Università degli Studi di Ferrara

DOTTORATO DI RICERCA IN
SCIENZE BIOMEDICHE E BIOTECNOLOGICHE

CICLO XXXI

COORDINATORE Prof. Paolo Pinton

Metformin inhibits Malignant Pleural Mesothelioma cell proliferation and induces apoptosis in MPM cells by targeting Notch-1

Settore Scientifico Disciplinare BIO/13

Dottoranda
Dott.ssa Marika Rossini

Tutori
Prof. Mauro Tognon
Prof.ssa Paola Rizzo

Anni 2015/2018

INDEX

INTRODUCTION

1. Malignant Pleural Mesothelioma (MPM)

Malignant Pleural Mesothelioma (MPM) is a lethal and aggressive tumor, representing the most common primary malignancy of the pleura, about 70-80% of all cases (Figure1). Rarely, other serosal membranes also coated with mesothelium, such as peritoneum (peritoneal mesothelioma), pericardial (mesothelioma pericardial), and tunica vaginalis (tunica vaginalis mesothelioma), are affected.

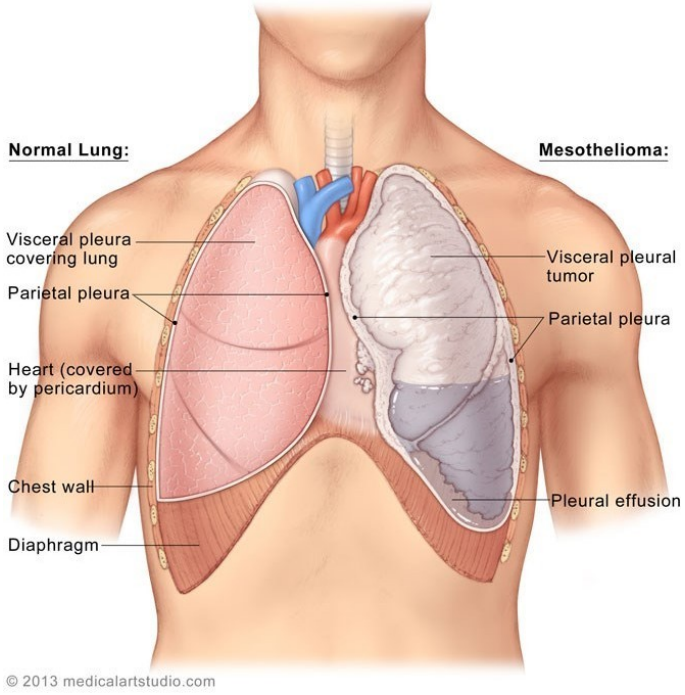


Fig. 1: Diagram of Normal Lung and Malignant Pleural Mesothelioma (MPM) ***(2013 medicalartstduio.com)*****.**

Although this malignancy is rare, its incidence is significant with an estimated number of about 40,000 deaths each year worldwide for asbestos-related MPM (1,2) due to the augmented use of these carcinogenic mineral fibers (3,4). Asbestos refers to a group of mineral silicate fibers that were used commercially in the 1970s, with physical properties causing disease (5). The International Agency for Research on Cancer (IARC) assessed that all forms of asbestos (actinolite, amosite, anthophyllite, tremolite, crocidolite and chrysotile) are carcinogenic to humans, causing mesothelioma. To date, about 400 forms of asbestos are known in nature, but only the six forms mentioned above are regulated, due to their current and widespread commercial use in many western countries, and newly industrializing

economies (4). The World Health Organization (WHO) estimates that 125 million people annually around the world are exposed to asbestos, both in the workplace and at home. Moreover, asbestos, related to its context (commercial, mineralogical, analytical, regulatory), is defined differently, missing the cancer causing property of some minerals (5). Numerous scientific evidences provide a clear and strong association between asbestos and MPM (6–9). Previous studies have reported several cases of MPM in individuals exposed to erionite, considered the most potent carcinogenic mineral fiber, but not regulated, because it is not defined asbestos (10).

A widely accepted view assumes that the interaction between asbestos fibers and human pleural mesothelial (HM) cells is the first step toward MPM. Asbestos fibers enter the pleura and depending on the size, type of fibrous mineral and length of exposure, cause inflammation (11), inducing the activation of nuclear factor-kappa B (NF-kB) signaling, increasing cell survival and proliferation through loss of tumor suppressor genes, oncogenes activation and DNA damage (12,13). To date, the molecular mechanism(s), through which asbestos influences the selection of this HM sub-population(s), is not completely explained and it remains to be fully understood (14).

Several epidemiological studies demonstrated (15) that exposure to asbestos and other carcinogenic mineral fibers is not only the cause of MPM among subjects with low levels or no history of occupational asbestos exposure (7), indicating a role of para-occupational exposure to asbestos. The term para-occupational exposure refers to asbestos-exposed workers clothes, asbestos-containing commercial products, asbestos-containing buildings and natural asbestos in the soil, indicating that asbestos is becoming an environmental contaminant. Both para-occupational exposure and direct (occupational) exposure have shown to increase the risk of mesothelioma (16,17), possibly in combination with other co-factors in the MPM onset (18).

In vitro and *in vivo* studies*,* together with recent immunological investigations, have shown an association between MPM and the oncogenic Simian Virus 40 (SV40) (19–22), suggesting a transforming synergistic action between asbestos fibers and SV40 (23–25). Some other etiologies or co-factors, alone or with asbestos, have been linked to MPM carcinogenesis, such as genetic predisposition and ionizing radiation exposure, potentially contributing to MPM development (15,26,27).

MPM develops with a long-term latency period of about 25 to 70 years from the first asbestos exposure, with a poor prognosis and median survival of less than one year from the time of

diagnosis (9,28). In the setting of occupational asbestos exposure, the majority of affected patients are 60 years old, with peaks of the age-specific incidence at 80-84 years for men and 75-79 for women (29), with a prevalence higher among males compared to females (with a male-female ratio of approximately 4:1 - 8:1) (7,30).

MPM is histologically heterogeneous (31) and it can be distinguished in three main subtypes (32), according to the predominant cellular component and different biological behavior. Epithelioid mesothelioma, the most common form (50–70 % of cases), is characterized by polygonal or cuboidal cells similar to carcinomas; the sarcomatoid type (10–20%), with a spindle cell morphology, similar to sarcomas and the mixed or biphasic type (30%), composed of both epithelioid and sarcomatoid forms within the same tumor (33). The correct identification of the MPM histological subtype facilitates the differential diagnosis and influences subsequent prognosis with its therapeutic decisions (34,35).

2. Molecular mechanisms underlying MPM

A large number of studies conducted in the last two decades has led to the identification of several dysregulated biological processes that may play a significant role in MPM development, such as the increased rate of cell proliferation, the inhibition of apoptosis (36,37) and the alteration of intracellular Ca^{2+} homeostasis (38,39).

There is evidence that some of these molecular alterations, such as overexpression of adenosine A3 receptor (A3R) (40), purinergic receptor P2X7 (37), dysregulation of cellular (41) and circulating microRNAs (25,42) could be used to diagnose MPM.

2.1. Tumor suppressor genes in MPM

Tumor suppressor genes play a critical role in regulating the cell cycle. The inactivation and/or loss of their function is one of the fundamental event in the tumor development. Loss of heterozygosity (LOH), which commonly leads to unmasking a recessive tumor suppressor gene, seems to be a consistent feature in MPMs. Recent studies have discovered a germline mutation/inactivation in *BAP1* (BRCA1-associated protein 1) in MPM cases with a family history of cancer (43,44). *BAP1* is a tumor suppressor gene located on chromosome 3p21.3, encoding the deubiquitinating hydrolase that binds the RING finger domain of the BRCA1 protein, thought to be a regulator of many pathways related to cancer (45). Previous studies reported the involvement of *BAP1* several biological processes including regulation of cell

cycle, chromatin dynamics and response to DNA damage (46). The expression of BAP1 is ubiquitous and it interacts with tissue and cell type specific proteins in mediating metabolic stress response (47) and in promoting survival related to its de-ubiquitinating activity (48). As shown in a recent published study, the heterozygous germline BAP1 mutations (BAP1 +/-) induce cell metabolic changes linked to the increase aerobic glycolysis, leading to reprogramming of the activities and stabilization of favorable environment to carcinogenesis (49). The germline *BAP1* gene mutations lead to a short BAP1 protein, probably broken down prematurely, associated to various malignancies other than malignant mesothelioma such as, uveal melanoma (44,50) and melanocytic BAP1 associated intradermal tumors (MBAITs) (44). Somatic truncated *BAP1* mutations and aberrant *BAP1* expression are more common in sporadic MPM, with a frequency that varies widely among different histologic tumor types (43,51), correlating with survival, providing additional clinical significance by facilitating histological classification (52–54). Besides single point mutations in the *BAP1* gene, rearrangements, multiple alterations and copy number loss have also been found in the MPM pathogenesis (54,55). Different strategies resulted insufficient and less precise to identify the minute or larger chromosomal deletions, underestimating the frequency of genetic alterations in MPM (56). To date, it has been demonstrated that none of mesothelioma patients with germline *BAP1* mutation was an ex-exposed asbestos worker (57), demonstrating that the development of MPM is not always a consequent of asbestos exposure, underlining also the crucial role of genetic factors among risk factors of this disease.

The high incidence (around 25-60%) of the somatic *BAP1* mutations reported in MPM (58) is also associated with frequent alterations (aberrant expression, epigenetic silencing and point mutations) in other major tumor suppressor genes, such as *p16/Cdkn2a*, *p19/Arf* and *p19/Cdkn2b* (59). Their genetic alterations, independently by *BAP1* mutations, suggest an important role of these tumor suppressor genes, together with asbestos exposure, in the induction of mesothelial transformation *in vitro* and *in vivo* (60). Moreover, *in vivo* studies have shown that the inactivation of both *p16* and *p19/Arf* expression accelerated the initiation of asbestos-induced MPM with a decreased percent survival, comparing with the inactivation of either gene alone (61). Consistent with these data, whole exome sequencing of asbestos-induced MPM showed the homozygous loss of *Cdkn2A* and alterations in other tumor suppressor gene (62).

Another tumor suppressor gene frequently inactivated in MPM is neurofibromin 2 (*NF2)*. A study has found that 38% of MPM samples revealed *NF2* gene mutations, and 29.4% revealed deletions, with no *NF2* mutations in non-small cell lung cancer patients (63). The *NF2* gene product shows a high similarity in its sequence with some members of the ERM (Ezrin, Radixin, Moesin) protein family. The NF2 protein is a membrane stabilizing protein that propagates extracellular signals through several cell surface receptors and it is involved in the regulation of ion transporters by interacting with specific proteins (64).

Other studies in MPM have shown the lack of frequent mutations in two most notorious tumor suppressor genes: *p53* (60,65) and *pRb* (66). Nevertheless, the association between SV40 large tumor antigen protein (Tag) and both *p53* and *pRb* has been found in human mesothelioma specimens (67,68), with the inactivation of these important regulators of the cell proliferation and survival, resulting in the transformation of HM cells (21,69,70).

2.2. Oncogenes in MPM

Oncogenes promote transformation by driving cell proliferation and preventing apoptosis. Some of these genes are involved in the regulation of intracellular levels of calcium (Ca^{2+}), an important regulator of many physiological processes, including the regulation of apoptosis of cancer cells (39,71). The remodeling of intracellular Ca^{2+} homeostasis is a general characteristic of cancer cells, as a consequence of the activity of different proteins with altered functions (71). It is widely accepted that both the Bcl-2 and Akt proteins are cofactors of the Ca^{2+}-dependent pathways leading to apoptosis (72,73). An anti-apoptotic member of the Bcl-2 family of proteins and the oncogene *Akt* were found to be dysregulated in mesothelioma cells (74,75) and elevated levels of Akt activity were found in 65% of human mesothelioma specimens (76,77).

Several studies have shown that the aberrant expression and dysregulated activity of growth factors and their specific transmembrane receptors induce an increased mesothelioma cell proliferation (78). Epidermal Growth Factor Receptor (*EGFR)* is an oncogene closely involved in many cancer types, including MPM, and its gene product is a transmembrane glycoprotein belonging to the tyrosine kinase receptor family. The specific binding between EGFR and its ligand induces cell motility, cellular proliferation and inhibits apoptosis and expression of extracellular matrix proteins (79). Previous studies have shown the over-expression of *EGFR* in MPM tissues and cell lines (80,81), with a correlation between the

induction of its phosphorylation and the carcinogenicity of the asbestos fibers observed in rat pleural mesothelial cells (82).

Vascular Endothelial Growth Factor (VEGF) and its receptor (VEGFR) are overexpressed in MPM human samples, in which they may stimulate tumor growth and promote angiogenesis and lymphangiogenesis (83,84).

It is well known that inflammation contributes to tumorigenesis by promoting cell proliferation and activating anti-apoptotic pathways. Specifically, the hallmarks of asbestos fibers inhalation include early and chronic inflammation linked to generation of reactive oxygen species (ROS) that cause oxidative DNA damage, thus contributing to asbestos-mediated carcinogenesis (85). In addition, when asbestos fibers penetrate the pleura, HM cells undergo programmed cell necrosis, releasing into the extracellular space the High-Mobility Group Box-1 protein (HMGB-1), an abundant damage-associated protein that mediates chronic inflammation recruiting macrophages, which actively secrete Tumor Necrosis Factor (TNF)-α. The pro-inflammatory and pro-survival NF-κB pathway is subsequently activated, leading to resistance to apoptosis, transformation of HM cells towards the malignant phenotype (86,87) (Figure 2).

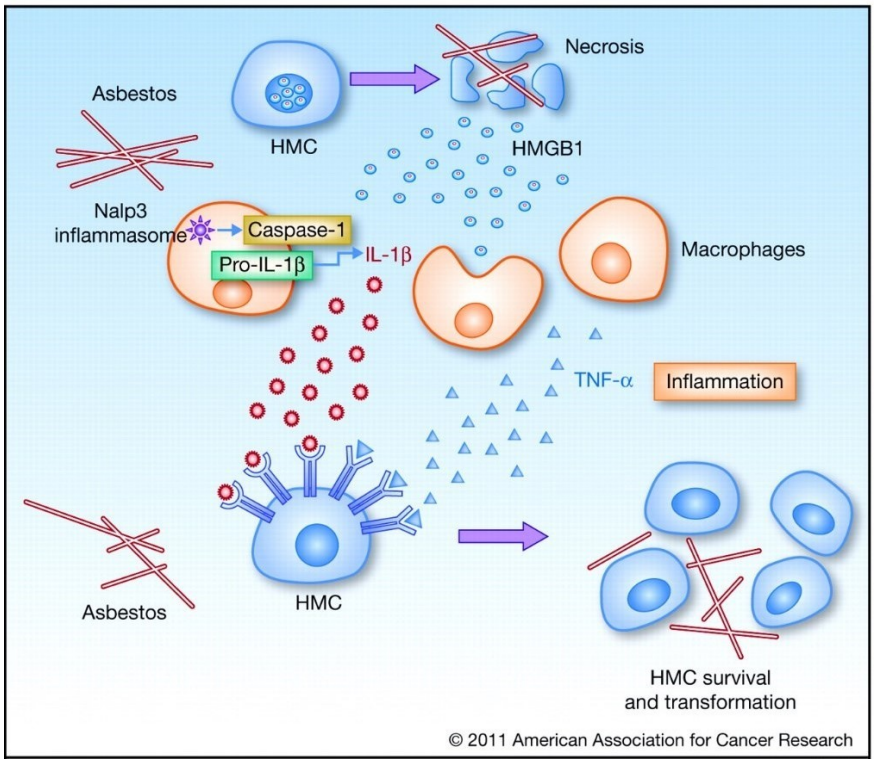


Fig. 2: Chronic inflammation and HM cell transformation ***(Carbone M. and Yang H., 2012)***

The decisive role of inflammation in MPM has been confirmed by another study that showed the increased concentrations of immune mediators in the sera of asbestos-exposed workers compared to health controls (88). Moreover, in alveolar macrophages of asbestos-exposed rats showed increased expression of Transforming Growth Factor (TGF)-β, indicating that the asbestosis of these cells contributes to fibrosis as well as to an inflammatory response. Furthermore, natural killer (NK) cells demonstrated impaired cytotoxicity, an immune-suppressive effect and tumorigenic effect upon exposure to asbestos (89). Consistently, functional alteration of NK cells and cytotoxic T lymphocytes upon asbestos exposure in MPM patients have been reported (90).

A recent study has reported data on the high specificity of HMGB1 protein in a hyper-acetylated isoform in serum of ex-exposed mesothelioma patients, selectively discriminating against their respective healthy control. This could suggest a role for HMGB1 as a serological biomarker (91).

3. The Notch signaling pathway

The Notch signaling pathway is a highly conserved evolutionary system of short-range intracellular communication that plays many key roles in the regulation of genes controlling a wide range of biological processes (92).

Notch signaling pathway has linear and simple molecular architecture, characterized by a small group of protein components, with a functional diversity and complexity. Simplicity derives from the absence of second messengers after proteolytic cleavage and activation of the signaling (93,94). The complexity arises from its ability to regulate a large number of downstream cellular effects during maintenance of self-renewing embryonic and adult tissues (95).

The *Notch* gene encodes the transmembrane receptor, highly conserved from invertebrates to mammals (95), that undergoes complex routing and modification events before it is presented in the cellular membrane in its functional conformation (96).

The maturation process of the Notch receptors is mediated by a direct contact between the extracellular domain of Notch receptors (four family members Notch1-4) and one of 5 canonical ligands (Delta-like 1, 3, 4 or Jagged 1, 2) on neighboring cells (95) (Figure 3). Notch receptors are proteolytically cleaved by furin-like convertase in the Golgi compartment on Site 1, resulting in a single-pass transmembrane protein to shuttle to the cell membrane. The mature form of protein contains an extracellular domain (N^{EC}), a single transmembrane domain (N^{TM}) and an intracellular domain (N^{IC}) (Figure 3).

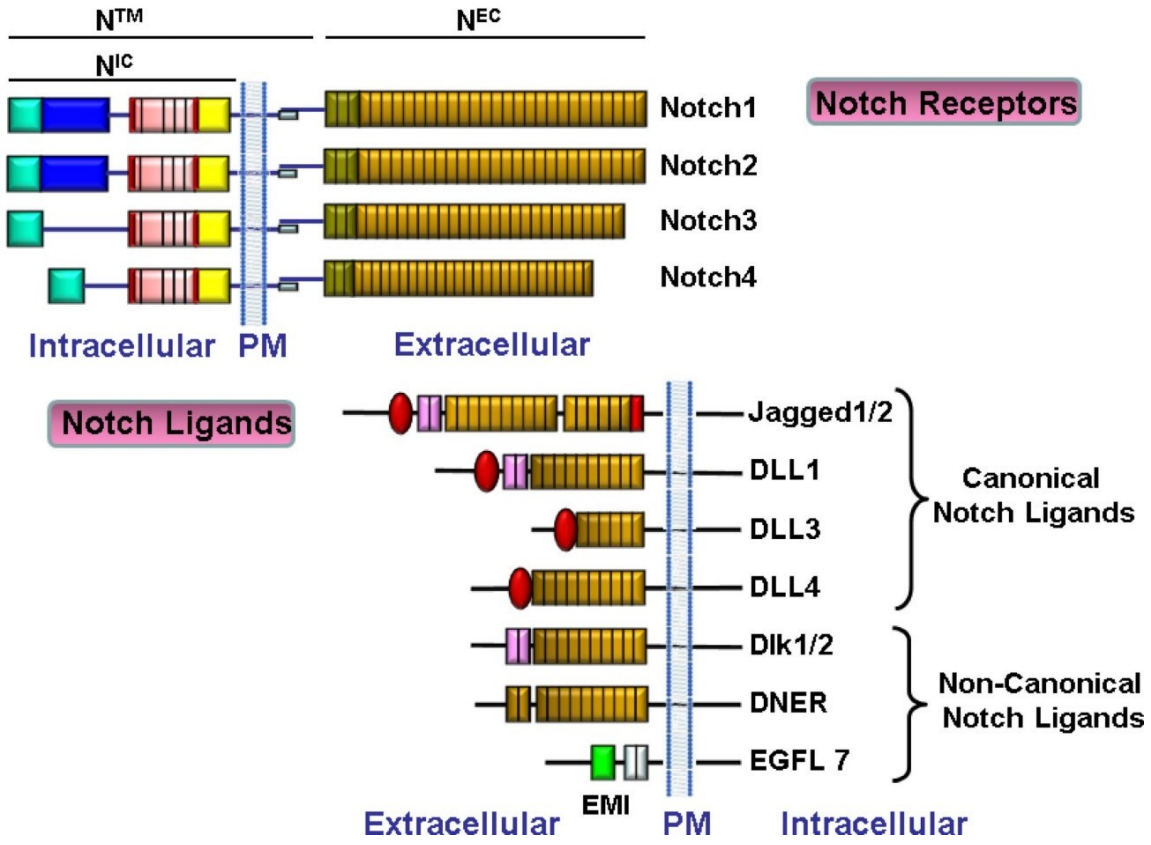


Fig. 3: Notch receptors and ligands. There are four mammalian Notch variations (Notch1-4) *(A)* Notch receptors are single-pass transmembrane proteins composed by a functional extracellular (N^{EC}) domain with different EGF repetitions; a transmembrane (N^{TM}) domain and an intracellular (N^{IC}) domain, characterized by a RAM domain, nuclear localization sequences (NLS), seven ankyrin repeats (ANK) and a transactivation domain (PEST). *(B)* The ligands can be divided in canonical Notch ligands (Jagged1/2, DLL1, 3 and 4) and non-canonical ligands (Dlk1/2, DNER and EGFL7) **(Espinoza I. and Miele L., 2013)**.

Receptor activation takes place by interaction of the extracellular domain of Notch with ligands present in the neighboring cells. This interaction triggers other two proteolytic cleavages on different sites of the protein, mediated by metalloproteases of ADAM family on Site 2 and followed by a γ-secretase complex-mediated cleavage on Site 3 at the cell membrane. The last cleavage allows the release of the intracellular domain of the Notch receptor (NICD), the active form of the receptor (94,97). NICD translocates into the nucleus and interacts with the transcription factor CSL (Suppressor of Hairless in *Drosophila,* Lag-

2 in *C. elegans* and CBF1/RBPJ-Jκ in mammals), converting the co-repressor complex into a potent transcriptional activator of downstream target genes (94,98). This constitutes the "canonical" Notch pathway (99,100) (Figure 4).

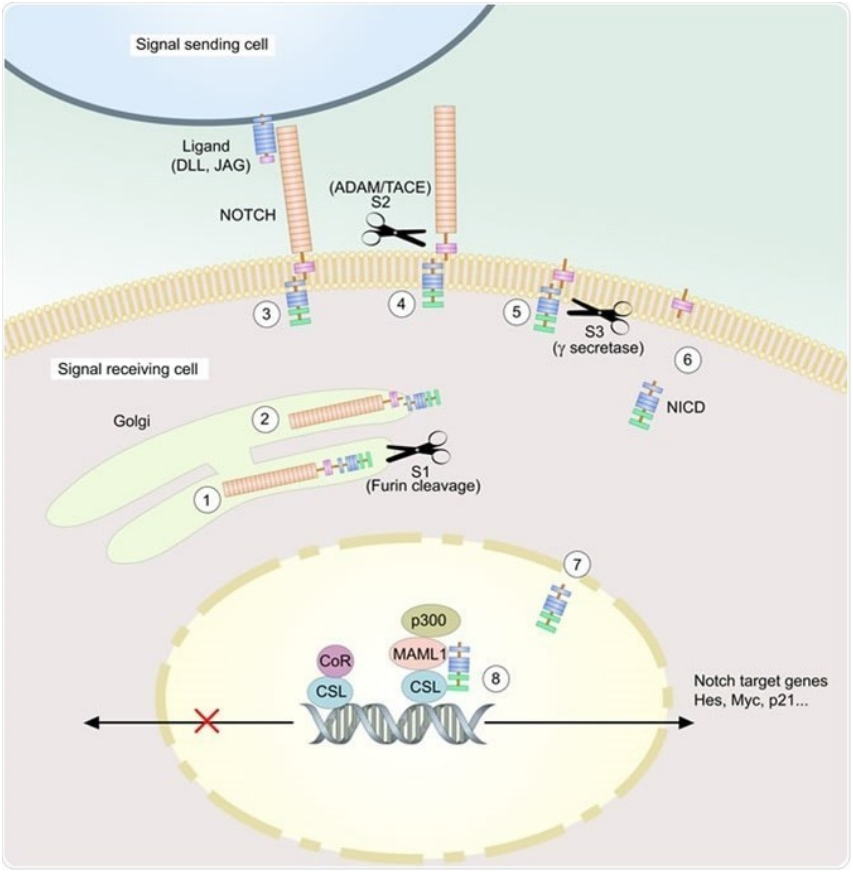


Fig. 4: Schematic representation of Notch signaling pathway (*Image Credit: Ellepigrafica/Shutterstock*).

Recently a "non-canonical" Notch pathway has been identified, which acts independently of CBF-1/CSL, playing important roles in normal and transformed cells (101–104).

There is a functional diversity among the Notch receptors, in particular among their intracellular active forms that induce the transcription of specific genes (95,105,106). There is evidence that, in breast carcinoma, Notch-2 has opposite effects on cell survival respect to Notch-1 and Notch-4. Moreover, the transcriptional activity of Notch-1 and Notch-3 is reduced by co-expression with the intracellular domain of Notch-2 (107). Detailed descriptions on the biochemical processes regulated by Notch and the implications of

dysregulation of this pathway in the development of cancer are reported in extensive reviews (108–110).

3.1. Notch and MPM

The normal tissue homeostasis is guaranteed and maintained by the balanced adjustment of these processes. A dysregulation of intracellular signaling pathways contributes to an extensive alteration of cell physiology causing excessive proliferation of cells. This signaling system disorder can be caused by mutations of proteins upstream or downstream of a signal transduction cascade or by a loss of function of negative regulators. It is well known that the Notch protein family is a critical regulator of differentiation programs during the normal development (108). Its ability to affect the cell proliferation and the response to apoptotic signals suggest that dysfunction of Notch proteins could be involved in the malignant transformation of some cell system. Indeed, alterations in the Notch signaling have been increasingly linked to many human cancer (110).

Since the biological effects of Notch activity seem to be tissue specific (92), the Notch signaling pathway has been found to be activated in a large number of solid tumors (110), such as breast (107), lung (111), prostate (112), gastric cancer (113), ovarian cancer (114), colon (115), leukemias (116), and in MPM human biopsies (117), but its role as tumor suppressor gene has also been reported in other tumors, such as squamous cells carcinoma (118,119).

The first evidence of the involvement of Notch signaling pathway in the development of tumors derives from the identification of a chromosomal translocation t(7;9)(q34;q34.3) in T-cell acute lymphoblastic leukemia (T-ALL). The chromosomal rearrangement resulted in constitutive Notch activity in T cells, due to the expression of a truncated Notch-1 protein lacking the extracellular subunit (116,120). This chromosomal translocation appeared in less than 1% of T-ALL cases, later it was discovered that over 50% of T-ALL examined cases had a variety of mutations activating Notch-1. These mutations may involve the heterodimerization domain (HD), promoting the proteolytic cleavage of the receptor in a ligand-independent manner, or may affect the PEST domain of regulation, leading to increased half-life of the protein due to the reduced interaction with the E3 ubiquitin ligase Fbw27/Sel-10.

Emerging evidences suggest that the Notch signaling network is frequently deregulated in human cancers, such as, characterized by over-expression of Notch receptors and their ligands.

Deregulated Notch receptors have been detected also in several MPM human biopsies, indicating a role for Notch in mesothelial cell transformation and/or MPM survival (121). In cell lines established from human MPM biopsies, elevated Notch-1 and reduced Notch-2 expression have been observed (117) respect their normal counterparts. Genetic and chemical modulation of the Notch pathway indicated that MPM cells are dependent on Notch signaling. Specifically in MPM cells, Notch-1 inhibits PTEN (phosphatase and tensin homolog) and activates the PI3K/Akt/mTOR signaling pathway indicating an oncogenic role of Notch-1 receptor in MPM cells and the involvement of its activation in the growth and survival of MPM cells (117). On the contrary, in the same cells, Notch-2 is a positive transcriptional regulation of PTEN and an inhibitor of the PI3K/Akt/mTOR signaling pathway and re-expression of Notch-2 was toxic to MPM cells (117). Previous studies have shown that SV40 activates Notch-1 leading to immortalization and transformation of primary HM cells (121–123). This indicates that Notch-1 can mediate the transformation process of mesothelial cells, downstream of mutagenic events caused by the exposure of carcinogenic factors, such as asbestos and viral infection (121,123). The same effect of SV40 on Notch-1 in HM cells has been reported in uterine cervical cancer with the infection of human papilloma virus (HPV) linked to the activation of Notch-1 (124).

3.2. The Notch signaling oncogenic network

Another aspect of Notch signaling which could enhance diversity of the signaling downstream response is the crosstalk between Notch and other signaling pathways. Numerous evidences suggest that Notch plays roles in both invertebrate and vertebrate development (125), operating not in isolation, but rather in cross-comunication with other molecular systems, often in context-specific manners. The deregulation of Notch signaling is frequently correlated also with more than fifty connection for the network in the tumor aggressiveness, including oncogenic pathways such as the transforming growth factor-β (TGF-β) (126), nuclear factor kappa-enhancer of light chain of activated B-cells (NF-κB) (127), phosphatidylinositol 3-kinase (PI3K/Akt) (128), Sonic hedgehog (Shh) (129), mammary target of rapamycin (mTOR) (130), estrogen receptor (ER) (131), growth factors

(132) and micro-RNA (133). These pathways are necessary for the survival of the cancer cells and it is becoming increasingly recognised the vision of an intricate network of signaling mechanisms that act no longer in an isolated manner. Notch is the nexus of a unique, pleiotropic and versatile signaling network that regulates and is regulated by a variety of cellular mechanisms highly dependent on cellular context. This reciprocity of communication and regulation among signaling pathways suggests that the feedback cycles represent important mechanisms of connection, useful for combining the individual signals in an interconnected network (134).

4. Current therapeutic approaches to MPM

4.1. Surgical treatment

Surgery is an essential option that, alone or in combination with chemo- and/or radiotherapy, attempts to eradicate the malignant tissue, trying to help the patient to relieve symptoms by reducing pain (135). Nevertheless, surgical resection of the tumor is controversial and limited to MPM patients with early stage disease and good cardiopulmonary functions (136). The intent and the role of surgical procedure influences the survival rate of MPM patients. In the analysis of the International Association for the study of Lung Cancer Mesothelioma Database, MPM patients undergoing curative-intent surgery showed a median survival that ranged from 20 to 12 months, strictly correlated with the stage of disease (stage I to IV). The MPM patients undergoing palliative-intent surgery showed 12 months of survival (137). A large study conducted with 14,288 patients has shown that surgery alone, compared with no treatment, is associated with a significant improvement in survival, but not radiation. The similar survival obtained with surgery alone has been observed after surgery and radiation combined (138). There are two type of surgery for MPM: 1) pleurectomy/decortication (P/D) removes radically all visible disease of the pleura or, if necessary, the entire organ affected by the tumor; 2) extrapleural pneumonectomy (EPP), a more radical surgical option, eradicates all macroscopic tumor through the removal of the areas surrounding it, including other mesothelial tissue (139). During both surgeries, lymph node sampling should be done to assess if the cancer has spread to the lymph nodes between the lungs. The optimal procedure for resection (EPP or P/D) of MPM depends on clinical factors and on individual surgical expertise. The authors of the study highlight similarities between the two approaches, concluding that there is no evidence to support the use of EPP or P/D (140). The

Mesothelioma and Radical Surgery (MARS), a multicenter randomized clinical trial, analyzed the advantage and the relevance of EPP approach, comparing the clinical outcomes and median survival between MPM patients assigned to EPP within trimodal therapy (chemotherapy, EPP and postoperative hemithorax irradiation) (about 14 months) and patients with chemotherapy, but no EPP approach (about 19 months) (141). Other groups reported data on mortality related both to EPP and to extended P/D, observing that both techniques can achieve prolonged median survival (142). When balancing these considerations, it has been underlined that surgery should be applied to obtain macroscopic compete resection limiting surgery-associated mortality. Furthermore, given the high rates of local failure/recurrence after surgery, would be appropriate the incorporation of intra-cavitary therapeutics into the multimodality treatments (MMT) protocol (142).

4.2. Radiotherapy

Radiation therapy is relatively common for MPM. Several studies have shown that radiotherapy is unable to cure MPM (143), but administrated either pre- or post-operatively, in combination with other treatments/approaches or alone, is useful to control pain, limit tumor spreading, improving the 2-years rate of overall survival from 20% to 34% (144). Given the unique way that MPM spreads along the pleura, surrounding the lungs, adjacent to the heart, spine and other vital organs, it is extremely difficult to identify the effective radiation dose and the site of radiation. The spread of cancer cells and the formation of nodules can occur in 20-50% of MPM patients. To prevent spreading, prophylactic radiation has been used without a standardized clinical practice, due to mixed results obtained (145), probably explained by differences in surgical procedures, closely related to the ability to administer radiation (146). In the neoadjuvant setting, the optimization of the administration of high-dose radiotherapy to the hemithorax was allowed by the development of new intensity-modulated radiation therapy (IMRT), followed by early EPP, providing in selected MPM patients an improved median overall survival up to about 39 months (147,148). In contrast with these results, multicenter clinical trials observed the not promising outcomes of IMRT in trimodality approach, due to the high toxic effects, not supporting the routine use of hemithoracic therapy for MPM (149).

4.3. Chemotherapy

Chemotherapy drugs kill fast-growing cells. Systemic chemotherapy for management of MPM remains the only and primary treatment modality, despite the toxic effects of chemical drugs, as it has shown an increased median survival from 9 to 12 months in most advanced stage MPM patients, who are not candidates for aggressive surgery (150). Almost every chemotherapy regimen has been tested in mesothelioma (151). Although these treatments are no curative, they can alleviate symptoms, prolong survival and improve quality of life, depending on the histological differentiation, tumor stage and the patient's overall health when treatment begins (135). Of note early clinical trials of MPM patients included heterogeneous groups of patients with divergent risk factors and were therefore often not powerful enough to assess therapeutic efficacy of a particular treatment (152). Vogelzang et al. were the first to demonstrate that pemetrexed/cisplatin combination chemotherapy is more effective with a greater activity in MPM than cisplatin monotherapy or non-platinum containing combinations (153–155). A few other combinations were evaluated in randomized trials, but they did not demonstrate an incisive improvement of overall survival (156). New generation of antifolates (pemetrexed, raltitrexed) and novel platinum derivates (157) have shown low efficacy and limited outcomes, with a three months survival benefit in their combination over cisplatin alone (median survival ranged from 9 months to 12 months) in MPM patients with advanced disease (153).

4.4. Multimodality therapy

A number of non-randomized clinical trial have investigates the feasibility and outcomes of multimodality treatment for MPM, making important progresses by involving experienced multidisciplinary team recommended by other guidelines for mesothelioma patients (158,159), according to the 2007 UK Department of Health's Mesothelioma Service Framework and the British Thoracic Society's Statement on Mesothelioma (160). For obtaining more effective outcomes, therapeutic options include the combination of two or more different therapeutic approaches, such as surgery, radio- and chemotherapy, with type of agent, timing and modality still debated. Selected patients with operable disease and a good performance status should be considered candidates for the multimodality therapy. For each stage of MPM disease, different methods and therapeutic approaches are indicated. Patients with clinical stage I disease, with potential for surgical tolerance, surgery is

recommended, because the tumor is localized and non-metastatic, respect to the patients with advanced stage who are not operable because of impaired cardiopulmonary function that can be treated with chemotherapy (161). Patients with stage II, with larger tumor and localized also in nearby organs, such as the lung or diaphragm, lymph nodes, may also be involved and stage III where MPM has invaded a region or area, such as the chest wall, esophagus, or lymph nodes should be offered trimodal therapy with surgery, chemotherapy and radiotherapy. A recent study has confirmed that the combination of surgical treatment, such as extra-pleural pneumonectomy (EPP) and chemotherapy with radiotherapy led to a median survival up to 24 months (162). Data from the Cochrane Lung Cancer group's Specialised Register, Cochrane Central Register of Controlled Trials, Medline, Embase and the strength of the evidence collected by Abdel-Rahman et al., revealed a lack of available evidence to support the use of radical multimodality therapy in routine clinical practice, leaving considerable uncertainty regarding the choice of the correct therapeutic protocol and the right type of surgery for each individual patient (163).

5. New therapeutic approaches and novel molecular targets

Despite progresses reached, survival time and response rate to cytotoxic agents used for MPM treatment are still not satisfactory (160), due to the high variability in treatment outcome observed in cancer patients undergoing chemotherapy (158). Furthermore, as the disease is still diagnosed at an advanced stage, there is a strong need for precise indicators for early detection of MPM and for the identification of new targeted therapeutic approaches.

5.1. Circulating biomarkers of MPM

Analysis of liquid samples, such as serum and pleural effusion, represents a promising approach for the characterization of markers related to MPM progression, for their ease of collection (164). Recently, proteins (165–168), metabolites (169) and miRNAs (42) have been identified differentially expressed in the serum of MPM patients and could be used as biomarkers of the onset and progression of this disease.

Soluble mesothelin, a cell surface glycoprotein, is highly expressed in several human cancers, including mesothelioma (170). Several studies have shown a sensitivity of 84% for advanced status of MPM, a specificity of 95% and a correlation with histological subtype of the tumor (171–173), with higher levels in epithelioid subtype than sarcomatoid one (174).

Another highly conserved and promising biomarker is the circulating glycoprotein fibulin-3 (175). A study showed elevated fibulin-3 levels both in plasma (sensitivity of 100% and specificity of 94%) and pleural effusion (sensitivity of 84% and specificity of 93%) of MPM patients, distinguishing healthy individuals with asbestos exposure from patients with MPM disease (176). The prognostic potential of fibulin-3 is greater than mesothelin, which instead results more functional as diagnostic biomarker of MPM (166). It has been shown that the osteopontin levels, an extracellular cell adhesion protein, were significantly higher in serum of MPM patients than healthy asbestos exposed individuals (177), but it is unable to distinguish between MPM, benign pleural lesion or pleural metastatic carcinoma, associated with asbestos exposure, due to high number of false-positive (178).

A clinical study has demonstrated that total or hyper-acetylated isoform of the protein HMGB1 is a sensitive and specific biomarker that allows to distinguish early the serum samples of asbestos-exposed MPM patients from healthy unexposed individuals and other pleural diseases (91).

The discovery of microRNAs (miRNAs), small sequences of RNA involved in regulation of gene expression, has changed the diagnostic approach and therapy of many diseases, including cancer (41). MiRNAs regulate a plethora of cellular activities, such as proliferation, metabolism, apoptosis and angiogenesis. They are characterized by high stability, under different typology of sample treatment, processing and isolation (179–181). Circulating miRNAs moves through the circulatory system naked or inside microparticles, such as microvescicles, exosomes and apoptotic bodies, representing an innovative form of intracellular communication (42,182). The miRNAs expression profile has been found to be altered in several human cancers, thus pointing at their role in tumorigenesis, in particular in the cancer progression, as oncomiRNas and tumor suppressor miRNAs (183–185). Based on their characteristics, miRNAs could be used as measurable indicators for prognosis, diagnosis and valuation of cancer treatment results, including MPM (8,42).

A specific circulating miRNAs signature differentiating MPM patients from ex-exposed asbestos and healthy subjects has been identified (25,42,186). It has been proposed that the detection of circulating miRNAs, i.e. miR-197-3p, miR-1281 and miR-32-3p, in sera of MPM affected patients and ex-exposed asbestos workers could be used as a novel, predictive and non-invasive biomarkers for this disease (42). This characteristic could also help to project targeted therapies for MPM (8,187), exploiting the use of antagomir (oligonucleotide

sequences) or anti-miRNAs (mimetic miRNA) (41), to silence the overexpressed oncomiRs or substitute the lost miRNA in cancer, respectively (188,189) (Figure 5).

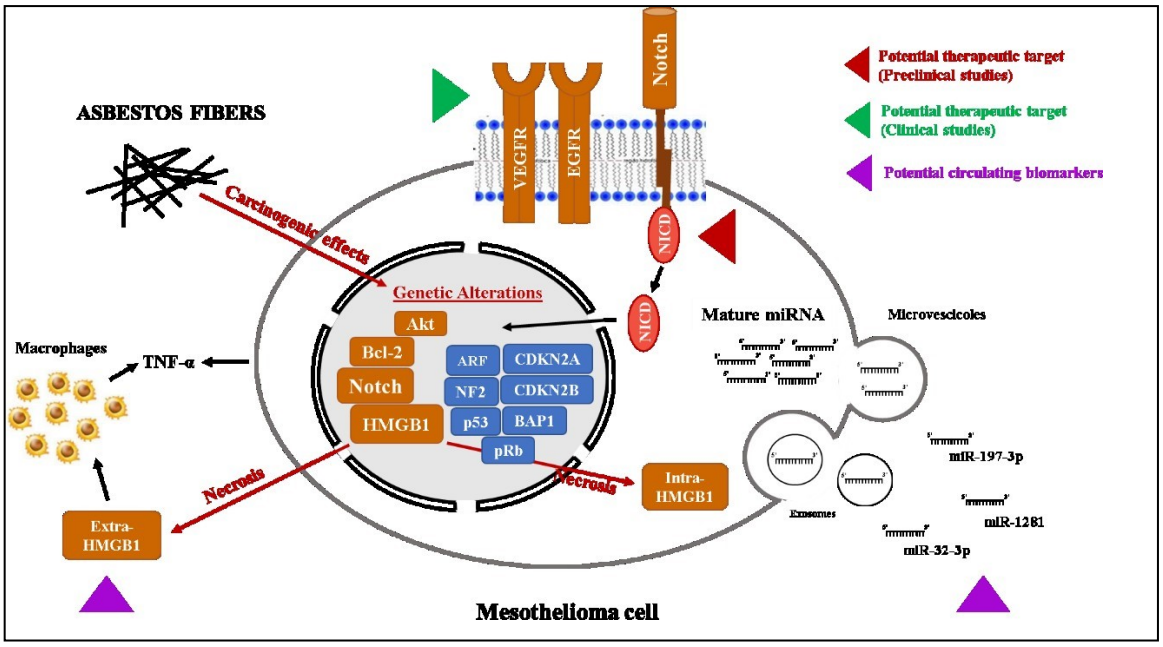


Fig. 5: Potential therapeutic targets and biomarkers in the mesothelioma *(Rossini M. et al., 2018).* Schematic representation mesothelioma cells under asbestos exposure. Genetic alterations, such as mutations on oncogenes and tumor suppressor genes, lead to the transformation of HM cells. Additionally, following necrosis caused by asbestos exposure, HMGB1 protein, translocates from the nucleus to the cytosol and extracellular space, triggering the inflammatory response, supporting the mesothelial cells transformation. All these events may contribute to the activation of the Notch signaling and it could represent a helpful target to stop the MPM progression. Changes in microRNA expression in MPM cells could represent a strategy for early diagnosis of mesothelioma. In the figure, the red and green arrows represent clinical and preclinical studies, respectively, aimed to target newly discovered pathways altered in MPM cells. The purple arrow indicates the novel potential circulating biomarkers under study for a no invasive MPM screening.

VEGFR (vascular endothelial growth factor receptor); EGFR (epidermal growth factor receptor); HMGB1 (high-mobility group protein 1); TNF-α (tumour necrosis factor-α).

6. Metformin as antineoplastic drug

Metformin (1,1-dimetilbiguanide hydrochloride) is the current first-line drug used as an oral biguanide in the treatment of type 2 diabetes mellitus (T2DM), with more than 120 million treated patients worldwide (190). It has been reported that patients with diabetes and untreated T2DM ones are associated with an increased cancer risk, attributed mostly to the growth promoting effect of chronic elevated plasma glucose and insulin levels (191–194). Insulin resistance and resultant hyperinsulinemia might indeed promote carcinogenesis directly through the insulin receptor or indirectly by increasing the levels of insulin-like growth factors (IGF). Metformin is known to act on liver, skeletal muscle and gut decreasing blood glucose level in diabetic patients with hyperglycemia without inducing hypoglycemia, with a good tolerance and minimum collateral effects (195).

The interest in potential anti-neoplastic and cancer preventive properties of metformin was based on numerous clinical studies that showed a significantly reduced incidence of neoplastic diseases and cancer mortality in diabetic patients treated with metformin compared to diabetic patients treated with another antidiabetic drug (196). Several clinical trials using metformin as a treatment also in non-diabetic cancer patients have produced encouraging results (197), although the findings vary depending on the intrinsic properties of the tumor. Afterwards, many groups focused their research on effects of metformin on cancer cells, both *in vitro*, alone or in combination with other drugs, and *in vivo*, demonstrating the efficacy of this biguanide in decreasing tumoral growth on various cancer cell lines and several cancers in animal models (198–202).

At the molecular level, different mechanisms of antitumor effects of metformin have been identified, but not all are yet currently full elucidated. It is now appreciated that the principal effect of metformin on cancer cells is the direct effect, through the inhibition of complex I of the electron transport chain (ETC) of mitochondria (195). Direct inhibition of complex I by metformin in cells leads to block of mitochondrial respiration and decrease cellular ATP levels, resulting in a compensatory increase in glycolysis. If the compensatory activation of glycolysis doesn't induce restoration of cellular ATP levels, the metabolic checkpoint AMP-kinase (AMPK) pathway becomes activated in order to potentiate catabolic metabolism and inhibit anabolic reactions (203–205). AMPK is an energy sensor that plays an important role in many pathways involved in restoring energetic balance within the cell, such as

proliferation, cell cycle regulation, cell polarity, apoptosis, and autophagy (205–207) (Figure 6).

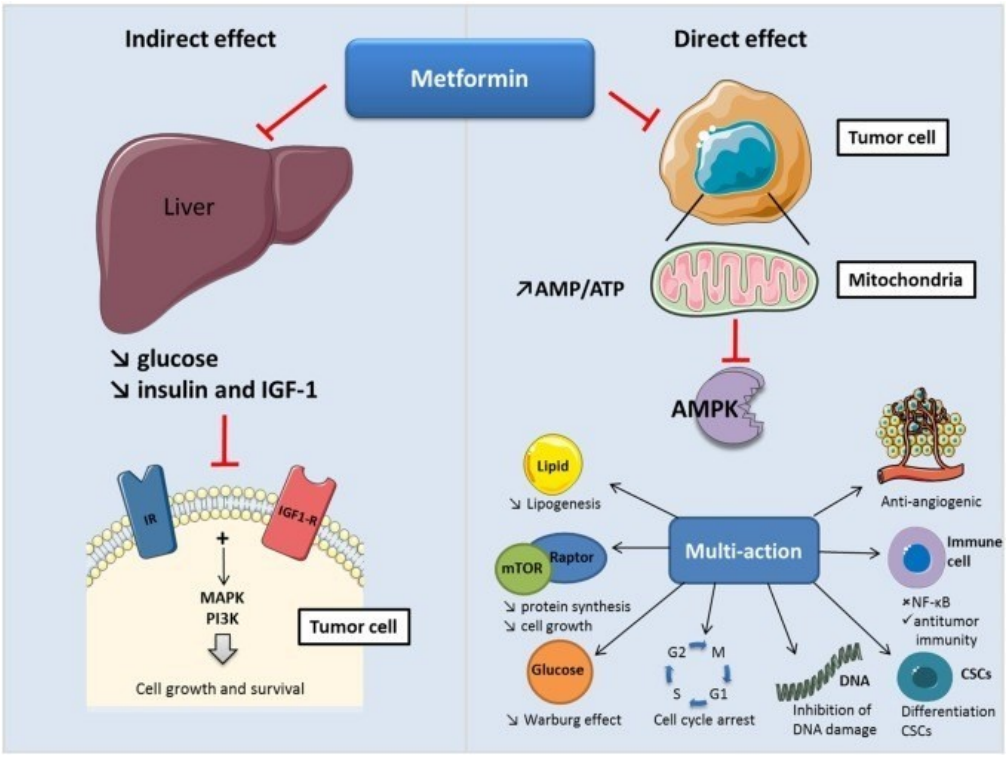


Fig. 6: Schematic representation of mechanisms of action of metformin (*Daugan M. et al., 2016).*

Metformin also inhibits mammalian target of rapamycin complex I (mTORC1), a nutrient-sensitive multiprotein complex whose core essential components include the protein kinase mTOR and scaffolding protein Raptor (208,209). This complex is implicated in many anabolic cellular processes essential for the cell growth. It is often activated in cancer cells and can be associated with cancer therapy resistance (210). Many different mechanisms have been discovered to explain the inhibition of mTORC1 complex (211).

Understanding the mechanism of action of metformin and the consequences of its action on cancer cells bioenergetics allows the identification of cancer types most susceptible to metformin action. Completed clinical trials have varied in outcomes depending on cancer type and its stage, trial design, timing and modality of metformin treatment (alone or combinatorial therapies).

6.1. Metformin and MPM

Mesothelioma is a disease resistant to conventional treatments. Previous studies showed that metformin has antineoplastic effects and its prescription has been reported to be associated with improved survival in the treatment of diabetic patients with several types of cancers (193–195). Nevertheless, an effect of metformin has not yet been examined in mesothelioma and there is no evidence to support trials of metformin in MPM patients. To date, there is just a preliminary study that put in correlation the effects of metformin and MPM patients with T2DM, reporting a lack of association between metformin use and improvement of survival in MPM patients (212). This study presented several limitations related to the small sample size, imprecise estimates of effects size and not complete data on mesothelioma characteristics for the study. Lack of adjustment for mesothelioma stage may have underestimated the association between metformin and survival if patients who received metformin prescription had a more advanced stage of MPM at diagnosis.

6.2. Targeting Notch pathway in MPM with metformin

There is an emerging realization that mutations in Notch genes and dysregulated Notch signaling pathway are linked to tumor initiation and development, depending on cell type. Numerous evidences show that inhibition of Notch signaling determines a reduction of tumor cell proliferation *in vitro* and arrests tumor growth *in vivo* (130,213), thus the targeting of Notch offers an attractive potential therapeutic strategy in oncology (Figure 5). Notch inhibition is able to shrink the tumor by increasing the apoptotic rate in the bulk of tumor, inhibiting the growth of cancer stem cells, responsible for tumor recurrence (214,215) and interfering with angiogenesis (216,217). To date, small molecules inhibiting γ-secretase, the proteolytic enzyme required for Notch activation, are under investigation in clinical trials (for a list of trials the reader is referred to clinicaltrials.gov) in combination with existing drugs (218). Other agents able to reduce angiogenesis by inhibiting Notch are also being developed, i.e. antibodies against Dll4 (219).

As previously discussed, Notch-1 is overexpressed in MPM and it is therefore possible to hypothesize the targeting of this receptor to prevent MPM progression (220) and cancer stem cells survival (221).

In addition, recent *in vivo* studies with transgenic mouse models of tissue specific manipulation of Notch signaling have begun to reveal the roles of Notch pathway in

regulating metabolism of several key metabolic organs (224). Another unanswered important consideration is about the upstream regulator of Notch signaling. A recent study indicates that the energy sensor AMP-activated protein kinase (AMPK), the important target of the action of metformin, regulates Notch signaling through mTORC1 under nutrient stress (225).

AIMS

Notch-1 signaling pathway is evolutionary conserved and it has the critical role for developmental cell processes, emphasizing the uniqueness of this molecular pathway. Numerous experiments and known data have underlined the involvement of Notch signaling deregulation in various diseases, such as leukemia and solid tumors solid, including MPM. A previous study demonstrated that mesothelioma cell lines and specimens have elevated Notch-1 expression compared with their normal counterparts and that Notch-1 pathway is required for malignant mesothelioma cell survival. At present time, the role of Notch-1 signaling in the onset/progression of MPM is not thoroughly investigated. To date, current treatments and approaches for mesothelioma do not significantly prolong survival and there is no standard second-line therapy, with very poor clinical outcomes. Many studies have demonstrated potential anti-neoplastic activity and cancer preventive properties of metformin, depending on the intrinsic properties of the cancer. This study was carried because there are not strong evidences in the role of metformin in the treatment of MPM. In this research, I investigated with different approaches the possible existence of a relation between the Notch-1 signaling pathway the anti-neoplastic action of metformin and MPM survival.

To this purpose, the experiments were conducted to analyze: (i) the anti-proliferative and pro-apoptotic effects of metformin; (ii) Notch-1 protein expression levels in human malignant pleural mesothelioma (MPM) cells and in human pleural mesothelial (HM) cells; (iii) the Notch-1 activation status after treatment with metformin and then (iv) the role of Notch-1 in MPM cells after metformin treatment combined with γ-secretase inhibitor (DAPT).

To perform these experiments, two cell lines of human MPM were chosen as model of study, MMP89 and IST-Mes2 cells, sarcomatoid and epitheliod subtype, respectively and primary human HM cells as normal control.

MATERIAL AND METHODS

1. Cell lines and culture conditions

Human malignant pleural mesothelioma (MPM) cell lines, MMP89 and IST-Mes2, were obtained from National Institute for Cancer Research c/o CBA (ILC, Genoa, Italy), and grown in DMEM Ham's F12 supplemented with 10% fetal bovin serum (FBS) (Euroclone). Primary pleural mesothelial (HM) cells were obtained from biopsies collected from non-oncologic patients affected by pneumothorax at the Surgical Clinic of the University/Hospital of Ferrara, Department of Thoracic Surgery. HM cells were grown in RPMI-1640 medium, 2 mM L-Glutamine, supplemented with 10% FBS. All cell lines were maintained in their respective media supplemented with antibiotics 100 units of Potassium Penicillin/ml and 100 μg of Streptomycin Sulfate/ml (Lonza), under sterile conditions, incubated at 37°C in a 5% CO_2-humidified atmosphere. The medium was changed twice a week and the cultures were passaged by Trypsin-EDTA according to their growth rate. For each experiment performed the cells were plated to 80-90% confluence.

2. Chemicals

Metformin and N-[N-(3,5-difluorophenacetyl-l-alanyl)]-S-phenylglycine t-butyl ester (DAPT) were purchased from Sigma-Aldrich. Metformin was resuspended in phosphate-buffered saline (PBS) to make the 1M stock solution that was used at concentration of 25 mM in all the experiments, with exception of some proliferation and apoptotic assays, in which scalar concentrations, ranging from 1 mM to 50 mM were used. DAPT was resuspended in dimethyl sulfoxide (DMSO) to make a 5 mM stock solution that was used at concentration of 10 μM in all of the experiments. Both metformin and DAPT were added to culture medium for each experiments.

3. Cell proliferation assay

Cell proliferation was evaluated to assess differences between treated and untreated cells, both in MPM cells and in their counterpart primary HM cells under the same conditions of treatment. Cells were counted and seeded in 96-well plates, ~10,000-6,000 cells/well (depending on the cell type), in duplicate and treated with different concentrations of metformin, DAPT and their combination. For metformin experiments, MPM cells and HM cells were treated for 24, 48 and 72h, initially with scalar doses 1, 5 and 10 mM and then

with 25 and 50 mM. For DAPT experiments, MPM cells were treated for 24, 48 and 72h with scalar doses, ranging from 1 µM up to 100 µM. For combined experiments, MPM cells were treated for 48h with scalar doses of metformin (1, 5, 10 and 25 mM) and with one concentration of DAPT (10 µM). During all the experiments, the control wells were exposed to the same concentration of PBS 1X (in the case of metformin treatment) and DMSO (in the case of DAPT treatment) to eliminate any possible effect of the vehicle on cell proliferation.

The alamarBlue assay was used to quantitatively measure the effect of the drugs on cell proliferation and its cytotoxicity in all cells. Therefore, subsequently, 5% of alamarBlue solution (Invitrogen) was added to each well, and after three hours of incubation, the absorbance was measured in an automated microplate reader (Sunrise Tecan reader) at two different wavelength, 570 nm and 620 nm. Relative cell proliferation of individual samples were expressed as a percentage, based on the ratio of the absorbance of treated cells to that of control cells treated with PBS 1X (100%).

4. Apoptosis assay

Cell apoptosis was evaluated by Annexin V assay. MPM and HM cells were seeded in a 6-well plates (1.0×10^6 cells per well) and, after 24h of growth, were treated with metformin (MMP89 25 mM; IST-MES2 and HM cells 25 - 50 mM) for 24h. Briefly, both MPM cell lines were treated with metformin for 24h, harvested by trypsin, washed twice with PBS, stained with 200 µL FITC-Annexin V and Propidium Iodide (according to the manufacturer's protocol, 4 µL of Annexin V-FITC and 1,5 µL of propidium iodide (PI)) (Sigma-Aldrich, Oakville, ON, Canada), and then incubated for 20 minutes at room temperature in the dark. Next, labeled cells were processed by flow cytometry. All early apoptotic cells (i.e., Annexin V positive, PI negative), necrotic/late apoptotic cells (i.e., double positive) and living cells (i.e., double negative) were detected by using a flow cytometer (Becton Dickinson, New Jersey, USA).

5. Western blot analysis

MPM cells, after treatment with metformin 25 mM for 24 and 48h, were collected, washed twice with ice-cold PBS, lysed with RIPA buffer (0,1% SDS, 1% NP40, Sodium

deoxycholate (Sigma-Aldrich)), supplemented with protease inhibitors (10 µg/ml Aprotinin (Sigma-Aldrich), 10 µg/ml Leupeptin (Sigma-Aldrich), 10 µg/ml Pepstatin A (Sigma-Aldrich), 1 mM 1mMphenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich) and 1 mM sodium orthovanadate (Sigma-Aldrich)) and incubated for 30 minutes on ice. The lysates were centrifuged at 14,000xg for 3 minutes at 4˚C. The concentration of protein lysates was quantified using the BCA protein assay Kit (ThermoFisher Scientific, Inc.). For each sample, 35 µg of protein extract were separated on 4-12% SDS-PAGE (Invitrogen by ThermoFisher Scientific, Inc) and transferred onto PVDF membranes (GE Healthcare) using the wet transfer system.

For immunoblotting analysis, the PVDF membranes were blocked with 5% non-fat milk at room temperature for 1h and then incubated over night at 4˚C with following primary antibodies: anti-cleaved Notch-1 (Val1744) (1:1,000), anti-pRPS6 (Ser240/244) (1:1,000), anti-Noxa (1:1,000), anti-Bcl2 (1:1000), anti-caspase-3 (1:1,000), anti-caspase-8 (1:1,000), anti-caspase-9 (1:1,000), anti-PARP (1:1,000) (all purchased from Cell Signaling), anti-Notch-1 (C-20) (1:500) (Santa Cruz Biotechnology, Inc.) and anti-β-actin (1:10,000) (Sigma). After washing the membranes three times for 10 minutes each with Tris-buffered saline (TBS) - Tween-20, they were incubated with HRP-conjugated secondary antibodies (1:3,000 dilution) [ImmunoReagent, Inc. GtxMu-003-DHRPX (goat anti-mouse IgG) (1:3,000) and GtxRb-003-DHRPX (goat anti-rabbit) (1:3,000)], purchased from Invitrogen. Proteins were visualized using an Enhanced Chemiluminescence-Plus kit (Thermo Scientific) and by Image Lab Software 4.0 (Bio-Rad).

6. Statistical analysis

The graphs were produced using GraphPad Prism 6.01 software. Statistical comparisons were performed using both one-way analysis of variance and two-away (multiple comparisons) (ANOVA). Data were expressed as the mean ± SEM. Significant differences were considered with values of $P<0.05$.

RESULTS

1. Metformin inhibits MPM cell proliferation in a dose- and time-dependent manner

In order to investigate the effects of metformin on MPM cells, the treatment conditions and cell proliferation rate were tested using the alamarBlue assay (Figure 1A-B). The toxic dose of metformin was determined treating both MPM cell lines with three different concentrations 1, 5 and 10 mM, for 24, 48 and 72h. The graphs related to these conditions show a very low sensitivity to the drug action both in MMP89 cells (Figure 1A) and IST-Mes2 cells (Figure 1B) analyzed.

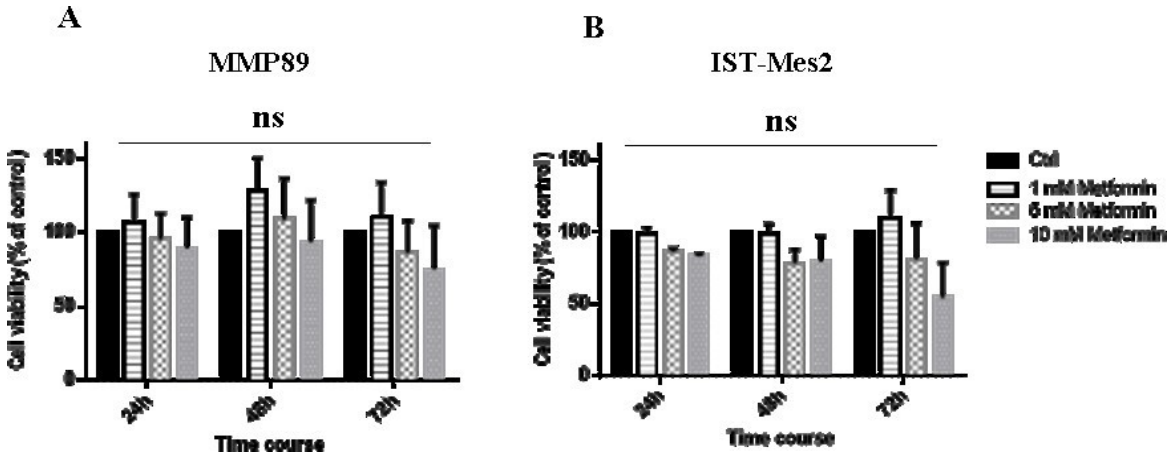


Fig. 1: MPM cell lines are not sensitive to low concentrations of metformin. *(A-B)* Cell viability of MMP89 and IST-Mes2 cells after treatment with 0, 1, 5 and 10 mM of metformin for 24, 48 and 72h. The results were presented as relative percentage to untreated control of each group (defined as 100%). Data represent mean ± SEM of three independent experiments in duplicate.

To determine whether MPM cells were susceptible to anti-proliferative effect of metformin, I decided to increase its concentration up to 25 mM and 50 mM, for 24, 48 and 72h as shown in figure 2. It is possible to observe that metformin reduced the proliferative capacity both MMP89 and IST-Mes2 cells in a dose and time-dependent manner compared to their respective untreated control (Figure 2C-D). In MMP89 cells, incubation with 25 mM metformin for 24h, 48h and 72h reduced the cell proliferation to 79,7%, 71,2% and 41,1%,

respectively. Increasing the concentration in the media, up to 50 mM metformin for 24, 48 and 72h, their cell proliferation reduced up to 37,4%, 37,3% and 32,1%, respectively (Figure 2C). The trend was the same for the IST-Mes2 cells with 25 mM metformin for 24, 48 and 72h, with a decrease of the cell proliferation up to 67,6%, 61,9% and 47,6%, respectively, and with 50 mM metformin up to 50,6%, 36,1% and 17,5%, respectively (Figure 2D). The difference in cell proliferation was greater both at 72h of treatment compared to the time points 24 and 48h, and with 50 mM of metformin concentration as compared to 25 mM. To verify if the metformin was also cytotoxic for the normal counterpart cells, it has been repeated the alamarBlue assay under the same experimental conditions in the HM cells (Figure 2E).

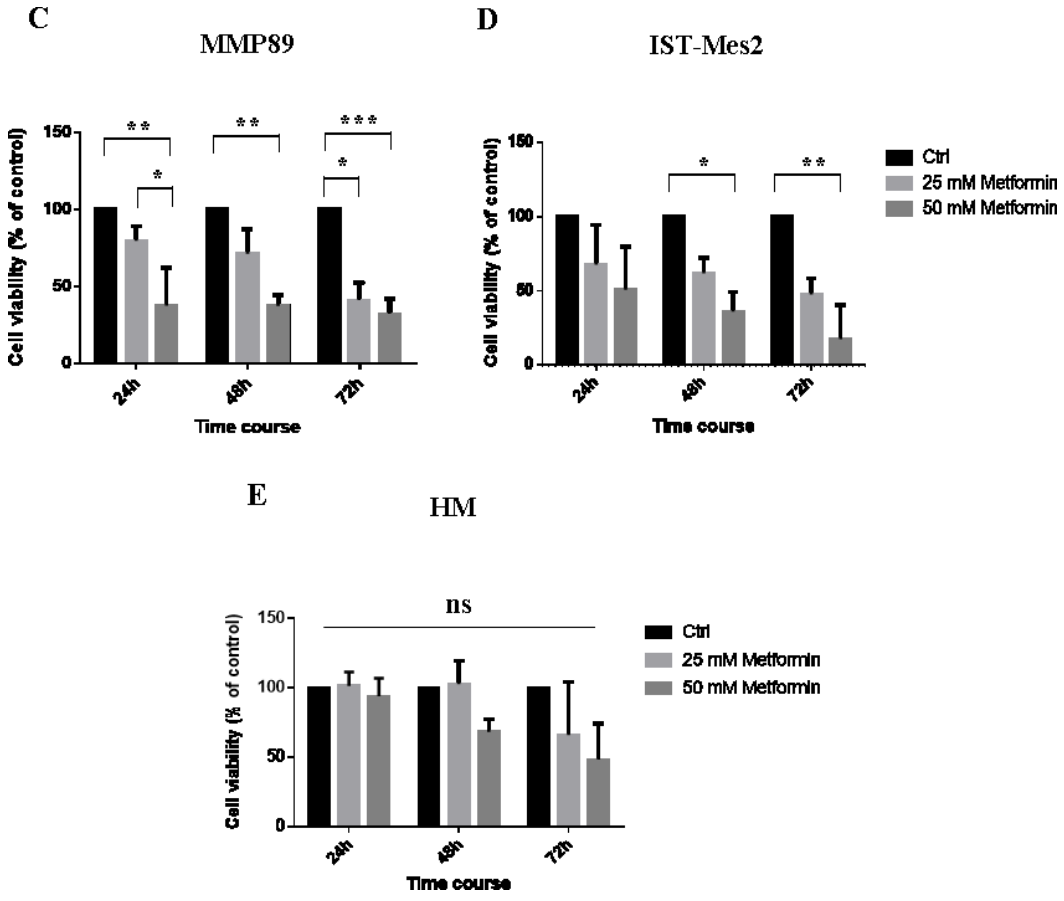
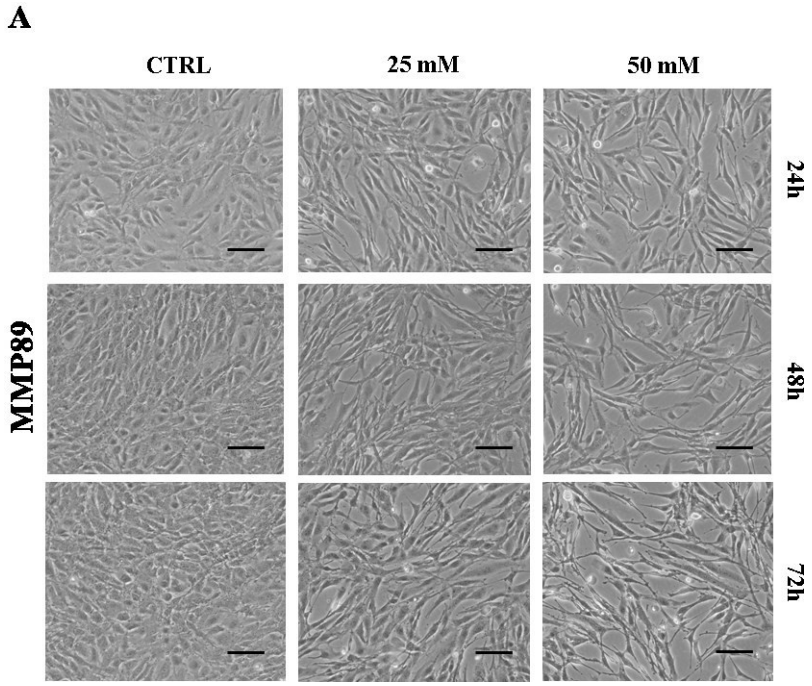


Fig.2: Metformin inhibited the MPM cell proliferation. *(C-D)* MPM cells treated with 25 and 50 mM of metformin for 24, 48 and 72h. *(E)* The same experiment was performed for human mesothelial (HM) cells to investigate the anti-proliferative effect of the metformin.

Cell viability was determined by AlamarBlue assay. The results were presented as relative percentage to untreated control of each group (defined as 100%). Data represent mean ± SEM of three independent experiments in duplicate. The *P* value was calculated compared to untreated control (*$P < 0.005$, **$P < 0.001$ and ***$P < 0.0001$).

After metformin treatment, under the same conditions of cell proliferation assay, the morphology and density of resultant cells were found to be both altered and reduced as compared with the cells growth in regular medium (Figure. 3).



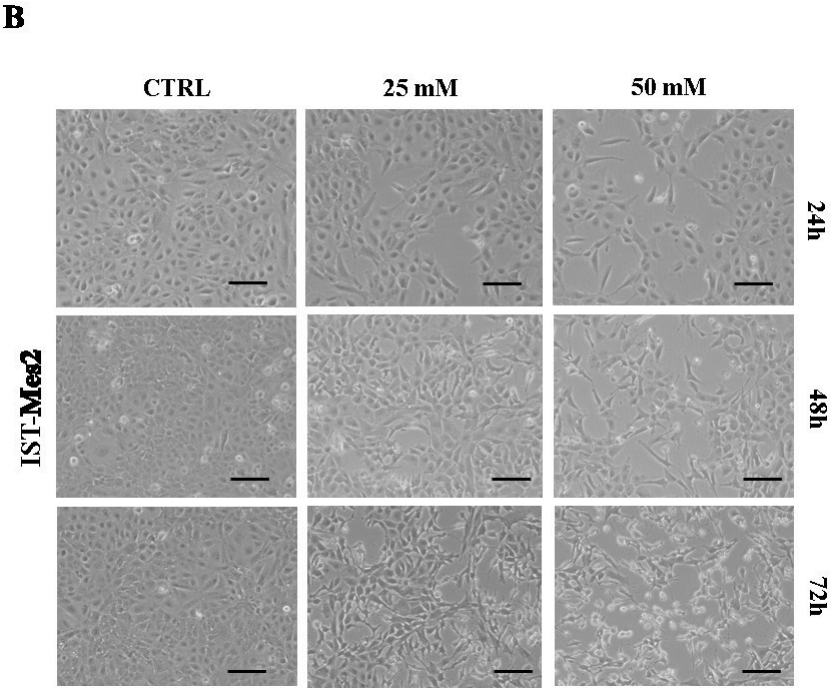


Fig. 3: Effect of metformin on morphology and proliferation of MPM cells. *(A, B)* MMP89 and IST-Mes2 cells grown in DMEM F12 with 10% FBS under different treatment conditions with metformin for 24, 48 and 72h. The controls with PBS 1X are present for both MPM cell lines. The images were taken under 10x objective. Scale bar: 25 µm.

For further experiments, it has been chosen 25 mM as metformin concentration, due to the important decrease on MPM proliferation without cytotoxic effects in HM cells. The hyper-activation of the PI3K-Akt pathway is a feature of a large majority of cancer cell types (226) and MPM (78). The anti-proliferative effect of metformin in some tumor cell types, is well described and requires inhibition of mTOR activation via the upregulation of AMPK activity (195). Thus, to identify differences between untreated and treated MPM cells with metformin, I assessed the mTOR activation via the phosphorylation status of the mTOR downstream target, S6 ribosomal protein (RPS6), by western blot. Phosphorylation of S6 ribosomal protein correlates with an increase in translation of mRNA transcripts that encode proteins involved in cell cycle progression, as well as ribosomal proteins and elongation factors necessary for translation (227). Metformin has previously been reported to cause a general inhibition of protein synthesis in different cancer cells (208,228,229). Here, metformin treatment caused a strong decrease in the phosphorylation of RPS6 protein in

both MPM cell lines, with 25 mM of concentration for 24-48h as show in the blot in the figure 4A, validating its anti-proliferative effect.

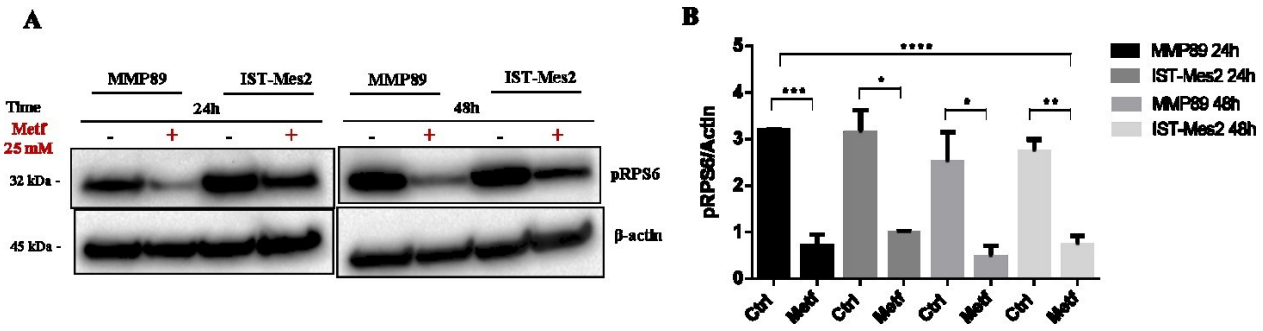


Fig. 4: Expression of pRPS6 in response to metformin exposure in MPM cells. *(A)* Lysates of MPM cells treated with 25 mM of metformin for 24 and 48h were subject to western blot using anti-pRPS6 and β-actin (loading control) antibodies. *(B)* Densitometric analysis relative to β-actin. Results are the mean ± SEM of 3 experiments (* and ** compared with the control group. *$P < 0.005$, **$P < 0.001$, ***$P < 0.0001$ and ****$P < 0.00001$).

2. Metformin induces apoptosis in MPM cells

To further investigate the anti-neoplastic effect of metformin in MPM cells, the preset thesis examined and compared the apoptotic features of MPM cells following metformin treatment. The results showed an increase in the number of floating cells, following metformin treatment compared to the untreated groups, which was suggestive of apoptosis (Figure 5). The percentage of apoptotic cells was determined by cell flow cytometric analysis following Annexin V/PI staining (Figure 5A-B). Following incubation with metformin for 24h, the extent of apoptosis in MMP89 cells markedly increased up to 42.12% with 25 mM metformin. Moreover, 50 mM of metformin was used as concentration of treatment on MMP89 for 24h, resulting in an important increase of apoptotic cell percentage (data not shown). IST-Mes2 cells were less sensitive to metformin, as the percentage of cells undergoing apoptosis only increased from 7.71% with 25 mM metformin to 11.16% with 50 mM metformin as shown in the graph in the figure 5B. The MPM treated cells underwent apoptosis in a dose-dependent manner, compared to their untreated counterpart cells, coherent with the alteration of cell morphology and in cell number showed in the

microscopic images (Figure 5E). The same experiment was performed with the HM cells, demonstrating the lack of cytotoxicity in non-cancer cells (Figure 5C-D).

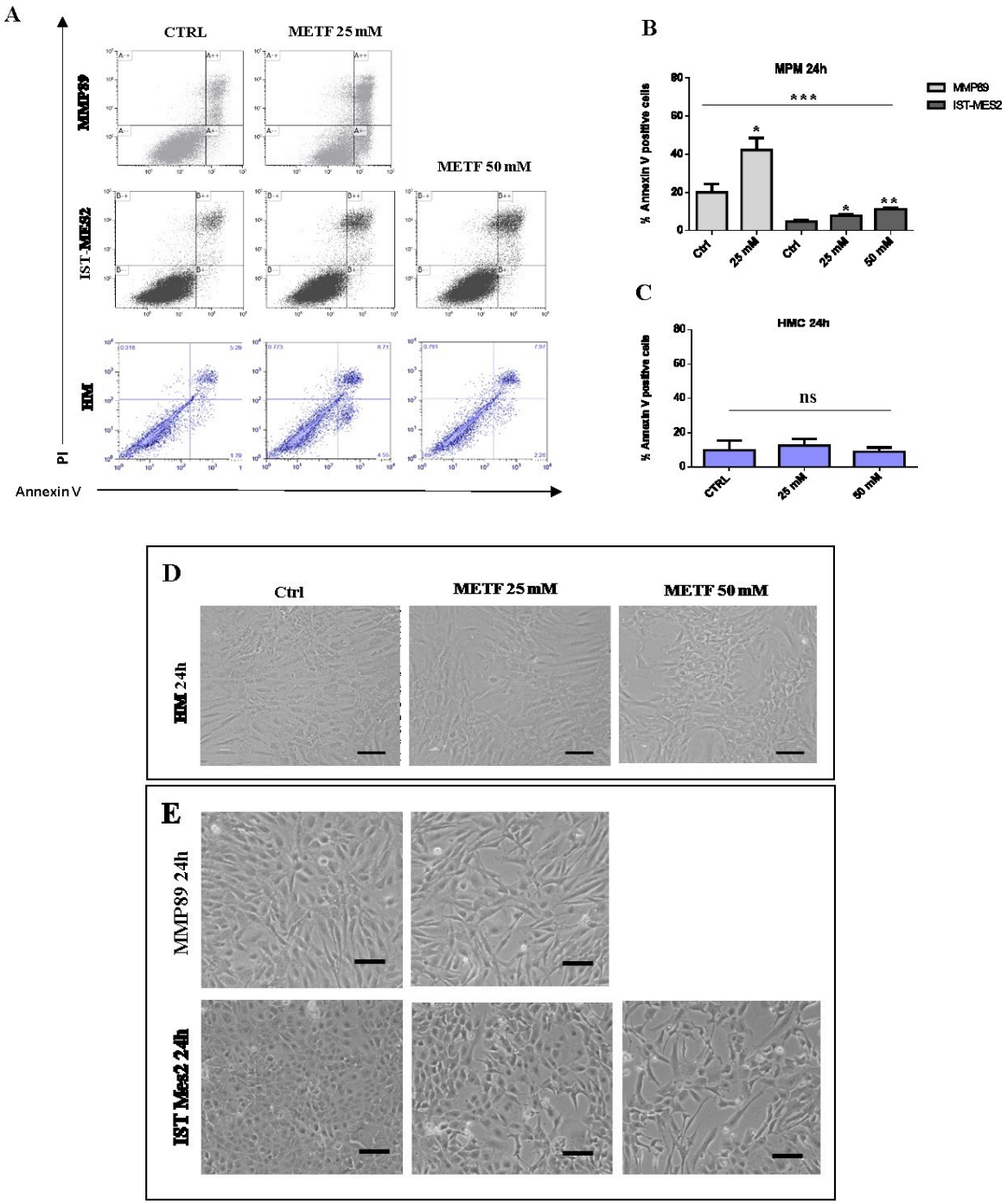


Fig. 5: Apoptotic effect of metformin in MPM cells but not in HM cells. *(A)* MMP89 cells were treated with 25 mM metformin, while IST-Mes2 and HM cells were treated at different doses, 25 and 50 mM of metformin, both for 24 h. Apoptosis was determined by

flow cytometry and the percentage was represented in the graph. Four fractions of the cells were identified: live cells in the early phase of apoptosis, late stage apoptosis and necrotic cells and quantitative analysis of individual fractions was represented. *(B-C)* Results are the mean ± SEM of 3 experiments (* and ** compared with the control group. *$P < 0.005$, **$P < 0.001$, ***$P < 0.0001$). *(D)* Images of HM cells showing no morphological alteration following treatment. *(E)* Images of MPM cells showing morphological alteration after 24h treatment with 25mM and 50 mM of metformin. All these images were captured under optical microscope (10x magnification). Scale bar: 25 µm.

The apoptotic effect was also validated by several other typical characteristics of apoptotic morphology of cells, including the condensed chromatin and micronucleation following nuclear staining with Hoechst-33342 with and without treatment with different concentrations (25 mM for MMP89 and 25-50 mM for IST-Mes2) of metformin for 24h (Figure 6).

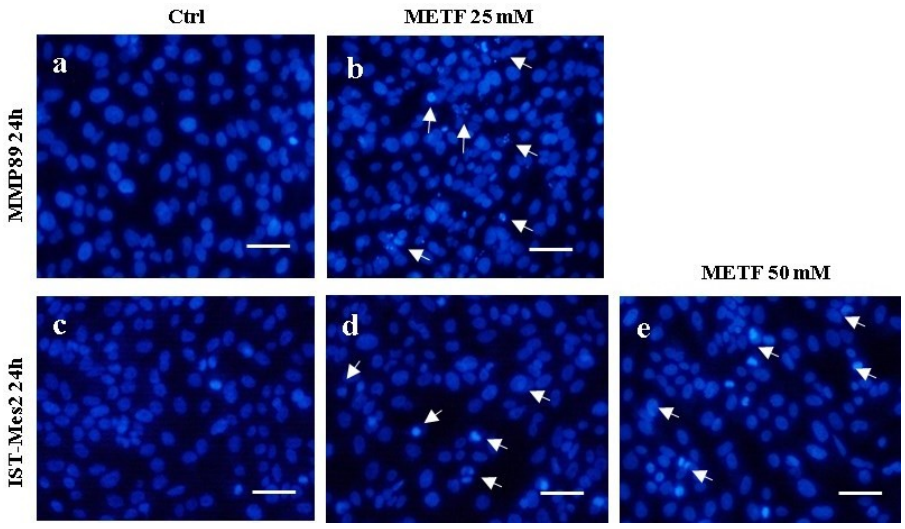


Fig. 6: Apoptotic effect in MPM cell lines. *(b)* MMP89 cells were treated with 25 mM and *(d-e)* IST-Mes2 cells with two different doses, 25 and 50 mM of metformin, both for 24 h. *(a-c)* Untreated MPM cells. Nuclear morphology was examined by staining the cells with Hoechst-33342. The images were captured under the fluorescence microscope (10x magnification). Scale bar: 25 µm.

The metformin-treated cells showed changed shape, from spindle (MMP89) and oval (IST-Mes2) to more elongated and stretched with a separation of the cells from the surface of monolayer. With Hoechst-33342, the nuclei appeared brighter, granular, blue fluorescence and typical changes of late apoptosis with small apoptotic bodies compared to control group (Figure 6). Also for apoptosis detection by Hoechst-33342 staining, were used different concentration of metformin 25 mM for MMP89 and 25-50 mM for IST-Mes2, because MMP89 resulted to much sensitive to 50 mM of metformin respect IST-mes2 that resulted more resistant (data not shown).

To corroborate the findings of the flow cytometric analysis and morphological evaluation previously described, cell lysates of cultured treated and untreated cells I investigated whether the metformin-induced apoptosis observed in MPM cells was a result of activation of the mitochondrial pathway, the caspases (3, 8 and 9), Bcl-2, PARP and Noxa proteins by using western blot analysis (Figure 7A-B).

Since the intrinsic apoptosis pathway is physically associated with mitochondria, the levels of conserved pro-apoptotic proteins that dysregulate the permeabilization of the outer mitochondrial membrane were measured.

Caspases are synthesized as inactive precursors (procaspases), which undergo proteolytic cleavage, playing an important role in apoptosis, as initiator (caspases 8 and 9) or effectors (caspase-3) depending on their point of enter into the apoptotic pathway (230). The blots in the figure 7A-B show a decrease of these caspases, in their total form, in both MPM cell lines treated with 25 mM metformin after 24h and, more evident, 48h of treatment, induced by mitochondrial dysfunction that leads the initiator caspase-9 in the cytosol inducing in turn the activation of the effectors caspase-3 and 8 (Figure 7B).

Poly(ADP-ribose) polymerase (PARP) is a family of proteins involved also in the apoptosis (231). PARP cleavage in early apoptosis is mediated by caspase-3, determining which cell death pathway has been activated (232). The blots in figure 7A-B showed a downregulated total form of PARP protein in both MPM cell lines, after treatment at 24 and 48h with 25 mM of metformin.

Bcl-2 protein has an important function in the control of activation of intrinsic apoptosis pathway (75,233,234) The family members consist of pro- and apoptotic members that induce opposing effects on the permeability of the mitochondria. The Bcl-2 family members have been divided into three subfamilies: Bcl-2 subfamily pro-survival, the Bax subfamily pro-apoptotic and BH3 subfamily pro-apoptotic.

I found that Bcl-2 is downregulated by metformin. Moreover, the apoptosis in both the MPM cell lines tested was consistently accompanied by another interesting observation that concerns the pro-apoptotic Noxa protein (235). Consistent with this observation, it has been shown here that, in MPM cell lines, metformin induces a strong Noxa protein up-regulation, thus suggesting that Noxa plays a critical role in metformin-induced MPM cell apoptosis (Figure 7).

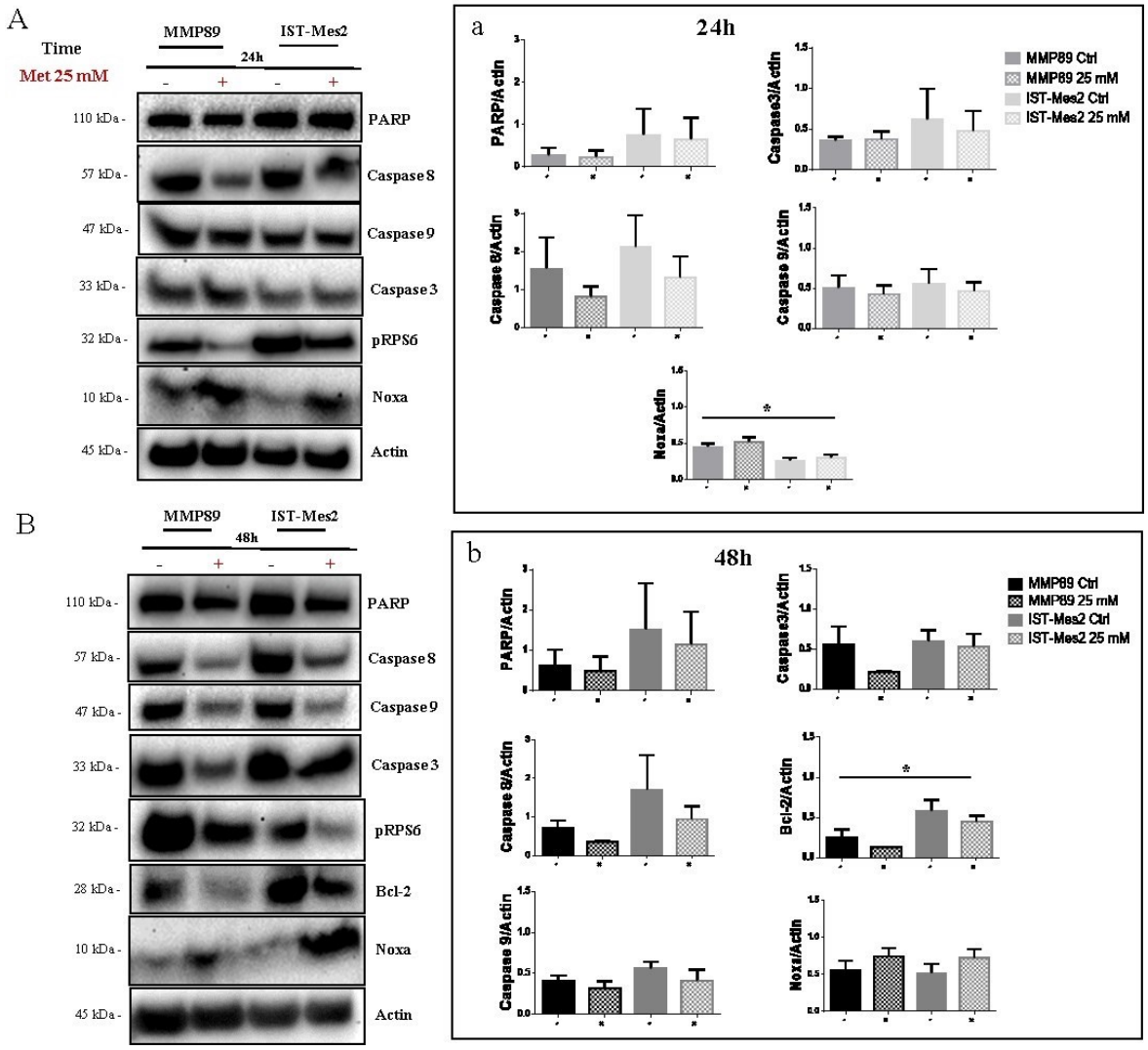


Fig. 7: Induction of pro-apoptotic proteins after metformin treatment in MPM cells. *(A-B)* 35 µg whole-cell lysate extracted form treated MPM cells were subjected to western blot analysis. There is a modulation of apoptotic proteins, PARP, caspase-8, -9, -3, Bcl-2 and Noxa in MPM cells after metformin treatment, specifically with 25 mM for 24 and 48h.

The band of pRPS6 protein was reported as positive control for the action of metformin on MPM cells. *(a-b)* Densitometric analysis calculated relative to β-actin protein (loading control). Results are the mean ± SEM of 3 experiments (* compared with the control group. *$P < 0.005$).

3. Notch-1 activation is increased in MPM cells

To investigate if Notch-1 could be a specific target of metformin action, we first evaluated the Notch1 basal level in both MPM cell lines. We detected the activation level of Notch1 signaling both in MMP89 and IST-Mes2 cell lines by western blot analysis. As shown in the blot (Figure 8) in both MPM cell lines we observed higher levels of the active form of Notch-1 (Cleaved Notch1/N1ICD) compared to HM cells. Notch-1 protein expression was detected by using a specific antibody directed against the C-terminal of Notch-1 and able to recognize the full length (FL), truncated transmembrane form (TM) and an antibody specific for the active form of Notch-1, as shown in the blot in the figure 8A.

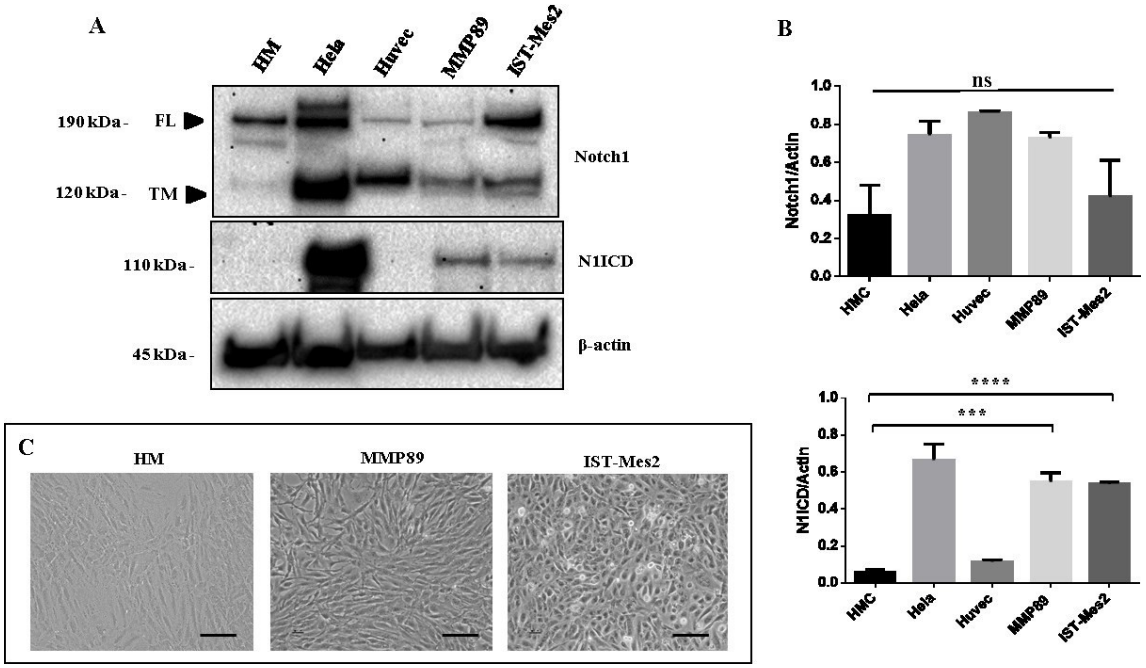


Fig. 8: Expression and activation level of Notch-1 in primary HM cells and MPM cell lines. (A) Western blot analysis of Notch-1 (FL and TM) and its active form cleaved Notch-1 (N1ICD). The blot shows the upregulation of N1ICD form in MPM cell lines compared with primary HM cells, where Notch-1 is expressed, but its activation is absent. The human cervical cancer cells (HELA) and Human Umbilical Vein Endothelial Cells (HUVEC) were

used as positive controls for Notch-1 protein and its active form. Protein samples (30 µg) were separated by 4-12% SDS-PAGE. (B) Densitometric analysis of the bands normalized to the β-actin band (loading control). Error bars represent the mean ± SEM for three independent experiments. The *P* value was calculated compared to untreated control (***$P < 0.0001$ and ****$P < 0.00001$). *(C)* Cytological features of MPM cells and primary HM cells. The images were captured under optical microscope (10x magnification). Scale bar: 25 µm.

4. Metformin downregulates Notch-1 signaling pathway in MPM cells

Since hyper-activation of Notch-1 is a stimulatory signal for mesothelioma development and progression, we wanted to investigate if the metformin influenced the levels of N1ICD in the MPM cells. Thus, the MMP89 and IST-Mes2 cells were treated with 25 mM metformin for 24h and 48h. After treatment western blot analysis was performed to assess the levels of N1ICD and truncated transmembrane form (TM) of Notch-1 proteins in cell lysates, as shown in Figure 9. Metformin treatment down-regulated N1ICD and Notch-1 protein levels in both MPM cells respect their untreated controls. As explained and shown before (Figure 4), in this thesis it has been used as positive control of action of metformin on MPM cells the antibody against the pRPS6 (Figure 9A).

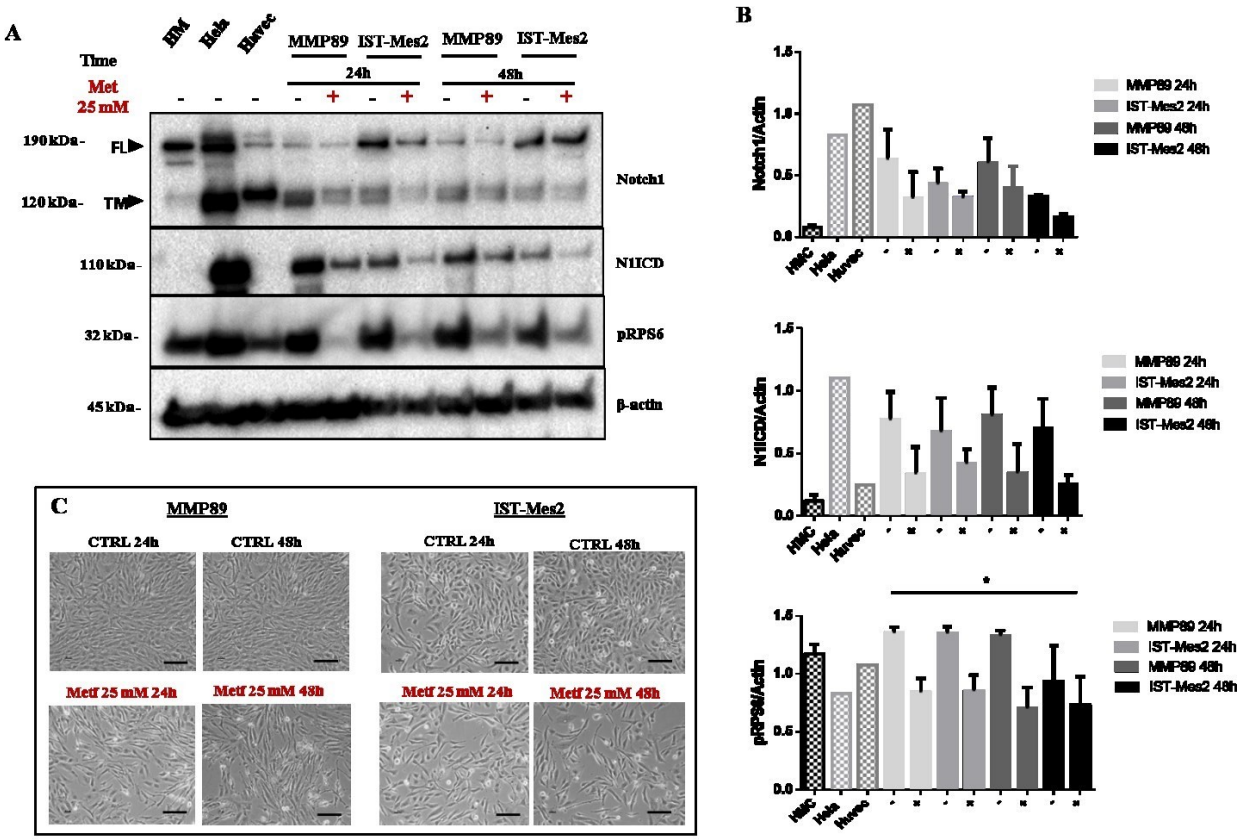


Fig. 9: Effect of metformin treatment on Notch-1 activation in MPM cells. *(A)* Western blot analysis showed downregulation of protein levels of Notch1 and its active form N1ICD after treatment with metformin 25 mM (24 and 48h) in both MPM cell lines, respect their untreated counterpart. pRPS6 antibody was used as positive control for the action of metformin. The human cervical cancer cells (HELA) and Human Umbilical Vein Endothelial Cells (HUVEC) were used as positive controls for Notch-1 protein and its active form. Protein samples (30 µg) were separated by 4-12% SDS-PAGE. *(B)* Densitometric analysis of the bands normalized to the β-actin protein band (loading control). Error bars represent the mean ± SEM for three independent experiments. The *P* value was calculated compared to untreated control. (**P* < 0.005). *(C)* Cytological features of untreated MPM cells and altered confluence and morphology of cells have been shown after metformin treatment. The images were captured under optical microscope (10x magnification). Scale bar: 25 µm.

5. Notch-1 inhibition in MPM cells with DAPT treatment

After evaluating the activation level of Notch-1 in MPM cells, it has been investigated whether γ-secretase inhibitor DAPT could prevent the release of the Notch-1 intracellular domain (N1ICD) in MPM cells. Western blot analysis showed that level of Notch-1 protein were downregulated by DAPT treatment in MMP89 and IST-Mes2 cells (Figure 10A). This finding indicated that the Notch-1 activation was efficiently suppressed by DAPT treatment in both MPM cell lines in a dose-dependent manner for 48h. Observing the blot in the figure 10A, DAPT blocked efficiently Notch-1 activation already at a 1 µM concentration. The concentration of 10 µM of γ-secretase inhibitor DAPT was used subsequently to effectively inhibit the Notch-1 activation because showed no cell cytotoxicity.

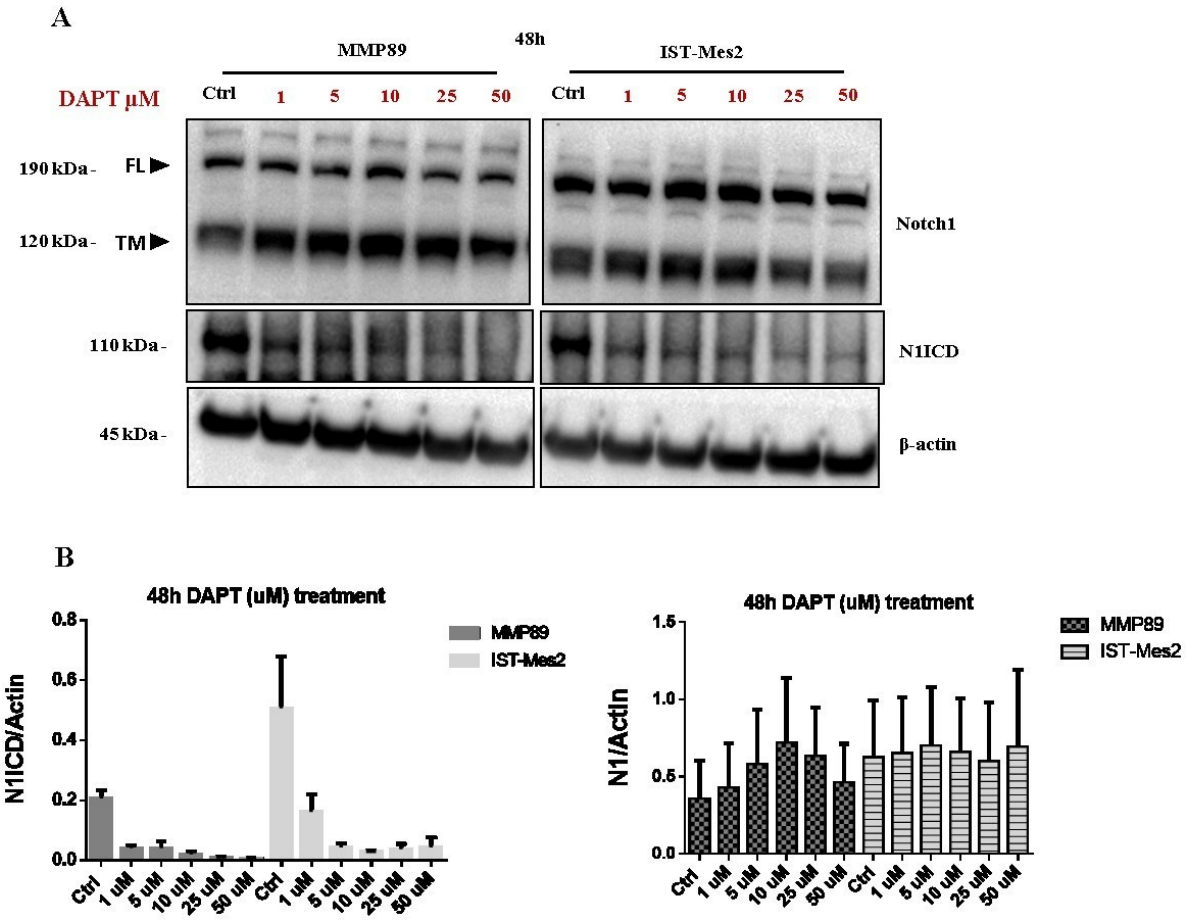


Fig. 10: Optimum treatment conditions to inhibit Notch-1 activation in MPM cells. MPM cell lines were treated with different concentration of DAPT (0, 1, 5, 10, 25 and 50 µM) for 48h. (A) The blot shows the activation level of Notch-1 for each condition of γ-

secretase inhibitor DAPT in MMP89 and IST-Mes2. (B) Densitometric analysis of the bands normalized to the β-actin band (loading control). Error bars represent the mean ± SEM for three independent experiments.

6. DAPT enhances the anti-proliferative effect of metformin on MPM cell lines

I then determined whether γ-secretase inhibitor DAPT was able to sensitize MPM cells to metformin-reduced cell proliferation, improving its anti-proliferative action. Initially MMP89 and IST-Mes2 cells were pre-treated with 10 μM DAPT for 24h and then they were treated with 10 μM DAPT combined with scalar doses of metformin up to 25 mM for 24, 48 and 72h. Cell proliferation was assessed for both MPM cell lines, as shown in the figure 11A and B. Data showed that the treatment with the γ-secretase inhibitor DAPT alone leads to a reduction of cell proliferation in both MPM cell lines, indicating an important implication of Notch-1 activation in MPM survival. When the MPM cells were treated combining DAPT with scalar doses of metformin, there was an evident decrease of cell proliferation in a time- and dose-dependent manner. Specifically, in the MMP89 cell line the proliferation was inhibited up to 48.28%, 47.16% and 23.68% respectively after 24, 48 and 72h of combined treatment with DAPT and 1 mM of metformin. In the IST-Mes2 cell line the proliferation was inhibited up to 48.57%, 46.44% and 51.11% respectively after 24, 48 and 72h of combined treatment with DAPT and 1 mM of metformin. Combined treatments with DAPT and low concentrations of metformin enhanced the metformin-reduced cell proliferation with a percentage comparable to treatments with 25 mM metformin alone as shown in the graphs in figure 2 and figure11.

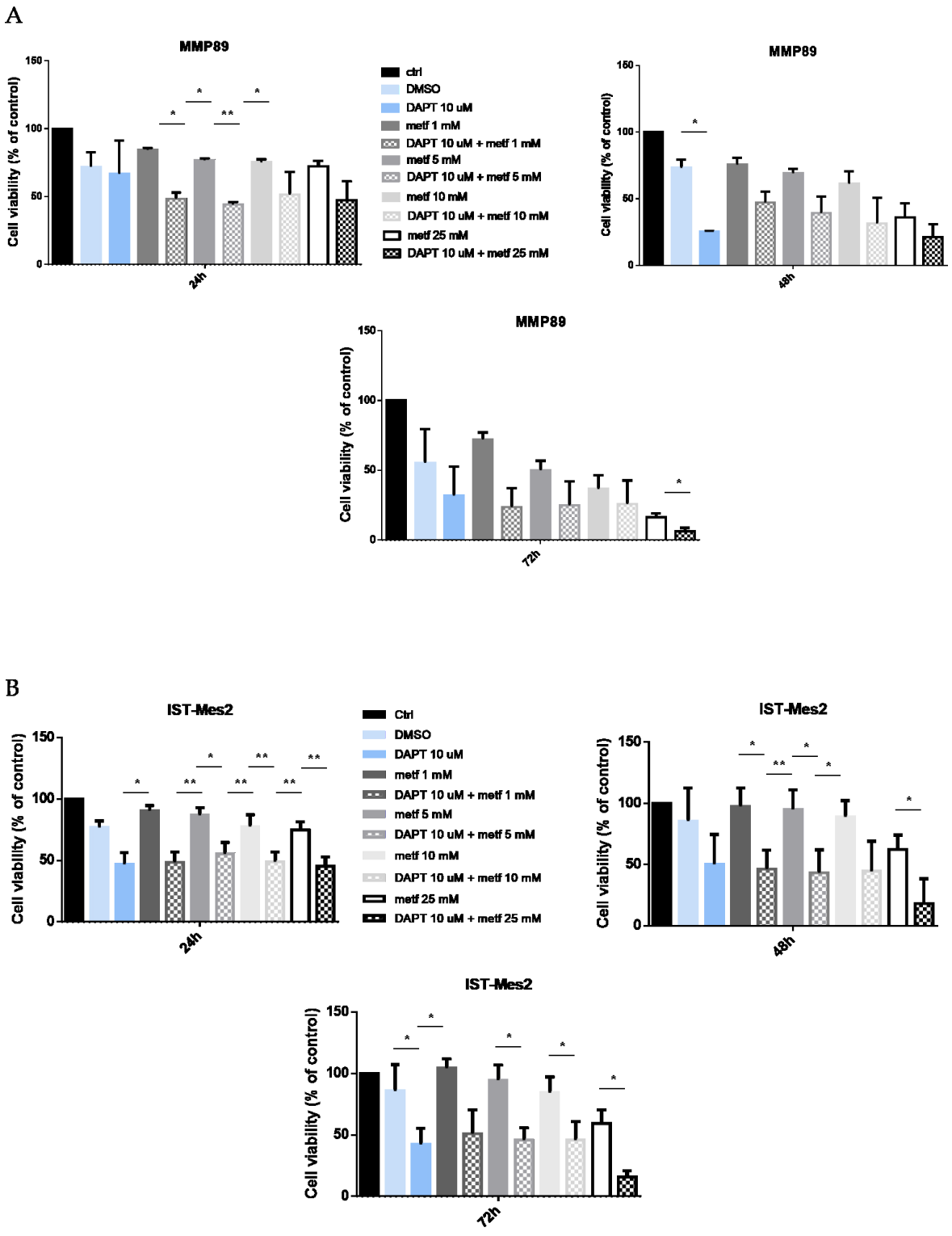


Fig. 11: Synergic effect of DAPT-metformin combination on MPM cell proliferation. MPM cells *(A)* MMP89 and *(B)* IST-Mes2 were treated with different concentrations of metformin (1, 5, 10 and 25 mM) and 10 µM of DAPT for 48h at 37°C. DMSO ≥ 99.9% on was used as the negative control. Error bars represent the mean ± SEM for three independent

experiments. The *P* value was calculated compared to untreated control. (*$P < 0.005$, and **$P < 0.001$).

DISCUSSION

The incidence of MPM has been increasing in the recent years, representing an enormous burden on public health worldwide, particularly in light of the prevalence of environmental asbestos fibers (7,17). Despite the significant progress in elucidating the molecular mechanisms of MPM pathogenesis, current screening tools detect the MPM in advanced stages with very underdog prognosis and poor overall survival (30,161). However, a deeper understanding of MPM pathogenesis has highlighted additional potential targets for therapy, together with new strategies and drugs under development, could determine the response to treatments, improving the outcome of MPM patients.

Due to the many roles that Notch signaling plays in normal development and postnatally, alterations of Notch-1 pathway have been described extensively in multiple human solid tumors (92) and hematological malignancies (116). Notch signaling is also implicated in MPM (117). In this study, we confirmed that Notch-1 activation is dysregulated in MPM cells (Figure 8).

In the present thesis, I also investigated the *in vitro* antineoplastic effect of anti-diabetic drug metformin on MPM cells, evaluating the possible role of the Notch-1 signaling as mediator of the mechanism of action of metformin.

In this context, epidemiologic evidence show that diabetes is strongly associated with both cancer incidence and mortality (194,196) and many retrospective analysis have shown that metformin possesses antineoplastic properties, improving survival in the treatment of diabetic patients with several types of tumors (190), with less toxicity compared to existing anti-cancer drugs. Currently, for lung cancer, metformin has been suggested to be a useful adjuvant agent to radio- and chemotherapy and most studies have indicated improved prognosis of lung cancer of diabetic patients treated with metformin, though the results are still inconsistent among different studies (236). To date, there are no studies reporting the association between metformin treatment and MPM progression. Only one study has investigated the association between the effect of metformin and survival in people with type 2 diabetes mellitus (T2DM) affected by MPM, compared to untreated diabetic ones with MPM. However, this investigation did not provide conclusive data, due to restricted sample size and unavailable information related to MPM stage and histological subtype (212).

In the present thesis, the relevance *in vitro* of antineoplastic activity of metformin on MPM cells, reported a particular interest in investigation of the oncogenic Notch-1 signaling pathway involved in mediating the reduction of MPM cell survival after treatment. For this purpose, I used as experimental models two MPM cell lines, representing the two different

histological subtypes, sarcomatoid MMP89 and epithelioid IST-Mes2 cells. I first conducted cell proliferation assay in MPM cells treated with metformin. In agreement with other previous reports in other tumor cell lines (237,238), metformin inhibited cell proliferation in a dose- and time-dependent manner (Figure 2). It has been widely reported that the major molecular mechanism of anti-proliferative effect of metformin on cancer cells is correlated with the increased activation of AMPK and inhibition of mTOR downstream signaling pathway, with a decrease in phosphorylation of S6K, RPS6 and 4E-BP1 (208,211). The mTOR pathway regulates cell growth, proliferation and survival (239). Consistently with some reports in literature, after treatment with metformin, I found a downregulation of RPS6phosphorylation in both MPM cell lines (Figure 4).

Apoptosis is an essential physiological process of cell death responsible for deletion of cells in normal tissue maintain proper cell homeostasis. However, defects in apoptotic pathways are now though to influence and to be essential for sustaining malignant phenotype with cancer cell proliferation. Most anticancer agents now in use were developed to selectively kill tumor cells, through the disruption of apoptotic programs (240). There are no published findings showing that metformin induces apoptosis in MPM. The results of this thesis revealed apoptotic effects of metformin in both MPM cell lines. In particular, in the Ist-mes2 cell line the response was dose-dependent (Figure 5). At molecular level, this effect was validated by the downregulation of anti-apoptotic protein Bcl-2 in both MPM cell lines. Moreover, a decrease of pro-apoptotic protein Noxa and the total forms of caspase-3, 8, 9 and PARP has been observed, suggesting the possible upregulation of their respective active forms following metformin treatments (Figure 7A-B), with no significant difference between the two subtypes. I then performed experiments to check the involvement of Notch-1 in the mechanism of action of metformin on MPM cells. In each experiment, after treatment with 25 mM of metformin, the activation level of Notch-1 (N1ICD – intracellular domain) was characterized by western blot analysis (Figure 9A). It was possible thus to observe a clear modulation of N1ICD, decreasing in both MPM cell lines treated with metformin added to their growth medium, thus suggesting a potential role of Notch-1 signaling in the mechanism of action of metformin.

Recent studies demonstrate that Notch signaling pathway may play critical roles in the regulation of anti-cancer drug-sensitivity and resistance. For these observations, the role of Notch-1 activation in both MMP89 and IST-Mes2 cell proliferation was investigated, through the inhibition of γ-secretase enzymatic activity with the DAPT inhibitor (Figure

10A). Indeed, γ-secretase is a critical proteinase for Notch-1 protein activation via nicastrin ectodomain binding to the N-terminus of Notch-1 protein and cleavage of Notch-1 (94). After treatment with scalar doses of γ-secretase inhibitor DAPT, it was possible observe a block of Notch-1 activation and thus a decreased levels of N1ICD in a dose-dependent manner (Figure 10A-B), showing the efficacy and specificity of this molecule in inhibiting Notch-1 activation in both MPM cell lines. Experiments were performed to investigate the combination of metformin and DAPT on the proliferation rate of MPM cells and the involvement of Notch-1 in this biological process. The pretreatment with γ-secretase inhibitor DAPT (10 μM) and the following treatment with metformin (scalar doses) induced an important reduction of MPM cell proliferation rate (Figure 11) compared to treatment with metformin only. Future studies need to confirm these current data before translating into *in vivo* investigations and then in clinical trials.

Downregulation of Notch-1 by multiple approaches appears to be a novel strategy for increasing drug-sensitivity of MPM cells to conventional chemotherapeutics. The inhibition of MPM cell proliferation and the induction of apoptosis could confirm a clear link between Notch-1 and metformin in MPM cells.

To date, metformin is still widely investigated by clinical trials for the treatment of an increasing number of human cancers (ClinicalTrial.gov; March 2018). This thesis provides new data on a novel therapeutic strategy based on the combination of two agents to treat MPM. Specifically, these results showed an additive stronger effect of metformin-DAPT combination compared to metformin alone, which has the value in reducing the chemotherapeutic agent dose.

In conclusion, our data showed that: (i) metformin inhibits MPM cell proliferation; (ii) metformin induces apoptosis in MPM cells; (iii) Notch-1 is dysregulated in two MPM cell lines compared to normal human mesothelial (HM) cells; (iv) metformin down-regulates Notch-1 and (v) the inhibition of Notch-1 by γ-secretase inhibitor DAPT induces an increase of MPM drug-sensitivity to metformin treatments.

These results suggest that metformin inhibits MPM cell proliferation by downregulating Notch-1 and induces programmed death in MPM cells. Hence, Notch-1 and metformin might represent novel targets for MPM therapy.

These interesting data lead to formulations of additional hypothesis and strategies that will be the subject of future works.

The *in vitro* results presented in this thesis could demonstrate a new molecular mechanism, among the others already studied and extensively explained, by which metformin performs its anti-proliferative action. Further studies will aim to find explanation about the modulation of Notch-1 signaling in response to metformin, a drug currently used worldwide against the type 2 diabetes for over a century.

Chemotherapy is the central pillar of systemic therapy for MPM and the goal today is to develop novel targeted chemotherapy agents, to be used either alone or in combination to increase efficacy and to minimize and/or avoid side effects (157,199,202). At the same time preclinical research together with clinical trials is investigating novel, more specific approaches: the combination of inhibition Notch-1 signaling with metformin could represent one of these new therapeutic approaches to treat MPM.

BIBLIOGRAPHY

1. Driscoll T, Nelson DI, Steenland K, Leigh J, Concha-Barrientos M, Fingerhut M, Prüss-Ustün A. The global burden of disease due to occupational carcinogens. *Am J Ind Med* (2005) **48**:419–31. doi:10.1002/ajim.20209
2. Grondin SC, Sugarbaker DJ. Malignant mesothelioma of the pleural space. *Oncol* (1999)
3. Mao W, Zhang X, Guo Z, Gao Z, Pass HI, Yang H, Carbone M. Association of asbestos exposure with malignant mesothelioma incidence in eastern China. *JAMA Oncol* (2017) doi:10.1001/jamaoncol.2016.5487
4. Carbone M, Kanodia S, Chao A, Miller A, Wali A, Weissman D, Adjei A, Baumann F, Boffetta P, Buck B, et al. Consensus report of the 2015 Weinman international conference on mesothelioma. *J Thorac Oncol* (2016) doi:10.1016/j.jtho.2016.04.028
5. Baumann F, Ambrosi JP, Carbone M. Asbestos is not just asbestos: An unrecognised health hazard. *Lancet Oncol* (2013) doi:10.1016/S1470-2045(13)70257-2
6. Qi F, Okimoto G, Jube S, Napolitano A, Pass HI, Laczko R, Demay RM, Khan G, Tiirikainen M, Rinaudo C, et al. Continuous exposure to chrysotile asbestos can cause transformation of human mesothelial cells via HMGB1 and TNF-α signaling. *Am J Pathol* (2013) doi:10.1016/j.ajpath.2013.07.029
7. Baumann F, Buck BJ, Metcalf R V., Mclaurin BT, Merkler DJ, Carbone M. The presence of asbestos in the natural environment is likely related to mesothelioma in young individuals and women from Southern Nevada. *J Thorac Oncol* (2015) doi:10.1097/JTO.0000000000000506
8. Chen Z, Gaudino G, Pass HI, Carbone M, Yang H. Diagnostic and prognostic biomarkers for malignant mesothelioma: an update. *Transl Lung Cancer Res* (2017) doi:10.21037/tlcr.2017.05.06
9. Carbone M, Bedrossian CWM. The pathogenesis of mesothelioma. *Semin Diagn Pathol* (2006) doi:10.1053/j.semdp.2006.08.002
10. Carbone M, Baris YI, Bertino P, Brass B, Comertpay S, Dogan AU, Gaudino G, Jube S, Kanodia S, Partridge CR, et al. Erionite exposure in North Dakota and Turkish villages with mesothelioma. *Proc Natl Acad Sci* (2011) doi:10.1073/pnas.1105887108
11. Kadariya Y, Menges CW, Talarchek J, Cai KQ, Klein-Szanto AJ, Pietrofesa RA, Christofidou-Solomidou M, Cheung M, Mossman BT, Shukla A, et al.

Inflammation-related IL1β/IL1R signaling promotes the development of asbestos-induced malignant mesothelioma. *Cancer Prev Res* (2016) doi:10.1158/1940-6207.CAPR-15-0347

12. Yang H, Bocchetta M, Kroczynska B, Elmishad AG, Chen Y, Liu Z, Bubici C, Mossman BT, Pass HI, Testa JR, et al. TNF- inhibits asbestos-induced cytotoxicity via a NF- B-dependent pathway, a possible mechanism for asbestos-induced oncogenesis. *Proc Natl Acad Sci* (2006) doi:10.1073/pnas.0604008103
13. Sartore-Bianchi A, Gasparri F, Galvani A, Nici L, Darnowski JW, Barbone D, Fennell DA, Gaudino G, Porta C, Mutti L. Bortezomib inhibits nuclear factor-κB-dependent survival and has potent in vivo activity in mesothelioma. *Clin Cancer Res* (2007) doi:10.1158/1078-0432.CCR-07-0536
14. Singh A, Pruett N, Hoang CD. In vitro experimental models of mesothelioma revisited. *Transl Lung Cancer Res* (2017) doi:10.21037/tlcr.2017.04.12
15. Carbone M, Yang H. Mesothelioma: recent highlights. *Ann Transl Med* (2017) doi:10.21037/atm.2017.04.29
16. Butnor KJ, Sharma A, Sporn TA, Roggli VL. Malignant mesothelioma and occupational exposure to asbestos: An analysis of 1445 cases. in *Annals of Occupational Hygiene* doi:10.1093/annhyg/46.suppl-1.150
17. Noonan CW. Environmental asbestos exposure and risk of mesothelioma. *Ann Transl Med* (2017) doi:10.21037/atm.2017.03.74
18. Bolognesi C, Martini F, Tognon M, Filiberti R, Neri M, Perrone E, Landini E, Canessa PA, Ivaldi GP, Betta P, et al. A molecular epidemiology case control study on pleural malignant mesothelioma. *Cancer Epidemiol Biomarkers Prev* (2005) doi:10.1158/1055-9965.EPI-04-0903
19. Cristaudo A, Foddis R, Vivaldi A, Buselli R, Gattini V, Guglielmi G, Cosentino F, Ottenga F, Ciancia E, Libener R, et al. SV40 enhances the risk of malignant mesothelioma among people exposed to asbestos: A molecular epidemiologic case-control study. *Cancer Res* (2005) doi:10.1158/0008-5472.CAN-04-2219
20. Carbone M, Pannuti A, Zhang L, Testa JR, Bocchetta M. A novel mechanism of late gene silencing drives SV40 transformation of human mesothelial cells. *Cancer Res* (2008) doi:10.1158/0008-5472.CAN-08-2332
21. Barbanti-Brodano G, Sabbioni S, Martini F, Negrini M, Corallini A, Tognon M. Simian virus 40 infection in humans and association with human diseases: Results

and hypotheses. *Virology* (2004) doi:10.1016/j.virol.2003.09.004

22. Gazdar AF, Carbone M. Molecular pathogenesis of malignant mesothelioma and its relationship to simian virus 40. *Clin Lung Cancer* (2003) doi:10.3816/CLC.2003.n.031
23. Kroczynska B, Cutrone R, Bocchetta M, Yang H, Elmishad AG, Vacek P, Ramos-Nino M, Mossman BT, Pass HI, Carbone M. Crocidolite asbestos and SV40 are cocarcinogens in human mesothelial cells and in causing mesothelioma in hamsters. *Proc Natl Acad Sci* (2006) doi:10.1073/pnas.0604544103
24. Carbone M, Rizzo P, Pass H. Simian Virus 40: The link with human malignant mesothelioma is well established. *Anticancer Res* (2000) doi:10.1016/j.ocecoaman.2013.07.006
25. Mazzoni E, Corallini A, Cristaudo A, Taronna A, Tassi G, Manfrini M, Comar M, Bovenzi M, Guaschino R, Vaniglia F, et al. High prevalence of serum antibodies reacting with simian virus 40 capsid protein mimotopes in patients affected by malignant pleural mesothelioma. *Proc Natl Acad Sci* (2012) doi:10.1073/pnas.1213238109
26. Ohar JA, Cheung M, Talarchek J, Howard SE, Howard TD, Hesdorffer M, Peng H, Rauscher FJ, Testa JR. Germline BAP1 mutational landscape of asbestos-exposed malignant mesothelioma patients with family history of cancer. *Cancer Res* (2016) doi:10.1158/0008-5472.CAN-15-0295
27. Goodman JE, Nascarella MA, Valberg PA. Ionizing radiation: a risk factor for mesothelioma. *Cancer Causes Control* (2009) doi:10.1007/s10552-009-9357-4
28. Carbone M, Ly BH, Dodson RF, Pagano I, Morris PT, Dogan UA, Gazdar AF, Pass HI, Yang H. Malignant mesothelioma: Facts, Myths, and Hypotheses. *J Cell Physiol* (2012) doi:10.1002/jcp.22724
29. Robinson BM. Malignant pleural mesothelioma: an epidemiological perspective. *Ann Cardiothorac Surg* (2012) doi:10.3978/j.issn.2225-319X.2012.11.04
30. Yang H, Testa JR, Carbone M. Mesothelioma epidemiology, carcinogenesis, and pathogenesis. *Curr Treat Options Oncol* (2008) doi:10.1007/s11864-008-0067-z
31. Husain AN, Colby T, Ordonez N, Krausz T, Attanoos R, Beasley MB, Borczuk AC, Butnor K, Cagle PT, Chirieac LR, et al. Guidelines for pathologic diagnosis of malignant mesothelioma. *Arch Pathol Lab Med* (2009) doi:10.5858/arpa.2012-0214-OA

32. Husain AN, Colby T V., Ordóñez NG, Allen TC, Attanoos RL, Beasley MB, Butnor KJ, Chirieac LR, Churg AM, Dacic S, et al. Guidelines for pathologic diagnosis of Malignant Mesothelioma: 2017 Update of the consensus statement from the International Mesothelioma Interest Group. *Arch Pathol Lab Med* (2018) doi:10.5858/arpa.2017-0124-RA
33. Geltner C, Errhalt P, Baumgartner B, Ambrosch G, Machan B, Eckmayr J, Klikovits T, Hoda MA, Popper H, Klepetko W, et al. Management of malignant pleural mesothelioma – part 1: epidemiology, diagnosis, and staging: Consensus of the Austrian Mesothelioma Interest Group (AMIG). *Wien Klin Wochenschr* (2016) doi:10.1007/s00508-016-1080-z
34. Brims FJH, Meniawy TM, Duffus I, De Fonseka D, Segal A, Creaney J, Maskell N, Lake RA, De Klerk N, Nowak AK. A novel clinical prediction model for prognosis in malignant pleural mesothelioma using decision tree analysis. *J Thorac Oncol* (2016) doi:10.1016/j.jtho.2015.12.108
35. Billé A, Krug LM, Woo KM, Rusch VW, Zauderer MG. Contemporary analysis of prognostic factors in patients with unresectable malignant pleural mesothelioma. *J Thorac Oncol* (2016) doi:10.1016/j.jtho.2015.10.003
36. Missiroli S, Bonora M, Patergnani S, Poletti F, Perrone M, Gafà R, Magri E, Raimondi A, Lanza G, Tacchetti C, et al. PML at Mitochondria-Associated Membranes Is Critical for the Repression of Autophagy and Cancer Development. *Cell Rep* (2016) doi:10.1016/j.celrep.2016.07.082
37. Amoroso F, Salaro E, Falzoni S, Chiozzi P, Giuliani AL, Cavallesco G, Maniscalco P, Puozzo A, Bononi I, Martini F, et al. P2X7 targeting inhibits growth of human mesothelioma. *Oncotarget* (2016) doi:10.18632/oncotarget.10430
38. Bononi A, Giorgi C, Patergnani S, Larson D, Verbruggen K, Tanji M, Pellegrini L, Signorato V, Olivetto F, Pastorino S, et al. BAP1 regulates IP3R3-mediated Ca 2+ flux to mitochondria suppressing cell transformation. *Nature* (2017) doi:10.1038/nature22798
39. Patergnani S, Giorgi C, Maniero S, Missiroli S, Maniscalco P, Bononi I, Martini F, Cavallesco G, Tognon M, Pinton P. The endoplasmic reticulum mitochondrial calcium cross talk is downregulated in malignant pleural mesothelioma cells and plays a critical role in apoptosis inhibition. *Oncotarget* (2015) doi:10.1016/j.foodqual.2017.06.021

40. Varani K, Maniero S, Vincenzi F, Targa M, Stefanelli A, Maniscalco P, Martini F, Tognon M, Borea AP. A3 receptors are overexpressed in pleura from patients with mesothelioma and reduce cell growth via Akt/nuclear factor-??B pathway. *Am J Respir Crit Care Med* (2011) doi:10.1164/rccm.201006-0980OC
41. Balatti V, Maniero S, Ferracin M, Veronese A, Negrini M, Ferrocci G, Martini F, Tognon MG. MicroRNAs dysregulation in human malignant pleural mesothelioma. *J Thorac Oncol* (2011) doi:10.1097/JTO.0b013e31820db125
42. Bononi I, Comar M, Puozzo A, Stendardo M, Boschetto P, Orecchia S, Libener R, Guaschino R, Pietrobon S, Ferracin M, et al. Circulating microRNAs found dysregulated in ex-exposed asbestos workers and pleural mesothelioma patients as potential new biomarkers. *Oncotarget* (2016) doi:10.18632/oncotarget.12408
43. Testa JR, Cheung M, Pei J, Below JE, Tan Y, Sementino E, Cox NJ, Dogan AU, Pass HI, Trusa S, et al. Germline BAP1 mutations predispose to malignant mesothelioma. *Nat Genet* (2011) doi:10.1038/ng.912
44. Carbone M, Ferris LK, Baumann F, Napolitano A, Lum CA, Flores EG, Gaudino G, Powers A, Bryant-Greenwood P, Krausz T, et al. BAP1 cancer syndrome: malignant mesothelioma, uveal and cutaneous melanoma, and MBAITs. *J Transl Med* (2012) doi:10.1186/1479-5876-10-179
45. Carbone M, Yang H, Pass HI, Krausz T, Testa JR, Gaudino G. BAP1 and cancer. *Nat Rev Cancer* (2013) doi:10.1038/nrc3459
46. Yu H, Pak H, Hammond-Martel I, Ghram M, Rodrigue A, Daou S, Barbour H, Corbeil L, Hebert J, Drobetsky E, et al. Tumor suppressor and deubiquitinase BAP1 promotes DNA double-strand break repair. *Proc Natl Acad Sci* (2014) doi:10.1073/pnas.1309085110
47. Baughman JM, Rose CM, Kolumam G, Webster JD, Wilkerson EM, Merrill AE, Rhoads TW, Noubade R, Katavolos P, Lesch J, et al. NeuCode Proteomics Reveals Bap1 Regulation of Metabolism. *Cell Rep* (2016) doi:10.1016/j.celrep.2016.05.096
48. Dai F, Lee H, Zhang Y, Zhuang L, Yao H, Xi Y, Xiao Z-D, You MJ, Li W, Su X, et al. BAP1 inhibits the ER stress gene regulatory network and modulates metabolic stress response. *Proc Natl Acad Sci* (2017) doi:10.1073/pnas.1619588114
49. Bononi A, Yang H, Giorgi C, Patergnani S, Pellegrini L, Su M, Xie G, Signorato V, Pastorino S, Morris P, et al. Germline BAP1 mutations induce a Warburg effect. *Cell Death Differ* (2017) doi:10.1038/cdd.2017.95

50. Harbour JW, Onken MD, Roberson EDO, Duan S, Cao L, Worley LA, Council ML, Matatall KA, Helms C, Bowcock AM. Frequent mutation of BAP1 in metastasizing uveal melanomas. *Science (80-)* (2010) doi:10.1126/science.1194472
51. Nasu M, Emi M, Pastorino S, Tanji M, Powers A, Luk H, Baumann F, Zhang YA, Gazdar A, Kanodia S, et al. High incidence of somatic BAP1 alterations in sporadic malignant mesothelioma. *J Thorac Oncol* (2015) doi:10.1097/JTO.0000000000000471
52. Yoshikawa Y, Sato A, Tsujimura T, Emi M, Morinaga T, Fukuoka K, Yamada S, Murakami A, Kondo N, Matsumoto S, et al. Frequent inactivation of the BAP1 gene in epithelioid-type malignant mesothelioma. *Cancer Sci* (2012) doi:10.1111/j.1349-7006.2012.02223.x
53. Bononi A, Napolitano A, Pass HI, Yang H, Carbone M. Latest developments in our understanding of the pathogenesis of mesothelioma and the design of targeted therapies. *Expert Rev Respir Med* (2015) doi:10.1586/17476348.2015.1081066
54. McGregor SM, Dunning R, Hyjek E, Vigneswaran W, Husain AN, Krausz T. BAP1 facilitates diagnostic objectivity, classification, and prognostication in malignant pleural mesothelioma. *Hum Pathol* (2015) doi:10.1016/j.humpath.2015.06.024
55. Shinozaki-Ushiku A, Ushiku T, Morita S, Anraku M, Nakajima J, Fukayama M. Diagnostic utility of BAP1 and EZH2 expression in malignant mesothelioma. *Histopathology* (2017) doi:10.1111/his.13123
56. Righi L, Duregon E, Vatrano S, Izzo S, Giorcelli J, Rondón-Lagos M, Ascoli V, Ruffini E, Ventura L, Volante M, et al. BRCA1- A ssociated protein 1 (BAP1) immunohistochemical expression as a diagnostic tool in malignant pleural mesothelioma classification: A large retrospective study. *J Thorac Oncol* (2016) doi:10.1016/j.jtho.2016.06.020
57. Carbone M, Flores EG, Emi M, Johnson TA, Tsunoda T, Behner D, Hoffman H, Hesdorffer M, Nasu M, Napolitano A, et al. Combined Genetic and Genealogic Studies Uncover a Large BAP1 Cancer Syndrome Kindred Tracing Back Nine Generations to a Common Ancestor from the 1700s. *PLoS Genet* (2015) doi:10.1371/journal.pgen.1005633
58. Xu J, Kadariya Y, Cheung M, Pei J, Talarchek J, Sementino E, Tan Y, Menges CW, Cai KQ, Litwin S, et al. Germline mutation of Bap1 accelerates development of asbestos-induced malignant mesothelioma. *Cancer Res* (2014) doi:10.1158/0008-

5472.CAN-14-1328

59. Buckler AJ, Klein WM, Bell DW, Lee WC, Altomare DA, Testa JR. pl6 Alterations and Deletion Mapping of 9p21-p22 in Malignant Mesothelioma. *Cancer Res* (1994)
60. Lecomte C, Andujar P, Renier A, Kheuang L, Abramowski V, Mellottee L, Fleury-Feith J, Zucman-Rossi J, Giovannini M, Jaurand MC. Similar tumor suppressor gene alteration profiles in asbestos-induced murine and human mesothelioma. *Cell Cycle* (2005) doi:10.4161/cc.4.12.2300
61. Altomare DA, Menges CW, Xu J, Pei J, Zhang L, Tadevosyan A, Neumann-Domer E, Liu Z, Carbone M, Chudoba I, et al. Losses of both products of the cdkn2a/arf locus contribute to asbestos-induced mesothelioma development and cooperate to accelerate tumorigenesis. *PLoS One* (2011) doi:10.1371/journal.pone.0018828
62. Sneddon S, Patch AM, Dick IM, Kazakoff S, Pearson J V., Waddell N, Allcock RJN, Holt RA, Robinson BWS, Creaney J. Whole exome sequencing of an asbestos-induced wild-type murine model of malignant mesothelioma. *BMC Cancer* (2017) doi:10.1186/s12885-017-3382-6
63. Andujar P, Pairon JC, Renier A, Descatha A, Hysi I, Abd-Alsamad I, Billon-Galland MA, Blons H, Clin B, Danel C, et al. Differential mutation profiles and similar intronic TP53 polymorphisms in asbestos-related lung cancer and pleural mesothelioma. *Mutagenesis* (2013) doi:10.1093/mutage/get008
64. Beltrami S, Kim R, Gordon J. Neurofibromatosis type 2 protein, NF2: An uncoventional cell cycle regulator. *Anticancer Res* (2013) doi:10.21873/anticanres. ???
65. Kumar K, Rahman Q, Schipper H, Matschegewski C, Schiffmann D, Papp T. Mutational analysis of 9 different tumour-associated genes in human malignant mesothelioma cell lines. *Oncol Rep* (2005)
66. Lee AY, Ras DJ, He B, Jablons DM. Update on the molecular biology of malignant mesothelioma. *Cancer* (2007) doi:10.1002/cncr.22552
67. Carbone M, Rizzo P, Grimley PM, Procopio A, Mew DJY, Shridhar V, De Bartolomeis A, Esposito V, Giuliano MT, Steinberg SM, et al. Simian virus-40 large-T antigen binds p53 in human mesotheliomas. *Nat Med* (1997) doi:10.1038/nm0897-908
68. De Luca A, Baldi A, Esposito V, Howard CM, Bagella L, Rizzo P, Caputi M, Pass HI, Giordano GG, Baldi F, et al. The retinoblastoma gene family pRb/p105, p107,

pRb2/p130 and simian virus-40 large T-antigen in human mesotheliomas. *Nat Med* (1997) doi:10.1038/nm0897-913

69. Tognon M, Martini F, Corallini A, Barbanti-Brodano G, Zur Hausen H. SV40 and human cancers (multiple letters). *Int J Cancer* (2004) doi:10.1002/ijc.20150
70. Rizzo P, Bocchetta M, Powers A, Foddis R, Stekala E, Pass HI, Carbone M. SV40 and the pathogenesis of mesothelioma. *Semin Cancer Biol* (2001) doi:10.1006/scbi.2000.0347
71. Marchi S, Pinton P. Alterations of calcium homeostasis in cancer cells. *Curr Opin Pharmacol* (2016) doi:10.1016/j.coph.2016.03.002
72. Marchi S, Rimessi A, Giorgi C, Baldini C, Ferroni L, Rizzuto R, Pinton P. Akt kinase reducing endoplasmic reticulum Ca2+release protects cells from Ca2+-dependent apoptotic stimuli. *Biochem Biophys Res Commun* (2008) doi:10.1016/j.bbrc.2008.07.153
73. Pinton P, Ferrari D, Rapizzi E, Di Virgilio F, Pozzan T, Rizzuto R. The Ca2+ concentration of the endoplasmic reticulum is a key determinant of ceramide-induced apoptosis: Significance for the molecular mechanism of Bcl-2 action. *EMBO J* (2001) doi:10.1093/emboj/20.11.2690
74. Cioce M, Canino C, Goparaju C, Yang H, Carbone M, Pass HI. Autocrine CSF-1R signaling drives mesothelioma chemoresistance via AKT activation. *Cell Death Dis* (2014) doi:10.1038/cddis.2014.136
75. Braun F, De Carné Trécesson S, Bertin-Ciftci J, Juin P. Protect and serve: Bcl-2 proteins as guardians and rulers of cancer cell survival. *Cell Cycle* (2013) doi:10.4161/cc.25972
76. Altomare DA, You H, Xiao GH, Ramos-Nino ME, Skele KL, De Rienzo A, Jhanwar SC, Mossman BT, Kane AB, Testa JR. Human and mouse mesotheliomas exhibit elevated AKT/PKB activity, which can be targeted pharmacologically to inhibit tumor cell growth. *Oncogene* (2005) doi:10.1038/sj.onc.1208744
77. Pinton G, Manente AG, Angeli G, Mutti L, Moro L. Perifosine as a potential novel anti-cancer agent inhibits EGFR/MET-AKT axis in malignant pleural mesothelioma. *PLoS One* (2012) doi:10.1371/journal.pone.0036856
78. Zhou S, Liu L, Li H, Eilers G, Kuang Y, Shi S, Yan Z, Li X, Corson JM, Meng F, et al. Multipoint targeting of the PI3K/mTOR pathway in mesothelioma. *Br J Cancer* (2014) doi:10.1038/bjc.2014.220

79. Ciardiello F, Tortora G. A novel approach in the treatment of cancer: Targeting the epidermal growth factor receptor. *Clin Cancer Res* (2001) doi:10.1158/1078-0432.ccr-06-0059
80. Jänne PA, Taffaro ML, Salgia R, Johnson BE. Inhibition of epidermal growth factor receptor signaling in malignant pleural mesothelioma. *Cancer Res* (2002)
81. Huang L, Cai M, Zhang X, Wang F, Chen L, Xu M, Yang K, Chen Z, Wang X, Fu L. Combinational therapy of crizotinib and afatinib for malignant pleural mesothelioma. *Am J Cancer Res* (2017)
82. Zanella CL, Posada J, Tritton TR, Mossman BT. Asbestos causes stimulation of the extracellular signal-regulated kinase 1 mitogen-activated protein kinase cascade after phosphorylation of the epidermal growth factor receptor. *Cancer Res* (1996)
83. Strizzi L, Catalano A, Vianale G, Orecchia S, Casalini A, Tassi G, Puntoni R, Mutti L, Procopio A. Vascular endothelial growth factor is an autocrine growth factor in human malignant mesothelioma. *J Pathol* (2001) doi:10.1002/path.824
84. Ohta Y, Shridhar V, Bright RK, Kalemkerian GP, Du W, Carbone M, Watanabe Y, Pass HI. VEGF and VEGF type C play an important role in angiogenesis and lymphangiogenesis in human malignant mesothelioma tumours. *Br J Cancer* (1999) doi:10.1038/sj.bjc.6690650
85. Carbone M, Yang H. Molecular pathways: Targeting mechanisms of asbestos and erionite carcinogenesis in mesothelioma. *Clin Cancer Res* (2012) doi:10.1158/1078-0432.CCR-11-2259
86. Pellegrini L, Xue J, Larson D, Pastorino S, Jube S, Forest KH, Saad-Jube ZS, Napolitano A, Pagano I, Negi VS, et al. HMGB1 targeting by ethyl pyruvate suppresses malignant phenotype of human mesothelioma. *Oncotarget* (2017) doi:10.18632/oncotarget.15152
87. Jube S, Rivera ZS, Bianchi ME, Powers A, Wang E, Pagano I, Pass HI, Gaudino G, Carbone M, Yang H. Cancer cell secretion of the DAMP protein HMGB1 supports progression in malignant mesothelioma. *Cancer Res* (2012) doi:10.1158/0008-5472.CAN-11-3481
88. Comar M, Zanotta N, Bonotti A, Tognon M, Negro C, Cristaudo A, Bovenzi M. Increased levels of C-C chemokine RANTES in asbestos exposed workers and in malignant mesothelioma patients from an hyperendemic area. *PLoS One* (2014) doi:10.1371/journal.pone.0104848

89. Nishimura Y, Maeda M, Kumagai-Takei N, Lee S, Matsuzaki H, Wada Y, Nishiike-Wada T, Iguchi H, Otsuki T. Altered functions of alveolar macrophages and NK cells involved in asbestos-related diseases. *Environ Health Prev Med* (2013) doi:10.1007/s12199-013-0333-y

90. Nishimura Y, Kumagai-Takei N, Matsuzaki H, Lee S, Maeda M, Kishimoto T, Fukuoka K, Nakano T, Otsuki T. Functional Alteration of Natural Killer Cells and Cytotoxic T Lymphocytes upon Asbestos Exposure and in Malignant Mesothelioma Patients. *Biomed Res Int* (2015) doi:10.1155/2015/238431

91. Napolitano A, Antoine DJ, Pellegrini L, Baumann F, Pagano I, Pastorino S, Goparaju CM, Prokrym K, Canino C, Pass HI, et al. HMGB1 and its hyperacetylated isoform are sensitive and specific serum biomarkers to detect asbestos exposure and to identify mesothelioma patients. *Clin Cancer Res* (2016) doi:10.1158/1078-0432.CCR-15-1130

92. Bigas A, Espinosa L. The multiple usages of Notch signaling in development, cell differentiation and cancer. *Curr Opin Cell Biol* (2018) doi:10.1016/j.ceb.2018.06.010

93. Bray SJ. Notch signalling: A simple pathway becomes complex. *Nat Rev Mol Cell Biol* (2006) doi:10.1038/nrm2009

94. Bray SJ, Gomez-Lamarca M. Notch after cleavage. *Curr Opin Cell Biol* (2018) doi:10.1016/j.ceb.2017.12.008

95. Siebel C, Lendahl U. Notch Signaling in Development, Tissue Homeostasis, and Disease. *Physiol Rev* (2017) doi:10.1152/physrev.00005.2017

96. Lai EC. Notch signaling: control of cell communication and cell fate. *Development* (2004) doi:10.4244/EIJV8I5A98

97. Ray WJ, Yao M, Mumm J, Schroeter EH, Saftig P, Wolfe M, Selkoe DJ, Kopan R, Goate AM. Cell surface presenilin-1 participates in the γ-secretase-like proteolysis of Notch. *J Biol Chem* (1999) doi:10.1074/jbc.274.51.36801

98. Nam Y, Weng AP, Aster JC, Blacklow SC. Structural requirements for assembly of the CSL·intracellular Notch1·Mastermind-like 1 transcriptional activation complex. *J Biol Chem* (2003) doi:10.1074/jbc.M301567200

99. Kopan R, Ilagan MXG. The Canonical Notch Signaling Pathway: Unfolding the Activation Mechanism. *Cell* (2009) doi:10.1016/j.cell.2009.03.045

100. Borggrefe T, Liefke R. Fine-tuning of the intracellular canonical Notch signaling

pathway. *Cell Cycle* (2012) doi:10.4161/cc.11.2.18995

101. D'Souza B, Meloty-Kapella L, Weinmaster G. *Canonical and non-canonical notch ligands*. (2010). doi:10.1016/S0070-2153(10)92003-6
102. Sanalkumar R, Dhanesh SB, James J. Non-canonical activation of Notch signaling/target genes in vertebrates. *Cell Mol Life Sci* (2010) doi:10.1007/s00018-010-0391-x
103. Ayaz F, Osborne BA. Non-Canonical Notch Signaling in Cancer and Immunity. *Front Oncol* (2014) doi:10.3389/fonc.2014.00345
104. Traustadóttir GÁ, Jensen CH, Garcia Ramirez JJ, Beck HC, Sheikh SP, Andersen DC. The non-canonical NOTCH1 ligand Delta-like 1 homolog (DLK1) self interacts in mammals. *Int J Biol Macromol* (2017) doi:10.1016/j.ijbiomac.2017.01.067
105. Gordon WR, Arnett KL, Blacklow SC. The molecular logic of Notch signaling - a structural and biochemical perspective. *J Cell Sci* (2008) doi:10.1242/jcs.035683
106. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: Cell fate control and signal integration in development. *Science (80-)* (1999) doi:10.1126/science.284.5415.770
107. Wang K, Zhang Q, Li D, Ching K, Zhang C, Zheng X, Ozeck M, Shi S, Li X, Wang H, et al. PEST domain mutations in Notch receptors comprise an oncogenic driver segment in triple-negative breast cancer sensitive to a γ-secretase inhibitor. *Clin Cancer Res* (2015) doi:10.1158/1078-0432.CCR-14-1348
108. Kushwah R, Guezguez B, Lee JB, Hopkins CI, Bhatia M. Pleiotropic roles of Notch signaling in normal, malignant, and developmental hematopoiesis in the human. *EMBO Rep* (2014) doi:10.15252/embr.201438842
109. Gu Y, Masiero M, Banham AH. Notch signaling: its roles and therapeutic potential in hematological malignancies. *Oncotarget* (2016) doi:10.18632/oncotarget.7772
110. Brzozowa-Zasada M, Piecuch A, Michalski M, Segiet O, Kurek J, Harabin-Słowińska M, Wojnicz R. Notch and its oncogenic activity in human malignancies. *Eur Surg - Acta Chir Austriaca* (2017) doi:10.1007/s10353-017-0491-z
111. Westhoff B, Colaluca IN, D'Ario G, Donzelli M, Tosoni D, Volorio S, Pelosi G, Spaggiari L, Mazzarol G, Viale G, et al. Alterations of the Notch pathway in lung cancer. *Proc Natl Acad Sci* (2009) doi:10.1073/pnas.0907781106
112. Zhu H, Zhou X, Redfield S, Lewin J, Miele L. Elevated Jagged-1 and Notch-1 expression in high grade and metastatic prostate cancers. *Am J Transl Res* (2013)

doi:10.1021/acs.est.5b03595

113. Brzozowa M, Mielanczyk L, Michalski M, Malinowski L, Kowalczyk-Ziomek G, Helewski K, Harabin-Slowinska M, Wojnicz R. Role of Notch signaling pathway in gastric cancer pathogenesis. *Wspolczesna Onkol* (2013) doi:10.5114/wo.2013.33765
114. Rose SL, Kunnimalaiyaan M, Drenzek J, Seiler N. Notch 1 signaling is active in ovarian cancer. *Gynecol Oncol* (2010) doi:10.1016/j.ygyno.2009.12.003
115. Zhang Y, Li B, Ji ZZ, Zheng PS. Notch1 regulates the growth of human colon cancers. *Cancer* (2010) doi:10.1002/cncr.25449
116. Weng AP, Ferrando AA, Lee W, Morris IV JP, Silverman LB, Sanchez-Irizarry C, Blacklow SC, Look AT, Aster JC. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science (80-)* (2004) doi:10.1126/science.1102160
117. Graziani I, Eliasz S, De Marco MA, Chen Y, Pass HI, De May RM, Strack PR, Miele L, Bocchetta M. Opposite effects of Notch-1 and Notch-2 on mesothelioma cell survival under hypoxia are exerted through the Akt pathway. *Cancer Res* (2008) doi:10.1158/0008-5472.CAN-08-0969
118. Nicolas M, Wolfer A, Raj K, Kummer JA, Mill P, Van Noort M, Hui C chung, Clevers H, Dotto GP, Radtke F. Notch1 functions as a tumor suppressor in mouse skin. *Nat Genet* (2003) doi:10.1038/ng1099
119. Nowell CS, Radtke F. Notch as a tumour suppressor. *Nat Rev Cancer* (2017) doi:10.1038/nrc.2016.145
120. Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD, Sklar J. TAN-1, the human homolog of the Drosophila Notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* (1991) doi:10.1016/0092-8674(91)90111-B
121. Bocchetta M, Miele L, Pass HI, Carbone M. Notch-1 induction, a novel activity of SV40 required for growth of SV40-transformed human mesothelial cells. *Oncogene* (2003) doi:10.1038/sj.onc.1206097
122. Bocchetta M, Di Resta I, Powers A, Fresco R, Tosolini A, Testa JR, Pass HI, Rizzo P, Carbone M, Resta I Di, et al. Human mesothelial cells are unusually susceptible to simian virus 40-mediated transformation and asbestos cocarcinogenicity. *Proc Natl Acad Sci U S A* (2000) doi:10.1073/pnas.170207097
123. Carbone M, Burck C, Rdzanek M, Rudzinski J, Cutrone R, Bocchetta M. Different

susceptibility of human mesothelial cells to polyomavirus infection and malignant transformation. *Cancer Res* (2003)

124. Lathion S, Schaper J, Beard P, Raj K. Notch1 Can Contribute to Viral-Induced Transformation of Primary Human Keratinocytes. *Cancer Res* (2003)
125. Baron M. An overview of the Notch signalling pathway. *Semin Cell Dev Biol* (2003) doi:10.1016/S1084-9521(02)00179-9
126. Blokzijl A, Dahlqvist C, Reissmann E, Falk A, Moliner A, Lendahl U, Ibáñez CF. Cross-talk between the Notch and TGF-?? signaling pathways mediated by interaction of the Notch intracellular domain with Smad3. *J Cell Biol* (2003) doi:10.1083/jcb.200305112
127. Gopalakrishnan N, Sivasithamparam ND, Devaraj H. Synergistic association of Notch and NFκB signaling and role of Notch signaling in modulating epithelial to mesenchymal transition in colorectal adenocarcinoma. *Biochimie* (2014) doi:10.1016/j.biochi.2014.09.020
128. Liu ZJ, Xiao M, Balint K, Smalley KSM, Brafford P, Qiu R, Pinnix CC, Li X, Herlyn M. Notch1 signaling promotes primary melanoma progression by activating mitogen-activated protein kinase/phosphatidylinositol 3-kinase-Akt pathways and up-regulating N-cadherin expression. *Cancer Res* (2006) doi:10.1158/0008-5472.CAN-05-3589
129. Ingram WJ, McCue KI, Tran TH, Hallahan AR, Wainwright BJ. Sonic Hedgehog regulates Hes1 through a novel mechanism that is independent of canonical Notch pathway signalling. *Oncogene* (2008) doi:10.1038/sj.onc.1210767
130. Efferson CL, Winkelmann CT, Ware C, Sullivan T, Giampaoli S, Tammam J, Patel S, Mesiti G, Reilly JF, Gibson RE, et al. Downregulation of Notch pathway by a γ-secretase inhibitor attenuates akt/mammalian target of rapamycin signaling and glucose uptake in an ERBB2 transgenic breast cancer model. *Cancer Res* (2010) doi:10.1158/0008-5472.CAN-09-3114
131. Rizzo P, Miao H, D'Souza G, Osipo C, Yun J, Zhao H, Mascarenhas J, Wyatt D, Antico G, Hao L, et al. Cross-talk between notch and the estrogen receptor in breast cancer suggests novel therapeutic approaches. *Cancer Res* (2008) doi:10.1158/0008-5472.CAN-07-5744
132. Purow BW, Sundaresan TK, Burdick MJ, Kefas BA, Comeau LD, Hawkinson MP, Su Q, Kotliarov Y, Lee J, Zhang W, et al. Notch-1 regulates transcription of the

epidermal growth factor receptor through p53. *Carcinogenesis* (2008) doi:10.1093/carcin/bgn079

133. Majidinia M, Darband SG, Kaviani M, Nabavi SM, Jahanban-Esfahlan R, Yousefi B. Cross-regulation between Notch signaling pathway and miRNA machinery in cancer. *DNA Repair (Amst)* (2018) doi:10.1016/j.dnarep.2018.04.002
134. Guruharsha KG, Kankel MW, Artavanis-Tsakonas S. The Notch signalling system: Recent insights into the complexity of a conserved pathway. *Nat Rev Genet* (2012) doi:10.1038/nrg3272
135. Bibby AC, Tsim S, Kanellakis N, Ball H, Talbot DC, Blyth KG, Maskell NA, Psallidas I. Malignant pleural mesothelioma: An update on investigation, diagnosis and treatment. *Eur Respir Rev* (2016) doi:10.1183/16000617.0063-2016
136. Cao C, Tian D, Park J, Allan J, Pataky KA, Yan TD. A systematic review and meta-analysis of surgical treatments for malignant pleural mesothelioma. *Lung Cancer* (2014) doi:10.1016/j.lungcan.2013.11.026
137. Rusch VW, Giroux D, Kennedy C, Ruffini E, Cangir AK, Rice D, Pass H, Asamura H, Waller D, Edwards J, et al. Initial analysis of the international association for the study of lung cancer mesothelioma database. *J Thorac Oncol* (2012) doi:10.1097/JTO.0b013e31826915f1
138. Taioli E, Wolf AS, Camacho-Rivera M, Kaufman A, Lee DS, Nicastri D, Rosenzweig K, Flores RM. Determinants of survival in malignant pleural mesothelioma: A surveillance, epidemiology, and end results (SEER) Study of 14,228 Patients. *PLoS One* (2015) doi:10.1371/journal.pone.0145039
139. Butchart EG, Ashcroft T, Barnsley WC, Holden MP. Pleuropneumonectomy in the management of diffuse malignant mesothelioma of the pleura. Experience with 29 patients. *Thorax* (1976) doi:10.1136/thx.31.1.15
140. Flores RM, Pass HI, Seshan VE, Dycoco J, Zakowski M, Carbone M, Bains MS, Rusch VW. Extrapleural pneumonectomy versus pleurectomy/decortication in the surgical management of malignant pleural mesothelioma: Results in 663 patients. *J Thorac Cardiovasc Surg* (2008) doi:10.1016/j.jtcvs.2007.10.054
141. Treasure T, Lang-Lazdunski L, Waller D, Bliss JM, Tan C, Entwisle J, Snee M, O'Brien M, Thomas G, Senan S, et al. Extra-pleural pneumonectomy versus no extra-pleural pneumonectomy for patients with malignant pleural mesothelioma: clinical outcomes of the Mesothelioma and Radical Surgery (MARS) randomised

feasibility study. *Lancet Oncol* (2011) doi:10.1016/S1470-2045(11)70149-8

142. Lapidot M, Freyaldenhoven S, Bueno R. New concepts in the treatment of malignant pleural mesothelioma. *J Thorac Dis* (2018) doi:10.21037/jtd.2018.02.75
143. Ung YC, Yu E, Falkson C, Haynes AE, Stys-Norman D, Evans WK. The role of radiation therapy in malignant pleural mesothelioma: A systematic review. *Radiother Oncol* (2006) doi:10.1016/j.radonc.2006.06.002
144. Rosenzweig KE. Malignant pleural mesothelioma: adjuvant therapy with radiation therapy. *Ann Transl Med* (2017) doi:10.21037/atm.2017.06.25
145. Clive AO, Taylor H, Dobson L, Wilson P, de Winton E, Panakis N, Pepperell J, Howell T, Stewart SA, Penz E, et al. Prophylactic radiotherapy for the prevention of procedure-tract metastases after surgical and large-bore pleural procedures in malignant pleural mesothelioma (SMART): a multicentre, open-label, phase 3, randomised controlled trial. *Lancet Oncol* (2016) doi:10.1016/S1470-2045(16)30095-X
146. Hiddinga BI, Rolfo C, van Meerbeeck JP. Mesothelioma treatment: Are we on target? A review. *J Adv Res* (2015) doi:10.1016/j.jare.2014.11.012
147. Opitz I. Management of malignant pleural mesothelioma-The European experience. *J Thorac Dis* (2014) doi:10.3978/j.issn.2072-1439.2014.05.03
148. Perrot M de, Wu L, Wu M, Cho BCJ. Radiotherapy for the treatment of malignant pleural mesothelioma. *Lancet Oncol* (2017) doi:10.1016/S1470-2045(17)30459-X
149. Stahel RA, Riesterer O, Xyrafas A, Opitz I, Beyeler M, Ochsenbein A, Früh M, Cathomas R, Nackaerts K, Peters S, et al. Neoadjuvant chemotherapy and extrapleural pneumonectomy of malignant pleural mesothelioma with or without hemithoracic radiotherapy (SAKK 17/04): A randomised, international, multicentre phase 2 trial. *Lancet Oncol* (2015) doi:10.1016/S1470-2045(15)00208-9
150. Bonelli MA, Fumarola C, La Monica S, Alfieri R. New therapeutic strategies for malignant pleural mesothelioma. *Biochem Pharmacol* (2017) doi:10.1016/j.bcp.2016.07.012
151. Tomek S, Emri S, Krejcy K, Manegold C. Chemotherapy for malignant pleural mesothelioma: Past results and recent developments. *Br J Cancer* (2003) doi:10.1038/sj.bjc.6600673
152. Curran D, Sahmoud T, Therasse P, van Meerbeeck J, Postmus PE, Giaccone G. Prognostic factors in patients with pleural mesothelioma: The European

organization for research and treatment of cancer experience. *J Clin Oncol* (1998) doi:10.1200/JCO.1998.16.1.145

153. Vogelzang NJ, Rusthoven JJ, Symanowski J, Denham C, Kaukel E, Ruffie P, Gatzemeier U, Boyer M, Emri S, Manegold C, et al. Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. *J Clin Oncol* (2003) doi:10.1200/JCO.2003.11.136
154. Van Meerbeeck JP, Gaafar R, Manegold C, Van Klaveren RJ, Van Marck EA, Vincent M, Legrand C, Bottomley A, Debruyne C, Giaccone G. Randomized phase III study of cisplatin with or without raltitrexed in patients with malignant pleural mesothelioma: An intergroup study of the European organisation for research and treatment of cancer lung cancer group and the National Cancer Institute . *J Clin Oncol* (2005) doi:10.1200/JCO.20005.14.589
155. Kelly K, Azzoli CG, Zatloukal P, Albert I, Jiang PYZ, Bodkin D, Pereira JR, Juhász E, Iannotti NO, Weems G, et al. Randomized phase 2b study of pralatrexate versus erlotinib in patients with stage IIIB/IV non-small-cell lung cancer (NSCLC) after failure of prior platinum-based therapy. *J Thorac Oncol* (2012) doi:10.1097/JTO.0b013e31824cc66c
156. Blomberg C, Nilsson J, Holgersson G, Edlund P, Bergqvist M, Adwall L, Ekman S, Brattström D, Bergström S. Randomized trials of systemic medically-treated malignant mesothelioma: A systematic review. *Anticancer Res* (2015)
157. Garland LL. Chemotherapy for malignant pleural mesothelioma. *Curr Treat Options Oncol* (2011) doi:10.1007/s11864-011-0152-6
158. Baas P, Fennell D, Kerr KM, van Schil PE, Haas RL, Peters S. Malignant pleural mesothelioma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* (2015) doi:10.1093/annonc/mdv199
159. Ettinger DS, Wood DE, Akerley W, Bazhenova LA, Borghaei H, Camidge DR, Cheney RT, Chirieac LR, D'amico TA, Dilling T, et al. NCCN Guidelines®: Insights malignant pleural mesothelioma, version 3.2016. *JNCCN J Natl Compr Cancer Netw* (2016) doi:10.6004/jnccn.2016.0087
160. Wiggins J, Brilton MG, Darlison L, Entwisle J, Greenstone MA, Hetzel M, Higgs C, Maskell N, Muers MF, Rudd R, et al. BTS statement on malignant mesothelioma in the UK, 2007. *Thorax* (2007) doi:10.1136/thx.2007.087619
161. Mott FE. Mesothelioma: a review. *Ochsner J* (2012)

162. Kishimoto T, Fujimoto N NH. Clinical pathological diagnosis, and treatment for pleural mesothelioma. Japanese. *Japanese J Cancer Chemother (pp 513-517), 2016Date Publ May 2016* (2016)
163. Abdel-Rahman O, Elsayed Z, Mohamed H, Eltobgy M. Radical multimodality therapy for malignant pleural mesothelioma. *Cochrane database Syst Rev* (2018) doi:10.1002/14651858.CD012605.pub2
164. Rolfo C, Castiglia M, Hong D, Alessandro R, Mertens I, Baggerman G, Zwaenepoel K, Gil-Bazo I, Passiglia F, Carreca AP, et al. Liquid biopsies in lung cancer: The new ambrosia of researchers. *Biochim Biophys Acta - Rev Cancer* (2014) doi:10.1016/j.bbcan.2014.10.001
165. Ostroff RM, Mehan MR, Stewart A, Ayers D, Brody EN, Williams SA, Levin S, Black B, Harbut M, Carbone M, et al. Early Detection of Malignant Pleural Mesothelioma in Asbestos-Exposed Individuals with a Noninvasive Proteomics-Based Surveillance Tool. *PLoS One* (2012) doi:10.1371/journal.pone.0046091
166. Creaney J, Dick IM, Robinson BW. Comparison of mesothelin and fibulin-3 in pleural fluid and serum as markers in malignant mesothelioma. *Curr Opin Pulm Med* (2015) doi:10.1097/MCP.0000000000000167
167. Creaney J, Dick IM, Robinson BWS. Discovery of new biomarkers for malignant mesothelioma. *Curr Pulmonol Reports* (2015) doi:10.1007/s13665-015-0106-8
168. Pass HI, Goparaju C, Espin-Garcia O, Donington J, Carbone M, Patel D, Chen Z, Feld R, Cho J, Gadgeel S, et al. Plasma biomarker enrichment of clinical prognostic indices in malignant pleural mesothelioma. *J Thorac Oncol* (2016) doi:10.1016/j.jtho.2016.02.006
169. Mairinger FD, Vollbrecht C, Flom E, Christian D, Schmid K, Kollmeier J, Popper HH. Folic acid phenotype (FAP) is a superior biomarker predicting response to pemetrexed-based chemotherapy in malignant pleural mesothelioma. *Oncotarget* (2017) doi:10.18632/oncotarget.16398
170. Hassan R, Ho M. Mesothelin targeted cancer immunotherapy. *Eur J Cancer* (2008) doi:10.1016/j.ejca.2007.08.028
171. Robinson BWS, Creaney J, Lake R, Nowak A, Musk AW, De Klerk N, Winzell P, Hellstrom KE, Hellstrom I. Mesothelin-family proteins and diagnosis of mesothelioma. *Lancet* (2003) doi:10.1016/S0140-6736(03)14794-0
172. Scherpereel A, Grigoriu B, Conti M, Gey T, Grégoire M, Copin MC, Devos P,

Chahine B, Porte H, Lassalle P. Soluble mesothelin-related peptides in the diagnosis of malignant pleural mesothelioma. *Am J Respir Crit Care Med* (2006) doi:10.1164/rccm.200511-1789OC

173. Pass HI, Wali A, Tang N, Ivanova A, Ivanov S, Harbut M, Carbone M, Allard J. Soluble Mesothelin-Related Peptide Level Elevation in Mesothelioma Serum and Pleural Effusions. *Ann Thorac Surg* (2008) doi:10.1016/j.athoracsur.2007.07.042
174. Lagniau S, Lamote K, van Meerbeeck JP, Vermaelen KY. Biomarkers for early diagnosis of malignant mesothelioma: Do we need another moonshot? *Oncotarget* (2017) doi:10.18632/oncotarget.17910
175. Rapisarda V, Caltabiano R, Musumeci G, Castrogiovanni P, Ferrante M, Ledda C, Lombardo C, Graziano ACE, Cardile V, Loreto C. Analysis of fibulin-3 after exposure to asbestos-like fibers. *Environ Res* (2017) doi:10.1016/j.envres.2017.03.055
176. Murray J. Fibulin-3 as a blood and effusion biomarker for pleural mesothelioma. *Thorax* (2013) doi:10.1136/thoraxjnl-2013-203396
177. Pass HI, Lott D, Lonardo F, Harbut M, Liu Z, Tang N, Carbone M, Webb C, Wali A. Asbestos Exposure, Pleural Mesothelioma, and Serum Osteopontin Levels. *N Engl J Med* (2005) doi:10.1056/NEJMoa051185
178. Grigoriu BD, Scherpereel A, Devos P, Chahine B, Letourneux M, Lebailly P, Grégoire M, Porte H, Copin MC, Lassalle P. Utility of osteopontin and serum mesothelin in malignant pleural mesothelioma diagnosis and prognosis assessment. *Clin Cancer Res* (2007) doi:10.1158/1078-0432.CCR-06-2144
179. Budhu A, Ji J, Wang XW. The clinical potential of microRNAs. *J Hematol Oncol* (2010) doi:10.1186/1756-8722-3-37
180. Reid G. MicroRNAs in mesothelioma: From tumour suppressors and biomarkers to therapeutic targets. *J Thorac Dis* (2015) doi:10.3978/j.issn.2072-1439.2015.04.56
181. Saito Y, Nakaoka T, Saito H. microRNA-34a as a Therapeutic Agent against Human Cancer. *J Clin Med* (2015) doi:10.3390/jcm4111951
182. Kinet V, Halkein J, Dirkx E, De Windt LJ. Cardiovascular extracellular microRNAs: Emerging diagnostic markers and mechanisms of cell-to-cell RNA communication. *Front Genet* (2013) doi:10.3389/fgene.2013.00214
183. Volinia S, Calin GA, Liu C-G, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, et al. A microRNA expression signature of human solid

tumors defines cancer gene targets. *Proc Natl Acad Sci* (2006) doi:10.1073/pnas.0510565103

184. Sethi S, Kong D, Land S, Dyson G, Sakr WA, Sarkar FH. Comprehensive molecular oncogenomic profiling and miRNA analysis of prostate cancer. *Am J Transl Res* (2013) doi:10.1158/1538-7445.AM2012-4599
185. Qi J, Wang J, Katayama H, Sen S, Liu SM. Circulating microRNAs (cmiRNAs) as novel potential biomarkers for hepatocellular carcinoma. *Neoplasma* (2013) doi:10.4149/neo_2013_018
186. Micolucci L, Akhtar MM, Olivieri F, Rippo MR, Procopio AD. Diagnostic value of microRNAs in asbestos exposure and malignant mesothelioma: systematic review and qualitative meta-analysis. *Oncotarget* (2016) doi:10.18632/oncotarget.9686
187. Smith B, Agarwal P, Bhowmick NA. MicroRNA applications for prostate, ovarian and breast cancer in the era of precision medicine. *Endocr Relat Cancer* (2017) doi:10.1530/ERC-16-0525
188. Kao SC, Fulham M, Wong K, Cooper W, Brahmbhatt H, MacDiarmid J, Pattison S, Sagong JO, Huynh Y, Leslie F, et al. A significant metabolic and radiological response after a novel targeted microRNA-based treatment approach in malignant pleural mesothelioma. *Am J Respir Crit Care Med* (2015) doi:10.1164/rccm.201503-0461LE
189. Shah MY, Ferrajoli A, Sood AK, Lopez-Berestein G, Calin GA. microRNA Therapeutics in Cancer — An Emerging Concept. *EBioMedicine* (2016) doi:10.1016/j.ebiom.2016.09.017
190. Zi F, Zi H, Li Y, He J, Shi Q, Cai Z. Metformin and cancer: An existing drug for cancer prevention and therapy (Review). *Oncol Lett* (2017) doi:10.3892/ol.2017.7412
191. Libby G, Donnelly LA, Donnan PT, Alessi DR, Morris AD, Evans JMM. New Users of Metformin Are at Low Risk of Incident Cancer. *Diabetes Care* (2009) doi:10.2337/dc08-2175.
192. Bodmer M, Meier C, Krähenbühl S, Jick SS, Meier CR. Long-term metformin use is associated with decreased risk of breast cancer. *Diabetes Care* (2010) doi:10.2337/dc09-1791
193. Giovannucci E, Harlan DM, Archer MC, Bergenstal RM, Gapstur SM, Habel LA, Pollak M, Regensteiner JG, Yee D. Diabetes and cancer: A consensus report. in

Diabetes Care doi:10.2337/dc10-0666

194. Noto H, Goto A, Tsujimoto T, Noda M. Cancer risk in diabetic patients treated with metformin: A systematic review and meta-analysis. *PLoS One* (2012) doi:10.1371/journal.pone.0033411
195. Foretz M, Guigas B, Bertrand L, Pollak M, Viollet B. Metformin: From mechanisms of action to therapies. *Cell Metab* (2014) doi:10.1016/j.cmet.2014.09.018
196. Evans JMM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. *Br Med J* (2005) doi:10.3906/fiz-1701-1
197. Bodmer M, Meier C, Krähenbühl S, Jick SS, Meier CR. Metformin, sulfonylureas, or other antidiabetes drugs and the risk of lactic acidosis or hypoglycemia a nested case-control analysis. *Diabetes Care* (2008) doi:10.2337/dc08-1171
198. Duo J, Ma Y, Wang G, Han X, Zhang C. Metformin synergistically enhances antitumor activity of histone deacetylase inhibitor trichostatin a against osteosarcoma cell line. *DNA Cell Biol* (2013) doi:10.1089/dna.2012.1926
199. Babcook MA, Shukla S, Fu P, Vazquez EJ, Puchowicz MA, Molter JP, Oak CZ, MacLennan GT, Flask CA, Lindner DJ, et al. Synergistic Simvastatin and Metformin Combination Chemotherapy for Osseous Metastatic Castration-Resistant Prostate Cancer. *Mol Cancer Ther* (2014) doi:10.1158/1535-7163.MCT-14-0451
200. Chen H, Yao W, Chu Q, Han R, Wang Y, Sun J, Wang D, Wang Y, Cao M, He Y. Synergistic effects of metformin in combination with EGFR-TKI in the treatment of patients with advanced non-small cell lung cancer and type 2 diabetes. *Cancer Lett* (2015) doi:10.1016/j.canlet.2015.08.024
201. Zhang Y, Feng X, Li T, Yi E, Li Y. Metformin synergistic pemetrexed suppresses non-small-cell lung cancer cell proliferation and invasion in vitro. *Cancer Med* (2017) doi:10.1002/cam4.1133
202. Xia C, Chen R, Chen J, Qi Q, Pan Y, Du L, Xiao G, Jiang S. Combining metformin and nelfinavir exhibits synergistic effects against the growth of human cervical cancer cells and xenograft in nude mice. *Sci Rep* (2017) doi:10.1038/srep43373
203. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* (2001) doi:10.1172/JCI13505
204. Miller RA, Chu Q, Xie J, Foretz M, Viollet B, Birnbaum MJ. Biguanides suppress

hepatic glucagon signalling by decreasing production of cyclic AMP. *Nature* (2013) doi:10.1038/nature11808

205. Hardie DG. AMP-activated protein kinase-an energy sensor that regulates all aspects of cell function. *Genes Dev* (2011) doi:10.1101/gad.17420111
206. Griss T, Vincent EE, Egnatchik R, Chen J, Ma EH, Faubert B, Viollet B, DeBerardinis RJ, Jones RG. Metformin Antagonizes Cancer Cell Proliferation by Suppressing Mitochondrial-Dependent Biosynthesis. *PLoS Biol* (2015) doi:10.1371/journal.pbio.1002309
207. Rena G, Hardie DG, Pearson ER. The mechanisms of action of metformin. *Diabetologia* (2017) doi:10.1007/s00125-017-4342-z
208. Dowling RJO, Zakikhani M, Fantus IG, Pollak M, Sonenberg N. Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. *Cancer Res* (2007) doi:10.1158/0008-5472.CAN-07-2310
209. Liu X, Chhipa RR, Pooya S, Wortman M, Yachyshin S, Chow LML, Kumar A, Zhou X, Sun Y, Quinn B, et al. Discrete mechanisms of mTOR and cell cycle regulation by AMPK agonists independent of AMPK. *Proc Natl Acad Sci* (2014) doi:10.1073/pnas.1311121111
210. Bar-Peled L, Sabatini DM. Regulation of mTORC1 by amino acids. *Trends Cell Biol* (2014) doi:10.1016/j.tcb.2014.03.003
211. Howell JJ, Hellberg K, Turner M, Talbott G, Kolar MJ, Ross DS, Hoxhaj G, Saghatelian A, Shaw RJ, Manning BD. Metformin Inhibits Hepatic mTORC1 Signaling via Dose-Dependent Mechanisms Involving AMPK and the TSC Complex. *Cell Metab* (2017) doi:10.1016/j.cmet.2016.12.009
212. Wu H, Walker J, Damhuis RA, Brewster DH, Wild SH. Metformin and survival of people with type 2 diabetes and pleural mesothelioma: A population-based retrospective cohort study. *Lung Cancer* (2016) doi:10.1016/j.lungcan.2016.07.020
213. De Keersmaecker K, Lahortiga I, Mentens N, Folens C, Van Neste L, Bekaert S, Vandenberghe P, Odero MD, Marynen P, Cools J. In vitro validation of γ-secretase inhibitors alone or in combination with other anti-cancer drugs for the treatment of T-cell acute lymphoblastic leukemia. *Haematologica* (2008) doi:10.3324/haematol.11894
214. Wang Z. Down-regulation of Notch-1 contributes to cell growth inhibition and apoptosis in pancreatic cancer cells. *Mol Cancer Ther* (2006) doi:10.1158/1535-

7163.MCT-05-0299

215. Zeng C, Xing R, Liu J, Xing F. Role of CSL-dependent and independent Notch signaling pathways in cell apoptosis. *Apoptosis* (2016) doi:10.1007/s10495-015-1188-z

216. Kuhnert F, Kirshner JR, Thurston G. Dll4-Notch signaling as a therapeutic target in tumor angiogenesis. *Vasc Cell* (2011) doi:10.1186/2045-824X-3-20

217. Garcia A, Kandel JJ. Notch: A key regulator of tumor angiogenesis and metastasis. *Histol Histopathol* (2012) doi:10.1016/j.biotechadv.2011.08.021.Secreted

218. Takebe N, Miele L, Harris PJ, Jeong W, Bando H, Kahn M, Yang SX, Ivy SP. Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: Clinical update. *Nat Rev Clin Oncol* (2015) doi:10.1038/nrclinonc.2015.61

219. Jia X, Wang W, Xu Z, Wang S, Wang T, Wang M, Wu M. A humanized anti-DLL4 antibody promotes dysfunctional angiogenesis and inhibits breast tumor growth. *Sci Rep* (2016) doi:10.1038/srep27985

220. Guazzelli A, Bakker E, Tian K, Demonacos C, Krstic-Demonacos M, Mutti L. Promising investigational drug candidates in phase I and phase II clinical trials for mesothelioma. *Expert Opin Investig Drugs* (2017) doi:10.1080/13543784.2017.1351545

221. Pattarozzi A, Carra E, Favoni RE, Würth R, Marubbi D, Filiberti RA, Mutti L, Florio T, Barbieri F, Daga A. The inhibition of FGF receptor 1 activity mediates sorafenib antiproliferative effects in human malignant pleural mesothelioma tumor-initiating cells. *Stem Cell Res Ther* (2017) doi:10.1186/s13287-017-0573-7

222. Uematsu K, Seki N, Seto T, Isoe C, Tsukamoto H, Mikami I, You L, He B, Xu Z, Jablons DM, et al. Targeting the Wnt signaling pathway with dishevelled and cisplatin synergistically suppresses mesothelioma cell growth. *Anticancer Res* (2007)

223. Woodard GA, Yang Y-L, You L, Jablons DM. Drug development against the hippo pathway in mesothelioma. *Transl Lung Cancer Res* (2017) doi:10.21037/tlcr.2017.06.02

224. Bi P, Kuang S. Notch signaling as a novel regulator of metabolism. *Trends Endocrinol Metab* (2015) doi:10.1016/j.tem.2015.02.006

225. Li H, Lee J, He C, Zou M-H, Xie Z. Suppression of the mTORC1/STAT3/Notch1 pathway by activated AMPK prevents hepatic insulin resistance induced by excess

amino acids. *Am J Physiol Metab* (2014) doi:10.1152/ajpendo.00202.2013

226. Fresno Vara JÁ, Casado E, de Castro J, Cejas P, Belda-Iniesta C, González-Barón M. P13K/Akt signalling pathway and cancer. *Cancer Treat Rev* (2004) doi:10.1016/j.ctrv.2003.07.007
227. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science (80-)* (2005) doi:10.1126/science.1106148
228. Rattan R, Giri S, Hartmann LC, Shridhar V. Metformin attenuates ovarian cancer cell growth in an AMP-kinase dispensable manner. *J Cell Mol Med* (2011) doi:10.1111/j.1582-4934.2009.00954.x
229. Shen P, Reineke LC, Knutsen E, Chen M, Pichler M, Ling H, Calin GA. Metformin blocks MYC protein synthesis in colorectal cancer via mTOR-4EBP-eIF4E and MNK-1-eIF4G-eIF4E signaling. *Mol Oncol* (2018) doi:10.1002/1878-0261.12384
230. Motadi LR, Misso NL, Dlamini Z, Bhoola KD. Molecular genetics and mechanisms of apoptosis in carcinomas of the lung and pleura: Therapeutic targets. *Int Immunopharmacol* (2007) doi:10.1016/j.intimp.2007.07.013
231. Soldani C, Scovassi AI. Poly(ADP-ribose) polymerase-1 cleavage during apoptosis: An update. *Apoptosis* (2002) doi:10.1023/A:1016119328968
232. Boulares a H, Yakovlev AG, Ivanova V, Stoica B a, Wang G, Iyer S, Smulson M, Chem MJB. Role of Poly (ADP-ribose) Polymerase (PARP) Cleavage in Apoptosis. *J Biol Chem* (1999) doi:10.1074/jbc.274.33.22932
233. Cao X, Rodarte C, Zhang L, Morgan CD, Littlejohn J, Smythe WR. Bcl2/bcl-xLinhibitor engenders apoptosis and increases chemosensitivity in mesothelioma. *Cancer Biol Ther* (2007) doi:10.4161/cbt.6.2.3626
234. Hata AN, Engelman JA, Faber AC. The BCL2 family: Key mediators of the apoptotic response to targeted anticancer therapeutics. *Cancer Discov* (2015) doi:10.1158/2159-8290.CD-15-0011
235. Ploner C, Kofler R, Villunger A. Noxa: At the tip of the balance between life and death. *Oncogene* (2008) doi:10.1038/onc.2009.46
236. Tsai MJ, Yang CJ, Kung YT, Sheu CC, Shen YT, Chang PY, Huang MS, Chiu HC. Metformin decreases lung cancer risk in diabetic patients in a dose-dependent manner. *Lung Cancer* (2014) doi:10.1016/j.lungcan.2014.09.012
237. Cerezo M, Tichet M, Abbe P, Ohanna M, Lehraiki A, Rouaud F, Allegra M,

Giacchero D, Bahadoran P, Bertolotto C, et al. Metformin Blocks Melanoma Invasion and Metastasis Development in AMPK/p53-Dependent Manner. *Mol Cancer Ther* (2013) doi:10.1158/1535-7163.MCT-12-1226-T

238. Zhang JW, Zhao F, Sun Q. Metformin synergizes with rapamycin to inhibit the growth of pancreatic cancer in vitro and in vivo. *Oncol Lett* (2018) doi:10.3892/ol.2017.7444
239. Laplante M, Sabatini DM. mTOR signaling. *Cold Spring Harb Perspect Biol* (2012) doi:10.1101/cshperspect.a011593
240. Lowe SW, Lin AW. Apoptosis in cancer. *Carcinogenesis* (2000) doi:10.1093/carcin/21.3.485

PUBLICATIONS PRODUCED DURING THE Ph.D PERIOD

1. Mazzoni E, Bononi I, Benassi MS, Picci P, Torreggiani E, Rossini M, Simioli A, Casali MV, Rizzo P, Tognon M, Martini F. Serum Antibodies Against Simian Virus 40 Large T Antigen, the Viral Oncoprotein, in Osteosarcoma Patients. *Front Cell Dev Biolol* (2018) doi: 10.3389/fcell.2018.00064
2. Bononi I, Mazzoni E, Pietrobon S, Torreggiani E, Rossini M, Violanti S, Perri P, Tognon M, Martini F. High prevalence of serum IgG antibodies reacting to specific mimotopes of BK polyomavirus, a human oncogenic polyomavirus, in patients affected by uveal melanoma. *J Cell Physiol* (2018) doi: 10.1002/jcp.26771
3. Rossini M, Rizzo P, Bononi I, Clementz A, Ferrari R, Martini F, Tognon MG. New Perspective on Diagnosis and Therapy of Malignant Pleural Mesothelioma. *Front Oncol* (2018) doi: 10.3389/fonc.2018.00091
4. Bononi I, Mazzoni E, Pietrobon S, Manfrini M, Torreggiani E, Rossini M, Lotito F, Guerra G, Rizzo P, Martini F, Tognon M. Serum IgG antibodies from healthy subjects up to 100 years old react to JC polyomavirus. *J Cell Physiol* (2018) doi: 10.1002/jcp.26457