released non-acetylated HMGB1 isoform, and that this isoform would be present in the sera of asbestos-exposed individuals without MM.

To test our hypothesis, we analyzed hyper- and non-acetylated HMGB1 in the supernatant of MM cell lines and HM cultures with or without exposure to asbestos, and in the serum samples from MM patients and from individuals with a strong occupational history of asbestos exposure, and from unexposed healthy individuals as controls. We also assessed whether serum levels of HMGB1 and its hyper-acetylated isoform would help discriminate MM pa-
tients from patients with benign or malignant non-MM pleural effusions. Moreover, we compared the diagnostic accuracy of total and hyper-acetylated HMGB1 to other proposed MM biomarkers: mesothelin, fibulin-3, and OPN.
2.2 Material and Methods

For the in vitro studies, HM were established in tissue culture from pleural fluid of patients with congestive heart failure as described [66]. HM in tissue culture were treated with crocidolite asbestos fibers, and tested between cell culture passages 2–5 [66]. MM cell lines, REN [285] and HP3 [286]—also referred to as PHI in our previous study [91]—have been characterized and authenticated previously [91, 285, 286]. Cells were cultured for 48 hours in serum-free media. Supernatants were concentrated 50× before analysis.

Briefly, we tested the levels of HMGB1 isoforms and other MM biomarkers respectively in serum and plasma from 60 patients who presented to the emergency room with pleural effusions: 13 of them were diagnosed with a benign pleural effusion; 22 of them were diagnosed with MM; and 25 of them with a malignant non-MM pleural effusion (all diagnoses were confirmed by cytology and, for the malignant effusions by histopathology and immunohistochemistry). In addition, we studied serum and plasma from insulation workers from New York City, U.S., part of the Selikoff cohort, with a strong history of occupational exposure to asbestos [287] (n=20); apparently healthy controls with no known history of asbestos exposure (n=20) from Liverpool, UK. MM patients and patients with other effusions were recruited in a hospital setting in New York City, U.S. from 2005 through 2013. Patients were enrolled, after giving consent, for participation in the approved Institutional Review Boards and blood procurement protocols. The samples analyzed were from non-consecutive randomly selected participants/patients, and collected prior to any treatment. To ensure a power of 80% to detect an effect size (Cohens $d$) of 1.0, with $\alpha=0.05$, we recruited at least
20 MM patients, 20 asbestos-exposed individuals and 20 healthy controls. HMGB1 isoforms present in our cell supernatants and in the human sera were blindly analyzed at the University of Liverpool by whole protein ESI-LC-MS. Post-translational modifications were confirmed by tandem mass spectrometry (LC-MS/MS), as described [202, 288, 289]. Briefly, prior to MS analysis, samples (50 µl of cell supernatants or 200 µl of human serum) were immunoprecipitated with 5 µg of rabbit anti-HMGB1 antibody (ab18256, Abcam, Cambridge, UK), and then subjected to trypsin (Promega, Madison, WI) or GluC (New England Biolabs, Ipswich, MA) digestion according to manufacturers instructions, and de-salted using ZipTip C18 pipette tips (EMD Millipore, Billerica, MA). Assay validation data showing the robustness of the protocol have already been extensively published elsewhere [202]. As a further control, total HMGB1 was also measured on the human samples using a commercially available ELISA kit (IBL International, Hamburg, Germany). Plasma levels of fibulin-3 (USCN, Wuhan, PRC), mesothelin (R&D, Minneapolis, MN), and OPN (R&D, Minneapolis, MN) were measured in MM patients and asbestos-exposed individuals with indicated commercially available ELISA kits following manufactures instructions. To measure plasma mesothelin, we did not use the FDA-approved SMRP Mesomark® kit (Fujirebio Diagnostics Inc., Malvern, PA), which is not available for research purposes in the U.S., but a different commercially available ELISA kit (R&D, Minneapolis, MN). In our hands, testing a subset of samples from this cohort, there is a very good correlation between the two kits ($R^2=0.75$, $P <0.0001$) (Figure 2.1). Using the Bartlett test, all the tested biomarkers presented significant heterogeneous vari-
Figure 2.1: Correlation between mesothelin levels measured with R&D and Mesomark® ELISA kits.

iances between groups. Therefore, the significance of two-group comparisons was calculated using the Mann-Whitney non-parametric test. $P$ values <0.05 were considered significant. Results were expressed as median, 1st quartile–3rd quartile. To combine the results of different biomarkers, we derived standardized scores from mean and standard deviation of common logarithm transformed results, and combined them using a logistic regression model [146]. Whenever present, cut-off values corresponded to the Youdens $J$ index (i.e., highest sum of sensitivity and specificity minus one) [290].
2.3 Results

2.3.1 Analysis of HMGB1 Isoforms *In Vitro* and *In Vivo*

Initially, we evaluated HMGB1 from concentrated supernatant of HM and MM cells in tissue culture. Unexposed HM did not release detectable HMGB1 into the extracellular space. When HM are exposed to 5 µg/cm² of crocidolite asbestos, ~60–70% of them undergo programmed necrosis within 48 hours [66]. Accordingly, in the supernatant of HM exposed to asbestos (Asb-HM), we consistently detected high levels of non-acetylated HMGB1, as expected from cells undergoing necrosis (Figures 2.2 and 2.4).

![MS analysis of HMGB1 from Asb-HM: only non-acetylated HMGB1](image)

Figure 2.2: MS analysis of HMGB1 from Asb-HM: only non-acetylated HMGB1.

Instead, in the supernatant of MM cells, we detected hyper-acetylated HMGB1, consistent with active secretion, as well as non-acetylated HMGB1, likely released by a fraction of MM cells undergoing necrosis, as they are grown in serum-free tissue culture condition (Figures 2.3 and 2.4).
Altogether, supernatants from MM cells showed higher levels of total (non-acetylated + hyper-acetylated) HMGB1 with a prevalence of the hyper-acetylated isoform, compared to the supernatants from HM exposed to asbestos that contained prevalently the non-acetylated isoform (Figure 2.4). The presence of non-acetylated HMGB1 in asbestos-exposed HM was associated with significant cell death with loss of the classic HM cobblestone appearance (Figure 2.5). The differences in levels of non-acetylated and hyper-acetylated HMGB1 observed in tissue cultures supported our hypothesis and prompted us to study HMGB1 in human sera. We compared the levels of total HMGB1 and its isoforms in the sera from 1) 20 unexposed healthy controls, 2) 20 insulation workers [287] that included individuals with 10 or more years of occupational asbestos exposure, who did not have pleural effusion or evidence of any malignancy at the time of sera collection, and 3) 22 MM patients who
Figure 2.4: Quantification of levels of HMGB1 isoforms in cell culture supernatants.

Figure 2.5: Microscopic photos (40×) of cells used for analysis.
had been diagnosed following the development of pleural effusion, a common presentation in MM.

In the serum samples from unexposed healthy controls, total levels of HMGB1 detected by MS were very low (1.4, 0.8–2.2 ng/ml), consistent with previously published HMGB1 values in healthy volunteers [202]. Total HMGB1 serum levels were significantly higher in asbestos-exposed individuals (10.2, 5.7–12.1 ng/ml) compared to the levels in unexposed controls ($P < 0.001$). MM patients had the highest levels of total HMGB1 (25.0, 15.7–36.6 ng/ml) when compared to either other group ($P < 0.001$) (Figure 2.6).

![Total HMGB1](image)

Figure 2.6: Quantification of total HMGB1 by MS in human samples. Horizontal dashed line represents optimal cut-off. *** $P < 0.001$.

The total levels of HMGB1 measured with a commercially available ELISA kit and with our MS protocol were very similar ($R^2=0.92$, $P < 0.0001$) (Figure 2.7), corroborating the reliability of our approach.

The levels of hyper-acetylated HMGB1 were very low in both the healthy controls (0.5,
0.3–0.7 ng/ml) and asbestos-exposed individuals (0.4, 0.3–0.6 ng/ml). MM patients showed significantly higher levels of hyper-acetylated HMGB1 (17.4, 10.3–21.9 ng/ml) compared to either other group ($P <0.001$) (Figure 2.8). Overall, hyper-acetylated HMGB1 comprised $\sim 10\%$ of the total HMGB1 in the sera of asbestos-exposed individuals, and $\sim 67\%$ of the total HMGB1 in the sera of MM patients (Figure 2.9).

Next, we evaluated the sensitivity and specificity of total and hyper-acetylated HMGB1 as potential biomarkers to discriminate MM patients from asbestos-exposed individuals and healthy controls. Both, total and hyper-acetylated HMGB1, showed exceptional accuracy in discriminating MM patients from healthy controls with a receiver operating characteristic (ROC) area under the curve (AUC) of 0.999 (95% CI 0.994–1.000) and 1.000 (95% CI 1.000–1.000), respectively. Comparing asbestos-exposed individuals to healthy controls, the AUC of total and hyper-acetylated HMGB1 were 0.964 (95% CI 0.893–1.000) and 0.574 (95% CI

Figure 2.7: Correlation between ELISA and MS values of total HMGB1 in human samples.
Figure 2.8: Quantification of hyper-acetylated HMGB1 by MS in human samples. Horizontal dashed line represents optimal cut-off. *** $P < 0.001$.

Figure 2.9: Ratio of HMGB1 isoforms
0.392–0.756), respectively (Table 2.1 and Figure 2.10).

<table>
<thead>
<tr>
<th></th>
<th>Total HMGB1</th>
<th>Hyper-acetylated HMGB1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUC (95% CI)</td>
<td>Cut-off (ng/ml)</td>
</tr>
<tr>
<td>MM vs. H</td>
<td>0.999 (0.994–1.000)</td>
<td>3.00</td>
</tr>
<tr>
<td>Asb vs. H</td>
<td>0.964 (0.893–1.000)</td>
<td>3.05</td>
</tr>
<tr>
<td>MM vs. Asb</td>
<td>0.830 (0.687–0.972)</td>
<td>15.75</td>
</tr>
</tbody>
</table>

Table 2.1: AUC and cut-off values of total and hyper-acetylated HMGB1 comparing healthy controls, asbestos-exposed individuals, and MM patients.

These data suggest that total HMGB1 is a reliable biomarker to discriminate individuals with asbestos-exposure and/or MM from healthy controls. Comparing MM patients and asbestos-exposed individuals (Figure 2.11), the AUC of total HMGB1 was 0.830 (95% CI 0.687–0.972); at specificity 100%, the sensitivity was 72.73% (for values >15.75 ng/ml, which also corresponded to the cut-off value); at sensitivity 100%, the specificity was 5%. The AUC of hyper-acetylated HMGB1, when comparing MM patients to asbestos-exposed individuals, was 1.000 (95% CI 1.000–1.000), with a cut-off value of 2.00 ng/ml. These results point to hyper-acetylated HMGB1 as a novel, sensitive and specific biomarker to discriminate MM patients from asbestos-exposed individuals.

We did not detect any significant difference in total or hyper-acetylated HMGB1 serum levels in MM patients with stage I-II vs. III-IV (Figure 2.12), suggesting that early lesions are also associated with increased HMGB1 levels and that hyper-acetylated HMGB1 may be a valuable screening tool for early detection of MM among asbestos-exposed cohorts.
Figure 2.10: AUC of total and hyper-acetylated HMGB1 comparing either asbestos-exposed individuals or MM patients vs. healthy controls
Figure 2.11: AUC of total and hyper-acetylated HMGB1 comparing either asbestos-exposed individuals or MM patients vs. healthy controls

Figure 2.12: Comparison of total and hyper-acetylated HMGB1 levels based on MM stage
2.3.2 HMGB1 Isoforms as Potential Novel Biomarkers

Next we sought to determine whether total and hyper-acetylated HMGB1 could also help differentiate MM patients from patients with pleural effusions due to other causes (Figure 2.13). Thirteen serum samples from patients with cytologically benign pleural effusions and 25 serum samples from patients with pleural effusions due to non-MM malignancy were available for these studies. We found that MM patients had significantly higher levels of total HMGB1 compared to patients with cytologically benign pleural effusions (6.4, 4.7–9.7 ng/ml; \( P < 0.001 \)) and malignant (non-MM) pleural effusions (6.7, 4.2–10.0 ng/ml; \( P < 0.001 \)). Similarly, levels of hyper-acetylated HMGB1 were significantly higher in the sera from MM patients compared to sera from patients with benign pleural effusions (5.2, 3.7–7.8 ng/ml; \( P < 0.001 \)) or malignant (non-MM) pleural effusions (5.7, 3.3–8.2 ng/ml; \( P < 0.001 \)). Next, we evaluated the sensitivity and specificity of total and hyper-acetylated HMGB1 to discriminate MM patients from patients with pleural effusions due to other causes. The AUC of total HMGB1 was 0.860 (95% CI 0.736–0.984); at specificity 100%, the sensitivity was 63.64%; at sensitivity 100%, the specificity was 10.53%. The AUC for hyper-acetylated HMGB1 was 0.837 (95% CI 0.709-0.966); at specificity 100%, the sensitivity was 59.09%; at sensitivity 100%, the specificity was 10.53%. Best cut-off values to discriminate MM patients from patients with benign or malignant non-MM pleural effusions were 11.35 ng/ml (sensitivity 81.82%, specificity 89.47%) and 9.70 ng/ml (sensitivity 77.27%, specificity 89.47%) respectively for total and hyper-acetylated HMGB1. Overall, levels of total and hyper-acetylated HMGB1 were helpful to distinguish MM patients from patients with pleural effusions due to
other causes.

We measured the levels of three previously proposed MM biomarkers (fibulin-3, mesothelin, and OPN) from the same asbestos-exposed individuals and MM patients and compared those biomarkers to total and hyper-acetylated HMGB1. All three biomarkers were significantly higher in MM patients compared to the asbestos-exposed individuals \( (P < 0.001) \) (Table 2.2, Figure 2.14A-C).

![Figure 2.13: Levels of total and hyper-acetylated HMGB1 in MM patients and patients with benign pleural effusions (Ben-PE) or malignant pleural effusions not caused by MM (Mal-PE). Horizontal dashed line represents optimal cut-off. AUC of total and hyper-acetylated HMGB1 comparing MM patients vs. patients with effusion due to other causes. *** \( P <0.001 \).](image)

Fibulin-3 had AUC of 0.959 (95% CI 0.905–1.000), mesothelin had AUC of 0.934 (95% CI
Table 2.2: Levels of fibulin-3, OPN, and mesothelin in the cohorts studied.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Fibulin-3, ng/ml</th>
<th>OPN, ng/ml</th>
<th>Mesothelin, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asb</td>
<td>(median, 1st–3rd quartile) 17.36, 6.91–22.14</td>
<td>(median, 1st–3rd quartile) 28.89, 15.12–37.05</td>
<td>(median, 1st–3rd quartile) 16.49, 12.08–19.40</td>
</tr>
<tr>
<td>MM</td>
<td>85.73, 46.25–128.07</td>
<td>90.20, 60.24–150.93</td>
<td>44.92, 29.59–106.31</td>
</tr>
</tbody>
</table>

However, none of these biomarkers reached the sensitivity and specificity achieved by hyper-acetylated HMGB1 (AUC of 1.000) to discriminate these two cohorts (Figure 2.13). When comparing MM patients to patients with pleural effusions due to other causes, the levels of fibulin-3 were significantly higher in MM patients vs. either benign pleural effusion or other malignant non-MM effusion ($P < 0.001$) (Figure 2.14G). Similarly, mesothelin levels were higher in MM patients vs. the benign effusion group ($P < 0.01$) or vs. the non-MM malignant effusion group ($P < 0.001$) (Figure 2.14H). Instead, OPN levels did not show significant difference among the groups (Figure 2.14I). Fibulin-3 had an AUC of 0.928 (95% CI 0.868–0.989), mesothelin had an AUC of 0.798 (95% CI 0.678–0.918), and OPN had an AUC of 0.502 (95% CI 0.348–0.657) (Figure 2.14J-L). Total HMGB1 (AUC=0.860) and hyper-acetylated HMGB1 (AUC=0.837), performed better than OPN and mesothelin in this comparison (Figure 2.13 and 2.14J-K).

Among MM patients, levels of total and hyper-acetylated HMGB1 did not correlate with any of the other biomarkers (Figure 2.15A–C and D–F respectively), suggesting independent roles of these molecules in MM. We therefore calculated combined values of biomarkers...
derived from logistic regression equations. AUC were calculated for those combinations of biomarkers with regression equations having pseudo-R² > 0.75 and all the parameters with P < 0.05. Combined values of fibulin-3 and either total or hyper-acetylated HMGB1 resulted in improved sensitivity and specificity in discriminating MM patients from patients with pleural effusions due to other causes, with AUC of 0.987 (0.967–1.000) and 0.981 (0.956–1.000), respectively. Best cut-off values to discriminate MM patients from patients with benign or malignant non-MM pleural effusions were for scores of -0.48 (sensitivity 95.45%, specificity 92.11%) and -0.22 (sensitivity 90.91%, specificity 92.11%) respectively for fibulin-3 with either total or hyper-acetylated HMGB1 (Figure 2.16 and Table 2.3).

In summary, hyper-acetylated HMGB1 showed the highest AUC in discriminating MM patients from asbestos-exposed individuals. The combination of fibulin-3 and HMGB1 increased the power of discrimination of MM patients from patients with pleural effusions due to other causes.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>AUC (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total HMGB1</td>
<td>0.860 (0.736–0.984) 0.987 (0.967–1.000)</td>
</tr>
<tr>
<td>Hyper-acetylated HMGB1</td>
<td>0.837 (0.709–0.966) 0.981 (0.956–1.000)</td>
</tr>
<tr>
<td>Fibulin-3</td>
<td>0.928 (0.868–0.989) -</td>
</tr>
</tbody>
</table>

Table 2.3: Combination of multiple biomarkers in the discrimination of MM patients from patients with effusions due to other causes.
Figure 2.14: Levels of fibulin-3, OPN, and mesothelin in asbestos-exposed individuals, MM patients, and patients with pleural effusions due to other causes and respective AUC. ** $P < 0.01$; *** $P < 0.001$. 
Figure 2.15: No correlation between levels of total or hyper-acetylated HMGB1 and other markers. Levels expressed in ng/ml.
Figure 2.16: Combination of fibulin-3 and HMGB1 in distinguishing MM patients from patients with pleural effusions due to other causes. *** $P < 0.001$. 
CHAPTER 3
*BAP1* HETEROZYGOSITY, ASBESTOS-INDUCED INFLAMMATION, AND MESOTHELIOMA

3.1 Background and Aim

None of the MM patients with germline *BAP1* mutations in our families had a personal or familial history of occupational exposure to asbestos [33,34]. We hypothesized that low-dose exposure to asbestos and other carcinogenic mineral fibers—such as the one deriving from unknown environmental sources, usually referred to as background exposure—would be sufficient to significantly increase the risk of MM in presence of germline *BAP1* mutations.

In this study, we used a murine model to test this hypothesis *in vivo* by exposing mice with and without germline *BAP1* heterozygosity—i.e., BAP1(+/-) and BAP1(+/+) mice—to either a standard dose of asbestos that is known to cause MM in ~30% of the animals, or to a dose ten times lower that is known to cause MM in only ~5–10% of the animals.

We also hypothesized that, given the crucial role of asbestos-induced inflammation in MM pathogenesis, BAP1(+/-) and BAP1(+/+) mice might have different asbestos-induced inflammatory responses at the cellular and humoral levels, in particular in the presence of exposure to low doses of asbestos.

The results here presented have also been recently reported to the scientific community in a formal peer-reviewed article [291].
3.2 Material and Methods

In our experiments, we used constitutive BAP1(+/−) mice (C57BL/6 background) generated by breeding mice with loxP sites flanking BAP1 exons 4 and 5 with mice expressing a constitutive general Cre deleter [271]. Identification of BAP1(+/−) mice from wild-type littermates was performed with a specific PCR reaction using DNA extracted from tail snips, followed by electrophoresis in 3% agarose gel. Primers and conditions of the PCR are reported in Table 3.1 and Table 3.2.

<table>
<thead>
<tr>
<th>Primer Sequence</th>
</tr>
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<tbody>
<tr>
<td><strong>Oligo 1</strong> CCATCAGTGACTACTGGGGAGCAAC</td>
</tr>
<tr>
<td><strong>Oligo 2</strong> ACAGATGGCTGGGCACATCTG</td>
</tr>
<tr>
<td><strong>Oligo 3</strong> GAACCCTCCGTTCATAGTGTTG</td>
</tr>
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</table>

Table 3.1: Primers used to genotype BAP1(+/−) mice.

<table>
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<tr>
<th>Step#</th>
<th>Temperature (°C)</th>
<th>Time</th>
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<tbody>
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<td>2</td>
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<td>5&quot;</td>
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<tr>
<td>3</td>
<td>65</td>
<td>40&quot;</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>1’</td>
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<tr>
<td>5</td>
<td><strong>Go to step#2</strong></td>
<td>39×</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>∞</td>
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</tbody>
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Table 3.2: PCR conditions used to genotype BAP1(+/−) mice.

Mice of 10–12 weeks old of either sex equally distributed in the experimental groups using a computational random number generator were used for our experiments. All the experiments
involving animals were reviewed and approved by the University of Hawaii Institutional Animal Care and Use Committee.

Mice received intraperitoneal injections of crocidolite asbestos fibers, whose extensive mineralogical characterization has been previously reported [66], or glass beads as a control. To mimic the chronic exposure to asbestos fibers, we injected asbestos and glass weekly, as opposed to single injections with higher doses. This protocol is associated with MM development while minimizing asbestos-induced fibrosis and animal deaths from non-malignant asbestos-induced conditions (Carbone et al., unpublished observations).

In the short-term study to investigate the chronic inflammatory milieu, BAP1(+/-) mice (n=7 per group) and BAP1(+/-+) (n=9 per group) were injected for 5 weeks with a low amount of crocidolite or glass beads (0.05 mg/week). Sample size was estimated hypothesizing a 60% difference in the levels of at least one cytokine. Cells and peritoneal lavages were collected one week after the last injection. Cells were blindly characterized with the following antibodies: CD45 (leukocytes; anti-CD45-BV711, 563709, BD Biosciences, San Jose, CA, USA), F4/80 (Mφ; anti-F4/80-AlexaFluor488, MCA497A488T, AbD Serotec, Raleigh, NC, USA), Ly-6G (neutrophils; anti-Ly6G-BV421, 562737, BD Biosciences), CD3 (T cells; anti-CD3-APC, 17-0032-80, eBioscience, San Diego, CA, USA) and B220 (B cells; anti-B220-PE, 561878, BD Biosciences).

The supernatants recovered from the peritoneal lavages were concentrated 45–60× using Amicon Ultra Centrifuge Filters (EMD Millipore Corporation, Billerica, MA, USA) with a 3,000 Dalton cut-off. Levels of 32 cytokines and chemokines (complete list in Table 3.3)
were detected in concentrated lavages using mouse cytokine multiplex kits (EMD Millipore Corporation). Given the small sample size, comparisons between groups were calculated using Mann-Whitney U test for rank comparisons. Results are presented as median, 1st quartile–3rd quartile.

<table>
<thead>
<tr>
<th>List of cytokines/chemokines analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eotaxin</td>
</tr>
<tr>
<td>RANTES</td>
</tr>
<tr>
<td>MIP-1</td>
</tr>
<tr>
<td>MCP-1</td>
</tr>
<tr>
<td>KC</td>
</tr>
<tr>
<td>IL-15</td>
</tr>
<tr>
<td>IL-12 (p40)</td>
</tr>
<tr>
<td>IL-7</td>
</tr>
<tr>
<td>IL-4</td>
</tr>
<tr>
<td>IL-1β</td>
</tr>
<tr>
<td>GM-CSF</td>
</tr>
</tbody>
</table>

Table 3.3: List of cytokines and chemokines analyzed.

In the long-term study to investigate the rates of MM development, BAP1(+/+) mice (n=50 per group) and BAP1(+/−) mice (n=25 per group) were injected for 10 weeks with a standard dose of crocidolite (0.5 mg/week), a low-dose of crocidolite (0.05 mg/week), or glass beads (0.5 mg/week). Sample size was estimated to detect a difference in MM incidence between the low-exposed groups ≥25%. Mice were monitored daily for clinical evidence of abdominal swelling, and killed by CO₂ asphyxiation in presence of unresponsiveness to pain stimuli, respiratory distress, gait instability, or in presence of an obvious pathological condition. After sacrifice, all the major organs were evaluated histologically. Formalin-fixed/paraffin-embedded samples were cut into 5-µm sections and stained with hematoxylin
and eosin (H&E) according to the standard procedure. The pathological diagnosis of MM was based on H&E staining and supported by WT1 nuclear staining in tumor cells. Tumors were also stained with a rabbit polyclonal anti-BAP1 antibody to evaluate presence and localization of BAP1. Survival curves of BAP1(+/−) mice and wild-type littermates after long-term exposure to asbestos fibers (standard and low dose) were compared using log-rank (Mantel-Cox) test.
3.3 Results

3.3.1 Identification and Characterization of BAP1(+/−) Mice

BAP1(+/−) mice are viable and do not show any significant behavioral or physical abnormality. Since homozygous \textit{BAP1} deletion is embryonically lethal [271], the colony is maintained by mating heterozygous and wild-type mice.

Identification of heterozygous mice is performed via a specific PCR-mediated amplification of two regions within the murine \textit{BAP1} gene that yield a longer product (503 base pairs) that identifies the deleted allele, and a shorter one (234 base pairs) that identifies the wild-type allele. Therefore, heterozygous mice present both bands, while wild-type mice only present the shorter band (Figure 3.1).

At a protein level, BAP1(+/−) mice express in their tissues—e.g., bone-marrow derived MΦ and mesothelial cells—∼50\% of the amount BAP1 found in their wild-type littermates, consistent with their genetic alteration (Figure 3.2).

![Figure 3.1: Genotyping of BAP1(+/−) and BAP1(+/+) mice.](image-url)
3.3.2 Deregulated Inflammation after Chronic Exposure to Low Doses of Asbestos in BAP1(+/–) Mice

We exposed BAP1(+/–) mice and wild-type littermates for 5 weeks to weekly injections with glass beads or a low amount of crocidolite asbestos fibers (0.05 mg/week). One week after the last injection, we euthanized the mice and performed a peritoneal lavage. Peritoneal cells were pelleted, fixed, and their immuno-phenotype analyzed via flow cytometry. The supernatant of the lavage was first concentrated and then used to measure the levels of a panel of cytokines and chemokines.

CD45+ leukocytes represented 95–99% of the total cells recovered in each group. In the glass control groups, Mφ and B cells represented the most abundant population, regardless of genotype (Table 3.4). Upon exposure to low-dose crocidolite fibers, we observed a significant increase in the total number of leukocytes and in the relative percentage of neutrophils (Ly-6G+ cells), and simultaneously, a significant decrease in the percentage of B
cells (B220+ cells) and Mφ (F4/80+ cells) (Table 3.4). This cellular inflammatory response was not significantly affected by the genotype. From these results we can conclude that prolonged exposure to asbestos fibers is, as expected, associated with significant alterations in the inflammatory response, mainly characterized by an influx of neutrophils in the peritoneal cavity of exposed animals.

Since the activity of different populations of immune cells strongly depends on the phenotype acquired during the maturation, we decided to further characterize the macrophage polarization in our mice, analyzing the expression of CD206 (a marker of M2 polarization) and CD86 (a marker of M1 polarization) (Figure 3.3).

Interestingly, we found that in the Mφ from BAP1(+/−) mice exposed to asbestos fibers, the normalized mean fluorescence (MFI) intensity for CD206 (marker of M2 macrophages) was significantly higher compared with controls (197.1%, 160.6–256.8 vs. 163.1%, 125.4–186.7; \( P < 0.05 \)), whereas the normalized MFI for CD86 (marker of M1 macrophages) was significantly lower compared with controls (74.6%, 57.6–90.3 vs. 95.8%, 77.4–109.1; \( P < 0.05 \)) (Figure 3.4A). Similarly, the percentage of M1 macrophages (CD206− CD86+ cells) was significantly lower in BAP1(+/−) mice compared to controls (43.2%, 28.9–44.9 vs. 67.3%, 46.7–78.2 of total macrophages; \( P < 0.05 \)), while the percentage of M2 macrophages (CD206+ CD86− cells) was significantly higher in BAP1(+/−) mice compared with wild-type littermates (3.8%, 2.1–6.8 vs. 1.2%, 0.5–3.6 of total macrophages; \( P < 0.05 \)). Double positive (CD206+ CD86+) macrophages, a transition state from M1 to M2, were also more represented in BAP1(+/−) mice compared with wild-type littermates (40.0%, 30.7–47.0 vs. 26.0% [13.3–37.6] of total macrophages; \( P < 0.05 \)).
<table>
<thead>
<tr>
<th>Cells</th>
<th>WT</th>
<th>WT</th>
<th>Het</th>
<th>Het</th>
<th>WT (G vs. A)</th>
<th>Het (G vs. A)</th>
<th>Glass (WT vs. Het)</th>
<th>Asbestos (WT vs. Het)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leukocytes (×10^6)</td>
<td>2.7, 1.3–3.6</td>
<td>6.1, 3.5–14.2</td>
<td>2.7, 1.3–4.9</td>
<td>8.5, 4.9–12.7</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
<td>ns</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>1.8, 1.6–2.4</td>
<td>13.0, 11.3–16.4</td>
<td>1.1, 0.8–2.2</td>
<td>10.4, 9.9–16.6</td>
<td>&lt;0.0001</td>
<td>&lt;0.001</td>
<td>ns</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>B cells (%)</td>
<td>20.4, 17.5–26.3</td>
<td>12.7, 9.9–14.2</td>
<td>19.4, 17.8–21.3</td>
<td>10.3, 8.6–12.6</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>ns</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>T cells (%)</td>
<td>7.0, 5.1–10.4</td>
<td>5.0, 3.8–6.4</td>
<td>6.4, 4.1–10.8</td>
<td>7.7, 4.3–8.4</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Mφ (%)</td>
<td>33.4, 27.0–38.5</td>
<td>21.3, 18.6–27.5</td>
<td>24.2, 20.1–45.2</td>
<td>19.2, 14.6–22.8</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
<td>ns</td>
<td>ns</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4: Peritoneal cells in glass or asbestos-exposed BAP1 heterozygous (Het) and wild-type (WT) mice. ns=not significant
tal macrophages; \( P < 0.05 \) (Figure 3.4B). The M2/M1 ratio (overall percentage of CD206+ cells divided by overall percentage of CD86+ cells) was also significantly higher in asbestos-exposed BAP1(+/−) mice compared with controls (0.54, 0.48–0.66 vs. 0.36, 0.16–0.56; \( P < 0.05 \) (Figure 3.4C).

Figure 3.3: Flow cytometry of \( \text{M} \phi \) subpopulations based on expression of CD206 (M2 marker) and CD86 (M1 marker).

Using a multiplexed ELISA assay, we then compared the levels of several cytokines and chemokines present in peritoneal lavages of these same mice. Despite having concentrated the peritoneal fluid, the levels of several analytes were below the lower limit of detection of our assay. Nevertheless, we were able to observe significant differences in BAP1 heterozygous and wild-type littermates in several analytes. Interestingly, whenever different, BAP1(+/−) mice constantly displayed lower levels of cytokines and chemokines compared to their wild-type littermates.

Specifically, the levels of granulocyte colony-stimulating factor (G-CSF), interleukin-5 (IL-
**Figure 3.4:** BAP1(+/−) mice have higher percentages and stronger expression of M2 macrophages marker CD206. *P < 0.05

5), interferon gamma-induced protein 10 (IP-10) and VEGF significantly increased following asbestos exposure, with similar extents in either genotype (Figure 3.5).

Instead, the levels of monocyte chemoattractant protein-1 (MCP-1) and leukemia inhibitory factor (LIF) were significantly lower in BAP1(+/−) mice compared to controls exposed to either glass (respectively 2.5 pg/ml [2.3–5.2] vs. 33.6 pg/ml [6.5–51.7], *P < 0.01, and 0.9 pg/ml [0.9–1.0] vs. 6.9 pg/ml [1.1–13.5], *P < 0.01) or asbestos (52.4 pg/ml [4.7–113.4] vs. 178.5 pg/ml [102.9–373.2], *P < 0.05, and 78.2 pg/ml [41.0–134.4] vs. 201.9 pg/ml [116.9–274.8], *P < 0.05). Also, peritoneal lavages from BAP1(+/−) mice exposed to asbestos contained significantly lower amounts of keratinocyte-derived chemokine (KC) compared with wild-type littermates (253.4 pg/ml [19.5–557.1] vs. 675.3 pg/ml [469.8–1741.5], *P < 0.05). Eotaxin levels were also significantly lower in BAP1(+/−) mice in the glass-exposed control group (1.73 ng/ml [1.11–2.06] vs. 3.27 ng/ml [1.94–3.92], *P < 0.05); the same trend, although non-significant, persisted following asbestos exposure (3.33 ng/ml [2.56–4.33] vs. 4.70 ng/ml [3.13–6.30], *P = 0.28). Even though the difference did not reach nominal significance (*P
Figure 3.5: Levels of peritoneal cytokines G-CSF, VEGF, IL-5, IP-10.
=0.08), also IL-6 levels differed between genotypes upon asbestos exposure, with BAP1 heterozygous mice showing lower levels of this analyte. Both IL-6 and leukemia inhibitory factor belong to the IL-6 family of cytokines; indeed their levels significantly correlated ($R^2=0.62$, $P<0.0001$) (Figure 3.6).

### 3.3.3 Mesothelioma Incidence and Overall Survival are Affected by BAP1 Status

During the 13 months of follow-up after the last injection, we did not observe MM or any other spontaneous tumor in the glass control groups. In mice exposed to asbestos fibers, MM was the only malignancy observed. In the low-dose group, crocidolite fibers caused pathologically confirmed MM in 9/25 (36.0%) BAP1(+/−) mice compared with 5/50 (10.0%) BAP1(+/+) mice ($P=0.010$). Similarly, in the standard-dose group, MM was diagnosed in 15/25 (60.0%) BAP1(+/−) mice compared with 14/50 (28.0%) BAP1(+/+) mice ($P=0.011$) (Figure 3.9A). Immunohistochemical staining of the tumors revealed expression of the mesothelial marker WT1, supporting the histologic diagnosis of MM (Figure 3.9B). BAP1 nuclear staining was absent in all MM analyzed arising from BAP1(+/−) mice and in 66.7% from BAP1(+/+) mice (Figure 3.9C). BAP1(+/−) mice had also a significantly shorter survival—i.e., life span—compared with BAP1(+/+) mice, both in the low-dose ($P<0.01$) and the standard-dose group ($P<0.001$) (Figure 3.9D).
Figure 3.6: Levels of peritoneal cytokines. (A) MCP-1, (B) LIF, (C) KC, (D) eotaxin, and (E) IL-6. (F) Correlation of IL-6 and LIF levels. * $P < 0.05$, ** $P < 0.01$
Figure 3.7: (A) MM incidence is higher in BAP1(+/–) mice; (B–C) Immunohistochemistry to confirm MM diagnosis and evaluate presence and localization of BAP1; (D) BAP1(+/–) have shorter survival curves. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
In summary, here we have shown that germline $BAP1$ heterozygosity is associated, in a genetically engineered murine model, with significant alterations in the asbestos-induced inflammatory response and increased MM risk even in the presence of low doses of asbestos.
Malignant mesothelioma is an aggressive cancer with dismal prognosis. Identifying individuals with higher risk of MM is a priority in the field. Two main populations have to be considered: 1) individuals professionally exposed to asbestos fibers; 2) individuals with genetic predisposition to MM and other cancers because of germline $BAP1$ mutations.

In chapter 2 of this manuscript, we reported that elevated serum levels of total HMGB1 differentiate asbestos-exposed individuals and MM patients from healthy unexposed controls. Moreover, we discovered that hyper-acetylated HMGB1 is a very sensitive and specific biomarker to discriminate MM patients from asbestos-exposed individuals and from healthy unexposed controls. These results were supported by $\textit{in vitro}$ experiments in which we found that asbestos-induced necrosis of HM leads to the passive release of non-acetylated HMGB1, whereas the supernatant of MM cells contains mostly hyper-acetylated HMGB1, which is the isoform that is actively secreted. We propose that HMGB1 and its hyper-acetylated isoform can be a valuable tool to identify among potentially exposed people, those individuals who have been exposed to asbestos, and to identify among them those who have developed MM.

The extremely high specificity and sensitivity (100%) of hyper-acetylated HMGB1 to identify MM patients from asbestos-exposed individuals exceeded our most optimistic expectations. In this setting, hyper-acetylated HMGB1 outperformed other previously proposed MM biomarkers [137,141].

We previously proposed total HMGB1 as a possible biomarker for asbestos exposure and
MM [88,91], but we did not investigate its possible value in differentiating MM patients from asbestos-exposed individuals. Here, we confirmed that the total serum levels of HMGB1 are elevated in both asbestos-exposed individuals and MM patients, and reliably distinguish these cohorts from unexposed healthy controls. We further discovered that MM patients have significantly higher total serum HMGB1 levels compared to asbestos-exposed individuals ($P < 0.001$). Although total HMGB1 levels are sensitive and specific to discriminate asbestos-exposed from unexposed individuals, the relatively low AUC of 0.830 when comparing MM patients with asbestos-exposed individuals would limit its clinical usefulness in identifying MM patients among large cohorts of asbestos-exposed individuals. Strikingly, hyper-acetylated HMGB1 reliably distinguished MM patients from individuals occupationally exposed to asbestos with 100% sensitivity and specificity. Identifying MM patients among the high-risk asbestos-exposed cohorts is extremely difficult, due to the lack of early phenotypical evidences of MM. The specificity and sensitivity of hyper-acetylated HMGB1 should significantly facilitate this task.

We also measured the levels of various biomarkers in patients with benign or malignant non-MM pleural effusions, and compared them to MM patients. We found that total and hyper-acetylated HMGB1 were second to fibulin-3 in distinguishing MM patients from patients with pleural effusions due to other causes, and performed better than mesothelin and OPN. However, the combination of fibulin-3 and HMGB1 resulted in even higher power of discrimination, suggesting a clinical advantage in measuring both proteins.

In our sample set, mesothelin and OPN performed better than in some previous stud-
ies [121,138]. Fibulin-3 data were consistent with the original report [141] and with additional studies [292,293], and were better than those reported by other groups [146,294]. A possible partial explanation to this observation is that most circulating biomarkers are very sensitive to variability introduced by sample preparation and handling, and the choice of adequate control groups. The problem of reproducibility in biomarker studies is very important, and standardization of protocols to collect and store samples is a necessary step that needs to be taken [295].

Of note, the serum samples from the exposed cohort tested here were from insulators with many years of exposure to high amounts of asbestos [287]. Further studies are needed to analyze whether HMGB1 levels are also increased at lower exposure levels to asbestos. Also, it has been shown that total HMGB1 levels in serum and plasma are elevated in severe trauma and septic shock. However, these are transient increases that occur only in severe circumstances that require hospitalization in intensive care. Under these conditions, HMGB1 levels return to baseline within hours to 7 days [199,200,296]. HMGB1 is also increased in patients with sepsis who succumbed to the infection [184], and in some cases of chronic autoimmune disease [189]. Specifically, patients with rheumatoid arthritis may have serum levels of HMGB1 above baseline [190,192], although the levels are more frequently increased in the synovial fluid than in the serum [297]. In contrast to all of the above, individuals heavily exposed to asbestos have sustained high HMGB1 serum levels—due to the bio-persistence of the asbestos fibers lodging in the tissues that cannot be removed—suggesting that serial longitudinal analysis of total HMGB1 will prove helpful to identify among potentially exposed
cohorts those individuals who have actually been exposed and are at risk of developing MM. Close follow up of these high-risk individuals, and testing for hyper-acetylated HMGB1 in their serum, should help detect MM at an earlier stage when it is more susceptible to therapy. Notably, the levels of total and hyper-acetylated HMGB1 were not influenced by stage, suggesting that HMGB1 isoforms might be a promising early MM detection biomarker.

*In vitro*, HMGB1 can be hyper-acetylated and released by monocytes and macrophages [164]. So far, among the non-malignant conditions, hyper-acetylated HMGB1 has only been detected in alcoholic liver disease (ALD) [288], acute acetaminophen-induced liver failure [202], and severe macrophage activation syndrome [289]. In the latter two conditions, however, the increase of serum HMGB1 is transient and limited to the acute crisis when patients are in intensive care. ALD might represent a possible confounding factor. However, a specific phosphorylation at serine 34 has been identified in circulating HMGB1 from ALD patients [288]. By contrast, HMGB1 phosphorylation was not detected in any of our samples (data not shown), using the same analytical methodology of detection, and by the same investigator who performed the ALD study [288]. Therefore, mass spectrometry, by revealing the presence of phosphorylated HMGB1 isoforms, can reliably distinguish the hyper-acetylated HMGB1 isoform found in ALD patients from the hyper-acetylated HMGB1 isoform found in MM patients.

Potential technical limitations of our results need to be considered: our sample size was relatively small, which could lead to an undesired over-fitting of the data; our patients and controls were not strictly matched for age, sex, ethnicity, smoking status, or other demo-
graphic/epidemiologic factors, potentially introducing unwanted confounding factors. Before total and hyper-acetylated HMGB1 can be introduced into the clinic as biomarkers of asbestos exposure and MM detection, our findings need to be independently validated in a larger cohort. Prospective longitudinal validation studies with matched case-controls will soon start to validate the results reported here in a larger cohort. In these studies, we will also simultaneously investigate whether HMGB1 levels can be affected, similar to the mesothelin, by clinical covariates such as kidney function or body-mass-index [298], and whether potential correlations exist between HMGB1 isoforms and other known markers of chronic inflammation, such as C-reactive protein or neutrophil-to-lymphocyte ratio. Moreover, further studies will be performed to investigate the potential role of specific HMGB1 isoforms as markers of prognosis or tumor recurrence after surgical cytoreduction, and as therapeutic targets. Specific reagents able to detect HMGB1 isoforms in a hospital setting will have to be developed. In this regard, we are currently trying to develop ELISA assays for specific HMGB1 isoforms. Nevertheless, the exceptional potential relevance of our findings to asbestos exposed individuals and MM patients warrants immediate attention.

In chapter 3 of this manuscript, we investigated whether low doses of asbestos would be associated with increased MM risk in the presence of germline \( BAP1 \) heterozygosity and whether deregulated inflammation could be a potential mechanism. Taken together, our results showed that germline \( BAP1 \) heterozygosity is indeed associated with a significantly altered peritoneal inflammatory response upon exposure to asbestos fibers and to an increased risk of MM following exposure to even minimal amounts of asbestos that rarely
cause MM in wild type animals. Heterozygous germline mutations of other important tumor suppressor genes, such as *BRCA1*, *CDKN2A*, and *RB1*, increase risk of cancer specifically to one or very few anatomical sites [299]. One of the few tumor suppressor genes whose germline heterozygosity, similarly to *BAP1*, is associated with increased risk of cancer to several sites is *TP53* [299]. Besides its well-known intrinsic functions, recently a novel non-cell-autonomous tumor suppressor effect of p53 has been described, via regulation of macrophage polarization and cytokine release [300]. Our results suggest that germline *BAP1* heterozygosity, similarly to *TP53*, influences *in vivo* macrophage polarization and cytokine release. Indeed, we found that BAP1(+/−) mice exposed to asbestos had significantly more M2-like pro-tumoral macrophages. We also identified the chemokines MCP-1 and KC, and two cytokines of the IL-6 family (IL-6 itself and LIF) as soluble mediators that are significantly reduced in BAP1(+/−) mice exposed to asbestos. Of note, levels of MCP-1 and IL-6 have been reported to increase following asbestos exposure and have been linked to asbestos pathogenesis [301,302]. Our results support these findings and also suggest that this inflammatory response might be associated with increased immunosurveillance, since lower levels of these and other inflammatory mediators in BAP1(+/−) mice are associated with higher M2/M1 macrophage ratio and higher MM incidence following asbestos exposure. BAP1 has been recently shown to regulate the myeloid stem cell compartment via complex alterations of the transcriptional profile, possibly via its interaction with transcriptional co-regulators such as HCFC1 and ASXL1 [271]. We are currently investigating the molecular mechanisms of the observed phenotype. Altogether, our results suggest a novel, complex model
of asbestos-induced MM pathogenesis, in which the chronic inflammatory response can have preferentially anti-tumoral or pro-tumoral roles, depending on the cellular and soluble mediators involved. To explain the observed intra- and inter-familial variability of cancer types in germline BAP1 mutated families, we hypothesized that MM might be more prevalent in individuals/families exposed to low levels of asbestos [260], levels that are considered only marginally carcinogenic for the population at large. Our results support our hypothesis, as we found that 36% of BAP1(+/–) mice exposed to low doses of asbestos developed MM, compared to 10% of wild type mice. Moreover, we found that MM is significantly more frequent in BAP1(+/–) mice exposed to standard doses of asbestos, as recently reported also by Xu et al. in an independent model [303]. It is not possible to directly compare the low-dose exposure in mice injected with a total of 0.5 mg of asbestos to indoor and/or outdoor environmental low-dose asbestos exposure in humans. However, our findings support our hypothesis that germline BAP1 heterozygosity increases susceptibility to the carcinogenic effects of low doses of asbestos. Based on these results, we suggest that prevention programs of MM in individuals carrying germline BAP1 mutations should focus on reducing exposure to even minimal indoor and/or naturally occurring outdoor sources of carcinogenic fibers, levels that are within the acceptable “safe” limits for the population at large (0.1 fibers/cc of air as an eight-hour time-weighted average, as per US Occupational Safety & Health Administration standards [304]). Finally, while our model focuses on asbestos as a trigger, this novel non-cell-autonomous tumor suppressive function of BAP1 may not be restricted to the peritoneal compartment or to the asbestos stimulation, and may contribute to the large
numbers and diverse types of tumors characteristics of the BAP1-TPS.

In conclusion, here we identified novel biomarkers of asbestos exposure and MM, and experimentally confirmed that minimal exposure to asbestos is sufficient to cause MM in presence of genetic predisposition. We are now also investigating the potential role of HMGB1 isoforms as biomarkers of MM in individuals with germline BAP1 mutations.
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