RESEARCH OF PREDICTIVE AND PROGNOSTIC TISSUE AND MOLECULAR MARKERS AND OF NEW THERAPEUTIC TARGETS IN MALIGNANT PLEURAL MESOTHELIOMA

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1. Abstract/Riassunto

1.1 Abstract

BACKGROUND: Malignant pleural mesothelioma (MPM) is an aggressive tumor with increasing incidence in industrialized countries, because of previous widespread asbestos exposure and long latency time before symptoms appearance. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) belongs to the tumor necrosis factor (TNF) family of death ligands; it was identified as a promising anticancer agent thanks to its property of killing cancer cells while sparing normal cells. Conflicting evidences about MPM resistance rather than sensitivity to TRAIL-induced apoptosis were previously reported. While TRAIL-dependent apoptosis is thought to be p53-independent, p53 wild type cancer cells can be sensitized to TRAIL through p53 activation. In contrast to most solid tumors, MPM cells frequently express wild type p53, thus p53 reactivation might be considered as an effective strategy to sensitize MPM cells to TRAIL-dependent apoptosis. DNA-damaging agents such as chemotherapy or radiotherapy and other agents targeting negative regulators of p53, may be considered as useful strategies to reactivate p53. Murine Double Minute 2 (MDM2) is a transcriptional target of p53: once activated, MDM2 binds p53 to the amino-terminus, targeting it for ubiquitylation and subsequent proteasomal degradation. Recently, many researchers have investigated a possible role of MDM2 in promoting tumor neoangiogenesis (Vascular Endothelial Growth Factor, VEGF; hypoxia inducible factor, HIF1alpha). Thus MDM2 might be a promising target for anticancer treatment because of its antiapoptotic and proangiogenetic role. The poor prognosis of

affected patients, the lack of effective treatment options, with particular reference to biologic drugs, the absence of predictive markers for targeted treatment and the lack of knowledge about the basis of different biological and clinical behaviour of the two main histologic subtypes, epithelioid *versus* non-epithelioid (sarcomatoid/biphasic), constitute the rationale for the present study.

AIMS: The first purpose of the study was to investigate new treatment options through preclinical evaluation of extrinsic apoptosis triggers (recombinant human Apo2L/TRAIL) in combination with intrinsic apoptosis inducers acting through the reactivation of p53, such as DNA-damaging agents (carboplatin/pemetrexed) or p53-MDM2 inhibitors (nutlin3-RG7112), both *in vitro* and *in vivo*. Moreover, the study aims to investigate new targets (MDM2, HIF1alpha, VEGF) for treatment in MPM tumor samples, testing possible different expression levels of such targets in the different histologic subtypes. Some morphological features, such as inflammation, necrosis and proliferation were quantified in the different histotypes and correlated with MDM2 and HIF1alpha. Finally, correlations between molecular data and clinical features were performed.

METHODS: Anticancer effects of rhApo2L/TRAIL (Amgen, Genentech) *plus* chemotherapy (Carboplatin/Pemetrexed) or nutlin3-RG7112 (Roche) was evaluated in different cell lines through annexin V and caspases assay, and in a Severe Combined ImmunoDeficiency (SCID) mouse model. p53 expression levels were evaluated through western blot. TRAIL receptors were evaluated through flow cytometry. Formalin-Fixed Paraffin Embedded (FFPE) chemonaive

tumor samples from MPM patients were analyzed: MDM2, VEGF and HIF1alpha mRNA and protein expression levels were investigated through RT-qPCR and immunohistochemistry (IHC) with specific antibodies, respectively. Proliferation was quantified through staining with Ki67 antibodies. Necrosis and inflammation were also quantified on histological sections using a grading score. Normal pleura samples from patients undergoing diagnostic surgery for non cancer disease were used as negative controls. Clinical data of the patients under study were collected in a password-protected database: age, gender, ECOG PS (Performance Status), EORTC score, stage, systemic treatments, surgery, radiotherapy, first progression and last follow-up date, status (alive/dead).

RESULTS: *In vitro* and *in vivo* results showed a significant increase of apoptosis in cell lines and reduction of tumor volume in animal models treated with rhApo2L/TRAIL plus chemotherapy or nutlin3-RG7112 compared with those receiving single treatments. This synergistic effect was dependent on the ability of chemotherapy or nutlin3-RG7112 to increase the expression of the TRAIL receptors DR4 and DR5 in a p53 manner. Higher MDM2 and HIF1alpha IHC expression was significantly associated with sarcomatoid/biphasic histologic subtype (p=0.010 and p=0.007, respectively) with positive correlation between MDM2 and HIF1alpha expression levels (correlation coefficient=0.533; p value= 0.00626). Proliferation index was significantly higher in sarcomatoid/biphasic compared with epithelioid samples (p=0.005) and also significantly higher in tumor samples with higher MDM2 expression (p=0.008). Clinical and pathological features or biomarker did not show any correlation with

prognosis, except for proliferation index and Progression Free Survival (PFS), even though the results of this exploratory analysis should be considered with caution because of the limited number of patients, the heterogeneous treatment received and the insufficient follow-up time in some patients.

CONCLUSION: Our preclinical *in vitro* and *in vivo* results confirm that reactivation of p53 by chemotherapy or p53-MDM2 inhibitors effectively sensitizes to TRAIL-dependent apoptosis in malignant pleural mesothelioma.

Our translational study in tumor samples from MPM patients confirmed different biological and pathological features and molecular targets expression in the two main histologic subtypes. It is tempting to speculate that MDM2 and Ki67 might be considered as further useful diagnostic tools to identify poor prognosis patients. Moreover, MDM2 and HIF1alpha might be considered as promising targets for anticancer treatment of MPM.

1.2 Riassunto

BACKGROUND: Il mesotelioma pleurico maligno (MPM) è una neoplasia aggressiva con incidenza in aumento nei paesi industrializzati per la pregressa esposizione ad amianto e il lungo periodo di latenza tra l'esposizione e la comparsa dei sintomi. TRAIL (*Tumor necrosis factor-related apoptosis-inducing ligand*) appartiene alla famiglia dei ligandi di morte apoptotica di TNF (*tumor necrosis factor*), ed è stato recentemente identificato come promettente agente antitumorale in considerazione della sua proprietà di uccidere le cellule tumorali, risparmiando le cellule normali. Evidenze contrastanti riportano la presenza di resistenza piuttosto che di sensibilità delle cellule di mesotelioma maligno all'apoptosi mediata da TRAIL. Sebbene l'apoptosi indotta da TRAIL (via

estrinseca dell'apoptosi) sembra essere indipendente da p53, alcune cellule tumorali portatrici di p53 *wild-type* possono essere sensibilizzate alla morte da TRAIL attraverso l'attivazione di p53 (via intrinseca dell'apoptosi). Contrariamente alla maggior parte delle neoplasie, le cellule di mesotelioma pleurico esprimono più frequentemente p53 *wild-type,* e quindi la riattivazione di p53 potrebbe essere una strategia efficace per sensibilizzare le cellule di mesotelioma all'apoptosi mediata da TRAIL. Agenti in grado di danneggiare il DNA (chemioterapia, radioterapia) ed altri agenti in grado di "down-regolare" gli inibitori di p53, possono essere considerati come valide strategie per riattivare p53. Murine Double Minute 2 (MDM2) è un bersaglio dell'attività trascrizionale di p53: una volta attivata, MDM2 lega il dominio ammino-terminale di p53 e la conduce al processo di ubiquitilazione e successiva degradazione proteasomica. Negli anni recenti, molti ricercatori hanno studiato un possibile ruolo di MDM2 nella attivazione di marcatori di neoangiogenesi tumorale (Vascular Endothelial Growth Factor, VEGF; hypoxia inducible factor, HIF1alpha), pertanto MDM2 potrebbe rappresentare un promettente bersaglio per il trattamento antitumorale in considerazione della sua possibile duplice attività antiapoptotica e proangiogenetica. La prognosi infausta dei pazienti affetti, l'assenza di opzioni terapeutiche efficaci, in particolare di farmaci biologici, l'assenza di marcatori predittivi di risposta ai farmaci a bersaglio molecolare, e la scarsità di conoscenze sui meccanismi che sottendono al diverso comportamento biologico e clinico dei due principali sottotipi istologici (epitelioide *versus* non-epitelioide), costituiscono il razionale del presente studio.

OBIETTIVI: Il primo obiettivo è stato valutare nuove opzioni terapeutiche attraverso studi preclinici *in vitro* ed *in vivo* con associazione di induttori della via estrinseca dell'apoptosi (rhApo2L/TRAIL) e induttori della via intrinseca dell'apoptosi che agiscono attraverso riattivazione di p53, come agenti danneggianti il DNA (carboplatino/pemetrexed) o inibitori del legame p53-MDM2 (nutlin3-RG7112). Secondariamente, lo studio si è proposto di ricercare l'espressione dei nuovi bersagli terapeutici (MDM2, HIF1alpha) nei campioni tumorali di pazienti affetti da mesotelioma maligno, e di valutarne la diversa espressione nei diversi sottotipi istologici. Inoltre, il progetto si è focalizzato sulla valutazione di alcuni parametri morfologici come infiammazione, necrosi ed indice proliferativo nei campioni tumorali dei diversi istotipi e sulla loro correlazione con MDM2 e HIF1alpha. Infine, sono state valutate le correlazioni tra dati molecolari e caratteristiche cliniche dei pazienti in studio.

MATERIALI E METODI: l'attività antitumorale di rhApo2L/TRAIL (Amgen, Genentech) in associazione a chemioterapia (Carboplatino/Pemetrexed) o nutlin3-RG7112 (Roche) è stata valutata in diverse linee cellulari attraverso il saggio di Annessina V e delle caspasi, e in un modello di topo Severe Combined ImmunoDeficiency (SCID). I livelli di espressione di p53 sono stati analizzati attraverso western blot. I recettori di TRAIL sono stati rilevati attraverso citofluorimetria. Campioni tumorali fissati in formalina e inclusi in paraffina da pazienti *chemonaive* sono stati analizzati con immunoistochimica e valutando l'espressione di mRNA per MDM2 e HIF1alpha. L'indice proliferativo è stato quantificato mediante anticorpo monoclonale di Ki67. La presenza di infiammazione e necrosi è stata valutata su sezioni istologiche. Campioni di

pleura normale da pazienti sottoposti a chirurgia toracica per patologia non oncologica sono stati utilizzati come controlli negativi. I dati clinici dei pazienti in studio sono stati raccolti un un database protetto da password: età, sesso, ECOG PS (Performance Status), score prognostico EORTC, stadio, trattamenti sistemici, chirurgia, radioterapia, prima progressione, data di ultimo follow-up e status (vivo/morto).

RISULTATI: I risultati *in vitro* ed *in vivo* mostrano un significativo aumento di apoptosi in linee cellulari e riduzione di volume tumorale in modelli animali trattati con rhApo2L/TRAIL in associazione a chemioterapia o nutlin3-RG7112, confrontato ai singoli trattamenti. Tale effetto sinergico è correlato all'incremento di espressione dei recettori di TRAIL (DR4 e 5) conseguente alla riattivazione di p53 da chemioterapia o nutlin3-RG7112. Abbiamo poi valutato i livelli di espressione di MDM2 e del suo possibile target HIF1alpha in campioni tumorali di pazienti affetti da mesotelioma. I livelli di espressione di MDM2 e HIF1alpha erano significativamente più elevati nel sottotipo istologico sarcomatoide/bifasico (*p*=0.010 and *p*=0.007, respectively), ed è stata osservata una correlazione positiva tra i livelli di espressione di MDM2 e HIF1alpha (coefficiente di correlazione =0.533; *p* = 0.00626). Infine, l'indice proliferativo (Ki67) si è dimostrato significativamente più elevato nel sottotipo istologico sarcomatoide/bifasico rispetto a quello epitelioide (*p*=0.005) e significativamente più elevato nei campioni con iperespressione di MDM2 (*p*=0.008). Per quanto riguarda gli obiettivi esploratori del progetto, nessuna correlazione prognostica è stata osservata per alcun parametro clinico o patologico o per diversi livelli di espressione dei biomarcatori in studio, mentre

è stata osservata una correlazione significativa tra i livelli di Ki67 e la sopravvivenza libera da progressione. I risultati di tale indagine esploratoria devono, comunque, essere considerati con cautela per la limitata dimensione campionaria, l'eterogeneità degli interventi terapeutici e l'insufficiente follow-up di alcuni pazienti.

CONCLUSIONI: I risultati *in vitro* e *in vivo* di questo progetto di ricerca dimostrano che la riattivazione di p53 con chemioterapia o molecole inibitrici del legame p53-MDM2 rappresenta un'efficace strategia per sensibilizzare all'apoptosi mediata da TRAIL. Lo studio traslazionale ha invece confermato diverse caratteristiche biologiche e patologiche così come differenti livelli di espressione di nuovi bersagli terapeutici nei due sottotipi istologici di MPM. MDM2 e Ki67 possono essere considerati come importanti ausili diagnostici per una migliore caratterizzazione dell'istotipo e soprattutto per identificare i tumori a peggiore prognosi. Inoltre, MDM2 e HIF1alpha potrebbero rappresentare promettenti bersagli per il trattamento del mesotelioma pleurico maligno.

2. Background

2.1 Malignant Pleural Mesothelioma

Malignant Pleural Mesothelioma (MPM) is an aggressive cancer involving pleural surfaces and, in the advanced stage, lung parenchyma, leading to a typicale clinical picture characterized by chest pain, dyspnoea and cough.

Although the main risk factor is asbestos exposure, other factors have a central role in MPM pathogenesis. The biological behaviour of MPM is determined by molecular alterations, such as oncosuppressor genes loss, like p16^{INK4A} and p14^{ARF}, while rare mutations or deletions of p53 and pRb, which are commonly involved in the pathogenesis of most cancer. Such molecular aberrations seem to be the basis of MPM resistance to systemic treatments currently adopted in the medical management of this disease. Surgery is feasible only in selected cases and current gold standard chemotherapy in unresectable disease is a platinum-based doublet with an antifolate agent, which shows a median overall and progression free survival of approximately 12 and 6 months respectively, and a response rate of 20-40%[1, 2]

High refractoriety to systemic treatment, rare and short-term complete responses make MPM a therapeutic challenge. Improved knowledge about molecular pathways lead to several clinical trials investigating biologic agents in the treatment of MPM, even though they have not found a precise placement in the therapeutic strategy yet.

2.1.1. Epidemiology

The incidence of MPM shows some difference among countries, and epidemiologic data are somewhere lacking. Whereas in some countries MPM and cancer registers are available, in other countries few areas are covered with sufficient information and relative rates are estimated by mortality data; elsewhere, only surveys of medical doctors and researchers are available. Such heterogeneous picture determines that not all available data have the same reliability.

In most European countries, such as France, Germany, Italy and Scandinavia, the incidence rate is between 11 and 20 cases on 1.000.000 inhabitants[3], and these rates may vary about 70 times according to the geographical area. In Italy, the National Mesothelioma Register (Registro Nazionale Mesotelioma, ReNaM) reports an incidence of 2.98 and 0.98 (rough rates, on 100.000 inhabitants, per year) in males and females, respectively.

Analysis on asbestos exposure and MPM incidence performed in several industrialized countries, showed a statistically significant correlation between the two variables[4, 5]. At the national level, the geographic distribution of MPM reflects the location of industries using asbestos such as shipyards, building construction, production of asbestos cement and construction/repair of railway cars; in Italy, mortality data for pleural cancer in males, between 1988 and 1997, showed wide variations between one province and another. Higher mortality rates, between 4 and 12/100.000, are shown in ares where the main shipyards and cement

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factories are located. Among the most affected provinces, Casale Monferrato, Savona, Genova, La Spezia, Alessandria, Gorizia and Trieste.

In Italy the extraction, import and marketing of asbestos were banned in 1992 but due to the long lag time (even 40 years) from exposure to clinical evidence of MPM the epidemiologic data foresee a sharp rise of MPM incidence and mortality in the next fifteen years[6]. European epidemiological surveys foresee the mortality peak for MPM in males between 2015 and 2020 [7],[8, 9].

The Veneto region is particularly interested by asbestos exposure because of its strong industrial development. Incidence is about 2.3/100.000/year, quite superior to the mean national and European incidence. Since 1987, about 2000 new cases of malignant pleural mesothelioma have been registered, with mean incidence over 80 new cases/year. Most of them are males with a previous occupational exposure, but many females living close to some industries or with asbestos exposed workers are also affected. Thus malignant mesothelioma has a strong social impact that deserves attention by the National Health Service (NHS).

2.1.2 Etiology and pathogenesis

Etiopathogenesis of MPM is attributable in 80% of the cases to asbestos exposure. Asbestos fibers are classified in two main groups, according to size and bio-persistance: serpentine (chrysotile) and amphibole (crocidolite, tremolite, anthofillite and amosite). Even if in the different experimental models all asbestos fibers have shown cancerogenetic potential[10], subsequent evidence confirm a weak potential of chrysotile

fibers[11], and currently the scientific community agree on a higher cancerogenetic potential of thinner and longer asbestos fibers and of the subtypes amphibole, compared to shorter and chrisotile fibers.

Asbestos fibers may directly damage the DNA of mesothelial cells, by laying down and penetrating in the pleura, and leading to a process characterized by damage, repair and local inflammation, pleural plaques or mesothelioma. Another mechanism implied in asbestos cancerogenesis is the reactive oxygen species (ROS) and cytokines production by macrophages[12], which determine DNA damage and an immunocompromised state. Moreover, asbestos fibers are able to interfer with mitotic processes, inducing aneuploidy and chromosomal aberrations, and to activate some kinases such as MAPK (mitogen-activated protein kinase)and ERK1 and 2 (extracellular signal-regulated kinases 1 and 2)[13].

The complex cancerogenetic process leading to plural mesothelioma might be triggered by other factors such as genetics, ionizing radiations, SV40 virus (Simian Virus 40) [14].

Loss of heterozygosity (LOH) is a frequent process in the pathogenesis of MPM, and the result is the loss or inactivation of several oncosuppressor genes.

Even though mutation and deletion of p53 and pRb tumour suppressor genes occur frequently in a lot of human cancers, they are extremely rare in malignant mesothelioma[15] Probably, a functional apoptotic defect causing MPM resistance to chemotherapy and radiotherapy occurs downstream p53 and pRb. The INK4a/ARF locus within 9p21 chromosome

encodes two proteins, p16INK4a and p14ARF , and previous series demonstrated deletion of this genome region in 70% of MPM cell lines. p16INK4a inhibits cyclinD-dependent kinase (CDK), preventing the phosphorilation and subsequent inactivation of pRb, whereas p14 promotes the degradation of Murine Double Minute 2 (MDM2) protein, that is responsible for p53 ubiquitination and inactivation. p53 leads the cell to apoptosis or inhibits the entrance into the cell cycle, whereas pRb arrests the cell in G1 phase *(see section 2.2.2).*

The most frequent chromosomal aberration in MPM is on chromosome 22, where the oncosuppressor gene NF2 (Neurofibromatosis 2) is located and the protein merlin is encoded. Mutations of NF2 gene have been observed in 40% of MPM cases[16-18]. In animal models with asbestos exposure, MPM is most commonly observed after inactivation of one NF2 allele, compared to wild type; moreover, the other allele is often loss in MPM, thus confirming its role as a gatekeeper[19, 20]. The loss of the remaining allele is often associated with the loss of INK4a/ARF locus, which might be responsible for a 'permissive' background.

The BCL2 family of genes which regulate the apoptotic process plays an important role in the cancerogenesis process; this family includes proapoptotic genes such as BAX, BAK and BAD, and antiapoptotic genes such as BCL2, BCLXL, MCL1. BCL2 is rarely expressed in MPM, but high mRNA levels of BCLXL have been observed in MPM cell lines and in tumor tissue samples [21-23], probably in order to contrast with the proapoptotic effect of BAX, expressed in MPM cell lines[15].

Many growth factors play a role in the pathogenesis of MPM, such as IGF-1 (*Insulin-like growth factor-1*), HGF (*hepatocyte growth factor*), bFGF (*basic fibroblast growth factor*), EGF (*epidermal growth factor*), VEGF (*vascular endothelial growth factor*), PDGF A e B (*platelet-derived growth factor A e B*), TNF (*tumor necrosis factor*). These factors come from the surrounding lung parenchyma, macrophages or mesothelial cells, after different stimula, inflammatory cytochines, asbestos, SV40 infection. Growth factors promote tumor growth, proliferation and invasiveness, but also the neoangiogenesis process which feeds the growing tumor with oxygen and metabolites *(see section 2.3).*

2.1.3 Histopathologic and clinical features and treatment

MPM usually arises between the fifth and seventh decade, more frequently in males (males:females equal to 3-5:1). Affected patients tipically come to the medical evaluation with pleural effusion (80%), and subsequent symptoms such as chest pain (60%) and exercise dyspnoea (50-70%). Other symptoms such as weight loss and fatigue might be present, especially in the advanced stages of the disease, and their presence at the diagnosis characterize a worse prognosis. MPM might spread to the abdomen through the diaphragm, and in the 30% of the patients the main complication is bowel occlusion, while rarely the invasion of liver or other organs is observed. About 10% of the patients might die because of myocardial or pericardial involvement. Distant metastases are uncommon, and generally more frequent in the sarcomatoid subtype. MPM patients usually die because of respiratory insufficiency o pneumonitis.

The 2004 WHO (World Health Organization) classification of pleural tumors have described three main MPM histologic subtypes: epithelioid (50-67%), sarcomatoid (7-21%) and biphasic (24-35%), each of them characterized by different biological and clinical behaviour[24] [25].

Epithelioid mesothelioma, associated to a more favourable prognosis, is characterized by cells distributed into three different patterns: solid, glandular and tubulo-papillar.

Sarcomatoid mesothelioma is biologically more aggressive, and characterized by fusiform cells organized in layers or folders (typical) or sometimes in a disorganized pattern associated with abundant stroma (desmoplastic).

The immunohistochemistry diagnosis uses panels which combine mesothelioma and cancer associated markers. The immunohistochemistry usually consists on subsequent steps, using first two markers for MPM (calretinin, podoplanin) and two for another cancer (usually lung adenocarcinoma: TTF1, CEA).

Surgical resection of MPM is feasible in a small percentage of patients, although selection criteria have not been identified yet in prospective series [26].

Pleurectomy/Decortication (P/D) leads to a significant but incomplete resection of the tumoral pleura, leaving the involved lung free to expand. This procedure has not a curative intent, but it may be considered in symptomatic patients in order to control pain and restrictive deficit[27].

Radical surgery consists on the complete resection of cancer tissue

macroscopically visible, and it may be achieved with extrapleural pneumonectomy (EPP): en-bloc resection of pleura, lung, pericardium, diapraghm, lymphnodes. In the attempt of reducing the incidence of local recurrences after extrapleural pneumonectomy, a multimodality approach with surgery followed by postoperative radiotherapy was explored in the past years. Extrapleural pneumonectomy allows higher doses of radiotherapy to the whole hemithorax by avoiding pulmonary toxicity and the result of this approach is a significant reduction of loco-regional relapses.

The issue of extrathoracic metastasis represents a major challenge in the management of the disease because of the impact on overall survival. Once a chemotherapy regimen shows activity in advanced malignant pleural mesothelioma, a subsequent step was the addition of such treatment to surgery and radiotherapy to improve the

systemic control of the disease. The success with surgical resection after neoadjuvant chemotherapy in stage IIIA non-small cell lung cancer has been the impetus for several groups to apply this strategy in malignant mesothelioma aiming at reducing the incidence of distant relapse. As well as in non-small cell lung cancer, neoadjuvant chemotherapy could maximize cytoreduction and increase the proportion of patients able to complete the following treatments. Furthermore, the difficult administration of both postoperative chemotherapy and radiotherapy in most patients induced many groups to introduce a trimodality approach

based on preoperative chemotherapy, surgery and postoperative radiotherapy in the attempt of improving compliance[28].

Recently the MARS (Mesothelioma And Radical Surgery) study, the first study which randomized between extrapleural pneumonectomy and no extrapleural pneumonectomy, showed no survival advantage and worse quality of life in those patients who underwent surgery. The trial had several limitations; patients were treated with different chemotherapy regimens, and in a relevant percentage of cases a sub-optimal chemotherapy was delivered. The heterogeneity of delivered chemotherapy could have unbalanced the two study arms. The study population was small but the conclusion of the trial raised the issue of a less invasive approach as suitable treatment of malignant pleural mesothelioma[29].

Chemotherapy is still the treatment option for the majority of MPM patients not suitable for surgical resection. Currently, the gold standard in the systemic treatment of MPM patients is a chemotherapy regimen based on a platinum-base doublet plus an antifolate agents such as pemetrexed or raltitrexed, which showed survival and response improvement compared with platinum single agent. These combinations showed median overall and progression free survival of approximately 12 and 6 months respectively, and a response rate of 20-40%[1, 2]. Carboplatin is considered a valid option in the systemic treatment of advanced pleural mesothelioma, with better toxicity profile compared to cisplatin [30-32].

After first-line treatment failure and disease progression or relapse, there is not a systemic regimen showing significant improvement in survival and quality of life, and few data of second line treatment are reported in small phase II studies or retrospective case series, thus raising some doubts about which might be the right drug for the right patient in previously treated subjects.

The improvement of knowledge about biological behaviour, molecular pathways and genetic alterations of MPM, lead to the preclinical and clinical investigation of new targeted agents in this setting. VEGF and other antiangiogenetic drugs, agents against other growth factors, HDAC and proteasome inhibitors, PI3K/mTOR inhibitors have been explored, although these drugs have not found a specific role in the therapeutic armamentarium of this disease[33].

2.2 Extrinsec and intrinsec apoptosis: TRAIL and p53-MDM2 pathway

2.2.1 Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and MPM.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) belongs to the tumor necrosis factor (TNF) family of death ligands inducing the extrinsic apoptotic pathway. Two surface death receptors (TRAIL-R1 or DR4 and TRAIL-R2 or DR5), two decoy non-functional receptors (TRAIL-R3 or DcR1 and TRAIL-R4 or DcR2) and the soluble decoy receptor osteoprotegerin were described [34-39]. After the binding of TRAIL to DR4/5 and the oligomerization of death receptors, the death-inducing

signalling complex (DISC) is formed, which includes also the Fas-associated death domain (FADD).

FADD recruits and initiates procaspase 8 to active caspase 8 which in turn cleaves and activates the effector caspases 3, 6 and 7.

TRAIL has been identified as a promising anticancer agent thanks to its property of killing cancer cells while sparing normal cells [40, 41], even though both sensitivity and resistance mechanisms to TRAIL-induced cell death are not completely clarified [42].

TRAIL-induced death program is successfully executed in "type I" cells, while "type II" cells need the activation of the intrinsic apoptotic pathway [43], through activation of the BH3-only protein, Bid, which moves to the mithocondria where contributes to Bax and Bak activation. The resulting mithocondrial pore formation leads to cytochrome c release into the cytosol with the final caspase 9 activation. Moreover, "type II" cancer cells seem sensitive to the synergistic effect of TRAIL, acting on the extrinsic pathway, and DNA damaging agents, targeting the intrinsic pathway; the mechanism of this synergy has been proposed to be via the upregulation of the TRAIL receptor DR5 [44-46].

Cancer cells are frequently resistant to TRAIL-dependent apoptosis through different mechanisms: mutations and disfunction of DR4 and DR5; defects of Fas-associated death domain (FADD) and caspase 8; overexpression of cellular FADD-like interleukin-1b-converting enzyme inhibitory protein (cFLIP); overexpression of the antiapoptotic proteins Bcl-2, Bcl-Xl, IAPs (Inhibitor of Apoptosis Proteins); loss of proapoptotic proteins Bax, Bak;

decreased release of second mithocondria-derived activator of caspases (Smac-Diablo); activation of mitogen-activated protein kinases (MAPK) or NFKB [42].

Monoclonal agonist antibodies directed against the DR4 and DR5 (Mapatumumab, Lexatumumab, Apomab, AMG655, LBY135) and recombinant human Apo2L/TRAIL (rhApo2L/TRAIL, Dulanermin) have been studied at a preclinical and clinical level both as single agents and in combination with chemotherapy [47-52].

Recombinant human (rh) Apo2L/TRAIL (Dulanermin), a receptor agonist which binds both DR4 and DR5[53, 54], showed antitumor activity *in vitro* and *in vivo* tumor models of different cancer types both as single agent and in combination with chemotherapy without any toxicity in normal cells [55-57], and was the first TRAIL agonist investigated in human clinical trials [51, 52, 58].

Conflicting evidences about MPM resistance rather than sensitivity to TRAIL-induced apoptosis were previously reported [49, 59]. A Swiss group showed apoptotic effects of TRAIL or the monoclonal antibodies Mapatumumab and Lexatumumab directed against DR4 and DR5 on 13 MPM cell lines; moreover, Mapatumumab (anti DR4) and Lexatumumab (anti DR5) sensitized MPM cell lines to the cytotoxic effects of Cisplatin, and cell death occurred through a synergistic cooperation of the two agents (Mapatumumab or Lexatumumab plus Cisplatin) probably through Reactive Oxygen Species (ROS) induction[49].

Increased apoptotic levels were demonstrated in four cell lines treated with the association of TRAIL and chemotherapy (Cisplatin, Doxorubicin, Gemcitabine or Etoposide), probably through p53-independent apoptotic pathway; no DR5 increase was observed at the basis of this sensitization[60].

Other studies showed a p53-dependent induction of DR4 and DR5 expression by Alpha-tocopheryl succinate, resulting in TRAIL sensitization [61]. While TRAIL-dependent apoptosis is thought to be p53-independent, p53 wild type cancer cells can be sensitized to TRAIL through p53 activation [62]. In contrast to most solid tumors, MPM cells frequently express wild type p53 [63], thus p53 reactivation through different strategies might be considered as an effective strategy to sensitize MPM cells to TRAIL-dependent apoptosis.

2.2.2 p53-MDM2 pathway and MPM.

The tumor suppressor p53 acts as a transcription factor regulating genes involved in DNA repair, metabolism, cell cycle arrest, apoptosis and senescence. It was defined as the 'guardian of genome' because of its ability of preserving the genomic integrity of the cell under stressed conditions; p53 disruption, subsequently, leads to increased cancer risk and to a worse cancer prognosis and treatment response.

Under unstressed conditions, p53 levels are kept low by a feedback interaction with the RING domain proteins murine double minute 2 (MDM2) and MDM4 (also known as MdmX). MDM2 is a target of p53's transcriptional activity, with E3 ubiquitin ligase activity: once activated,

MDM2 binds p53 to the amino-terminus, targeting it for ubiquitylation and subsequent proteasomal degradation which represents its main, and probably first known, function[64]. MDM2 inhibits p53 through multiple other inhibitory mechanisms, such as the prevention of the transcriptional coactivator recruitment, the inhibition of p53-DNA interaction and p53 indirect translation[65].

Under stressed condition - such as DNA damage- induced p53 decides cell fate outcomes among apoptosis, cell cycle arrest and senescence through specific gene transcription; p53's promoter selectivity, the levels of the protein itself, antiapoptotic proteins levels, specific cofactors recruitment for downstream genes transcription, post-translational p53 modifications seem to condition cell death rather than cell cycle arrest or senescence[66].

p53 is mutated in about 50% of the human cancers[67], while in tumors with wild-type p53 gene, the protein function may be lost because of overexpression of p53 regulatory proteins such as MDM2 and MDM4, or because of CDKN2A –encoding for ARF which binds to and rapidly degrades MDM2- deletions. MDM2 and MDM4 protein overexpression with or without increased gene copy number occurs in several cancer types, more frequently in those tumors with a wild-type p53[68].

MDM2 and MDM4 amplification have been shown in about 30% and 17% of soft tissue sarcomas (STS) respectively, while p53 mutations and CDKN2A deletions were described in about 20 and 15% of the cases, respectively[69-72]. A recent p53-pathway mapping in different STS

histologic subtypes, confirmed frequent p53 mutations in leiomyosarcomas, osteosarcomas and pleomorphic sarcomas, while frequent MDM2 amplifications in well differentiated liposarcomas and MDM4 in Ewing's sarcoma/PNETs; MDM2 and MDM4 coamplification was a common event in synovial sarcomas, Ewing's/PNET and osteosarcomas. Moreover, p53 mutations and MDM2 amplifications appeared as mutually exclusive events, which acquire particular relevance in patients selection for p53-reactivating treatments[73]. However, gene amplification is not the only mechanism sustaining MDM2 overexpression[74-76].

Transcriptional and post-transcriptional mechanisms have been proposed at the basis of MDM2 and MDM4 overexpression, thanks to the dense interaction network of these proteins[77]. Among post-transcriptional modifications, post-translational modifications acquire particular relevance in MDM2 regulation, and phosphorylation is one of the most commonly implied in different conditions. This post-translational modification leads to p53 ubiquitination and degradation and to the MDM2-p19[ARF] interaction prevention, finally to MDM2 nucleus-cytoplasm shuttling.

Heterogeneous data across tumor types have been reported about MDM2 overexpression with or without gene amplification, and about a possibile prognostic role of such marker[78] but its protoncogenic activity, both p53 dependent and independent, suggests this may be a promising target for treatment.

The majority of MPM tumor specimens have p53 wild type but present deletion of the locus INK4A/ARF (70-80%) that contains the genes p14/ARF

and p16/INK4A[79] p14/ARF is crucial in controlling cell proliferation. It is activated by oncogenic triggers and acts by binding to MDM2, sequestering it in the nucleolus and, so, inhibiting its functions as p53 negative regulator [80]. p14/ARF deletion has a significant role in driving MPM pathogenesis *in vivo* [81] Despite this, several evidences demonstrate that the p53 pathway, downstream p14/ARF, is functional and that p53 activation is able to induce apoptosis in absence of p14/ARF[82]. The introduction of p14 gene in INK4A/ARF-deficient MPM cell lines induced activation of p53 and subsequent cell cycle arrest and apoptosis[83].

Investigations on the MDM2-p53 interaction provided a basis for the design of novel small molecules targeting the MDM2 activity, possibly reactivating the wild-type p53 function. Previous evidence reported the discovery of a series of 4,5-dihydroimidazolines called Nutlins. Compound 1, also known as Nutlin-3a, has become a tool of choice to study p53 biology and therapeutic applications. Although these early lead compounds have shown good cellular activity and provided the mechanistic proof-of-concept for inhibiting p53-MDM2 interaction for cancer therapy, their pharmacological properties were suboptimal for clinical development[84]. Optimization efforts led to the discovery of a new member of the Nutlin3 family of MDM2 inhibitors, RG7112 which is currently being evaluated in human clinical trials.

RG7112 is the first clinical small-molecule MDM2 inhibitor designed to occupy the p53-binding pocket of MDM2. In cancer cells expressing wild-type p53, RG7112 stabilizes p53 and activates the p53 pathway, leading to

cell cycle arrest, apoptosis, and inhibition or regression of human tumor xenografts[85].

2.3 Neoangiogenesis and MPM

VEGF (Vascular Endothelial Growth Factor) is an autocrine growth factor released by MPM cells which binds endothelial cell receptors and induces new blood vessels formation. VEGF levels are higher in MPM patients than in healthy individuals or patients with non neoplastic pleural-pulmonary disease or patients with other solid malignancies; high levels of this growth factor are associated to microvessels density and poor prognosis. These data suggest that VEGF may be a proper target for MPM treatment[33, 86, 87]. Treatment of MPM cell lines with rhVEGF (recombinant human VEGF) induces MPM proliferation and this effect is abrogated by using VEGF blocking antibodies[88, 89] demonstrating that VEGF has a role both in angiogenesis and cell proliferation. These results offer a rationale for the use of antiangiogenic therapies in MPM patients. Nevertheless, antiangiogenic therapy in MPM did not achieve the expected results. Bevacizumab, a recombinant humanized monoclonal antibody that inhibits the binding of VEGF to its receptors, was clinically investigated in MPM. Simultaneous administration of Bevacizumab plus cisplatin/gemcitabine, Cisplatin/Pemetrexed or Carboplatin/Pemetrexed did not improve the overall survival of MPM patients[90-93]. These results demonstrate that, although anti-VEGF target therapy may be a promising strategy for MPM treatment, it is important to individuate a new molecular marker to predict the efficacy of anti-angiogenic therapies.

Recently, many researchers have investigated a possible role of MDM2 in promoting tumor neoangiogenesis through the regulation of VEGF expression and probably other factors involved in this biological process. Physiologically, MDM2 seems to be entailed in exercise-induced muscles vascularization[94]. In pathological condition, hypoxia might induce expression of MDM2 which in turns binds and stabilizes Hypoxia-Inducible Factor (HIF)-1alpha, a transcription factor responsible for VEGF transcription[95]. In agreements with this observation, LaRusch and co-workers demonstrated that the inhibition of MDM2-HIF-1alpha interaction by Nutlin-3a reduces VEGF mRNA expression[96].

An alternative mechanism by which MDM2 regulates VEGF it was proposed by Zhou and colleagues. They demonstrated that MDM2 binds the 3'UTR of VEGF and stabilizes VEGF mRNA. In their work they suggested that hypoxia provokes MDM2 translocation from the nucleus to the cytoplasm where MDM2 interacts with VEGF mRNA inducing high levels of VEGF in the cells[97].

The same mechanisms seems to be at the basis of VEGF expression regulation in breast cancer cell lines, independenty from the p53 status. Moreover, the administration of MDM2 inhibitors in nude mice injected with breast cancer cells is able to reduce tumor volume compared with untreated controls[98].

3. Aims

The general purpose of the present study comes from unanswered scientific questions and unmet medical needs in the knowledge and medical management of Malignant Pleural Mesothelioma.

Considering the poor prognosis of affected patients, the lack of effective treatment options, with particular reference to biologic drugs, and absence of predictive markers for targeted treatment, the first purpose was to investigate new treatment options through preclinical evaluation of extrinsic apoptosis triggers (recombinant human Apo2L/TRAIL) in combination with intrinsic apoptosis inducers acting through the reactivation of p53.

Moreover, the study aims at investigating new targets for treatment in MPM cell lines and tumor samples, investigating possible different expression levels of such targets in the different histologic subtypes.

Finally, considering the lack of knowledge about the genetic and molecular mechanisms at the basis of different biological behaviour of the two main mesothelioma histologic subtypes (epithelioid and sarcomatoid/biphasic) the project tried to put on evidence some difference between different histologic subtypes of mesothelioma samples in terms of pathological features. We achieved the project's aims according to the following timelines (Gannt Chart):

		Year 1												Year 2												Year 3										
Months		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
PRELIMINARY STAGE		░	░	░	░	░																															
study design		▓																																			
grant application			▓																																		
funding achievement				▓																																	
teamwork definition				▓	▓																																
study planning					▓	▓																															
AIM 1.1							░	░	░	░	░	░	░	░	░	░	░	░	░	░	░	░	░	░												
ethical approval							▓	▓																													
in vitro studies									▓	▓	▓																										
in vivo studies											▓	▓	▓	▓																						
data analysis														▓	▓	▓	▓	▓	▓																	
abstract presentation									▓																												
paper writing, submission and publication																										▓	▓	▓	▓								
AIM 1.2																			░	░	░	░	░	░	░	░	░	░	░	░	░	░				
ethical approval																			▓	▓																
in vitro studies																					▓	▓	▓													
in vivo studies																								▓	▓	▓	▓									
data analysis																											▓	▓	▓	▓						
abstract presentation																															▓					
AIM 2																				░	░	░	░	░	░	░	░	░	░	░	░	░	░	░	░	░
ethical approval																				▓	▓															
patients recruitment																					▓	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓				
clinical data collection																					▓	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓				
tumor samples collection																					▓	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓	▓				
MDM2, HIF1alpha IHC analysis																										▓	▓	▓	▓	▓	▓	▓	▓			
MDM2, HIF1alpha RTqPCR analysis																										▓	▓	▓	▓	▓	▓	▓	▓			
inflammation, necrosis, proliferation analysis																										▓	▓	▓	▓	▓	▓	▓	▓			
data analysis																															▓	▓	▓	▓	▓		
abstract presentation																																		▓		
thesis work writing and conclusion																																	▓	▓	▓	▓

3.1 Preclinical evaluation of the anticancer activity of the extrinsic apoptosis activator (rhApo2L/TRAIL) in combination with intrinsic apoptosis triggers acting through p53 activation

Even though TRAIL-induced apoptosis is believed to be p53-independent, several and complex interactions between the two pathways were reported suggesting that targeting p53 might be a promising strategy to

sensitize tumors with wild-type p53 (e.g. MPM) to TRAIL-dependent cell death [15, 62].

The first aim of this study is to investigate the anticancer effects of rhApo2L/TRAIL (Amgen/Genentech) in combination with p53 reactivating agents such as chemotherapy and p53-MDM2 inhibitors, employing epithelioid and sarcomatoid MPM cell lines and an *in vivo* preclinical model.

3.1.1. First, we investigated the anticancer activity of rhApo2L/TRAIL *plus* the current gold standard chemotherapy regimen, a platinum-based doublet associated with the antifolate agent pemetrexed. We furthermore investigated if the improved cytotoxicity after the combination of rhApo2L/TRAIL plus chemotherapy was actually p53-dependent.

3.1.2. We also explored the association of rhApo2L/TRAIL *plus* a new member of the Nutlin3 family of MDM2 inhibitors, RG7112 (Hoffmann-La Roche Inc).

3.2 Translational study of the identification of pathological and molecular differences in chemonaive tumor samples from different MPM histologic subtypes (epithelioid *versus* non-epithelioid)

Druggable molecular difference between epithelioid and sarcomatoid MPM has not been identified so far. Preliminary results suggest that MDM2 might promote tumor growth through apoptosis inhibition and neoangiogenesis (VEGF and HIF1alpha) induction. According to our preliminary data, this protein might be expressed at different levels in the two MPM histologic subtypes.

Through the analysis of chemonaive patients samples the aim of this study is to describe the different MPM histologic subtypes in terms of:

- possible molecular targets for treatment– MDM2, VEGF, HIF1alpha- at mRNA and protein level; the correlation of MDM2 and neoangiogenesis markers expression level have been explored.

- pathological features –necrosis, inflammation and proliferation index- and their correlation with MDM2 expression levels

- as exploratory endpoints we aim at describing a possible prognostic and/or predictive role of MDM2 and other markers/histological features.

4. Methods

4.1 Preclinical evaluation of the anticancer activity of the extrinsic apoptosis activator (rhApo2L/TRAIL) in combination with intrinsic apoptosis triggers acting through p53 activation (chemotherapy; RG7112)

4.1.1.Cell lines and primary cultures

Peripheral Blood Mononuclear Cells (PBMC) were isolated from peripheral blood of healthy donors using Ficoll-Paque PLUS (GE HEALTHCARE, Little Chalfont, Buckinghamshire, U.K.) according to the manufacturer's protocol. We employed three cell lines of epithelioid derivation (ZL55, H28, M14K), three biphasic cell lines (ZL5, SPC111, MSTO-211H) and the sarcomatoid cell line ZL34. PBMC and MPM cell lines were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 (Gibco-Life Technologies, Carlsbad, CA, U.S.); Human Foreskin Fibroblasts (HFF) were grown in Modified Dulbecco's Eagle Medium (DMEM) (Gibco-Life Technologies, Carlsbad, CA, U.S.); both mediums were supplemented with 2 mM L-

glutamine, 1 mM sodium pyruvate, 10% FBS and 1% (w/v) penicillin/streptomycin (Invitrogen-Life Technologies, Carlsbad, CA, U.S.). All cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. One MPM primary culture (MPM1801) of sarcomatoid mesothelioma was established from fresh human pleural mesothelioma surgical specimen. Specimens have been obtained from the Thoracic Surgical Unit (University of Padua), after patient's informed consent signature. The project was submitted for approval to the Ethical Committee of Istituto Oncologico Veneto and to the Ethical Committee for animal studies of the University of Padua.

4.1.2 Annexin V staining:

MPM cells were seeded into 12-well plates in 1.0 mL/well of complete RPMI 1640 and treated with Carboplatin/Pemetrexed (27uM and 42 uM respectively) for 48 hours or Nutlin3a 10uM for 24 hours and/or rhApo2L/TRAIL (Dulanermin, Amgen Inc, Thousand Oaks, CA, U.S.; Genentech Inc, South San Francisco, CA, U.S.) 50 ng/mL for 24 hours.

In vitro chemotherapy concentrations were defined according to the dose inducing the higher cell death in MPM cell lines with the lower cell death in normal cells (PBMC and fibroblasts).

Thus, we choose concentration of Carboplatin 27uM and Pemetrexed 42uM inducing 10% of apoptosis in ZL55; the same concentration of Carboplatin induced about 5% of apoptosis in ZL34, while Pemetrexed as single agent showed no apoptosis induction with any tested concentration (0-100 uM) (data not shown). *In vitro* rhApo2L/TRAIL concentrations were

defined according to previous data showing that these are able to reach similar blood concentrations [99].

Time and sequence of exposure to chemotherapy and rhApo2L/TRAIL were established according to previous data with other TRAIL agonists and considering the pharmacokinetic of the drugs under study (shorter half-life of rhApo2L/TRAIL compared to agonistic antibodies or to chemotherapy) [49]'[53] and the doubling times of cell lines (20.89 hours for ZL55 and 28.12 hours for Zl34, data not shown).

The Annexin V assay was performed using Annexin-V-Fluos and PI (Roche, Basel, Switzerland) according to the manufacturers' instructions. Cells were collected, centrifuged, and then resuspended in 300 uL of Annexin-binding buffer, followed by incubation with 1 uL of Annexin V-Fluos and 1uL of PI for 10 minutes at room temperature. Cells positive for Annexin V/PI were detected by flow cytometry using a FACSCalibur apparatus and CellQuest software (BD Biosciences San Jose, CA, U.S.). Where indicated cells were pre-treated with the ROS scavenger N-Acetyl-Cysteine (NAC 100 uM) overnight. Specific Apoptosis was calculated by the following formula: (percentage of Annexin V positive cells in treated samples- percentage of Annexin V positive cells in untreated samples) / (100- percentage of Annexin V positive cells in untreated samples)* 100.

Drug interactions were quantified by determining the combination index (CI) using the CompuSyn software (ComboSyn, Inc., Paramus, NJ), where CI< 1, CI=1, and CI > 1 indicated synergistic, additive, and antagonistic effects, respectively.

4.1.3 Caspases Assay:

Caspases Assay was performed using Fluorometric Homogenous Caspase Assay (Roche, Basel, Switzerland). MPM cells were seeded into 96-well plates in 0.1 mL/well of complete RPMI 1640, treated with Carboplatin/Pemetrexed (27uM and 42 uM respectively) for 48 hours or Nutlin3a 10 uM for 24 hours and/or rhApo2L/TRAIL (50 ng/mL) for 24 hours, and then incubated with DEVD-Rhodamine 110. Upon cleavage of the substrate by activated caspases, fluorescence of the released Rhodamine 110 was measured using Victor microplate reader (PerkinElmer, Waltham, Massachusetts, U.S.) with an excitation wavelength of 480 nm and emission wavelength of 520 nm. Specific caspases activity was calculated by the following formula: (fluorescence intensity of treated samples- fluorescence intensity of untreated samples) / (fluorescence intensity of untreated samples).

4.1.4 Western blot:

Tissue specimens were processed by cryogenic grinding with mortar and pestle to obtain a fine powder. The tissue powders and the cell lines were lysed in Mammalian Cells Disruption Buffer Paris-Kit (Ambion-Life Technologies, Carlsbad, CA, U.S.) supplemented with Phosphatase Inhibitor Cocktail (Roche, Basel, Switzerland) and Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). Protein concentration was determined by the Coomassie (Bradford) Protein Assay Kit (Thermo Scientific, Waltham, Massachusetts, U.S.) using bovine serum albumin as standard, and equal amounts of proteins were analyzed by SDS-PAGE (12%

acrylamide/bis-acrylamide). Gels were electroblotted onto polyvinylidenedifluoride membranes (Amersham-GE HEALTHCARE, Little Chalfont, Buckinghamshire, U.K.). In immunoblot analysis, membranes were blocked for 1 hour with 5% non-fat dry milk in Tris Buffered Saline (TBS) containing 0.1% Tween-20, and incubated at 4°C over night with primary antibody direct against p53 (Santa Cruz Biotechnology), p21 (Cell Signaling) and p53 (Abcam) and anti-b-actin antibody (Sigma) used as loading control, followed by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Dallas, Texas, U.S.). Finally, the membranes were incubated with chemiluminescence reagents (Supersignal Pico; Pierce-Thermo Scientific, Waltham, Massachusetts, U.S.) and revealed using Chemidoc XRS System (Biorad, Hercules, CA, U.S.).

4.1.5 Flow cytometry analysis:

Surface expression of TRAIL receptors was evaluated by indirect immunostaining using the primary antibodies DR4, DR5, DcR1 and DcR2 (Alexis Biochemicals, San Diego, CA, U.S.) followed by Alexa Fluor Goat anti-mouse immunoglobulin G (IgG H+L) (Life Technologies, Life Technologies, Carlsbad, CA, U.S.). Non-specific fluorescence was assessed using normal mouse IgG followed by secondary antibody. Flow cytometry analysis was performed using a FACSCalibur apparatus and CellQuest software (BD Biosciences San Jose, CA, U.S.).Relative expression of TRAIL-R was calculated by the following formula: percentage of positive cells x mean fluorescence intensity (MFI).

4.1.6 Transfections:

The siRNA pool (25nM) for p53 (RIBOXX-Life Science, Dresden-Radebeul, Germany) and/or the wild-type p53 expression vector (200 ng) were transiently transfected in MPM cell lines using LIPOFECTAMINE 2000 (Invitrogen-Life Technologies, Carlsbad, CA, U.S.), according to the manufacturers' instructions. The expression levels of p53 were evaluated 24 hours after transfection by western blot analysis.

4.1.7 In vivo experiments:

4.1.7.1. rhAPO2L/TRAIL plus chemotherapy

In vivo experiments were performed in accordance with the Padua University Ethic Committee for Animal Testing.

60 SCID male mice at the 6th week were implanted subcutaneously (sc) in the right flank with $2x10^6$ ZL55 (30 mice) or ZL34 cell lines (30 mice) suspended in 0.1 ml volume of RPMI. When tumor volume reached 50 mm^3, mice were randomized in four groups (N=6 mice/group) and treated by intraperitoneal (IP) injection: not treated (NT, vehicle 100uL on day 1); Carboplatin/Pemetrexed (CP, 75 mg/Kg and 100 mg/Kg respectively on day 1); rhApo2L/TRAIL (T, 60 mg/Kg on days 1, 2 and 3); CPT (CP, 75 mg/Kg *plus* 100 mg/Kg respectively on day 1 *plus* T, 60 mg/Kg on days 1, 2 and 3).

rhApo2L/TRAIL schedule and dose were established according to previous studies (data on file, Amgen Inc, Thousand Oaks, CA/Genentech Inc, South San Francisco, CA, 2009).

Tumor volumes were measured with a caliper every third day; volumes were calculated using the modified ellipsoidal formula: $1/2(length \times width^2)$. Mice were suppressed at the 21th day or when tumor volume

reached 500 mm^3. Delta volume was calculated by the following formula: (tumor volume at the day n- tumor volume at the day 1)/tumor volume at the day 1*100.

4.1.7.2. rhAPO2L/TRAIL plus RG7112

30 SCID mice at the 6th week were intraperitoneally (IP) injected with 5x10^6 ZL34 cells previously transduced with lentiviral vector containing a plasmid encoding for Luciferase. 3 weeks post injection the mice were randomized in 4 treatment groups and treated with RG7112 (Hoffman-La Roche Inc.) or vehicle (days 1-21) by gavage and/or rhAPO2L/TRAIL (days 1-3) by IP. Tumor size was assessed once a week by in vivo bioluminescence using Xenogen bioluminescence imaging after IP injection of D-luciferin in each mouse. The mice were suppressed at the 22th day. Average Radiance [p/s/cm²/sr] was proportional to the number of ZL34 cells expressing the LUC gene and D Average radiance was used as indicator of tumor growth and calculated by the following formula: (Average Radiance at the day n- Average Radiance at the day 1)/Average radiance at the day 1.

4.2 Translational study of the identification of pathological and molecular differences in chemonaive tumor samples from different MPM histologic subtypes (epithelioid *versus* non-epithelioid)

4.2.1 Patients samples and data collection

We retrospectively collected and analyzed epithelioid, biphasic and sarcomatoid malignant pleural mesothelioma samples from the diagnostic biopsy. In order to perform RT-qPCR and immunohistochemistry the following tumor samples were required:

- 1 hematoxylin and eosin stained slide and paraffin-embedded tumor block or 5-10 paraffin embedded tumor sections in IHC slides (for immunohistochemistry analysis)

- paraffin embedded tumor block or 5 sections (10 u) in eppendorf RNASE/DNASE free (for RT-qPCR).

All the samples (*N*=38) were analyzed for protein expression levels of MDM2 and HIF1alpha through IHC and for inflammation, necrosis and Ki67 levels; mRNA expression levels of MDM2 and HIF1alpha levels were quantified through RT-qPCR in 32 tumor samples (20 epithelioid and 12 sarcomatoid/biphasic) where RNA was available. VEGF IHC and RT-qPCR analysis were performed in a training set of 27 tumor samples (17 epithelioid and 10 sarcomatoid/biphasic). As negative controls we used normal pleural from 4 non cancer patients undergoing thoracic surgery.

Complete clinical information about patients enrolled in the study were collected: age, gender, ECOG PS, EORTC score, stage, systemic treatments, surgery, radiotherapy, first progression and last follow-up date, status (alive/dead).

To perform the statistical analyses, all data collected were recorded in a computer data base (Microsoft Excel) with a protection of password.

We collected the informed consent to the data processing of the data subjects included in the research if, during the study, it was possible to give them the information and to get the related consent, and in particular, when they turned to our Institute also for follow up reasons. The operations of collection, storage, preservation, and circulation of the

biological samples as well as all the data processing operations regarding health data of the persons involved in the study were uniformed to the security and organizational measures defined in the document "Guidelines on data protection in medical and biomedical research".

Eight slices of 10 μm sections/sample have been collected in 1,5 ml of a microcentrifuge tube and incubated in xylene at elevated temperatures to solubilize and remove paraffin from the tissue, then washed in alcohol solutions to remove the xylene. Total RNA extraction of the deparaffinized samples have been performed using RecoverAll Total Nucleic Acid Isolation Kit (Ambion) according to manufacturer's protocol.

4.2.2 mRNA expression analysis.

Reverse transcription of total mRNA was performed using 500 ng of total RNA/sample by SuperScript II Reverse Trancriptase (Invitrogen) according to the manufacturer's protocol. Quantitative analysis of MDM2, VEGF, and HIF-1 alpha genes have been performed by LightCycler 480 Real time PCR System and LightCycler 480 SYBR Green I Master Mix (Roche) according to the manufacturer's protocol using specific primers for each genes (Sigma). As internal reference we used B2-microglobulin, GAPDH and b-actin. The Real Time reaction was carried out as follows: 95°C for 5 min, 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. All reaction have been run in triplicate and the quantitation of gene expression performed using the ΔΔCT calculation as previously described.

4.2.3 Immunohistochemistry and pathologic assessment

Serial sections of 4 micron were immunostained with the monoclonal Ki67, MDM2, HIF 1alpha and VEGF antibodies. The strong dark stained Ki67 and MDM2 and HIF1alpha nuclei were counted and expressed as % of total cell number. The counting of VEGF positive cells was based on the number and staining intensity of positive cells (from 0 to 300). The presence of necrosis and inflammation were evaluated on hematoxylin eosin sections and quantified using a score system from 0 to 3 (0: absent; 1: <10%; 2 from 10 to 20% and 3>20% of the whole tumor section examined).

4.3. Statistics

4.3.1 Preclinical studies

In vitro and *in vivo* studies results were expressed as mean +/- standard error and +/- standard deviation, respectively. Mann Whitney and ANOVA test (followed by post-hoc LSD test) were used to compare different treatment groups *in vitro* and *in vivo*, respectively. A p value ≤ 0,05 was considered as significant.

4.3.2 Translational studies

Low *versus* high expression levels of each marker and pathological parameter have been identified over and under the median value, and data presented as % of patients with high/low expression levels in each histological subtype. Kruskall-Wallis test have been performed to evaluate a different expression of molecular markers and pathological parameters in the two main histological subtypes, and to assess different pathological features in tumor tissues with high/low MDM2 expression. The correlation

between MDM2 and HIF1alpha expression levels (and possibly VEGF), and between RNA and protein expression levels of each marker have been investigated through the linear correlation analysis of Spearman. Overall and progression free survival curves have been designed according to Kaplan-Meier method.

The univariate analysis allowed to select the molecular marker and/or morphological parameter to be analyzed with the clinical features in the multivariate analysis for the assessment of the impact on PFS and OS (Cox Regression Proportional Hazards Model).

PFS has been assessed from the date of enrolment to the date of disease progression to the first-line (or relapse) or to the date of death, whichever occurred first. OS has been assessed from the date of enrolment to the date of death.

5. Results

5.1 Preclinical evaluation of the anticancer activity of rhApo2L/TRAIL in combination with chemotherapy (carboplatin *plus* pemetrexed)[100] or p53-MDM2 inhibitor RG7112

5.1.1 rhApo2L/TRAIL triggers apoptosis in MPM cells but not in normal cells.

The induction of apoptosis by rhApo2L/TRAIL treatment was tested in seven MPM cell lines (3 epithelioid: ZL55, H28, M14K; 1 sarcomatoid: ZL34; 3 biphasic: MSTO-211, SPC111, ZL5) and one short-term primary culture of sarcomatoid MPM cells established from a patient (MPM1801). Cells were treated with 50 ng/ml rhApo2L/TRAIL for 24 hours and apoptosis was

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measured by Annexin V staining and Fluorometric Homogenous Caspase Assays. The results showed a significant, even if heterogeneous sensitivity of MPM cells to TRAIL treatment, independent from the histotype. Interestingly, this effect was specific in MPM cells, as significant death was not observed in control cells (HFF and PBMC)(Figure 1a and b).

5.1.2 Carboplatin and Pemetrexed or Nutlin3a enhance the pro-apoptotic effects of rhApo2L/TRAIL on MPM cell lines

As Carboplatin and Pemetrexed (CP) are the cornerstone of current MPM therapies, we next tested whether these drugs might synergize with rhApo2L/TRAIL (T). Apoptosis was measured by Annexin V staining and flow cytometry. Cell lines ZL34 and ZL55 were selected as representative of sarcomatoid and epitheliod MPM, respectively. Results showed a significant (p < 0.001) synergistic effect of the combination of these drugs compared with no treatment or with CP or T as single agents (Figure 2 a and c). These results were also confirmed when apoptosis was assessed with the caspases assay (Figure 2b). A similar effect was shown in all the tested cell lines (3 epithelioid, 3 biphasic and 1 sarcomatoid) and in the sarcomatoid primary culture (Figure 3).

Previous studies [49] suggested that sensitivity to TRAIL might be dependent on the levels of reactive oxygen species (ROS). However, we did not observe any difference in specific cell death when both ZL34 and ZL55 cell lines were treated with the ROS scavenger NAC (Figure 4).

We then analyzed *in vitro* effects of rhAPO2L/TRAIL plus the MDM2-p53 inhibitor Nutlin3a by Annexin V and Caspases assay. Apoptosis assay

performed in eight MPM cell lines, representing the three different histotypes (epithelioid, biphasic and sarcomatoid), showed a synergistic anticancer effect of Nutlin3a plus rhAPO2L/TRAIL. Higher synergistic effect was shown in sarcomatoid cell (Figure 2d).

5.1.3 p53 activation by Carboplatin and Pemetrexed sensitizes to TRAIL-dependent apoptosis in vitro

We next investigated the mechanisms at the basis of the sensitization to TRAIL-dependent apoptosis induced by CP. Considering that both Carboplatin and Pemetrexed induced DNA damage resulting in p53 activation and that p53 is not mutated in most MPM cases, we tested the effect of CP on p53 levels in the ZL55 and ZL34 cell lines. Results indicated an increase in p53 levels in ZL55 and ZL34 cell lines following CP treatment (Figure 5a). To test whether this increase in p53 levels accounts for the ability of CP to sensitize to TRAIL-induced apoptosis, we investigated cell death in ZL55 and ZL34 cell lines treated with CP and/or T after p53 knock–down by siRNAs. Results demonstrated that the siRNA treatment induced a significant, specific knock-down of p53 expression (Figure 5a and b). Interestingly, p53 silencing resulted in a significant reduction of cell death induced by CP and T (Figure 5c); importantly, this effect was reverted by cotransfection with a vector coding for wild-type p53 (Figure 5c). We observed no or weak p53 expression in five tumor tissues from MPM patients; in contrast, p53 was readily detected in the ZL34 and ZL55 cell lines after p53 vector (Figure 5b).

5.1.4 p53 activation increases the expression of TRAIL receptors in vitro

To explore the TRAIL-sensitizing effect of p53 activation, we next investigated whether p53-inducing treatments enhance the expression of the TRAIL receptors DR4/DR5, DcR1/DcR2. Results showed that the ZL55 and ZL34 cell lines expressed higher levels of DR4 and DR5 in response to CP treatment (Figure 6a). Interestingly, knock-down of p53 expression resulted in a significant reduction of CP-induced DR4 and DR5 expression in MPM cells (p< 0.05), while no reduction of DR4 and DR5 expression was observed in MPM cells cotransfected with the p53 siRNA plus wild-type p53 expression vector (Figure 7). Taken together, these data provide evidence of a causal link between CP treatment, p53 activation, increased expression of DR4 and DR5 receptors and sensitivity to TRAIL. The detection of DcR1 and DcR2 levels in ZL34 and ZL55 cell lines treated or not with CP seemed not relevant.

When we explored the mechanism at the basis of the synergism between rhAPO2L/TRAIL and Nutlin3a in sarcomatoid cell lines, we observed the activation of p53, confirmed also by the activation of the two p53 targets p21 and inhibition of surviving. Additionally, p53 activation by Nutlin3a increased the expression of DR4/DR5 TRAIL death receptors (Figure 6b).

5.1.5 Antitumor activity of Apo2L/TRAIL + chemotherapy or RG7112 in preclinical animal models of MPM

To test the *in vivo* efficacy of Apo2L/TRAIL as single agent and in combination with CP, we employed a preclinical model based on the subcutaneous injection of the ZL55 and ZL34 MPM cell lines in SCID mice.

Thirty SCID mice were inoculated with ZL55 and 30 SCID mice were inoculated with ZL34 cells. Twenty-four mice had a measurable tumor and were randomized in the four treatment groups (N=6/group). Mice inoculated with ZL55 cells showed a statistically significant reduction of tumor volume at every time point in the three treatment groups (CP; T; CPT) compared to not treated (NT) mice; moreover, tumor volume was significantly reduced in mice treated with CPT compared to CP at the 21th day (p< 0.05) (Figure 8a).

Mice inoculated with ZL34 cells showed a statistically significant reduction of tumor volume at every time point in T and CPT treatment groups compared to not treated; at the day 21 we observed a statistically significant difference of tumor volume between all three treatment groups and untreated mice and between CPT and CP groups (Figure 8b).

No significant difference was observed between mice treated with CP compared to T (Figures 8 a,b).

To test the *in vivo* efficacy of Apo2L/TRAIL in combination with RG7112, we employed a preclinical model based on the intraperitoneal injection of ZL34 MPM cell lines in SCID mice. 30 SCID mice were inoculated with ZL34 cells.

Tumor growth in mice injected with ZL34 cells was significantly reduced in mice treated with RG7112 plus rhApo2L/TRAIL compared with mice treated with RG7112 or Apo2L/TRAIL as single agents (Figure 9).

	Year 1												Year 2												Year 3											
Months	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
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5.2 Translational study of the identification of pathological and molecular differences in chemonaive tumor samples from different MPM histologic subtypes (epithelioid *versus* non-epithelioid)

Formalin-fixed paraffin embedded tumor samples from Video-Assisted Thoracic Surgery (VATS)-guided pleural biopsies of 38 treatment-naive MPM patients who were referred to our Center for diagnosis and treatment over the years, were collected and analyzed. Our case series included 25 epithelioid, 7 biphasic and 6 sarcomatoid mesothelioma samples (25 epithelioid and 13 non-epithelioid). The last patient was included in November, 30th 2014; all alive patients were censored for survival analysis in December, 15th 2014. In 5 patients no survival follow-up was available. Patients' clinical features are summarized in Table 1.

5.2.1 MDM2, HIF1alpha, VEGF expression levels in epithelioid versus non-epithelioid MPM samples

Nuclear expression of MDM2 and HIF1alpha was higher in sarcomatoid/biphasic tumor samples (77% and 77% of tumor samples) and lower in epithelioid samples (68% and 64% of tumor samples, respectively); similar results were observed when we investigated mRNA expression levels of MDM2 but not of HIF1alpha (Table 2 and 3). No expression of MDM2 and HIF1alpha was observed in normal pleura samples.

Higher MDM2 and HIF1alpha IHC expression levels were significantly associated with sarcomatoid/biphasic histologic subtype (p=0.010 and p=0.007, respectively) (Figure 10 and 11). When we analyzed mRNA expression levels of MDM2 we observed a correlation trend with histologic subtype (higher levels in sarcomatoid/biphasic samples), although not statistically significant (p=0.067); no correlation was observed between HIF1alpha mRNA expression levels and histologic subtype (p=0.2), and between RNA and protein expression levels of MDM2(p=0.3) and HIF1alpha (p=0.9).

Among the 18 tumor samples with high MDM2 expression at the IHC, only 9 showed high levels of mRNA expression at the RT-qPCR analysis, suggesting mechanisms other than gene amplification sustaining protein overexpression.

Importantly, when we assessed the correlation between nuclear IHC expression levels of MDM2 and HIF1alpha, we observed a statistically

significant positive correlation (correlation coefficient=0.533; *p* value= 0.00626).

No significant difference between the two main histologic subgroups was observed when we analyzed VEGF protein and mRNA expression levels in the training set of 27 tumor samples (Table 4), therefore we considered this marker not worthy of further investigation through either IHC or RT-qPCR. Moreover, VEGF expression was not completely negative in normal pleural samples, probably because of a consequence of a pro-inflammatory phenotype of such controls.

5.2.2 Inflammation, necrosis and proliferation index levels in epithelioid versus non-epithelioid MPM samples

When we investigated different pathological features in tumor samples and compared epithelioid and sarcomatoid/biphasic subtypes, we observed more frequently low levels of inflammation in sarcomatoid samples (62% of tumor samples) and low levels of necrosis among epithelioid tumor samples (72% of tumor samples). Differently, proliferation index was more frequently low in epithelioid (72%) and high in sarcomatoid/biphasic (77%) samples (Table 5). No statistically difference in terms of inflammation (*p*=0.112) and necrosis (*p*=0.07) levels was observed between epithelioid and sarcomatoid/biphasic samples (Figure 12 a,b and d); proliferation index was significantly higher in sarcomatoid/biphasic compared with epithelioid samples (*p*=0.005) (Figure 12 c and d).

When we divided the whole patients population in two subgroups according to different MDM2 expression levels (high *versus* low), we observed that proliferation index was also significantly higher in tumor samples with higher MDM2 expression (*p*=0.008, data not shown).

5.2.3 Exploratory endpoints: prognostic and predictive role of MDM2 expression levels and other markers/histological features

As exploratory analysis, we assessed the impact of some clinical and pathological features on OS and PFS. Unfortunately, 5 patients were lost to follow-up and no survival data were available, thus reducing the sample size and impairing this analysis. Moreover, other weak points were that some sarcomatoid/biphasic mesothelioma patients had no sufficient follow-up time (less than 6 months) and different local or systemic treatments were considered.

No statistically significant difference in terms of OS (p=0.3) or PFS (p=0.7) was observed between epithelioid and sarcomatoid/biphasic samples, even if a trend towards a longer OS and PFS was observed in epithelioid (median OS 80 weeks; median PFS 48 weeks) compared with sarcomatoid/biphasic MPM (median OS 56 weeks; median PFS 40 weeks) (Figure 13). No statistically significant difference in terms of OS (p=0.5) or PFS (p=0.3) between high and low MDM2-expressing samples was observed. A trend towards a longer OS and PFS was seen in samples with lower MDM2 expression levels (median OS 96 weeks; median PFS 48 weeks) compared with sarcomatoid/biphasic MPM (median OS 60 weeks; median PFS 36 weeks).

Finally we analyzed the impact of different clinical (EORTC prognostic score, gender, histology, surgery, subsequent chemotherapy lines) and pathological features (inflammation, necrosis, proliferation index) and marker expression levels (MDM2 and HIF1alpha) on OS and PFS in a multivariate analysis: we observe no significant impact of any covariate on OS, while proliferation index significantly influences PFS ($p= 0.007$).

The results of this exploratory analysis might be read with caution due to the small number of patients, the heterogeneity of local and systemic treatment and the insufficient follow-up for a consistent part of sarcomatoid/biphasic MPM patients. Future investigation in a wider sample size is warranted.

Months	Year 1												Year 2												Year 3											
---	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
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patients recruitment																																				
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6. Discussion

Malignant pleural mesothelioma shows high refractoriety to chemotherapy and radiotherapy, thus median overall survival and progression free survival are about 12 and 6 months respectively in patients not eligible for surgery. Since 2003, when antifolate agents were introduced in the clinical management of

this disease, the gold standard chemotherapy is a Platinum-based doublet *plus* Pemetrexed or Raltitrexed[1, 2]. Surgery is feasible in highly selected cases, and patients suitable for trimodal treatment (neoadjuvant chemotherapy, surgery, postoperative radiotherapy) achieve overall survival longer than 25 months [28]. After the standard first-line Pemetrexed/Platinum combination there is not a defined regimen for the second line treatment of MPM, and the clinical benefits are uncertain[101-104]. Recent studies tested biologic agents that target key oncogenic pathways, including phosphatidylinositol3-kinase (PI3K)/mammalian target of Rapamycin (mTOR) pathways, histone deacetylases (HDAC), Nuclear Factor kB (NFkB) and neoangiogenesis. However, no one of these therapies proved to significantly impact the natural history of this neoplasm, thus reinforcing the need for new drugs to improve prognosis of MPM patients.

TRAIL is a member of the TNF superfamily which has recently emerged as a potentially interesting anticancer agent because of its ability to kill cancer cell lines while sparing many normal cells[40, 55, 105]. Several studies showed that MPM cells are resistant to TRAIL *in vitro*, although this resistance can be overcome by combining chemotherapy with alpha-tocopherylsuccinate, anisomycin, HDAC inhibitors, proteasome inhibitors and FLIP siRNA [49, 60, 61, 106-109]. Increased apoptotic levels were demonstrated in four cell lines treated with the association of TRAIL and chemotherapy (Cisplatin, Doxorubicin, Gemcitabine or Etoposide), probably through p53-independent apoptotic pathway; no DR5 increase was observed at the basis of this sensitization[60]. Recently, another study showed apoptotic effects of TRAIL or

the monoclonal antibodies Mapatumumab and Lexatumumab directed against DR4 and DR5 on 13 MPM cell lines; this effect was enhanced by Cisplatin probably trough the induction of ROS [49]. Other studies showed a p53-dependent induction of DR4 and DR5 expression by Alpha-tocopheryl succinate, resulting in TRAIL sensitization [61]. While TRAIL-dependent apoptosis is thought to be p53-independent, p53 wild type cancer cells can be sensitized to TRAIL through p53 activation [62]. In contrast to most solid tumors, MPM cells frequently express wild type p53 [63].

Recombinant human (rh) Apo2L/TRAIL (Dulanermin), a receptor agonist which binds both DR4 and DR5[53, 54], showed antitumor activity in *in vitro* and *in vivo* tumor models of different cancer types both as single agent and in combination with chemotherapy without any toxicity in normal cells [55-57], and was the first TRAIL agonist investigated in human clinical trials [51, 52, 58]. In the present study we investigated proapoptotic effects of rhApo2L/TRAIL combined to different intrinsic apoptosis triggers: first the DNA-damaging agents Carboplatin and Pemetrexed[100], then the p53-MDM2 inhibitor nutlin3-RG7112. To our knowledge, this is the first *in vivo* study of a TRAIL agonist in mesothelioma.

We observed heterogeneous sensitivity of seven mesothelioma cell lines and one primary culture to rhApo2L/TRAIL treatment; higher or lower sensitivity to the TRAIL agonist did not seem to be dependent on the histologic subtype. These data seem in line with previous studies of TRAIL agonists *in vitro,* and might be explained by the variable expression levels of antiapoptotic proteins such as Bcl2 and IAPs-family proteins, or other proteins involved in the

apoptotic pathway[49]. These proteins have been previously suggested as useful predictive markers of sensitivity to TRAIL-dependent apoptosis.

We observed higher apoptotic levels when both epithelioid and sarcomatoid cell lines were treated with chemotherapy and rhApo2L/TRAIL compared to Carboplatin/Pemetrexed and rhApo2L/TRAIL alone. This effect seemed to be dependent upon p53 but not on increased ROS levels. Furthermore we showed that p53 activation leads to increased expression of the DR4 and DR5 receptors. Previous evidences showed that chemotherapy-induced p53 activation lead to antiapoptotic proteins downregulation (such as survivin or Mcl1) and proapoptotic targets upregulation (such as Bax) [49, 110].

Contrary to previous evidences[63], preliminary data from next-generation sequencing of 123 MPM samples showed that p53 was among the five most frequently altered genes [111], although the overall frequence of such mutations may be considered low. Our results in a primary culture of sarcomatoid MPM (MPM1801) showed no mutation in p53 gene. Thus, based on our results, it is tempting to speculate that p53 mutational status in MPM samples may be a predictive marker of sensitivity to the combination of chemotherapy and rhApo2L/TRAIL. This hypothesis will be thoroughly tested in further studies in p53 knock-out models. *In vivo* studies showed that the association of Carboplatin/Pemetrexed with rhApo2L/TRAIL significantly reduced tumor growth compared to Carboplatin/Pemetrexed in both cell lines tested. Considering the short-term responses achieved with currently used chemotherapy, especially in the neoadjuvant setting where tumor shrinkage and distant relapses prevention assume particular relevance, the synergistic

effect of chemotherapy combined to rhApo2L/TRAIL might improve patients prognosis. Moreover, similar antitumor effects of rhApo2L/TRAIL and chemotherapy *in vivo*, might suggest a role for such agent at the disease progression after the first line treatment, where a valid option is still lacking in patients eligible for further treatments.

Taken together our findings suggest that rhApo2L/TRAIL combined to standard chemotherapy as first line treatment and as single agent in the second line setting might prove to be an effective drug in the treatment of MPM, considering also its low toxicity profile.

On the same way, we adopted the combination of rhApo2L/TRAIL *plus* nutlin3-RG7112, as alternative strategy to reactivate p53 thus sensitizing to TRAIL-dependent apoptosis *in vitro* and *in vivo.* Given the central role of MDM2 in regulating p53 activity and stability, developing small-molecule inhibitors of MDM2 could offer a novel approach to treating cancers. While nutlin3, the first developed compound targeting the p53-MDM2 complex was optimal for previous studies of p53 biology, and was effectively adopted in our *in vitro* studies, this compound was not optimal in clinical studies, thus recent efforts lead to a new molecule belonging to the nutlin family of MDM2 inhibitors, RG7112, which is currently in clinical development[84] (clinicaltrials.gov NCT00559533; NCT00623870). The high genetic plasticity characteristic for human tumors, especially at advanced stages, increases the chances for acquired resistance to most single agent therapies including MDM2 antagonists. Therefore, combination approaches to cancer therapy are extensively sought.

In vitro studies confirmed p53 activation by nutlin3, with subsequent upregulation of TRAIL receptors and final sensitization to extrinsec apoptosis, thus confirming previous results by other groups [112]. *In vitro* studies showed the higher synergism between the two molecules in sarcomatoid cell lines, thus raising the question about different MDM2 expression levels in epithelioid *versus* non-epithelioid (sarcomatoid and biphasic) MPM. This scientific question was someway reinforced by previous evidence of MDM2 overexpression with or without gene amplification in soft tissue sarcomas [70-73] and by the evidence of higher effectiveness of RG7112 in killing cancer cells overproducing MDM2 protein as a result of MDM2 gene amplification [85]. Previous results from our *in vitro* studies showed higher MDM2 mRNA levels in sarcomatoid cell lines, compared with epithelioid (Figure 15). Translational studies performed on clinical samples from affected patients confirmed these data.

Sarcomatoid/biphasic mesothelioma are characterized by higher aggressive biological behaviour, higher resistance to systemic treatments, more frequent distant spread and poor prognosis. Our *in vivo* results in the sarcomatoid animal model showed remarkable reduction in tumor growth with the combination under study (rhApo2L/TRAIL plus nutlin3-RG7112). These findings might open new scenarios to the medical treatment of such histologic MPM subtype, with a well-tolerated alternative to chemotherapy, worthy of clinical investigation. Moreover a systemic targeted treatment according to a specific mesothelioma histotype could be planned.

Recently MDM2 disregulation in merlin-deficient tumors has been reported. However to best of our knowledge, different expression level of MDM2 in

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different mesothelioma histotypes have not been investigated, so far. On the same way, specific morphological features (inflammation, necrosis, proliferation index) have not been carefully investigated in epithelioid *versus* sarcomatoid/biphasic MPM.

Our results showed that strong MDM2 overexpression- mainly in IHC- was significantly correlated with sarcomatoid/biphasic histotype even if the mRNA levels of MDM2 did not reach the statistically significant value. The correlation test between mRNA and protein analysis was negative. These results might be explained in different ways. First of all, literature data confirm that MDM2 protein overexpression is not only regulated by gene amplification, but also by other mechanisms, such as transcriptional and post-transcriptional modifications[77]. Secondarily, IHC detected only strong nuclear expression, while RT-qPCR quantified mRNA derived from the whole tumor sample. Moreover, there are pools within a cell of active and inactive MDM2 that do not directly correlate with overall MDM2 expression but which may reflect different isoforms or modified forms of the protein. Moreover, other factors could influence intracellular MDM2 expression level such as altered rates of transcription, mRNA stability, enhanced translation, and diminished destruction of the protein all will affect intracellular levels of MDM2. The prognostic significance of MDM2 overexpression are quite controversial in the literature [78]. We found a trend towards a negative prognostic and predictive significance of high MDM2 strong nuclear expression levels. In our case series, this lack of correlation with survival data may be related to several factors such as the small sample size, especially for sarcomatoid/biphasic samples whose

survival data were available in a small number of patients; the insufficient follow-up time of the last patients might have some impact on prognosis. This underlines the importance to validate our data in a prospective study with a larger and more homogenous population. Our results reported a statistically significant correlation of high Ki67 levels with sarcomatoid/biphasic histology and with high MDM2-expressing tumors, and Ki67 was the only covariate significantly affecting progression free survival to the first line treatment, at the multivariate analysis.

Thus, from our results, it is tempting to speculate that MDM2 and Ki67 might be considered as important diagnostic parameters to characterize MPM with poor prognosis.

Recently, nuclear grading in epithelioid mesothelioma has been shown as a simple, practical, and cost-effective prognostic tool that better stratifies clinical outcome and time to recurrence than currently available clinicopathologic factors [113]. Our preliminar data on 25 epithelioid tumor samples where survival data were available, showed only a trend towards a prognostic value of nuclear grading, although not statistically significant, probably because of the small sample size; in fact, we observed a median overall survival of 128, 96 and 60 weeks (p value= 0.5), in those patients with G1 (N=7), G2 (N=9) and G3 (N=9) nuclear grading, respectively; median progression free survival was 55, 36 and 58 weeks (p=0.2), in patients with G1, G2 and G3 nuclear grading, respectively.

To date, no literature data are available about different levels of inflammation and necrosis across mesothelioma histotypes; the prognostic significance of necrosis in mesothelioma was reported by other groups, even though these

results were limited to biphasic [87] or epithelioid histotype [114]. Our results did not show any significant difference, even though a trend towards higher necrosis features in sarcomatoid/biphasic tumors was detected. This finding may be due to a different biological behaviour of the two cancer types. The sarcomatoid histotype would present necrosis following a failure blood supply, as usually occurs in tumours with great proliferative index.

Overexpression of tumor neoangiogenesis markers has been previously described as a prognostic factor in MPM patients, with particular reference to VEGF [86, 87]. MDM2 has been suggested as a possible regulator of neoangiogenesis, both through direct regulation of VEGF and through stabilization of HIF1alpha, responsible for VEGF transcription [95-98].

Our results did not show significant difference of VEGF expression between epithelioid and non-epithelioid mesothelioma samples both at the protein and mRNA level. These results, together with recent evidence from negative clinical studies with Bevacizumab, a monoclonal antibody targeting VEGF, lead us to leave the investigation of this marker in MPM.

One innovative finding of our study was the significant correlation between HIF1alpha expression levels and the sarcomatoid/biphasic histotype, as much as the correlation between expression levels of MDM2 and HIF1alpha.

These results are of particular relevance further supporting the different biological behaviour of sarcomatoid/biphasic *versus* epithelioid MPM. As MDM2, HIF1alpha might be another promising target for antiangiogenetic treatments in MPM because monoclonal antibodies against VEGF has failed in the systemic treatment of mesothelioma. Further efforts should be made in

order to clarify the role of other potential markers involved in the complex process of neoangiogenesis.

7. **Conclusion and summary of topic results**

Our preclinical *in vitro* and *in vivo* results confirmed that reactivation of p53 by chemotherapy or p53-MDM2 inhibitors effectively promotes TRAIL-dependent apoptosis in malignant pleural mesothelioma:

- rhApo2L/TRAIL combined to standard chemotherapy as first line treatment and as single agent in the second line setting might prove to be an effective drug in the treatment of MPM, considering also its low toxicity profile.

- rhApo2L/TRAIL *plus* nutlin3-RG7112 showed remarkable anticancer effect, expecially in the highly aggressive sarcomatoid models.

Our translational study in tumor samples from MPM patients confirmed different biological and pathological features and molecular targets expression in the two main histologic subtypes:

- MDM2 and HIF1alpha strong nuclear protein expression and proliferation index are higher in sarcomatoid/biphasic samples. Proliferation index is higher in sarcomatoid and MDM2-overexpressing samples, and higher Ki67 levels correlate with shorter progression free survival.

From our results, it is tempting to speculate that MDM2 and Ki67 might be considered interesting markers to characterize MPM with poor prognosis.

- For the first time, our study showed a significant correlation between expression levels of MDM2 and HIF1alpha. This has relevant therapeutic implications expecially for possible targeted therapies aimed to the use of new antiangiogenetic and proapoptotic treatments.

8. Appendix: figures and tables
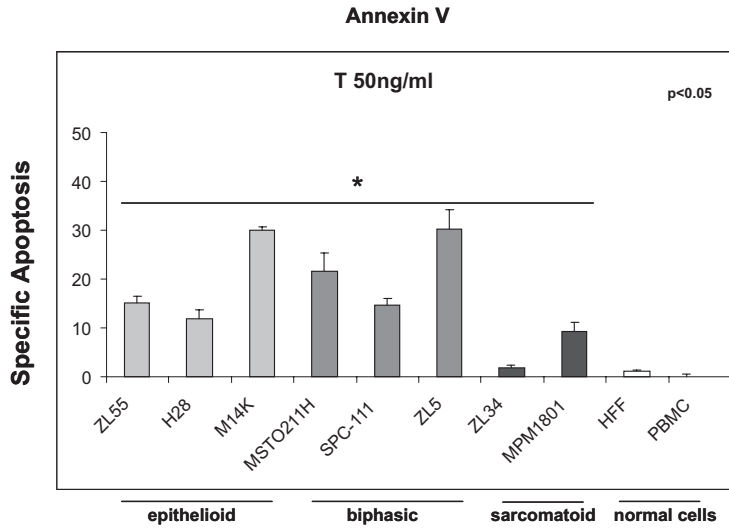
Figure 1a. *Anticancer effects of rhAPO2L/tumor necrosis factor-related, apoptosis-inducing ligand (TRAIL) in malignant pleural mesothelioma (MPM) cell lines and primary culture. MPM cell lines and control cells (human foreskin fibroblasts and peripheral blood mononuclear cells) were treated with rhAPO2L/TRAIL (T) 50 ng/ml for 24 hours and Annexin V staining was evaluated. Results were represented as mean ± SE of three different experiments running in duplicate. *Statistically significant difference of specific apoptosis between MPM cells treated with rhAPO2L/TRAIL compared with untreated cells ($p < 0.05$) by Mann-Whitney test.*
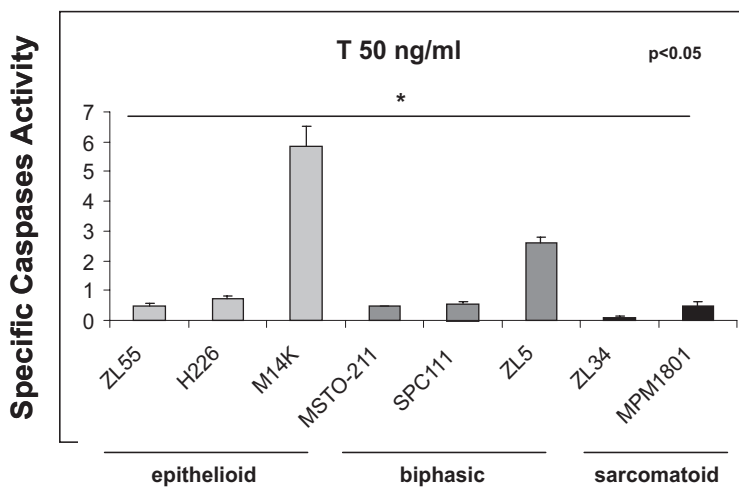
Figure 1b. *Anticancer effects of rhAPO2L/TRAIL in MPM cell lines and primary culture (Caspases assay).*

2a.
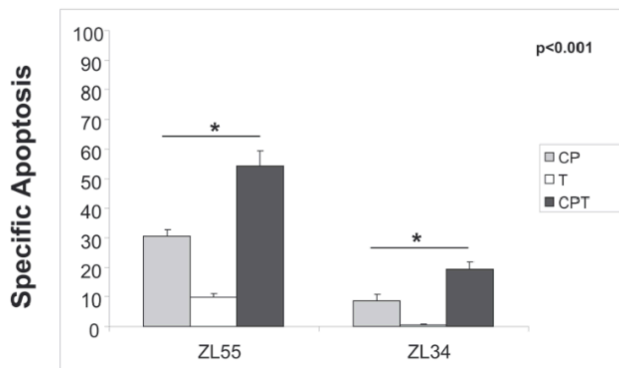
2b.
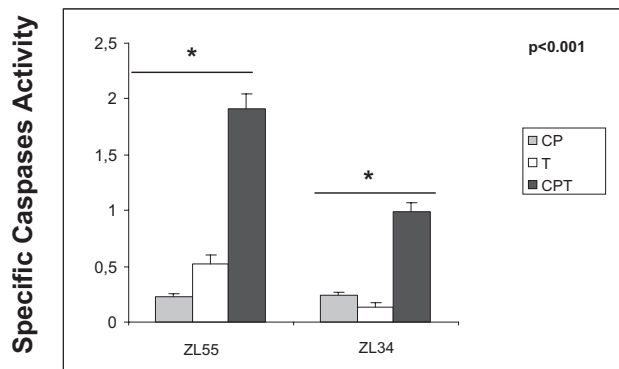
Figure 2a and 2b

ZL55 and ZL34 cell lines were previously treated with Carboplatin plus Pemetrexed (CP) for 24 hours followed by rhAPO2L/TRAIL (T) 50 ng/ml for 24 hours and apoptosis induction was evaluated by AnnexinV/PI staining (2a) and caspases assay (2b). Mean of Specific Apoptosis ± SE of three independent experiments running in triplicate are given.*statistically significant difference of Specific Apoptosis and specific caspases activity between MPM cells treated with CPT compared with MPM cells treated with CP (p< 0.001) by Mann-Whitney test.
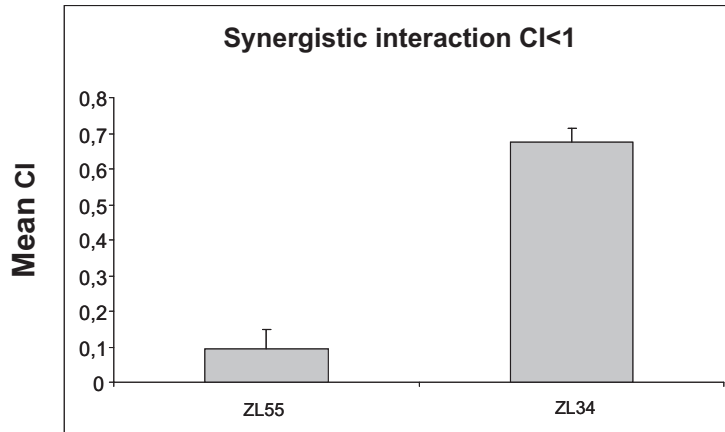
Figure 2c

ZL55 and ZL34 cell lines were treated with different concentration of Carboplatin/Pemetrexed (in constant ratios) and rhAPO2L/TRAIL. The synergistic apoptosis induction of the combinations was calculated using the CI methods and represented as the mean of CI values at FA (fraction affected) 0.25, 0.5, 0.75 and 0.9. Results were represented as mean ± SE of three different experiments running in triplicate.
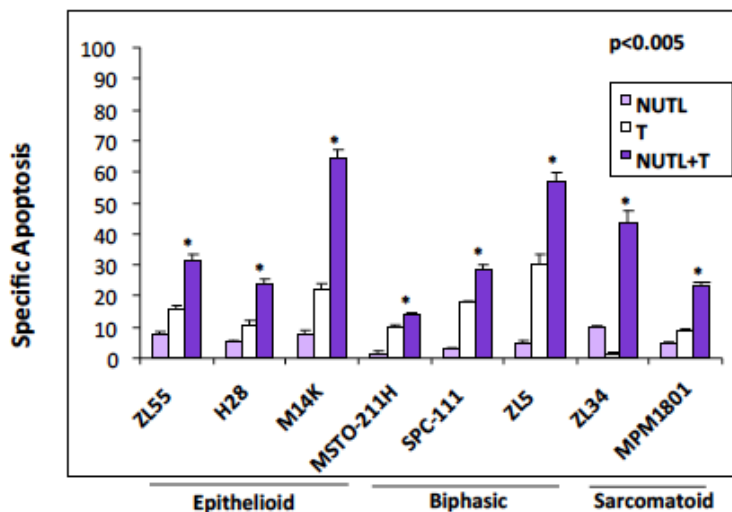
Figure 2d

*MPM cell lines were treated with Nutlin3a (NUTL) 10uM and Apo2L/TRAIL (T) 50ng/ml for 24 hours and analyzed by AnnexinV staining. Specific Apoptosis was calculated by the following formula: (percentage of Annexin V positive cells in treated samples- percentage of Annexin V positive cells in untreated samples) / (100- percentage of Annexin V positive cells in untreated samples)*100. The results were represented as means ± SE of 3 different experiments running in triplicate.* statistically significant difference of Specific Apoptosis between cells treated with NUTL+T compared with cells treated with T (p< 0.05) (Mann-Whitney test)*

63
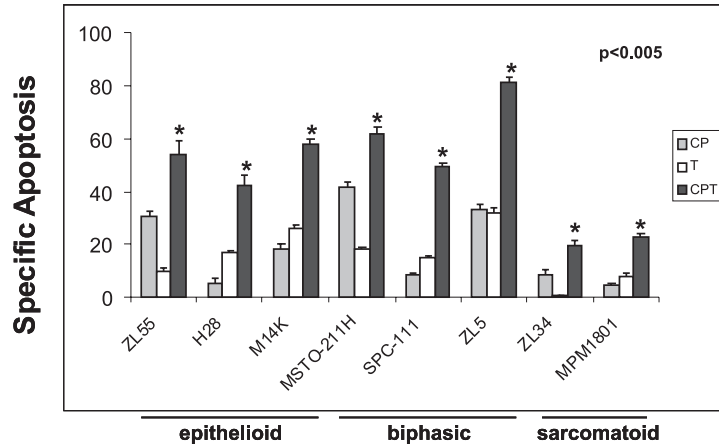
Figure 3

MPM cell lines were previously treated with Carboplatin plus Pemetrexed (CP) for 24 hours followed by rhAPO2L/TRAIL (T) for 24 hours and Specific Apoptosis was detected by Annexin V staining. Results were represented as mean ± SE of three different experiments running in duplicate.

*statistically significant difference of Specific Apoptosis between MPM cells treated with CPT compared with MPM cells treated with CP (p< 0.005) by Mann-Whitney test.
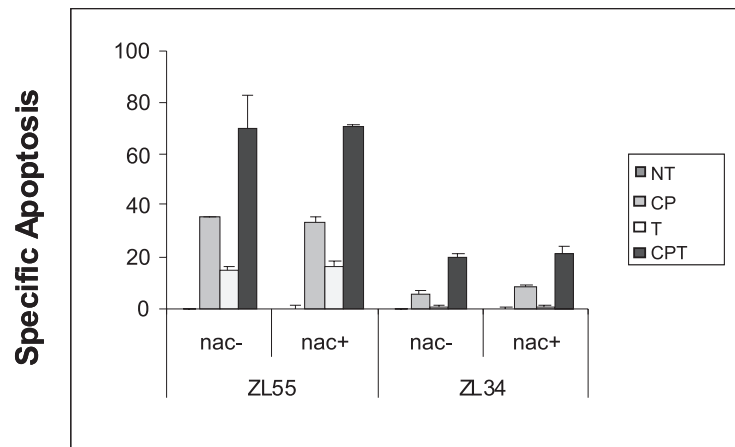
Figure 4

MPM cells were previously incubated with 100 uM NAC overnight and then treated or not (NT) with Carboplatin/Pemetrexed (CP) and/or rhAPO2L/TRAIL (T). Specific Apoptosis was detected by Annexin V staining. Results were represented as mean ± SE of three different experiments running in duplicate
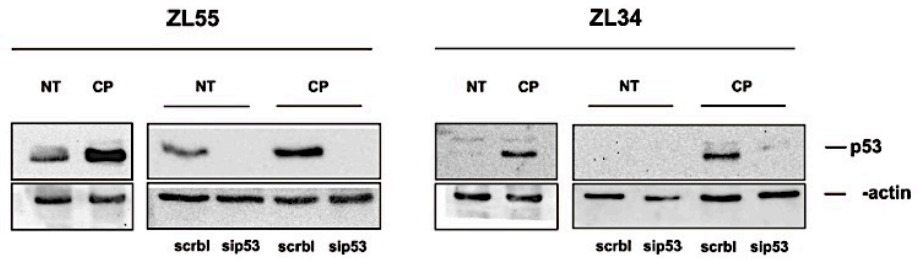
Figure 5a

Western blot analysis of p53 protein expression levels in ZL55 and ZL34 cell lines treated or not with CP for 24 hours. Where indicated, cells were previously treated with 25nM of negative control scrambled sequence (scrbl) or siRNA-p53
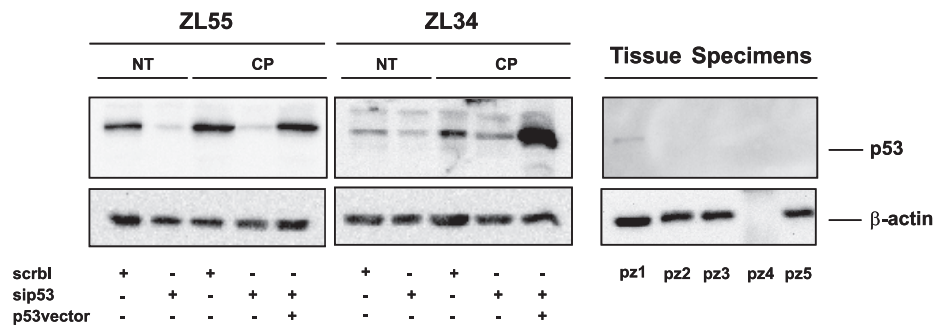
Figure 5b

MPM cell lines were treated with scrbl or siRNA-p53 (25 nM) in presence or not of wild-type p53 vector (200 ng) for 24 hours followed by CP stimulation and western blot analysis of p53 protein expression levels was performed (left panel). Western blot analysis of p53 protein expression levels in tissue specimens of 5 MPM patients (right panel). We were unable to detect proteins expression in patient 4 probably due to degradation of the sample
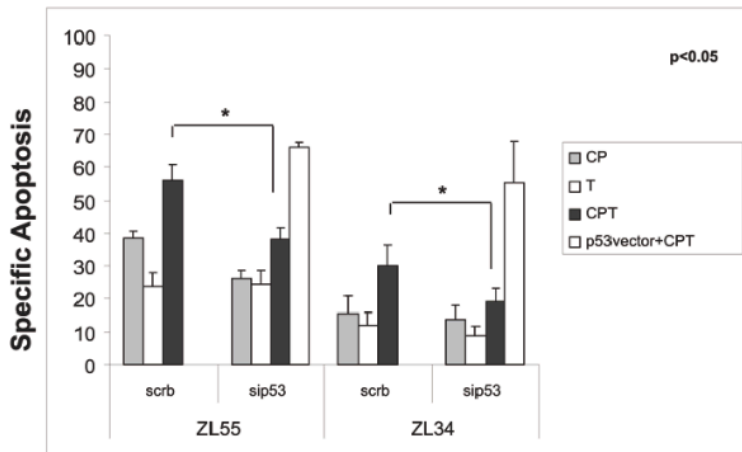
Figure 5c

*MPM cell lines were treated with scrbl or siRNA-p53 (25 nM) in presence or not of wild-type p53 vector (200 ng) for 24 hours followed by CP and/or T stimulation and Annexin V/PI assay was performed. Results were represented as mean ± SE of three different experiments running in duplicate. *statistically significant difference of Specific Apoptosis between MPM cells treated with scrbl plus CPT compared with cells treated with siRNA-p53 plus CPT (p< 0.05) by Mann-Whitney test.*
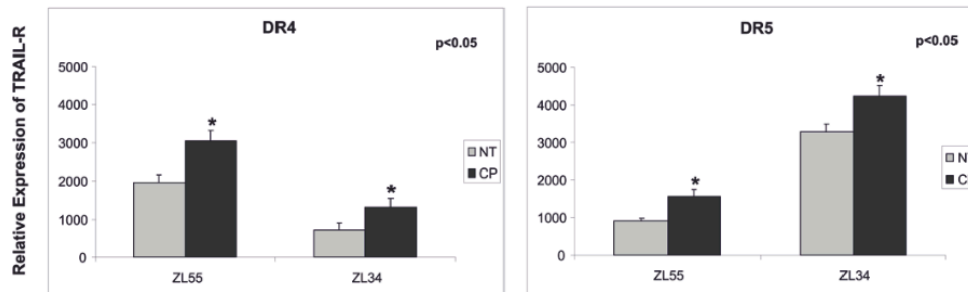
Figure 6a

*Flow cytometry analysis of TRAIL-Receptors expression levels in ZL55 and ZL34 cell lines treated with Carboplatin/Pemetrexed (CP) for 24 hours. The graph bar represents the mean of relative expression of TRAIL-R (for DR4 and DR5) ± SE of three independent experiments running in triplicate. * statistically significant difference of receptor expression levels between MPM cells untreated (NT) compared to MPM cells treated with CP (p< 0.05) by Mann-Whitney test.*
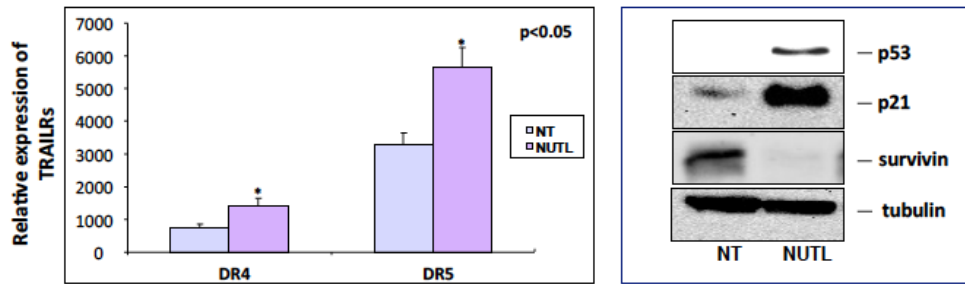
Figure 6b

*ZL34 cell lines were treated with NUTL 10uM for 24 hours: A) Flow cytometry analysis of DR4/DR5 TRAIL-Receptors expression levels. Relative expression of TRAIL-R was calculated by the following formula: percentage of positive cells x mean fluorescence intensity (MFI). The graph bar represents the mean of relative expression of TRAILR ± SE of three independent experiments running in triplicate. * statistically significant difference of receptor expression levels between MPM cells untreated (NT) compared to MPM cells treated with NUTL (p< 0.05) (Mann-Whitney test). B) Western blot analysis of p53, p21, survivin and tubulin (as loading control) expression levels.*
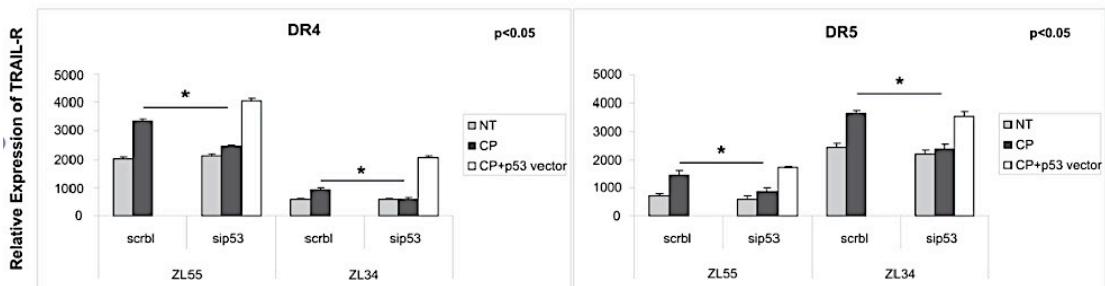
Figure 7

*Flow cytometry analysis of TRAIL-Receptors expression levels in ZL55 and ZL34 cell lines treated with negative control scrambled sequence (scrbl) or siRNA-p53 (25 nM) in presence or not of wild-type p53 vector (200 ng) for 24 hours followed by CP treatment. A) Representative dot blot representing MPM cells treated with scrbl plus CP versus siRNA-p53 plus CP. p≤ 0.001 indicate differences in fluorescence by Kolmogorov-Smirnov Statistics. B) The results were represented as mean of relative expression of TRAIL-R ± SE of three different experiments running in duplicate. * statistically significant difference of receptor expression levels between MPM cells treated with scrbl plus CP compared to MPM cells treated with siRNA-p53 plus CP (p< 0.05) by Mann-Whitney test.*
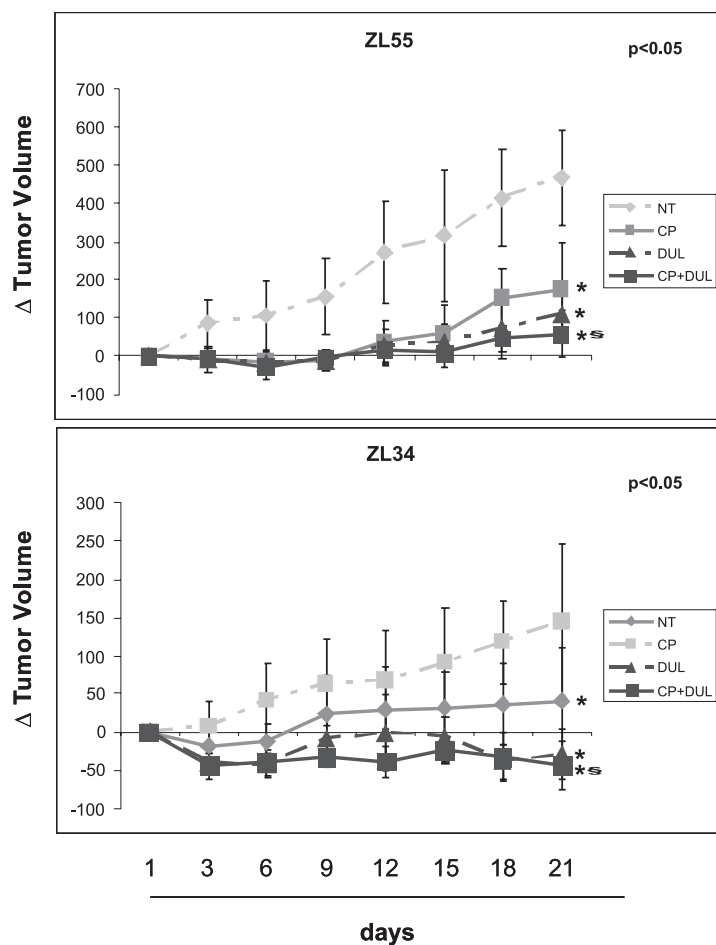
Figure 8a and 8b

*Mice were subcutaneously injected with $2x10^6$ of ZL55 (8a, upper graph) or ZL34 (8b, lower graph) cell lines and randomized in four treatment groups (n=6/group). When tumor volumes reached $50mm^3$ (day 1), each group received by intraperitoneal injection vehicle or Carboplatin/Pemetrexed (CP) (on day 1) or rhAPO2L/TRAIL (Dulanermin, DUL) (on days 1,2,3) or CP+DUL (on day 1 and days 1,2,3 respectively). Tumor volumes were recorded every third day. The results were represented as mean of delta volume ± SD. * statistically significant difference of delta tumor volume between treated groups and untreated controls (p<0.05). § statistically significant difference of delta tumor volume between CPT and CP (p<0.05) by analysis of variance (ANOVA) followed by LSD post hoc test.*

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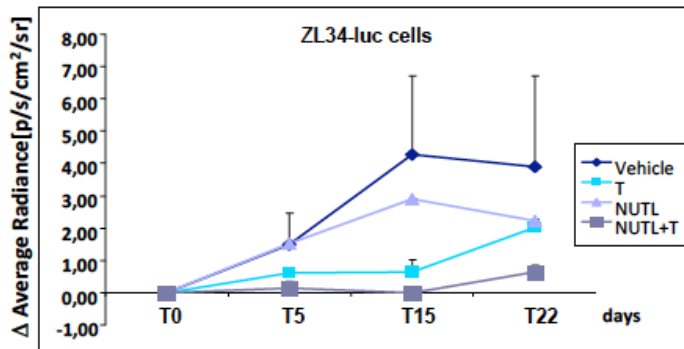
Figure 9

Mice were intraperitoneally (IP) injected with 5×10^6 ZL34 cells previously transduced with lentiviral vector containing a plasmid encoding for Luciferase. 3 weeks post injection the mice were randomized in 4 treatment groups and treated with Nutlin3 3a or vehicle (days 1-21) by gavage and/or APO2L/TRAIL (days 1-3) by IP. Tumor size was assessed at the indicated time point by in vivo bioluminescence using Xenogen bioluminescence imaging after IP injection of D-luciferin in each mouse. The mice were suppressed at the 22th day. Average Radiance [p/s/cm²/sr] was proportional to the number of ZL34 cells expressing the LUC gene and D Average radiance was used as indicator of tumor growth and calculated by the following formula: (Average Radiance at the day n- Average Radiance at the day 1)/Average radiance at the day 1
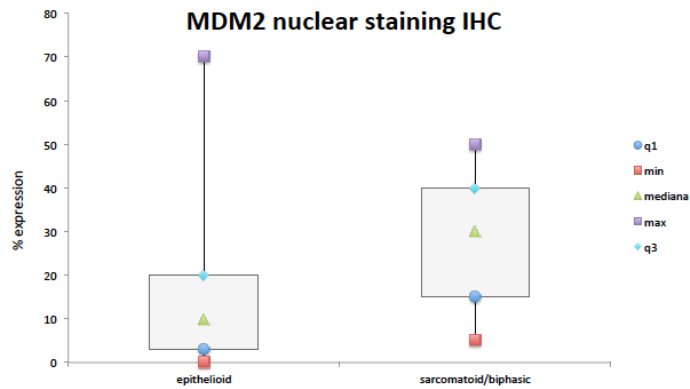
Kruskal-Wallis One Way Analysis of Variance on Ranks:

MDM2	N	median	25%	75%	p value
Epithelioid	25	10	2.5	20	/
Sarcomatoid/Biphasic	13	30	12.5	40	/
Epithelioid vs Sarcomatoid/Biphasic	/	/	/	/	0.010

Figure 10. *Expression levels of MDM2 in epithelioid versus sarcomatoid/biphasic samples.*
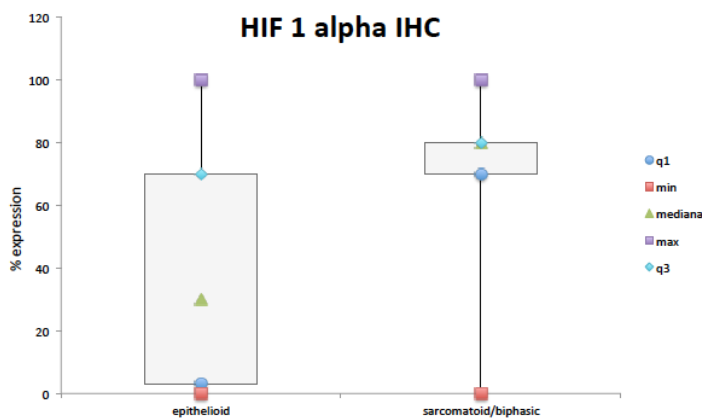
Kruskal-Wallis One Way Analysis of Variance on Ranks:

HIF1alpha	N	median	25%	75%	p value
Epithelioid	25	30	3	70	/
Sarcomatoid/Biphasic	13	80	50	90	/
Epithelioid vs Sarcomatoid/Biphasic	/	/	/	/	0.007

Figure 11. *HIF1alpha expression levels in epithelioid versus sarcomatoid/biphasic samples.*

a.   b.   c.
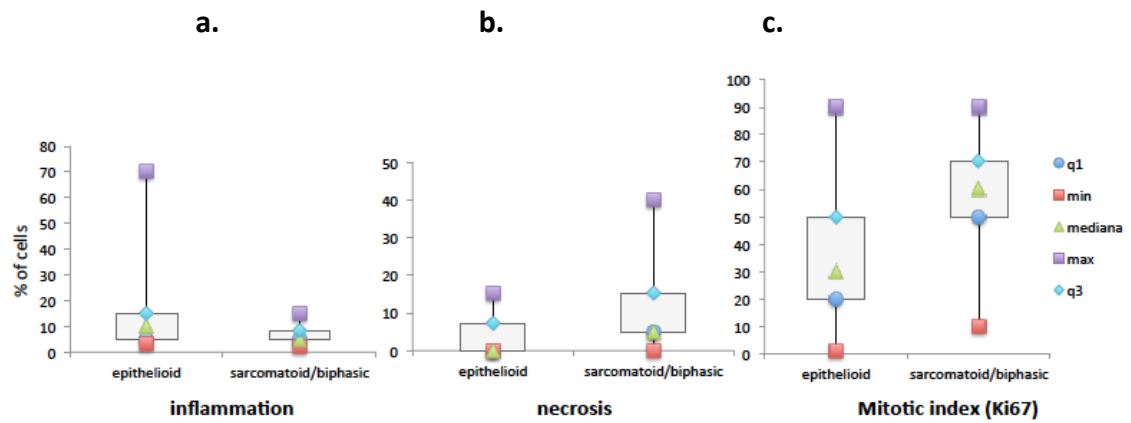
d.

Kruskal-Wallis One Way Analysis of Variance on Ranks:

	N	median	25%	75%	*p* value
inflammation					
E	25	10	5	20	
S/B	13	5	5	9	
E *vs* S/B					0.112
necrosis					
E	25	0	0	8.5	
S/B	13	5	4	22.5	
E *vs* S/B					0.07
Ki67					
E	25	30	20	50	
S/B	13	60	50	70	
E *vs* S/B					0.005*

Figure 12. *Inflammation (a), necrosis (b) and proliferation index (c) in epithelioid versus sarcomatoid/biphasic samples (d).*

E:epithelioid; S/B: sarcomatoid/biphasic; * statistically significant
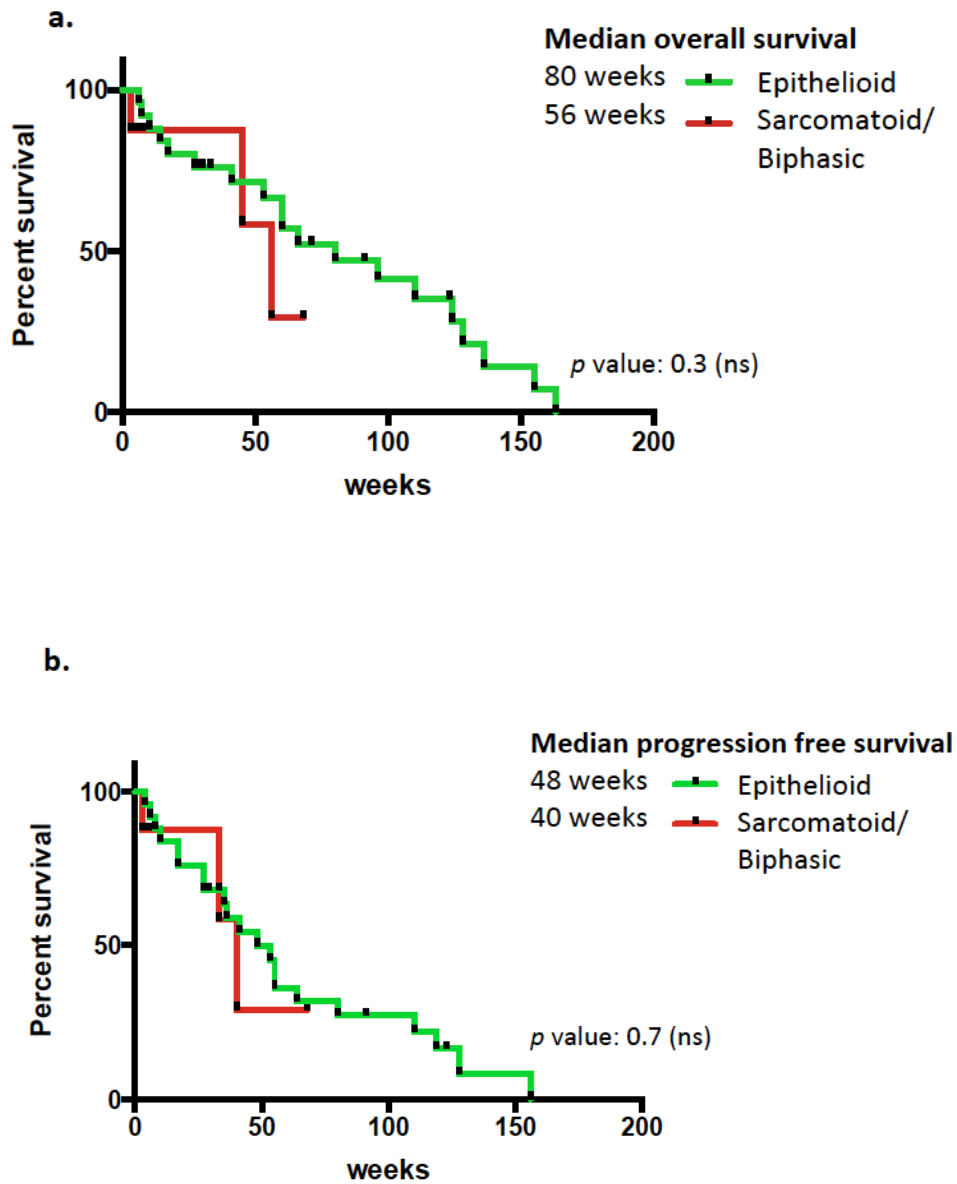
Figure 13. *Overall survival (a) and Progression Free Survival (b) in epithelioid versus sarcomatoid/biphasic MPM patients.*
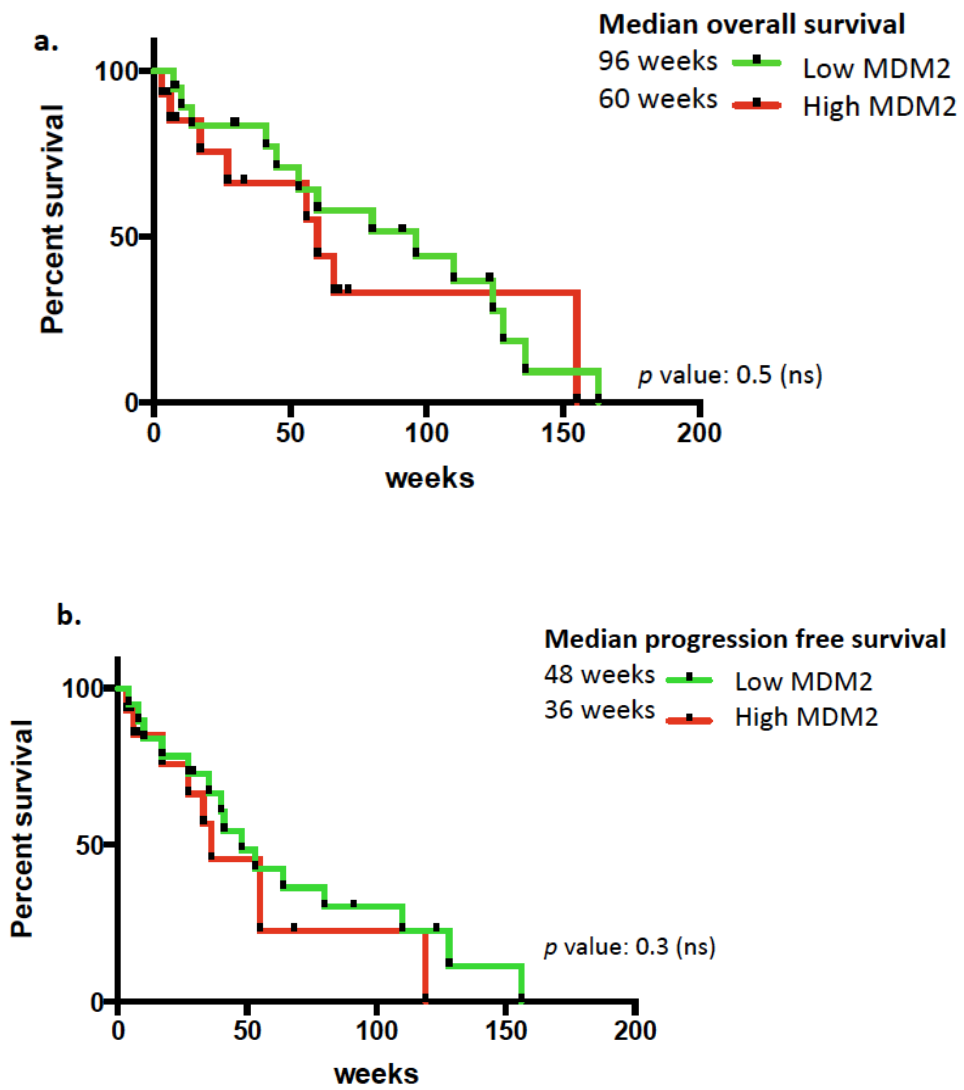
Figure 14. *Overall survival (a) and Progression Free Survival (b) in low MDM2 versus high MDM2-expressing MPM patients.*
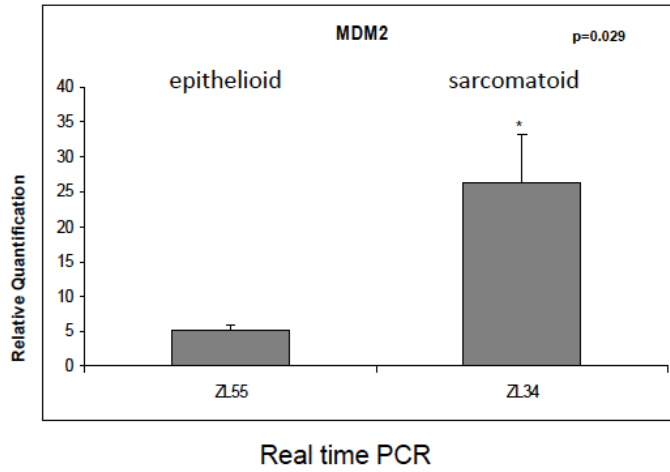
Figure 15. *mRNA MDM2 expression levels in epithelioid versus sarcomatoid mesothelioma cell lines.*

Table 1. Patients' clinical features (*N*=38)

Patients' characteristic		Study population *N*=38*
Age		Median 68; Range (36-89)
Gender	Male	31 (82%)
	Female	7 (18%)
Histology	Epithelioid	25 (66%)
	Biphasic	7 (18%)
	Sarcomatoid	6 (16%)
Stage (*N*=20)*	I	1 (5%)
	II	7 (35%)
	III	9 (45%)
	IV	3 (15%)
ECOG PS	0	10 (26%)
	1	24 (63%)
	2	4 (11%)
EORTC score	High	28 (74%)
	Low	6 (16%)
	Unknown	4 (10%)
Surgery	No	29 (76%)
	EPP	3 (8%)
	P/D	6 (16%)
Response to first-line chemotherapy (*N*=22)*	CR	1 (4%)
	PR	2 (9%)
	SD	14 (64%)
	PD	5 (23%)

Table 2. MDM2 expression levels at IHC and RT-qPCR through patients' samples.

MDM2	IHC strong nuclear staining	RT-qPCR
EPITHELIOID	*N=25*	*N=20*
Low	68%	65%
High	32%	35%
SARCOMATOID/ BIPHASIC	*N=13*	*N=12*
Low	23%	25%
High	77%	75%
NORMAL PLEURA (N=4)	negative	negative

Table 3. HIF1alpha expression levels at IHC and RT-qPCR through patients' samples.

HIF1alpha	IHC	RT-qPCR
EPITHELIOID	N=25	N=20
Low	64%	50%
High	36%	50%
SARCOMATOID/ BIPHASIC	N=13	N=12
Low	23%	50%
High	77%	50%
NORMAL PLEURA (N=4)	negative	negative

Table 4. VEGF expression levels at IHC across tumor samples.

VEGF	IHC strong	RT-qPCR
EPITHELIOID	N=17	N=17
Low	65%	60%
High	35%	40%
SARCOMATOID/ BIPHASIC	N=10	N=10
Low	67%	55%
High	33%	45%
NORMAL PLEURA (N=4)	variable	variable

Table 5. Inflammation, necrosis and proliferation index across tumor samples.

EPITHELIOID (N=25)	INFLAMMATION	NECROSIS	Ki67
Low	48%	72%	72%
High	52%	28%	28%
SARCOMATOID/ BIPHASIC (N=13)			
Low	62%	69%	23%
High	38%	31%	77%

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10. European curriculum vitae

Curriculum Vitae Europass	
Personal Information	
Surname(s) / Name(s)	**PASELLO GIULIA**
Address	VIA R. TOGNANA 16
	31100 TREVISO (TREVISO)
Mobile	003932069808 3
E-mail	giulia.pasello@ioveneto.it
Nationality	Italian
Date of birdth	17/10/1979
Gender	Female
Occupational Field	**Medical Oncology**
Work experience	
Dates	07/01/2014-current position
Occupation or position held	Associate Medical Director
Main activities and responsabilities	Clinical and research activity in thoracic oncology
Name and address of employer	Second Medical Oncology Unit, Istituto Oncologico Veneto, Via Gattamelata 64, 35128 Padua (Italy)
Type of business or sector	Medical Oncology
Dates	01/02/2010- 06/01/2014

Occupation or position held	Medical Oncologist (with contract)
Main activities and responsabilities	Clinical and research activity in thoracic oncology
Name and address of employer	Second Medical Oncology Unit, Istituto Oncologico Veneto Via Gattamelata 64, 35128 Padua (Italy)
Type of business or sector	Medical oncology
Dates	01/07/2009 - 01/12/2009
Occupation or position held	Fellow
Main activities and responsabilities	Basic research about malignant pleural mesothelioma
Name and address of employer	Molecular Oncology laboratories, University of Zurich (Switzerland), Prof. Rolf Stahel
Type of business or sector	Molecular Oncology
Dates	01/07/2008 - 31/12/2008
Occupation or position held	Resident in Medical Oncology
Main activities and responsabilities	Clinical management of oncological pediatric patients
Name and address of employer	Pediatric oncohematology, University of Padua (Italy)
Type of business or sector	Pediatric Oncology and Haematology
Dates	01/07/2007 - 31/12/2009
Occupation or position held	Resident in Medical Oncology
Main activities and responsabilities	Clinical management and research in Oncology
Name and address of employer	Second Medical Oncology Unit, Istituto Oncologico Veneto Padua (Italy)

Type of business or sector	Medical Oncology
Dates	01/01/2006 - 30/06/2007
Occupation or position held	Resident in Medical Oncology
Main activities and responsabilities	Basic and translational research in oncology
Name and address of employer	Oncological and Surgical Science Department, Oncology Section, University of Padua (Italy)
Type of business or sector	Molecular Oncology
Education and Training	
Dates	01/01/2012-currently ongoing
Title of qualification awarded	PhD
Principal subjects/occupational skills covered	Neoangiogenic biomarkers and therapeutic targets in malignant pleural mesothelioma
Dates	01/01/2006 - 31/12/2010
Title of qualification awarded	Residency in Medical Oncology
Principal subjects/occupational skills covered	Medical Oncology
Name and type of organisation providing education and training	Residency School in Medical Oncology (University of Padua) Padua (Italy)
Dates	01/10/1999 - 11/10/2005
Title of qualification awarded	Graduation in medicine and surgery
Name and type of organisation providing education and training	University of Ferrara (Ferrara, Italy)
Other training experiences	March 2014: admitted at the 13th ESO ESMO Masterclass in Clinical Oncology where she attended plenary lectures regarding state-of-the-art clinical evaluation and treatments in oncology. As a selected

participant the applicant presented a research abstract which was selected for publication and had the opportunity to present and discuss with the faculty clinical cases of thoracic cancer.

Personal skills and competences	
Mother tongue(s)	**Italian**
Other language(s)	**English:** excellent reading, writing, speaking
Organisational skills and competences	Clinical and translational research projects design and conduction.
Additional informations	

- 2010-2011: recipient of ESMO (European Society for Medical Oncology) translational research fellowship award

- 2012: recipient of IMIG (International Mesothelioma Interest group) travel award

-2014 'Saro Leggio' award for translational study of malignant pleural mesothelioma

- 2011- current position: National AIOM (Assicioazione Italiana di Oncologia Medica) young working group elected member

- Since January 2014 she is lecturer at the fith year of Specialization School of Medical Oncology with the teaching 'Epidemiology, diagnosis and staging of neuroendocrine tumors', and supervisor of several students attending the Specialization School in Medical Oncology

Participation as a speaker and scientiific committee to several national conferences

Summary of scientific and research activity

Several research and scientific activities have been done during the three years of PhD program. First, the scientific literature study about the main object of the project – malignant pleural mesothelioma- lead to the study design which took into account the main unmet medical needs and scientific questions in the specific context.

After study design and planning and working group constitution, a grant application was performed in order to receive support for preclinical and traslational studies. The European Society for Medical Oncology (ESMO) translational research fellowship award was received in 2012 and renewed also for the second year. The project was mainly divided into two aims. Preclinical studies about new drug combinations for the treatment of the disease under study were performed during the first two years, while the third year was focused on translational studies investigating treatment targets and morphological features on patients samples. In July 2014 the first paper reporting data from this project was published, and currently two more papers are under review.

Malignant mesothelioma was the main field of interest during the three years of the PhD program, both as clinical management and research studies, and several papers were published as single-center thoracic oncology group and in collaboration with other specialized center.

Scientific publication with impact factor (2012-2014)

1° year

- Gridelli C, Gallo C, Morabito A, Iaffaioli RV, Favaretto A, Isa L, Barbera S, Gamucci T, Ceribelli A, Filipazzi V, Maione P, Rossi A, Barletta E, Signoriello S, De Maio E, Piccirillo MC, Di Maio M, Rocco G, Vecchione A, Perrone F; G-STEP Investigators "Phase I-II trial of gemcitabine-based first-line chemotherapies for small cell lung cancer in elderly

patients with performance status 0-2: the G-STEP trial" J Thorac Oncol. 2012 Jan;7(1):233-42

- Di Maio M, Leighl NB, Gallo C, Feld R, Ciardiello F, Butts C, Maione P, Gebbia V, Morgillo F, Wierzbicki R, Favaretto A, Alam Y, Cinieri S, Siena S, Bianco R, Riccardi F, Spatafora M, Ravaioli A, Felletti R, Fregoni V, Genestreti G, Rossi A, Mancuso G, Fasano M, Morabito A, Tsao MS, Signoriello S, Perrone F, Gridelli C; TORCH Investigators."Quality of life analysis of TORCH, a randomized trial testing first-line erlotinib followed by second-line cisplatin/gemcitabine chemotherapy in advanced non-small-cell lung cancer." J Thorac Oncol. 2012 Dec;7 (12):1830-4

- Pasello G, Marulli G, Polo V, Breda C, Bonanno L, Loreggian L, Rea F, Favaretto A. "Pemetrexed plus carboplatin or cisplatin as neoadjuvant treatment of operable malignant pleural mesothelioma (MPM)" Anticancer Res. 2012 Dec;32 (12):5393-9.

2° year

- Pasello G, Ceresoli GL, Favaretto A "An overview of neoadjuvant chemotherapy in the multimodality treatment of malignant pleural mesotelioma". Cancer Treat Rev. 2013 Feb;39 (1):10-7

- Marulli G, Di Chiara F, Braccioni F, Perissinotto E, Pasello G, Gino Favaretto A, Breda C, Rea F. "Changes in pulmonary function tests predict radiological response to chemotherapy in malignant pleural mesothelioma" Eur J Cardiothorac Surg. 2013 Jul; 44 (1):104-10

-Pasello G, Urso L, Conte P, Favaretto A. "Effects of Sulfonylureas on Tumor Growth: A Review of the Literature". Oncologist. 2013;18(10):1118-25

3° year

- Pasello G, Urso L, Silic-Benussi M, Schiavon M, Cavallari I, Marulli G, Nannini N, Rea F, Ciminale V, Favaretto A. "Synergistic antitumor activity of recombinant human Apo2L/tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in combination with carboplatin and pemetrexed in malignant pleural mesothelioma."J Thorac Oncol. 2014 Jul;9(7):1008-17.

- Ceresoli GL, Grosso F, Zucali PA, Mencoboni M, Pasello G, Ripa C, Degiovanni D, Simonelli M, Bruzzone A, Dipietrantonj C, Piccolini E, Beretta GD, Favaretto AG, Giordano L, Santoro A, Botta M."Prognostic factors in elderly patients with malignant pleural mesothelioma: results of a multicenter survey."Br J Cancer. 2014 Jul 15;111(2):220-6.

- Marulli G, Verderi E, Zuin A, Schiavon M, Battistella L, Perissinotto E, Romanello P, Favaretto AG, Pasello G, Rea F."Outcomes and prognostic factors of non-small-cell lung cancer with lymph node involvement treated with induction treatment and surgical resection."Interact Cardiovasc Thorac Surg. 2014 Aug;19(2):256-62

- Schiavon M, Marulli G, Nannini N, Pasello G, Lunardi F, Balestro E, Perissinotto E, Rebusso A, Saetta M, Rea F, Calabrese F. "COPD-related adenocarcinoma presents low aggressiveness morphological and molecular features compared to smoker tumours". Lung Cancer. 2014 Dec; 86(3):311-7.

- Pasello G, Carli P, Canova F, Bonanno L, Polo V, Zago G, Urso L, Conte PF, Favaretto A. "Epirubicin *plus* Paclitaxel regimen as second line treatment of Small-Cell Lung Cancer (SCLC) patients" IN PRESS

Book chapter (2012-2014)

- Giulia Pasello and Adolfo Favaretto. Neoadjuvant Chemotherapy in Malignant Pleural Mesothelioma. Malignant Mesothelioma Chapter 5 December 2012.

http://dx.doi.org/10.5772/47880

- Giovanni Luca Ceresoli, Giulia Pasello, Fiorella Calabrese.

ESMO book: Thoracic Tumours –Essentials for Clinicians: Malignant Pleural

Mesothelioma

March 2014

<u>Conference participation (2012-2014)</u>

-advances in medicine 23 march 2012, Padova

- 2012 american society of clinical oncology (asco) meeting 1-5 june 2012, Chicago (il)

- primo congresso italo-greco di oncologia medica, Padova, 10-11 may 2012:

"treatment of malignant pleural mesothelioma: multimodality management and new

perspectives" (<u>speaker</u>)

-aiom post asco review: updates and news from the annual meeting in chicago,

Bologna 16-17 june 2012: "lung cancer: asco poster review" (<u>speaker</u>)

- endothelial progenitors cells from discovery to therapy 21 june 2012, Padova

- hot topics in oncologia toracica: stato dell'arte sulla terapia medica del mesotelioma

pleurico, Bergamo 22 june 2012 (<u>speaker</u>)

- the 11th international mesothelioma interest group (imig) conference 11-14

september 2012, Boston (ma) (<u>speaker</u>)

- malignant pleural mesothelioma: clinical pathological and legal features 8 november

2012, Padova(<u>speaker</u>)

- corso nibit: immunobioterapia nelle neoplasie toraciche, Padova 23 november 2012

(<u>speaker</u>)

<u>-</u> xii congresso nazionale fonicap (forza operativa nazionale interdisciplinare contro il

cancro al polmone), Verona 30 november-1 december 2012 (<u>speaker</u>)

- corso nazionale aiom e siapec-iap "marcatori biomolecolari nella terapia

personalizzata dei tumori: indicazioni cliniche e di laboratorio", Napoli 22 march 2013

- "antiangiogenic therapy: recent advances and future directions in oncology", Padova

23 april 2013

- corso educazionale macroregionale aiom giovani "metodologia della ricerca clinica in

oncologia", Verona 3-4 may 2013 (speaker)

- convegno aiom giovani veneto: "tumori rari: tra empirismo ed evidenza", Padova 21

june 2013 (speaker)

- convegno nazionale aiom giovani "news in oncology 2013", Perugia 5-6 july 2013

(speaker)

- corso di formazione del personale medico in oncologia generale, Bugando medical

centre, Mwanza, Tanzania, 30 september 2013 – 04 october 2013 (speaker)

- congresso fonicap le terapie biologichenel nsclc: update dopo iaslc 2013, Verona 9

december 2013 (speaker)

- 2014 esmo-christie lung cancer course, manchester (uk) 12-14 february 2014

-13th eso-esmo masterclass in clinical oncology, 8 march 2014-13 march 2014,

Ermatingen Switzerland

- hot topics in oncologia toracica:il mesotelioma pleurico, Bergamo 24 may 2014

(speaker)

- aiom post-asco 2014, Bologna 13-14 june 2014 (speaker)

- convegno nazionale aiom giovani "news in oncology 2014", Perugia 11-12 july 2014

(speaker)

- 2014 esmo meeting, Madrid 26 september 2014- 30 september 2014 (poster

presenter)

- chest tumors: what's new from asco-esmo meetings? Padova 9-10 october 2014
(speaker)

- the 12th international mesothelioma interest group (imig) conference 21 october
2014-24 october 2014 Cape Town Africa (poster presenter)

- corso macroregionale aiom di metodologia della ricerca, Torino 7-8 november 2014
(speaker)

Awards and grants:

- 2010-2011 and 2011-2012: recipient of ESMO (European Society for Medical
  Oncology) translational research fellowship award

- 2012: recipient of IMIG (International Mesothelioma Interest group) travel
  award

- 2014: premio per la ricerca 'Saro Leggio' (AIRPP)

Teaching activity

- professor with contract 5° year of residency school in medical oncology with
  the teaching: epidemiology, diagnosis and staging of neuroendocrin tumors

Research activity

- -Coinvestigator of more than 30 clinical trials on lung cancer, pleural
  mesothelioma and thymic cancer

- Principal investigator of preclinical studies on the anticancer activity of new
  drugs combinations in malignant pleural mesothelioma.

- Principal investigator of retrospective studies on lung cancer and pleural
  mesothelioma.