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# Expression analysis of High Mobility Group Box 1 (HMGB1) in histological samples of malignant pleural mesothelioma and its clinical significance

Eltjona Rrapaj, Elena Trisolini, Luca Bertero, Michela Salvo, Rossella Indelicato, Silvano Andorno, Josè M Garcia-Manteiga, Ottavio Rena, Renzo L Boldorini

## Introduction

Malignant mesothelioma (MM) is a rare and aggressive tumour arising from the neoplastic transformation of mesothelial cells lining the pleural and peritoneal cavities, or, less commonly, the pericardium. Malignant Pleural Mesothelioma (MPM) accounts for about 70% of all mesotheliomas (Bridda A, et al. *MedGenMed*, 2007). Causal relationship with asbestos or asbestos-like fibers exposure has been established from time (Wagner et al, 1960), although other potential carcinogenic agents, including infection by Simian Virus 40, radiation exposure (Yang et al, 2008) and genetic predisposition (Cheung M. et al, *Cancer Genetics*, 2013) should be considered. The estimated annual incidence of MPMs in Europe (15–33 cases per million inhabitants) is expected to further increase over the next 20 years because of the long latency between exposure to risk factors and disease development (Peto et al, 1999).

The definite diagnosis of MPM is based on the histologic and immunohistochemistry evaluation of pleural biopsies (Husain A.N. et al, *Arch Pathol Lab Med*, 2013). Patients with MPM have poor prognosis (Fennell, D. A., et al, 2008; Vogelzang et al, 2003), with a median survival ranging from 8 to 12 months (Vogelzang et al, 2003). The gold standard of treatment is a combination of cisplatin (DDP) and pemetrexed (Davidson, B. et al, *Human Pathology*, 2015), extending the patients' survival, on average, only 12 weeks. The European Organisation for Research and Treatment of Cancer has indicated that the main predictors of a negative prognosis are a poor performance status (PS), high white blood cell counts, male gender, non-epithelioid subtype, anaemia, and thrombocytosis (Curran et al, 1998). However, unlike other solid tumours, there are not yet specific tissue biomarkers with a prognostic significance useful in clinical practice.

High mobility group B1 (HMGB1) is a highly conserved nuclear protein, acting as a chromatin-binding factor that binds non-specifically to DNA and facilitates the assembly of transcriptional protein on specific DNA targets (Lotze M.T. et al, *Nat Rev Immunol*, 2005; Muller S. et al, *EMBO J*. 2001). In addition, HMGB1 can be released into the extracellular environment, where it exerts crucial functions in inflammation and carcinogenesis through different membrane receptors,

including the receptor for advanced glycation end products (RAGE), Toll-like receptors (TLR)-2, TLR-4 and TLR-9 (Yang H et al, *Biochim Biophys Acta*, 2010; Gebhardt C et al. *J Exp Med*. 2008; Mittal D, et al. *EMBO J*. 2010). The increased expression of HMGB1 stimulates cellular proliferation and metastasis in several cancers, including breast carcinoma (Brezniceanu ML. et al, *FASEB J*. 2003) and hepatocellular carcinoma (Kawahara N, *Cancer Res* 1996), melanoma (Poser I, *Mol Cell Biol* 2003), gastric (Akaike H, *Anticancer Res*. 2007) and colorectal cancer [Volp K, *Gut* 2006]. Previous studies indicated that HMGB1 plays a crucial role in the carcinogenesis of asbestos-exposed primary human mesothelial cells, facilitating or leading to mesothelioma initiation and progression (4, 9) (18). Another study indicates that HMGB1 is highly expressed and secreted by malignant mesothelioma cells, establishing an autocrine circuit that supports the malignant phenotype (Jube et al, 2012). These findings suggest an important role of HMGB1 in development and progression of mesothelioma, at least in preclinical studies. In addition, patients with peritoneal mesothelioma showed higher serum levels of HMGB1 compared to the controls, suggesting that this protein may be a useful biomarker for early diagnosis of malignant mesothelioma in clinical practice. However, powerful studies evaluating the prognostic significance of HMGB1 in tissue samples of MPM are, to the best of our knowledge, still lacking. In this work, we evaluated both protein expression and mRNA levels of HMGB1 by means of immunohistochemistry and qPCR from a large series of histological samples of MPM, in order to investigate its potential role as a novel prognostic biomarker.

## **Materials and methods**

### **Tumour samples**

Our study included biopsy samples from 170 patients with histologic and immunohistochemistry diagnosis of MPM from April 2005 to December 2014. All the samples were collected by means of video-assisted thoracoscopy performed at the Thoracic Surgery Unit at Maggiore hospital of Novara (Italy). For comparison, 8 biopsies of normal mesothelium (NM) (pleura sampled at the time of bullectomy in patients with pneumothorax), and 6 biopsies of reactive mesothelium (RM) (from patients with pleuritis) were also examined. Tumour and control samples were immediately fixed in formalin for 24 h, embedded in paraffin, and routinely processed for histology and immunohistochemistry. The diagnosis of MPM was based on standard histologic and

immunohistochemistry criteria, including positivity to calretinin, cytokeratins 5 and 6, Wilms Tumor 1 and negativity to carcinoembryonic antigen, thyroid transcription factor 1, and BerEP4. Clinical data were obtained retrospectively, and only those patients with adequate biopsy tissue and available clinical data were included in the study.

All the tumour samples were classified according to the WHO classification of pleural tumours (Travis et al, 2004), clinically and pathologically staged based on the TNM staging system (Sobin et al, 2009). The patients' Performance Status at the time of diagnosis was graded using the Eastern Cooperative Oncology Group (ECOG) scale (Oken et al, 1982), and the patients with a PS of 0–2 underwent therapeutic protocols indicated by the referring oncologist. One hundred five patients received cisplatin plus pemetrexed and 25 patients were chemotreated with a single agent (cisplatin). Forty patients did not receive treatment due to their PS (>2) or patients' refusal or because the advanced tumour. Haematoxylin/eosin-stained slides of the pleural biopsies/tumor fragments and corresponding formalin-fixed, paraffin-embedded blocks were reviewed by a pathologist (RB) to select a representative area with more than 50% of tumour cells.

### **ImmunoHistochemistry**

IHC analysis was performed in 170 MPM tissues, using standard protocols. The 3 µm thick sections were baked for 1 h at 65 °C and were deparaffinized with xylene and rehydrated with graded ethanol to distilled water. For epitope retrieval, slides were submerged in heated citrate buffer and exposed to microwave treatment for 30 minutes at 650 Watts. HMGB1 immunostaining process was performed on a DAKO Autostainer (Dako, Carpinteria, CA). The endogenous peroxidase activity of tissue sections was blocked by incubation with 3% H<sub>2</sub>O<sub>2</sub> for 5 minutes. The incubation with primary antibody was performed for 60 minutes at room temperature, using anti-HMGB1 (clone ab18256, Abcam; dilution 1:500) rabbit polyclonal antibody. Subsequently, the reaction was revealed with Envision Dual Rabbit/mouse detection system, using 3'-diaminobenzidine tetrahydrochloride (DAB) as chromogen. The slides were counterstained with hematoxylin. Negative controls were obtained by replacing the primary antibody with PBS. Normal liver parenchyma and reactive lymph nodes were selected as positive controls.

### **Evaluation of staining**

The expression of HMGB1 in MPMs was scored using the semi-quantitative system derived from Soumaoro (Soumaoro LT et al. Clin Cancer Res 2004) for both the percentage of positive cells and

the intensity of staining. In all the positive cases, the HMGB1 immunostaining was detected in the nuclei (N) and/or in the cytoplasm (C) of the tumour cells, therefore we applied independently three scoring methods (N, C and mean N+C) according to the system proposed by Koo et al, 2009 for the evaluation of p16 immunohistochemistry expression. The extent of staining was scored as 0 (0%), 1 (1-25%), 2 (26%-50%), 3 (51%-74%), and 4 ( $\geq 75\%$ ), according to the percentage of the positive staining. Then we evaluated the intensity of the staining and grouped them into the following four categories: no staining (score = 0), weak staining detectable above background (score = 1), moderate staining (score = 2), and intense staining (score = 3). The final index was obtained by the sum of the intensity and percentage scores for each subgroup.

For the purpose of statistical evaluation, samples with a final staining score  $>3$  were considered as high expression of HMGB1, whereas samples with a final score 0-3 were considered as low expression.

Each sample was independently scored by two pathologists. If an inconsistency occurred, slides were reviewed jointly by two pathologists and consensus reached.

### **RNA extraction, Reverse transcription and Quantitative Real Time PCR (qPCR)**

The neoplastic area was manual macrodissected from FFPE tissue blocks and 5  $\mu\text{m}$  thick sections were collected in a 1,5 mL tube for the RNA extraction process. After deparaffinization with xylene, RNA was isolated by using the RecoverAll Total Nucleic Acid Isolation Kit (ThermoFisher) following the manufacturer instructions. A total of 93 ng of mRNA per sample were reverse transcribed to cDNA by TaKaRa PrimeScript™ RT reagent Kit using 200 pmol of random examers for each reaction. Quantitative real time polymerase chain reaction qRT-PCR was performed in triplicate with 2  $\mu\text{l}$  of cDNA, 1x TaqMan® Gene Expression Master Mix and 1x of Taqman Gene Expression assay (Assay ID: HMGB1:-----, Life Technologies) in a final reaction volume of 10  $\mu\text{l}$ .

Samples were amplified by the ABI 7500 real-time PCR machine (Applied Biosystems) under the following thermal profile: initial incubation at 95°C for 20 seconds, 40 cycles of denaturation at 95°C for 15 seconds followed by annealing and extension at 60 °C for 30 seconds. Assay results were normalized to 18S rRNA (Eucaryotic 18S rRNA Endogenous Control; Life Technologies) and gene expression quantification was performed by  $\Delta\Delta\text{CT}$  methods using Sequence detector system 7500 software v2.0.4. A pool of normal pleuras (mesothelial cells) obtained from pneumothorax samples was used as a calibrator.

## Statistical analysis

Patients characteristics were described in terms of number and percentage, median and range. Disease-specific survival (DSS) was defined as the time from diagnosis to death or until April 2017, date of last follow-up for alive patients. Survival analyses were undertaken using the Kaplan-Meier method and curves were compared by the log-rank test. The association between IHC staining score or mRNA levels and the clinicopathological characteristics of the patients was analyzed respectively by chi-square test. The correlation between *HMGB1* gene expression and HMGB1 protein expression by IHC was analyzed by Pearson's test. All the statistical analysis were performed using STAT11 and GraphPad prism (version...; GraphPad software, Inc, La Jolla, CA) statistical software. The level of significance was set at  $P=0.05$ .

## Patients and clinicopathological characteristics

The main demographic and clinicopathologic characteristics of the patients included in the study are summarized in [Table 1](#): 118 patients were male (69,4 %) and 52 were female (30,6%), their mean age at diagnosis was 68,49 years  $\pm$  9,99 (SD) (range 27 - 91, median 70 years). Eighty-six patients (50,6 %) had previous exposure to asbestos at work. The histologic types of MPMs were: 125 (73,53 %) epithelioid, 23 (13,53 %) biphasic, and 22 (12,94 %) sarcomatoid. According to the 2009 TNM classification of malignant tumours by the International Union Against Cancer, 104 patients (61,2%) were in stage I-II, and 66 (38,8%) in stage III- IV. Eastern Cooperative Oncology Group PS (performance status) was 0–2 in 146 (85,9%) patients, and 3-4 in 23 (13,53%) patients. One-hundred-five patients (61,8%) were treated with platinum plus pemetrexed and 25 (14,7%) with platinum alone; whereas 40 (23,5%) patients received best supportive care alone since they refused therapy or because their PS was  $>2$ .

Follow-up data after surgery were obtained from all the patients. At the end of the study, 6 (3,53 %) patients were still alive with a median follow-up of 56 months (range 27-94 months), whereas 164 patients had died. The median DSS of the cohort was 12 months (range 1-94 months).

Informed consent was obtained from all patients before surgery, and this investigation was approved by the Research Ethics Committee of “Maggiore della Carità” Hospital of Novara.

<b>Variables</b>	<b>Number of patients (%)</b>
<b>Age, mean <math>\pm</math> SD</b> 68,49 years $\pm$ 9,99 (SD)	
<68	68 (40%)
$\geq$ 68	102 (60%)
<b>Gender</b>	
male	118 (69,4%)
female	52 (30,6%)
<b>Other malignancies</b>	
no	149 (87,6%)
yes	21 (12,4%)
<b>Asbestos exposure</b>	
no	80 (47,1%)
yes	86 (50,6%)
not available data	4 (2,4%)
<b>Histologic subtype</b>	
Epithelioid	125 (73,53%)

Biphasic	23 (13,53%)
Sarcomatoid	22 (12,94 %)
<b>ECOG score</b>	
0-2	146 (85,9%)
>2	23 (13,53%)
not available	1 (0,57%)
<b>Clinical stage</b>	
I-II	104 (61,2%)
III-IV	66 (38,8%)
<b>Treatment</b>	
Untreated	40 (23,5%)
Platinum	25 (14,7%)
Platinum&Pemetrexed	105 (61,8%)
<b>Smoking status</b>	
Smoker	86 (50,6%)
Non Smoker	65 (38,22%)
not available data	19 (11,18%)
<b>Status at follow-up</b>	
Alive	6 (3,5%)
Death secondary to MPM	164 (96,5%)
<b>DSS (mean)</b>	
DSS(<16 months)	106 (62,35%)
DSS (≥16 months)	64 (37,65%)

**Table 1:** Clinicopathological characteristics of the studied 170 MPM patients.

### HMGB1 protein expression by immunohistochemistry

In tumour samples, HMGB1 immunostaining was found in 158 cases (93 %); the positivity was heterogeneous in tumour cells and in all the positive samples there were tumour cells stained in the nucleus or cytoplasm only, mixed with tumour cells stained both in the nucleus and the cytoplasm. Conversely, only nuclear HMGB1 immunostaining was found in normal pleura (Fig.1.B), and only nuclear and cytoplasmic staining in mesothelial cells of the inflamed pleura (Fig. 1.A) (Table 2).

As shown in Table 3, the score of HMGB1 staining in the cytoplasm was low in 88 (51,76%) (Fig.1.D) cases and high in 82 (48,24%) (Fig.1.H). The score of nuclear staining was low in 43 (25,29%) (Fig. 1.D) cases and high in 127 (74,71%) (Fig.1.E., F., G., H.), whereas the total HMGB1 score (mean N+C) was low in 69 (40,59%) (Fig.1.C., D.) cases and high in 101 (59,41%) (Fig. 1.E., F., G., H).



<b>Total HMGB1 score= Mean of Nuclear and Cytoplasmic score</b>	<b>N° of cases</b>
0-3 low Total score	69 (40,59%)
4-7 high Total score	101 (59,41%)
<b>Cytoplasmic HMGB1 score</b>	
0-3 low Cytoplasmic score	88 (51,76%)
4-7 high Cytoplasmic score	82 (48,24%)
<b>Nuclear HMGB1 Score</b>	
0-3 low Nuclear score	43 (25,29%)
4-7 high Nuclear score	127 (74,71%)

Table 2: Distribution of cases according to different immunostaining scores.

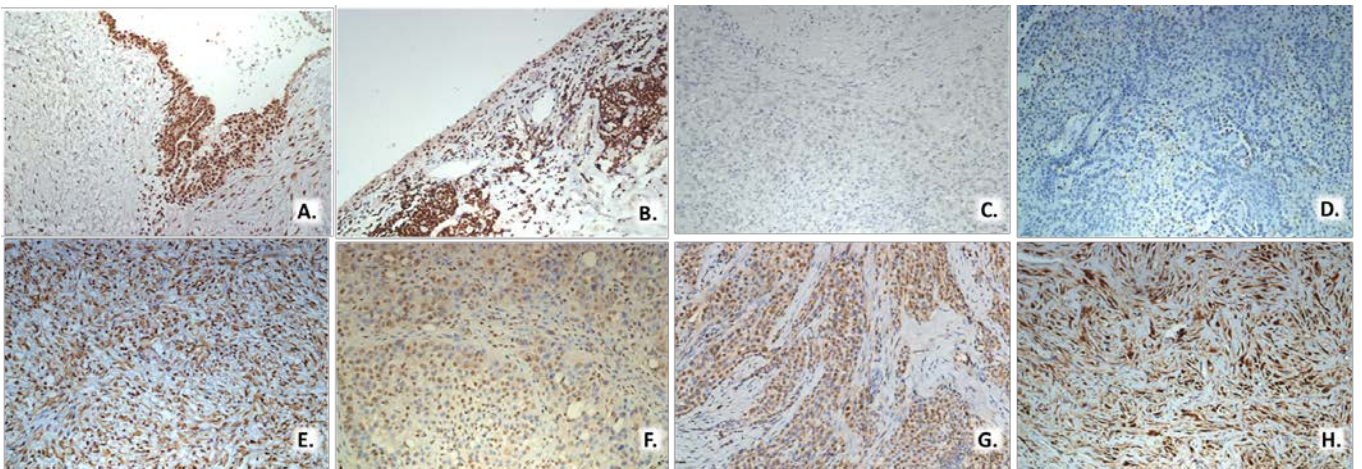


Figure.1. Representative IHC staining for HMGB1 in MPM cases (C ,D , E, F, G, H), inflamed pleura (A ) and normal pleura (B ). (original magnification A,B, X100; C–H, X200).

<b>Total HMGB1 score = Mean of Nuclear and</b>	<b>N° (%)</b>

Cytoplasmic score	
<b>Total cases of normal pleuras evaluated</b>	8 (100%)
• High total score (4-7)	1 (12,5%)
• Low total score (0-3)	7 (87,5%)
<b>Total cases of inflamed pleuras</b>	6 (100%)
• High total score (4-7)	4 (66,7%)
• Low total score (0-3)	2 (33,3%)

Table 3: Distribution of inflamed and normal pleuras according to different immunostaining scores.

### Correlation between HMGB1 scoring by immunohistochemistry and clinicopathological characteristics

The association between the total score of HMGB1 expression and clinicopathological characteristics of MPM was examined by chi-square test. Total HMGB1 expression was significantly associated with gender ( $p < 0,0001^*$ ), tumour clinical stage ( $p = 0,0049^*$ ), ECOG PS ( $p = 0,0139^*$ ), and DSS ( $p = 0,0036^*$ ), (Table 4). No significant correlation between total HMGB1 protein expression and age, asbestos exposure, other previous malignancies, smoking status, histologic subtype and treatment was found.

Clinicopathological characteristics	Total (N+C) HMGB1 expression		
	low IHC score (0-3)	high IHC score (4-7)	p
<b>Age</b>			
<68	32 (47,06%)	36 (52,94%)	0,2022
≥68	37	65	
<b>Gender</b>			
male	102	68	<0,0001*
female	137	33	
<b>Other malignancies</b>			
no	64	85	0,094
yes	5	16	

<b>Asbestos exposure</b>			
no	31	49	
yes	35	51	0,354
not available data	3	1	
<b>Histologic subtype</b>			
Epithelioid	47	78	
Biphasic	14	9	0,1028
Sarcomatoid	8	14	
<b>ECOG score</b>			
0-2	65	81	
>2	4	19	<b>0,0139*</b>
not available	0	1	
<b>Clinical stage</b>			
I-II	51	53	
III-IV	18	48	<b>0,0049*</b>
<b>Treatment</b>			
Untreated	15	25	
Platinum	12	13	0,6898
Platinum and pemetrexed	42	63	
<b>DSS (mean)</b>			
DSS (<16)	34	72	
DSS (≥16)	35	29	<b>0,0036*</b>

**Table 4: Statistical correlation between clinicopathological characteristics and total HMGB1 expression.**

#### **Correlation between *HMGB1* gene expression and clinicopathological characteristics**

The *HMGB1* gene expression analysis was successfully achieved in 110 cases (:::%), since tumour tissue was exhausted in the remaining cases after the use for standard diagnostic and immunohistochemistry procedures. The median of the relative expression level of the *HMGB1* gene (RQ value=1,7) was used as cut-off value to discriminate the patients into high-expression (RQ ≥1,7; score=1) and low-expression groups (RQ<1,7; score=0) (Table 7). Furthermore, the

correlation between the gene expression levels of HMGB1 and the clinicopathologic characteristics of patients was investigated. As summarized in **Table 8**, there was no significant correlation between *HMGB1* gene expression and age ( $p= 0,85$ ), gender ( $p= 0,84$ ), history of previous malignancies ( $p= 0,7677$ ), asbestos exposure ( $0,6293$ ), histologic subtype ( $0,6535$ ), clinical stage ( $p>0,999$ ), smoking status ( $p=0,1662$ ) and DSS ( $p=0,6844$ ). Conversely, high expression levels of *HMGB1* were positively correlated with ECOG score ( $p = \mathbf{0,0317^*}$ ), and treatment ( $p=\mathbf{0,0045^*}$ ).

<b>HMGB1 gene expression</b>	<b>N° (%)</b>
Low-expression (RQ value<1,7) score=0	55 (32,35%)
High-expression (RQ value≥1,7)	55 (32,35%)
Not evaluated	60 (35,3%)

**Table 7** Distribution of MPM patients according to different mRNA expression.

<b>Clinicopathological characteristics</b>	<b>HMGB1 mRNA expression</b>		<b>p</b>
	<b>Low-expression ( &lt; 1,7)</b>	<b>High-expression (RQ ≥ 1,7)</b>	
<b>Age</b>			
<68	22	23	
≥68	33	32	0,85
<b>Gender</b>			
male	38	37	0,84
female	17	18	
<b>Other malignancies</b>			
no	49	48	
yes	6	7	0,7677
<b>Asbestos exposure</b>			
no	29	24	
yes	25	30	0,6293
not available data	1	1	
<b>Histologic subtype</b>			
Epithelioid	43	41	
Biphasic	9	6	0,2322
Sarcomatoid	3	8	
<b>ECOG score</b>			
0-2	72	42	
>2	8	13	<b>0,0317*</b>
not available	0	0	
<b>Clinical Stage</b>			
I-II	32	32	>0,999
III-IV	23	23	

<b>Treatment</b>			
Untreated	12	18	
Platinum	13	8	<b>0,0045*</b>
Platinum&Pemetrexed	76	29	
<b>Smoking status</b>			
Smoker	27	31	
Non smoker	26	17	0,1662
Not available data	2	7	
<b>DSS (mean)</b>			
DSS (<16)	36	38	0,6844
DSS (≥16)	19	17	

**Table 8.** Correlation between the clinicopathological characteristics and gene expression of *HMGB1*.

### **Correlation between clinicopathological characteristics and DSS**

In our cohort, as expected, patients with: low clinical stage (stage I-II versus stage III-IV;  $p < 0,0001$ ); low ECOG score (ECOG 0-2 versus ECOG >2;  $p < 0,0001$ ); treated with chemotherapy (treated with chemotherapy versus untreated;  $p < 0,0001$ ); epithelioid subtype (epithelioid versus non-epithelioid subtype;  $p < 0,0001$ ); younger than 70 years (age  $\leq 70$  versus age  $> 70$ ) ( $p=0,0050$ ), showed significantly better DSS.

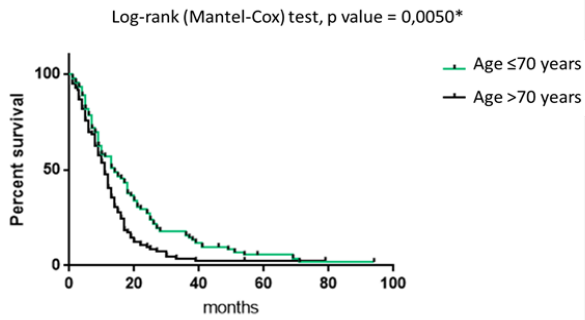
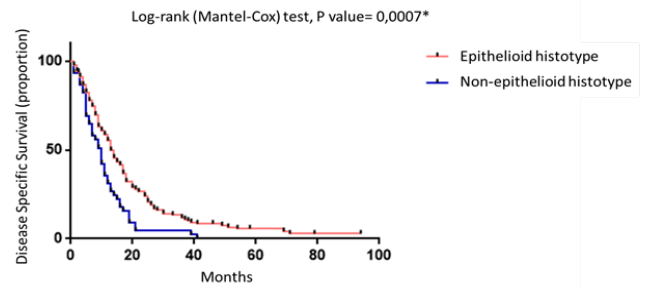
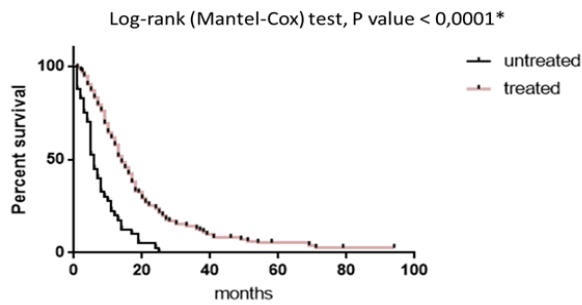
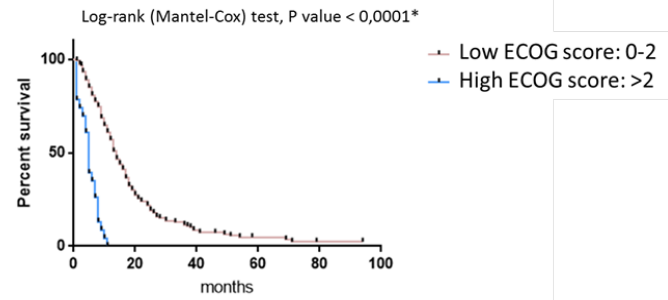
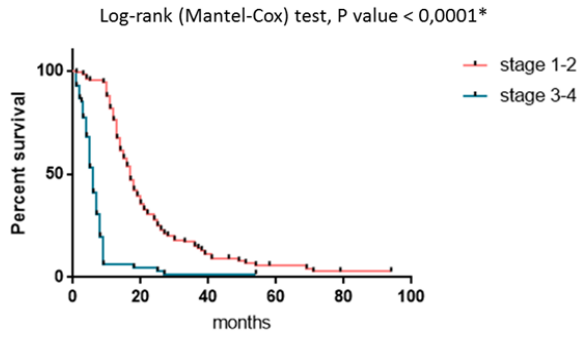


Figure 2:

### **Correlation between HMGB1 scoring by immunohistochemistry and DSS**

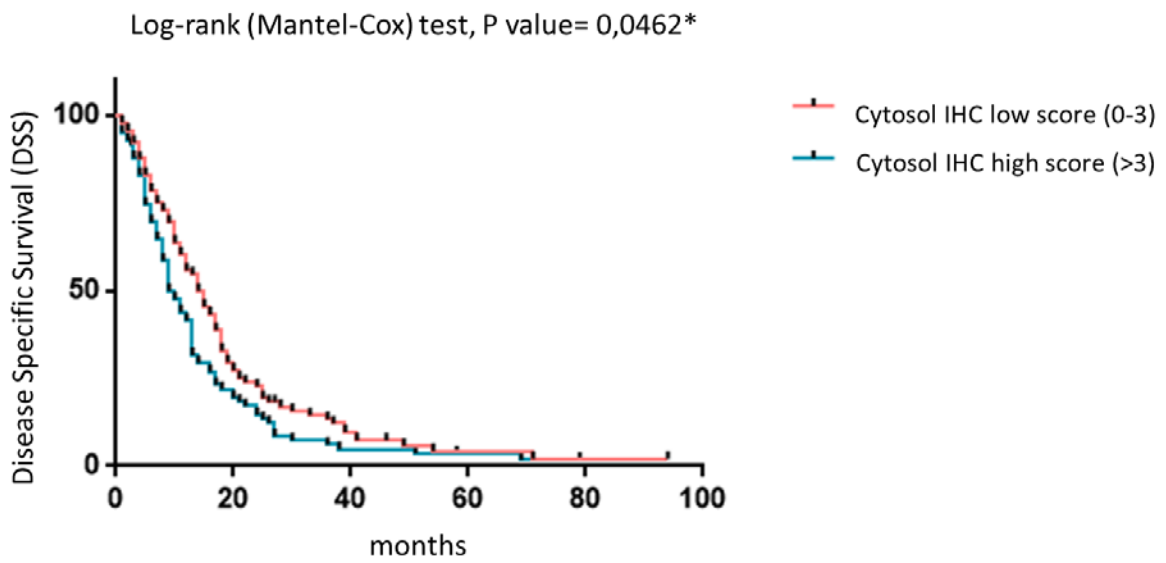
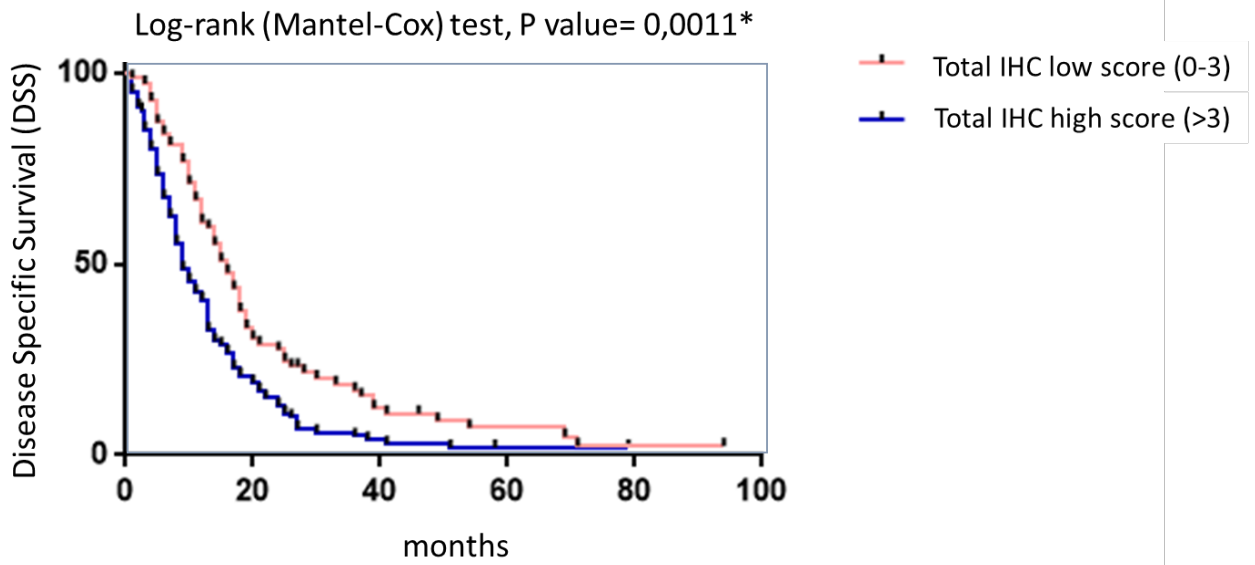
We investigated the relationship between the total score of HMGB1 and the DSS by means of Kaplan-Meier analysis. Patients with high total score had significantly worse DSS than patients with low total score ( $p = 0,0011^*$ ) (Figure 3 ).

Then, we analysed the relationship between the cytoplasmic HMGB1 score and DSS: patients with high score had worse DSS than patients with low HMGB1 score ( $p = 0,0462$ ). Conversely, the expression levels of nuclear HMGB1 score, did not show any statistically significant correlation with DSS.

Moreover, we investigated the prognostic value of HMGB1 expression in subgroups of patients with MPM, subdivided by age ( $> 70$  versus  $\leq 70$ ), clinical stage ( I-II versus III-IV), previous history of other malignancies (not versus yes), histologic subtype (epithelioid versus non-epithelioid), ECOG score (0-2 versus  $>2$ ), and treatment (untreated versus treated with chemotherapy).

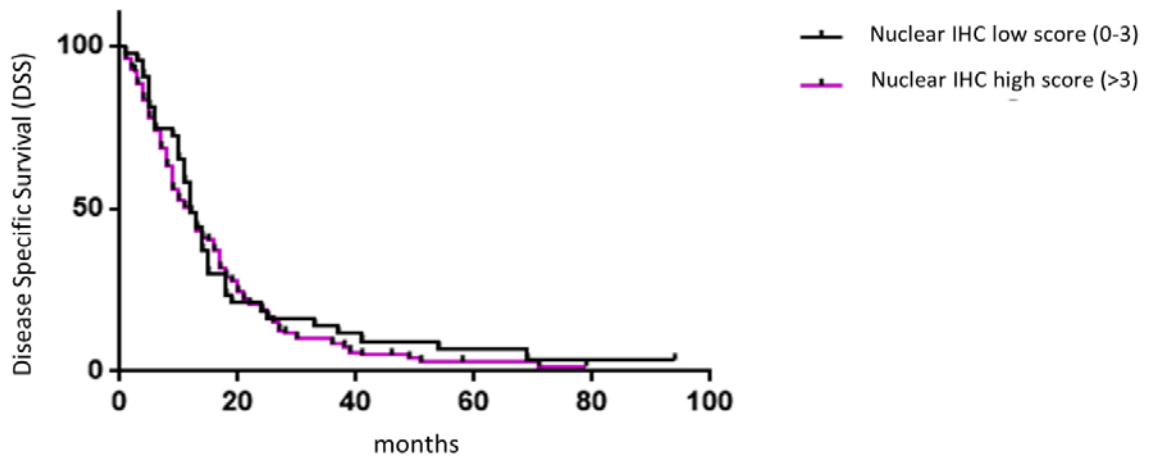
High levels of total score of HMGB1 expression was correlated with worse DSS in patients older than 70 years ( $p=0,0014^*$ ), with no previous history of other malignancies ( $p=0,0008^*$ ), in the subgroups of patients treated by chemotherapy ( $p=0,0097^*$ ) and untreated ( $p=0,0006$ ) and both in the subgroups of patients with epithelioid ( $p=0,0039^*$ ) and non-epithelioid subtypes ( $p=0,0035^*$ ).

Interestingly, high levels of cytoplasmic HMGB1 expression were associated with worse DSS in untreated patients ( $p=0,0167^*$ ) and in the subgroup of patients with non-epithelioid ( $p=0,0058^*$ ) mesothelioma, whereas nuclear score of HMGB1 did not show any correlation with DSS in any of the subgroups analysed.

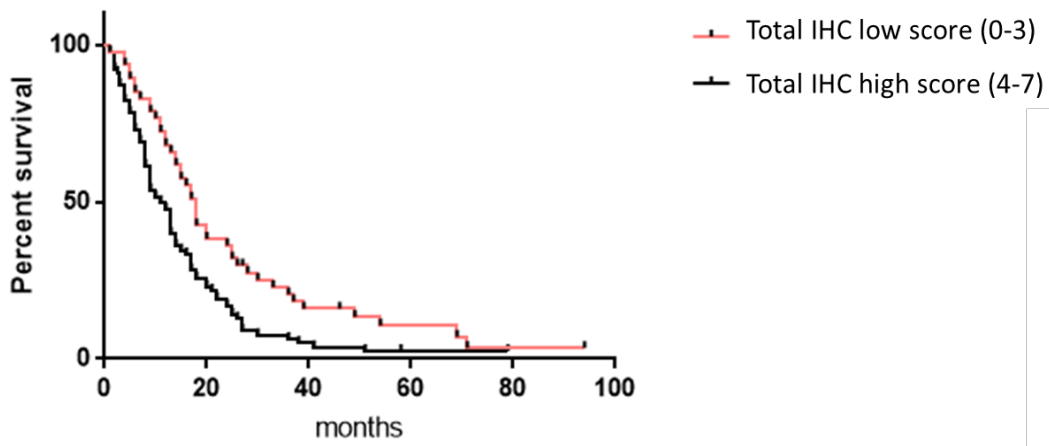




Log-rank (Mantel-Cox) test, P value= 0,5167

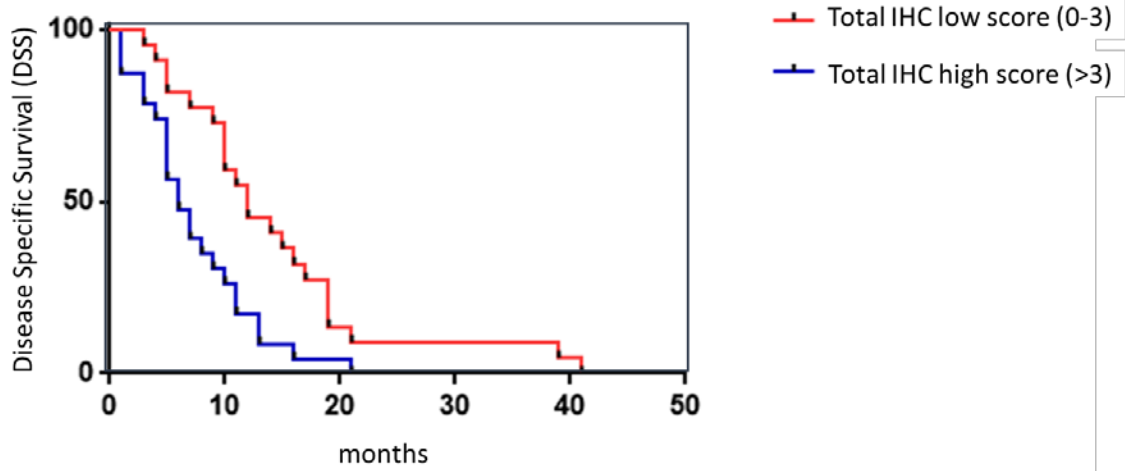


Epithelioid subtype of population, n°=  
Log-rank (Mantel-Cox) test, p value= 0,0039\*



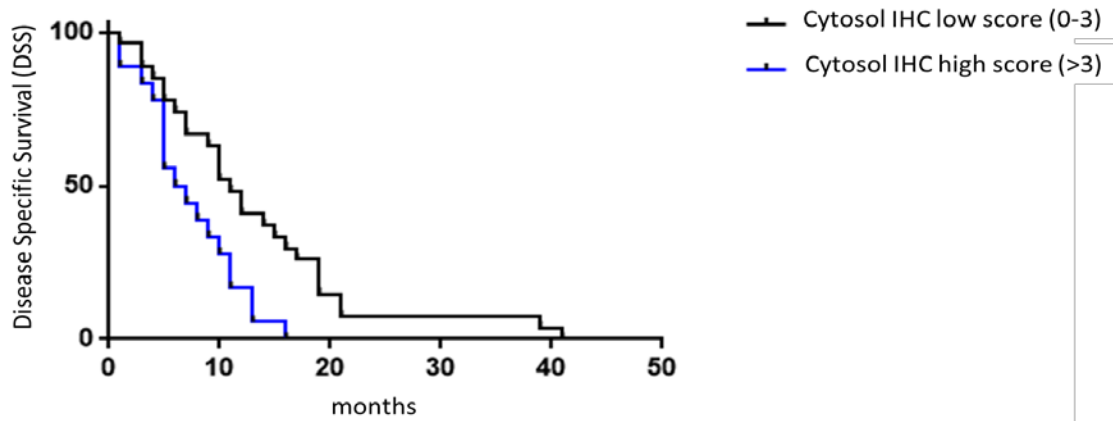
Non-epithelioid population: N° of patients=45

Log-rank (Mantel-Cox) test, P value= 0,0035\*



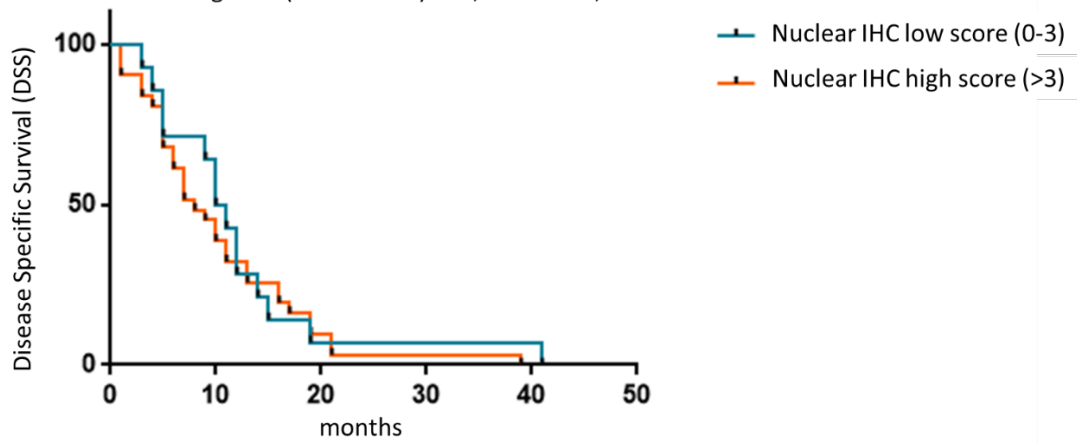
Non-epithelioid population: N° of patients=45

Log-rank (Mantel-Cox) test, P value= 0,0058\*



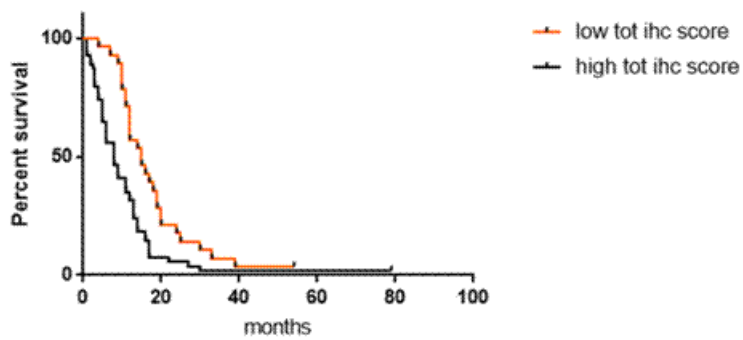
Non-epithelioid population: N° of patients=45

Log-rank (Mantel-Cox) test, P value= 0,6058

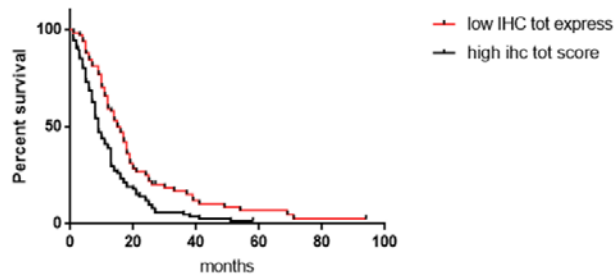


High score of age using median age as cutoff = 82

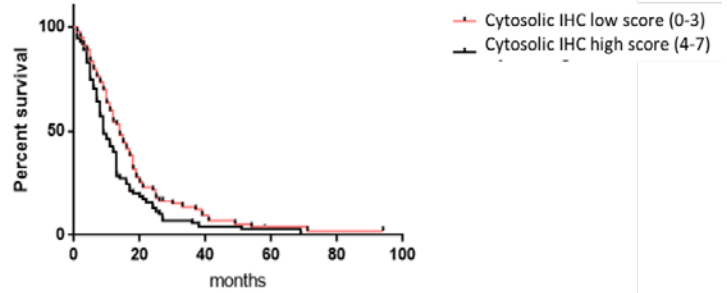
Log-rank (Mantel-Cox) test, p value= 0,0014\*



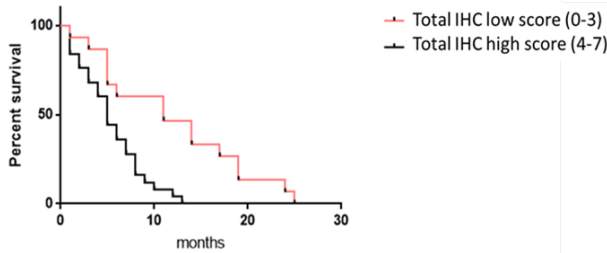
Subgroup of patients with no other malignancies, p value=0,0008\*



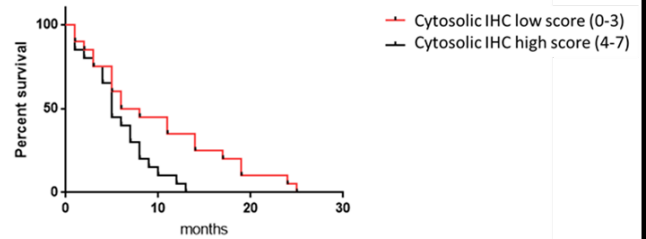
Subgroup of patients without other malignancies, n° 150  
Log-rank (Mantel-Cox) test, p value= 0,0304\*



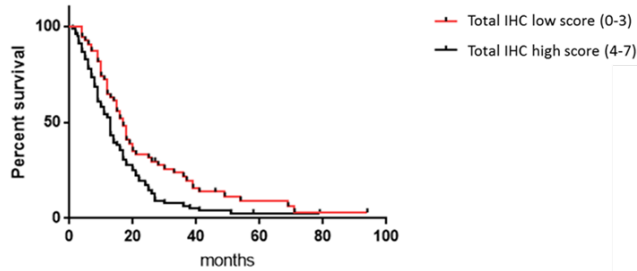
Untreated group of patients, n°=40  
Log-rank (Mantel-Cox) test, p value=0,0006\*



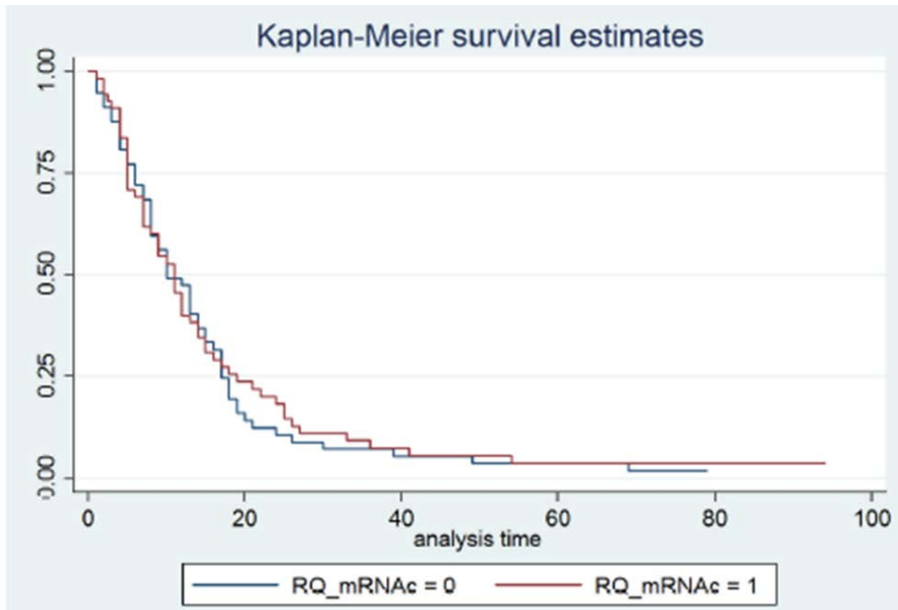
Untreated group of patients, n°=40  
Log-rank (Mantel-Cox) test, P value= 0,0167\*



Treated group of patients (uni- and multimodality chemotherapy), n°130  
Log-rank (Mantel-Cox) test, P value= 0,0097\*



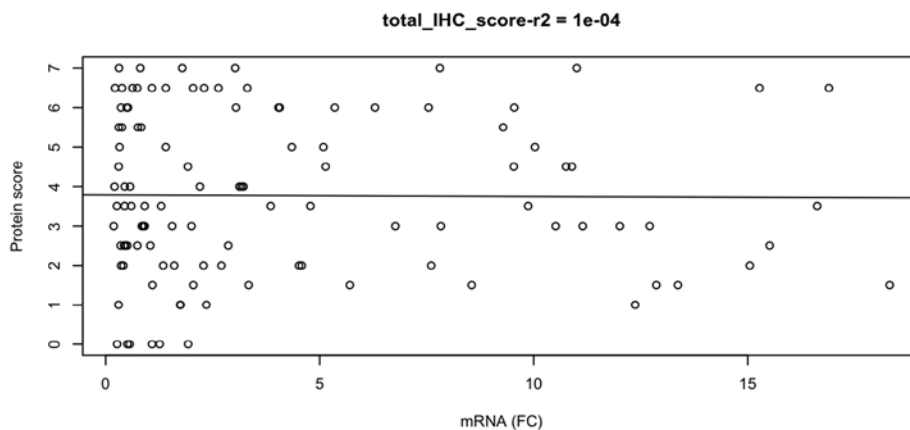
The correlation between *HMGB1* gene expression and DSS was also investigated. The Kaplan-Meier survival curves generated for the low-expression and high-expression groups of patients indicated that there was no significant difference ( $P = 0,718$ ) in terms of DSS between the 2 groups.



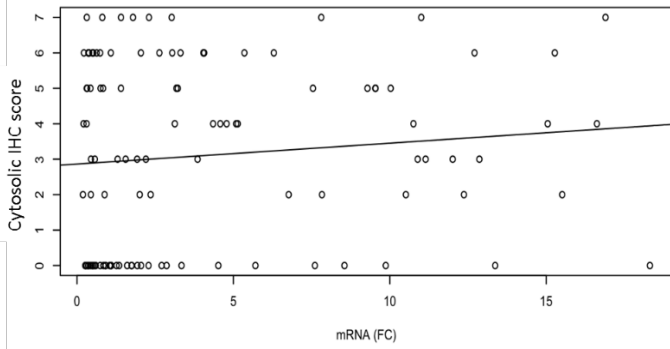
**Fig. 16:** No statistically significant difference in DSS between downregulated and overexpressed levels of HMGB1 mRNA.  $P=0,718$ .

**Correlation between HMGB1 gene expression and HMGB1 scoring by immunohistochemistry**

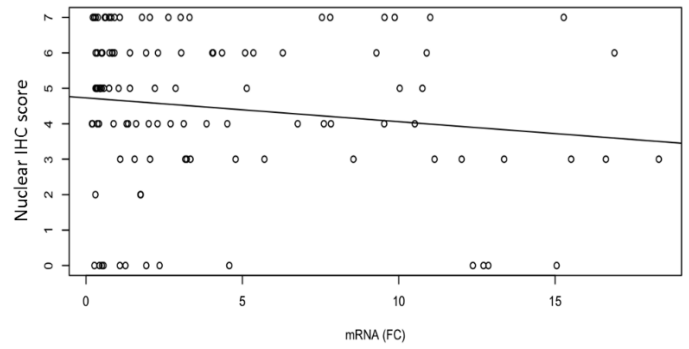
Finally, we analyzed the statistical correlation between the HMGB1 scoring and gene expression into the cohort of 110 MPM cases, by means of Pearson test. No statistically significant correlation was found between the HMGB1 gene expression and HMGB1 scoring, evaluated in terms of total score (Fig. a); nuclear score (Figure. ); cytoplasmic score (Figure. ); percentage of nuclear positivity; intensity of nuclear positivity; percentage of cytoplasmic positivity and percentage of nuclear positivity.



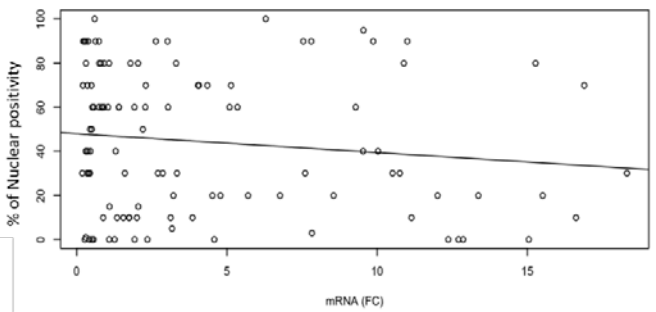
Cytosolic IHC score –  $r^2=0,0116$



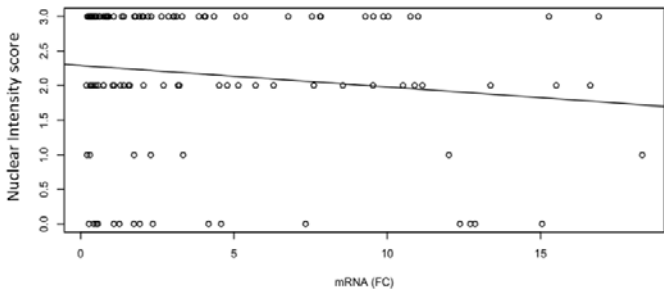
Nuclear IHC score –  $r^2=0,0212$

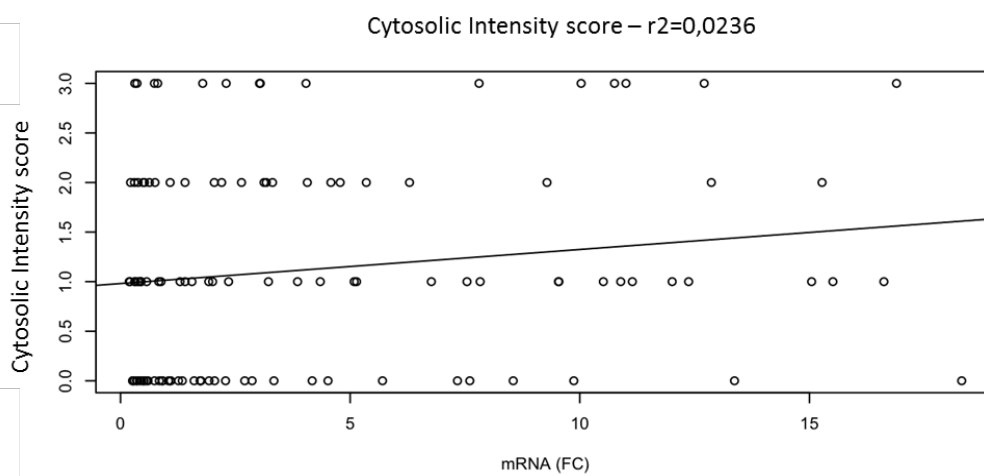
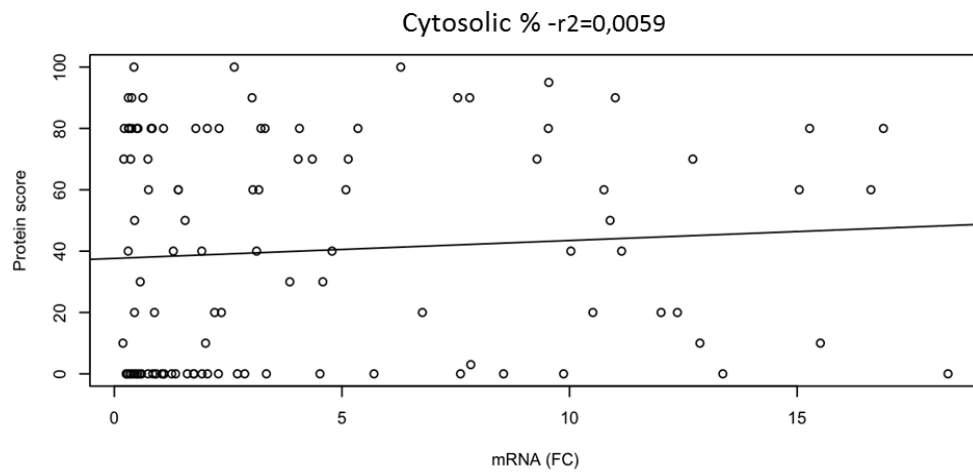


Nuclear % -  $r^2=0,0161$



Nuclear Intensity score –  $r^2=0,0185$





## Univariate and multivariate analysis

The variables putatively associated with survival were individually analyzed with a univariate Cox proportional hazards regression model (Table 4).

Significant variables in the univariate analysis (median age, *HMGB1* gene expression, clinical stage, ECOG, treatment...) were added to a multivariate model and backward stepwise elimination was applied at the 5% significance level. Median age ( $\leq 70$  versus  $>70$  years), clinical stage (I-II versus III-IV), histologic subtype (epithelioid versus non-epithelioid) and interestingly, total and cytoplasmic HMGB1 score were the only significant predictors. The deviation from the proportional hazards assumption was tested by the regression of scaled Schoenfeld residuals against the logarithm of time.

Table 4. Univariate and multivariate analysis of DSS for the cohort of 170 MPM patients.

	Univariate analysis	
Variable of patients with MPM	HR (95% CI)	P
Age	(- 2,53) (- 0,52- 0,06)	<b>0,012*</b>
Gender (male/female)	(-0,73) (-6,821 - 3,150482)	0,47
Other malignancies (no/yes)	1,11 (-7,00 – 6,99)	0,99
Asbestos exposure (no/yes)	(-1,11) (-7,2346 - 2,04224)	0.271
Histological subtype [epithelioid/non-epithelioid (biphasic and sarcomatoid)]	(-2,81) [-12,35- (-2,15)]	<b>0,006*</b>
ECOG score (0-2/>2)	(-3,96) [(-19,4)- (-6,5)]	<b>0,000*</b>
Clinical stage (I-II/III-IV)	(-7,2) [(-19,2)- (-10,9)]	<b>0,000*</b>
Treatment type [untreated/treated (cispatin and cisplatin and pemetrexed)]	4,16 (5,7- 16,04)	<b>0,000*</b>
Smoking status (no/yes)	(-0,22) [(-5,74)- 4,6]	0,823
HMGB1 gene expression_RQ value [low espression (2 - $\Delta\Delta Ct < 1,7$ )/high expression (2 - $\Delta\Delta Ct \geq 1,7$ )]	0,26 [(-5,11)- 6,66]	0,795
HMGB1 Total IHC score (low expression (0-3)/ high expression (4-7)	(- 3,31) [(-12,2)- (-3,1)]	<b>0,001*</b>
HMGB1 Citosol IHC score (low expression (0-3)/ high expression (4-7)	(- 2,2) [(-0,82)- (- 0,04)]	<b>0,03*</b>

Histological subtype [epithelioid/non-epithelioid (biphasic and sarcomatoid)]	(-2,9) [(-8,99)- (-1,82)]	<b>0,003*</b>
ECOG score (0-2/>2)		
Clinical stage (I-II/III-IV)	(-5,9) [(-2,3)- (-1,13)]	<b>0,000*</b>
Treatment type [untreated/treated (cispatin and cisplatin and pemetrexed)]	5,4 (5,65 - 12,1)	<b>0,0001*</b>
HMGB1 Total IHC score (low expression (0-3)/ high expression (4-7)	(-2,43) [(-1,2)- (0,13)]	<b>0,015*</b>
HMGB1 Citosol IHC score (low expression (0-3)/ high expression (4-7)	(-2,21) [(-0,98)- (-0,06)]	<b>0,027*</b>



## Discussion

Malignant pleural mesothelioma (MPM) is a very aggressive tumour. Indeed, the average disease-specific survival after histologic diagnosis is currently 8-12 months with little improvement in patients treated with chemotherapy (Carbone M, et al. *Journal of cellular physiology*. 2012; Vogelzang NJ, et al. *J Clin Oncol*. 2003). According to the Cancer and Leukaemia Group B, and the European Organization for Research and Treatment of cancer, a poor performance status (PS greater than 2), the non-epithelioid histology, the advanced clinical stage (III and IV) and the patients' age older than 75 years were identified as poor prognostic factors of MPM (Moore J.A. et al. *Orphanet Journal of rare diseases*. 2008). Moreover, there have been several reports concerning potential serum markers which could be clinically useful as prognostic factors in MPM, such as mesothelin (Schneider J et al. *Journal of thoracic oncology*. 2008; Hollevoet K, et al. *J Clin Oncol*. 2012), Vascular Endothelial Growth Factor, osteopontin (Pass HI, et al. *N Engl J Med*. 2005) and Fibulin-3 (Pass HI et al. *N Engl J Med*. 2012). In addition, Tabata et al, also reported that high serum levels of high mobility group box 1 (HMGB1) were related to a poor prognosis in MPM, suggesting its use in clinical management of MPM (Tabata et al. *BMC Cancer* 2013).

However, unlike other malignant tumours, tissue biomarkers detectable by immunohistochemistry or by molecular biology techniques and able to predict the prognosis of MPM are still lacking.

A recent meta-analysis by Wu et al, indicated that the overexpression of HMGB1, when detected by immunohistochemistry, is significantly associated with poor overall survival and progression-free survival in several types of malignant tumours, like gastric cancer, colorectal cancer, hepatocellular carcinoma, pancreatic cancer, nasopharyngeal carcinoma, head and neck squamous cell carcinoma, esophageal cancer and gynaecologic tumours (Wu T et al. *Oncotarget*. 2016). In addition, Jube et al, found a statistically significant correlation between tumor stage and HMGB1 expression levels in the cytoplasm of mesothelioma cells. However, their results were obtained in

a small cohort of the patients with MPM and need more powerful studies to be definitively confirmed.

On these bases, the main goals of our study were: a) to assess if the expression levels of HMGB1, evaluated by immunohistochemistry and qPCR in tissue samples from a large series of MPM, were related to the patients' survival and b) to evaluate if HMGB1 could be useful as a prognostic biomarker in clinical practice.

In this work, we demonstrated that high expression levels of HMGB1, evaluated by immunohistochemistry, in cancer cells of clinical samples of MPM were significantly correlated to a poor DSS. This result was obtained either when the score was calculated as total score (nuclear plus cytoplasmic) and cytoplasmic score alone, in both the univariate and multivariate analyses and both in the entire cohort and in the clinical and pathologic subgroups of patients. Conversely, the expression levels of nuclear HMGB1 score alone did not show any statistically significant correlation with DSS.

HMGB1, is a nuclear protein constitutively expressed in both cancer and normal cells and acts as chromatin-binding factor that bends DNA, promoting access to several transcriptional proteins (Kang R. et al. *Molecular Aspects of Medicine*. 2014). Some studies have demonstrated that HMGB1 could be actively shuttled between the nucleus and the cytoplasm of tumour cells (Gardella S. *EMBO Rep*. 2002): following various stressors (e.g., cytokine, chemokine, heat, hypoxia, H<sub>2</sub>O<sub>2</sub>), HMGB1 translocates from the nucleus to the cytoplasm, whose main role is to function as a positive regulator of autophagy (Tang et al., 2010c). At the extracellular level, HMGB1 functions as a cytokine during inflammation, cell differentiation, cell migration, and seems also to play a role in tumor metastasis development ( M.T. Lotze et al, *Nat. Rev., Immunol.* 5. 2005; Muller S., *EMBO J.* 2001; X.D. Dong et al. *J. Immunother.* 2007; J.E. Ellerman et al. *Clin. Cancer Res.* 13. 2007).

Since in our study the cytoplasmic but not nuclear overexpression of HMGB1 was significantly associated with poor prognosis, we can speculate that in malignant mesothelioma, HMGB1 when translocated from the nucleus to the cytoplasm of the tumour cells could be subsequently released into the extracellular matrix, enhancing tumour cell survival and proliferation. Tang D. et al, suggested that tumour cells overexpressing HMGB1 might release it in the extracellular medium, and extracellular HMGB1 might be associated with some of the hallmarks of cancer, including unlimited replicative potential, neoangiogenesis, evasion of programmed cell death, self-sufficiency in growth signals, insensitivity to inhibitors of growth, inflammation, and tissue invasion (Tang D. et al, *Biochimica et Biophysica Acta*. 2010). Furthermore, Jube et al, 2012 demonstrated, by means of in vivo and in vitro experiments, that MPM cells overexpressing HMGB1 in the cytoplasm might release it in the extracellular medium, promoting cancer cell survival and proliferation in an autocrine manner.

Our study did not show any statistically significant correlation with DSS when the detection method of qRT-PCR was used. Moreover, no statistically significant correlation was found between the *HMGB1* gene expression and HMGB1 immunohistochemistry scoring. These results were similar to the results obtained by Soldevilla et al and by Ueda et al in colorectal cancers, and could be tentatively explained by the presence of inflammatory cells, also expressing HMGB1, mixed with cancer cells in tissues used for the evaluation of *HMGB1* gene expression. Alternatively, post-transcriptional or post-translational modifications of the *HMGB1* mRNA or the protein itself could contribute to explain this discrepancy. Among the first, one of the most important is the regulation of gene expression by microRNA, which can inhibit the transduction process; among others, acetylation, sulphonation, methylation and oxide reduction mechanisms can modify the structure and functions of HMGB1. (41)

In conclusion, we have demonstrated that the expression levels of HMGB1, in the cytoplasm of mesothelioma cells are inversely correlated with DSS in MPM, when assessed by immunohistochemistry. To the best of our knowledge, this study represents the most significant analysis in terms of the number of patients enrolled, and attempts to evaluate the possible usefulness of HMGB1 in the clinical management of patients with MPM.