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Identification of a new potential plasmatic biomarker panel for the diagnosis of malignant pleural mesothelioma

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Keywords: Malignant pleural mesothelioma; respiratory disease; asbestos exposure; circulating biomarkers

Abstract

Background: *Malignant pleural mesothelioma (MPM) is a rare, highly aggressive tumor strongly associated with asbestos exposure and characterized by poor prognosis. Currently, diagnosis is based on invasive techniques; thus, there is a need to identify non-invasive biomarkers to detect the disease. In the present study, we measured the plasmatic concentrations of Mesothelin, Fibulin-3, and HMGB1 protein biomarkers and of hsa-miR-30e-3p and hsa-miR-103a-3p Extracellular-Vesicles- embedded micro RNAs (EV-miRNAs). We tested the ability of these biomarkers to discriminate between MPM and PAE subjects alone and in combination.* **Methods:** *The study was conducted on a population of 26 patients with MPM and 54 healthy subjects with previous asbestos exposure (PAE). Mesothelin, Fibulin-3, and HMGB1 protein biomarkers were measured by the enzyme-linked immunosorbent assay (ELISA) technique; the levels of hsa-miR-30e-3p and hsa-miR-103a-3p EV-miRNAs was assessed by real-time quantitative PCR (qPCR).* **Results:** *The most discriminating single biomarker resulted to be Fibulin-3 (AUC 0.94 CI 95% 0.88-1.0; Sensitivity 88%; Specificity 87%). After investigating the possible combinations, the best performance was obtained by the three protein biomarkers Mesothelin, Fibulin-3, and HMGB1 (AUC 0.99 CI 95% 0.97-1.0; Sensitivity 96%; Specificity 93%).* **Conclusions:** *The results obtained contribute to identifying new potential non-invasive biomarkers for diagnosing MPM. Further studies are needed to validate the evidence obtained to assess the reliability of the proposed biomarker panel.*

1. Introduction

Malignant pleural mesothelioma (MPM) is an aggressive and highly lethal cancer originating from the pleura's mesothelial cells after asbestos exposure [1, 2]. Although MPM is considered a rare malignancy with an incidence of 1:100,000, about 40,000 deaths worldwide have been estimated to occur each year globally for asbestos exposures, with a long latency of between 30 and 50 years [3-5].

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MPM diagnosis is still conducted by thoracoscopic biopsy, an invasive and costly approach [6, 7]. The dismal prognosis of MPM is attributable to different factors, such as late diagnosis mainly due to typically subtle and nonspecific clinical symptoms, unpredictable tumor growth, and minimal response to current treatment protocols, the average survival time being 13 months [8-10].

In this scenario, the identification of reliable non-invasive biomarkers of disease to be used in high-risk populations is challenging [11, 12]. Several strategies have been explored to develop robust minimal invasive tests starting from liquid biopsies [11, 13]. Circulating biomarkers are found in different body fluids, such as serum, plasma, and pleural effusions, with the potential to diagnose MPM early, even before clinical imaging techniques [11, 13, 14]. Various studies have investigated different types of potential circulating biomarkers. Mesothelin, Fibulin-3, and High Mobility Group B1 protein (HMGB1) are the most promising ones to be further validated and tested in combination in independent populations [15]. Circulating nucleic acids, such as DNA, RNA, and microRNAs (miRNAs), have also been examined as potential MPM biomarkers [16]. In particular, miRNAs embedded within circulating extracellular vesicles (EV-miRNA) might be particularly interesting. Indeed, E.V.s are membrane-bound structures that contain several bioactive molecules, such as miRNA, and are released by cells to promote intercellular communication [17, 18]. Thus, it is plausible that specific EV-miRNA signatures may represent the active crosstalk between MPM cells and immune cells rather than a passive result of miRNA accumulating in plasma as a waste product [19].

In a previous study, we measured the expression of 754 circulating EV-miRNAs in 23 patients with MPM and 19 cancer-free subjects with past asbestos exposure (PAE). We identified the two EV-miRNA signatures, i.e., hsa-miR-30e-3p and hsa-miR-103a-3p, as the best discriminating combination between MPM and PAE groups [20]. In the present study, we evaluated, in a larger population, the plasmatic expression of the EV-miRNA signature previously identified and that of the circulating Mesothelin, Fibulin-3, and HMGB1 protein biomarkers. Moreover, we tested the discriminating potential of different combinations of the examined biomarkers and presented evidence that the combination of Mesothelin, Fibulin-3, and HMGB1 showed the best discrimination performance.

2. Methods

2.1 Study population

The study population includes patients with MPM and subjects with past occupational asbestos exposure. The MPM patients were enrolled at the Thoracic Surgery Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, between October 2013 and August 2016. MPM diagnosis was performed on pleural biopsies collected during video-assisted thoracoscopy surgery. Tissue specimens were classified according to the TNM staging system established by the International Mesothelioma Interest Group (IMIG) and the International Association for the Study of Lung Cancer (IASLC) [21-23]. The 54 PAE subjects underwent a clinical surveillance program in the same study period as MPM subjects at the Occupational Health Unit Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy, as established by the Italian Law Dlgs 81/2008.

2.2. Asbestos exposure assessment

As previously described, information on detailed asbestos exposure in both occupational and environmental settings was collected through a standardized questionnaire administered to each subject by trained interviewers [20, 24]. Demographic, lifestyle, and smoking information was also collected in that context.

2.3. Blood collection, plasma separation, E.V. isolation, and EV-miRNA extraction

Each study participant was asked to donate a 7.5 ml blood sample, collected in 3K-EDTA Vacutainer plastic tubes (Becton Dickinson, New Jersey, USA) and processed within 3 h of the blood draw. None of the MPM patients underwent surgery,

chemo- or radiotherapy before blood collection. Blood was centrifuged at 400 g for 15 min to separate the plasma fraction from the blood cells. For EV-miRNA analysis, plasma samples were centrifuged three times at 1000 g, 2000 g, and 3000 g for 15 min at 4 °C to remove cell debris and aggregates. Supernatants were ultracentrifuged at 110000 g for 2 h at 4 °C. EV-miRNAs were isolated as previously described [20]. Briefly, miRNAs were isolated with the miRNeasy purification kit (Qiagen Hilden, Germany) following the manufacturer's instructions and eluted in 25 µl of elution buffer. miRNA quality after purification was analyzed with the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using Agilent RNA 6000 Pico Kit. Isolated miRNAs were concentrated with Concentrator Plus (Eppendorf, Hamburg, GER) to 6.7 µl and stored at -80 °C until use.

2.4. Protein analysis

Plasmatic mesothelin was measured using the enzyme-linked immunosorbent assay (ELISA) kit MESOMARK (Fujirebio Diagnostics, Inc., Malvern, PA, USA) as described previously [25]. ELISA for Fibulin-3 coding protein was conducted according to the manufacturer's instructions using the Human EFEMP1 PicoKine ELISA Kit (Boster Bio, Pleasanton, CA, USA) [26]. The HMGB1 protein levels were assessed by the kit HMGB1 express ELISA (IBL International GmbH, Hamburg, Germany), as reported by Handke N.A. and colleagues [27]. Absorbance was measured at 450 nm using the microplate reader Sinergy HT (Santa Clara, CA, USA). All samples were tested in duplicate.

2.5. EV-miRNA analysis

Expression quantification of hsa-miR-30e-3p and hsa-miR-103a-3p EV-miRNAs was determined by Custom TaqMan™ microRNA assay (Thermo Fisher Scientific, Waltham MA, USA) following standard procedures. Briefly, each miRNA was analyzed in triplicate, and RNU48 was used for data normalization. Specific reverse transcription of miRNAs was performed following standard procedures. R.T. was performed using a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA), and the cDNAs were pre-amplified. After the pre-amplification product was diluted at 1:8, quantitative RT-PCR was run in a Quant Studio 12K Flex Fast Real-Time PCR System (Thermo Fisher Scientific), according to the manufacturer's protocol. miRNA expression was calculated by the comparative cycle threshold (ΔCT) method and analyzed with SDS software (Thermo Fisher Scientific).

2.6. Statistical analysis

Data were expressed as mean and standard deviation when normally distributed, otherwise by the median, with the minimum, maximum, and first and third quartiles. Frequencies and percentages were calculated for categorical variables. The differences between the MPM patients and PAE subjects' groups were compared using Pearson's chi-square test, Fisher's Exact test for categorical data, or t-test or Mann-Whitney U-test for continuous variables, as appropriate. Spearman correlation coefficients were obtained from each pair of plasmatic biomarkers. To evaluate the levels of biomarkers for the TNM stages, histotype, and survival, we applied ANOVA models after the log transformation of biomarkers. We reported the geometric means of biomarker and the p-values of comparisons between each TNM stage and stage I, set as the reference category, and the overall p-value of the differences across the four stages.

Univariate logistic regression was performed to investigate the association between potential MPM-associated risk factors (i.e., gender, age, body mass index (BMI), and smoking habits) on the risk of MPM. Multivariate logistic regression, adjusted for gender, age, BMI, and smoking habits, was performed to investigate the association between each plasmatic biomarker and the risk of MPM. The estimated effects were reported as odds ratios (OR) and 95% Confidence Intervals (CI) associated with a unit increase of each biomarker.

Receiver-Operating Characteristic (ROC) curves were generated to evaluate the diagnostic ability of each biomarker to distinguish between subjects with MPM and PAE subjects, and the area under the ROC curve was computed to assess their

discriminant performance. Sensitivity, specificity, true positive (T.P.), and true negative (T.N.) values for MPM were calculated for each biomarker of interest and their combination.

We thus investigated the discrimination ability of the combination of Mesothelin, Fibulin-3, and HMGB-1, comparing to that generated by each of them taken alone, or in combination, irrespectively to the abovementioned covariates. Statistical significance was defined as $p<0.05$. Statistical analyses were performed with SAS software, version 9.4, and R software, version 3.6.3.

3. Results

3.1. Characterization of the study participants

The study participants include 26 patients with MPM and 54 subjects with past asbestos exposure, whose main characteristics are reported in Table 1. Most subjects in each group were males (77% MPM; 87% PAE). The mean age was 71.3 (±7.8) years for patients with MPM and 64.8 (±6.0) for PAE subjects. Smoking habits and the categorical distributions of BMI means did not differ ($p=0.417$; $p=0.084$) between the two groups. Asbestos exposure was established in 57.7% of MPM cases, most of which (n=14) had occupational exposure. Duration of exposure and time since last exposure were higher in patients with MPM than in PAE subjects ($p=0.036$ and 0.006, respectively) and patients with MPM showed a longer latency (years since first exposure to the diagnosis of MPM or blood collection for PAE subjects; $p=0.006$). The most frequent histological MPM types were epithelioid (n=14) and biphasic (n=10). 81% of MPM was T1-T2, and 21 cases (19 in the T1-T2 size category; 2 in T4) had no metastases at diagnosis. The TNM was also determined and reported in Table 1.

3.2 Expression profiles of plasmatic biomarkers

Expression profiles of the plasmatic levels of Mesothelin, Fibulin-3, and HMGB1 proteins, EV-miRNA hsa-miR-103a-3p, and hsa-miR-30e-3p are reported in Table 2. All the protein biomarkers showed higher expression in MPM patients than in PAE controls. On the contrary, both hsa-miR-103a-3p and miR-30e-3p expression was lower in MPM cases (fold change 0.57 and 0.76, respectively). In particular, the greatest difference between MPM and PAE was observed for Mesothelin and hsa-miR-103a-3p.

Moreover, we investigated the possible correlations of the tested biomarkers with the TNM subgroups and histotype. As reported in Supplementary Tables S1 and S2, no differences were found. We further tested the correlation between the different biomarkers and observed that the two EV-miRNAs hsa-miR-30e-3p and hsa-miR-103a-3p were the most correlated ($r=0.97$), followed by Fibulin-3 and HMGB1 ($r=0.41$), as reported in Figure 1.

3.3. Discrimination between patients with MPM and PAE subjects

By logistic regression, we estimated the odds of being a patient with MPM for each measured biomarker and the covariates considered in the analyses (i.e., gender, BMI, and smoking habits) (supplementary tables S3 and S4). Moreover, to examine the discrimination ability between patients with MPM and PAE subjects, we fitted multiple logistic regression models adjusted for gender, age, BMI, and smoking habits and calculated adjusted receiver operating characteristic (ROC) curves for each one of the biomarkers taken into consideration (Table 3; Figure 2). The best discriminating biomarkers were Fibulin-3, HMGB-1, and Mesothelin, and the areas under the curve (AUC) were, respectively, 0.94, 0.92, and 0.88. Thus, we investigated the discrimination ability of the different combinations of these three biomarkers. The use of all three proteins generated an AUC of 0.99, with a Sensitivity of 96% and a Specificity of 93%, slightly higher than the combination of Mesothelin and Fibulin-3 only (AUC=0.98, Sensitivity 92%, specificity 91%). In addition, the estimate of ROC difference between the combination only of Fibulin-3 and Mesothelin, with that of the three biomarkers HMGB-1, Fibulin-3 and Mesothelin did not reach significance ($p= 0.674$; Supplementary Table S5).

Table 1. Characteristics of MPM and PAE subjects.

	MPM n=26	PAE n=54	p-value
Gender			0.333[§]
Male	20 (77%)	47 (87%)	
Female	6 (23%)	7 (13%)	
Age, years, (mean±SD)	71.3±7.8	64.8±6.0	<0.001[w]
Smoking habits			0.417[c]
Non-smokers	8 (31%)	25 (46%)	
Former smokers	15 (58%)	24 (44%)	
Smokers	3 (12%)	5 (9%)	
Categorical BMI			0.084[c]
Underweight (BMI < 18.5)	16 (62%)	19 (35%)	
Lean (18.5 ≤ BMI < 25)	7 (27%)	24 (44%)	
Overweight (BMI > 25)	3 (12%)	11 (20%)	
Asbestos exposure categorization			
Occupational	14 (53.8%)	54 (100%)	
Environmental	1 (3.8%)	0 (0%)	
Unknown	11 (42.3%)	0 (0%)	
Duration of exposure, years, median (min, Q1, Q3, max)	25 (1, 17, 32, 47)	11 (0, 6, 25, 40)	0.036*
Latency years, median (min, Q1, Q3, max)	55 (24, 47, 60, 67)	44 (12, 37, 49, 59)	0.006*
Histology			
Epithelioid	14 (54%)		
Biphasic	10 (38%)		
Sarcomatoid	2 (8%)		
Tumor size			
T1-T2	21 (81%)		
T3	0 (0%)		
T4	5 (19%)		
Lymph Node Status			
N0	10 (38%)		
N1	8 (31%)		
N2	8 (31%)		
Metastases at diagnosis			
No	21 (81%)		
Yes	5 (19%)		
TNM staging			
I	8 (31%)		
II	6 (23%)		
III	7 (27%)		
IV	5 (19%)		

*S.D., standard deviation; min, minimum, max, maximum, Q1, Q3, first-third quartile; [c]p-value from Pearson's chi-square test. [w]p-value from t-test. [§]p-value from Fisher's Exact test. *p-value from Mann-Whitney U-test. Tissue specimens were classified according to the TNM staging system established by IMIG and IASLC.*

Table 2. Description of protein and EV-miRNA in MPM and PAE subjects.

	Mean±standard deviation		
Biomarker	**MPM n=26**	**PAE n=54**	**p-value**
Mesothelin, ng/ml	3.8±5.8	0.9±0.5	0.015[w]
Fibulin-3, ng/ml	53.9±18	44.6±2.9	0.014[w]
HMGB1, ng/ml	12±11.2	5.4±5.2	0.008[w]
hsa-miR-103a-3p	568.2±408.7	1001.4±364.2	0.001[*]
hsa-miR-30e-3p	368.7±264	484.7±171.5	0.021[*]

[w]*p-value from t-test.*
[*]*p-value from Mann-Whitney U-test.*

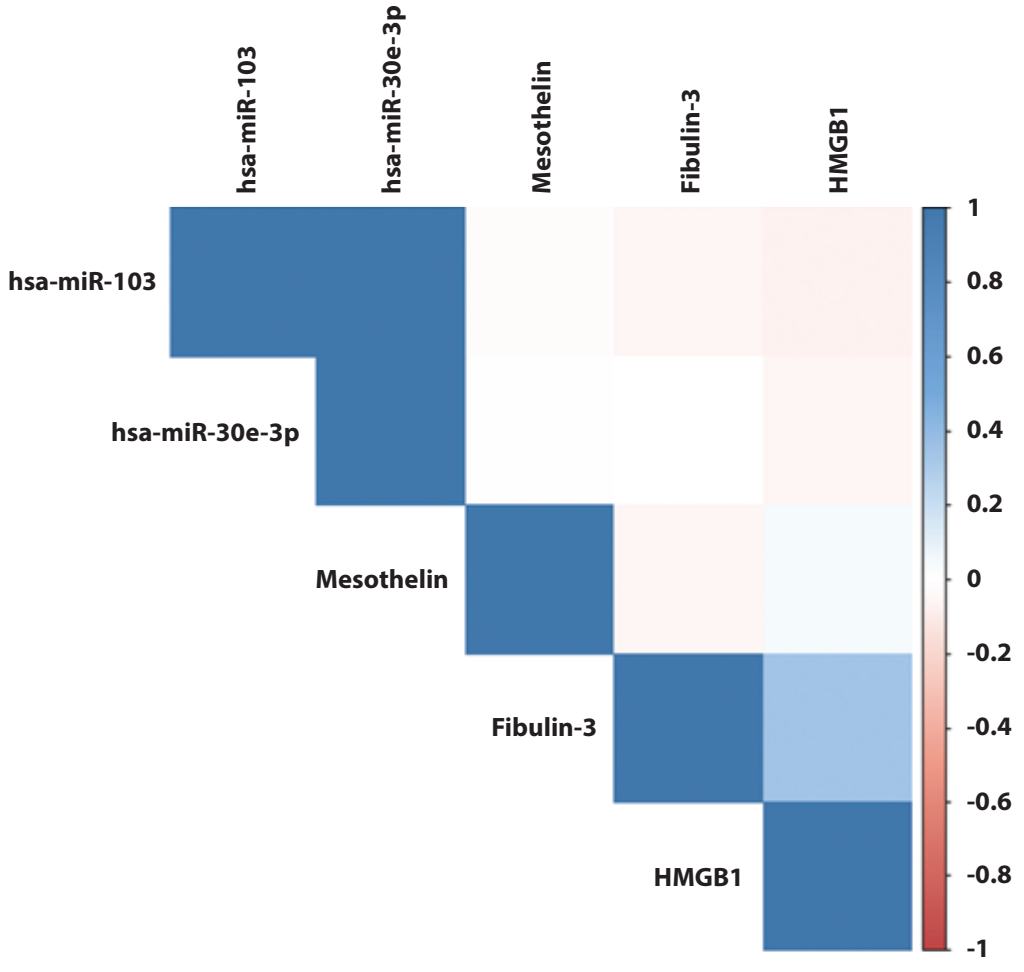

Figure 1. Correlation plot of plasmatic biomarkers (proteins and EV-miRNAs). Pairwise correlation between biomarkers. Colored squares represent the amount of Pearson correlation within each pair of variables.

Table 3. Sensitivity and specificity of predictive variables associated with MPM.

Biomarker	TP	TN	Sensitivity (%)	Specificity (%)	AUC (95% CI)
Mesothelin	20	43	77	80	0.88 (0.79, 0.96)
Fibulin-3	23	47	88	87	0.94 (0.87, 1.0)
HMGB1	24	43	92	80	0.92 (0.86, 0.97)
hsa-miR-30e-3p	16	42	62	78	0.81 (0.71, 0.91)
hsa-miR-103a-3p	22	32	85	59	0.81 (0.71, 0.91)
hsa-miR-30e-3p	21	42	81	78	0.90 (0.83, 0.98)
hsa-miR-103a-3p					
Mesothelin	22	47	85	87	0.94 (0.89, 1.0)
HMGB1					
Fibulin-3	24	46	92	85	0.95 (0.91, 1.0)
HMGB1					
Mesothelin	24	49	92	91	0.98 (0.97, 1.0)
Fibulin-3					
Mesothelin	25	50	96	93	0.99 (0.97, 1.0)
Fibulin-3					
HMGB1					

TP: True positives; TN: true negatives; AUC, Area Under the Curve; CI, confidence interval.

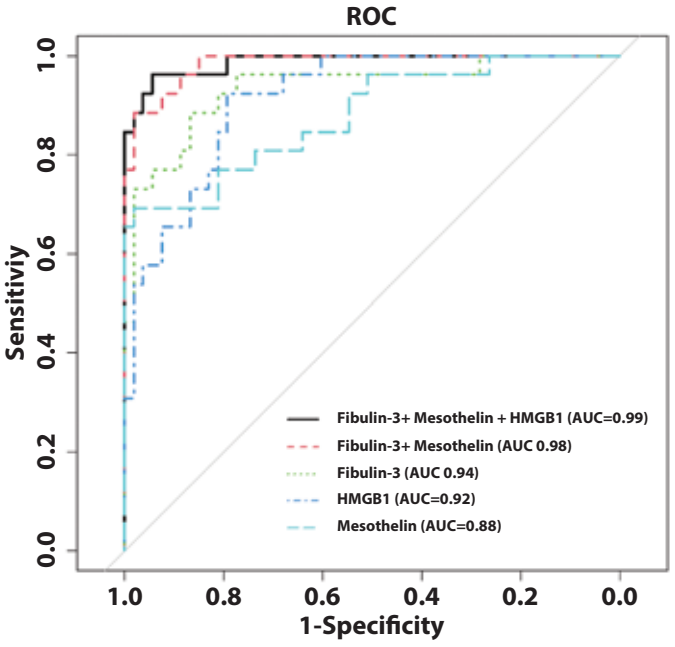

Figure 2. ROC curves of the three most discriminating biomarkers taken alone (i.e., HMGB1, Fibulin-3, and Mesothelin) and in combination (i.e., Mesothelin+Fibulin-3+HMGB1, and Mesothelin+Fibulin-3). Models were adjusted for sex, age, smoking habits, and BMI.

4. Discussion

MPM is a rare malignant tumor strongly associated with asbestos exposure. It is characterized by poor prognosis, and diagnosis is based on invasive techniques. In such a scenario, there is a need to identify reliable non-invasive early biomarkers in subjects at high risk. In particular, an increasing number of studies have explored using different biomarker panels to overcome the poor sensitivity and specificity of single markers and improve the predictive disease power in a diagnostic setting [15].

In the current study, we measured the Mesothelin, Fibulin-3, and HMGB1 protein biomarkers and the two hsa-miR-30e-3p and hsa-miR-103a-3p EV-miRNA and assessed their ability, alone and in combination, to discriminate between 26 MPM and 54 PAE subjects.

Mesothelin is the most studied diagnostic molecular biomarker for MPM [28]. Generally, it is expressed at low levels by normal mesothelial cells while it is overexpressed both in the membrane-bound- and in the soluble form in different cancers, such as MPM, ovarian, and lung cancers [29]. To assess plasmatic mesothelin levels, we used the ELISA technique taking advantage of the MesoMark assay, as did most of the studies in the field [15]. As reported in the literature, we detected higher concentrations of plasmatic mesothelin in MPM compared to healthy PAE subjects. Although Mesothelin showed a good discrimination power (AUC 0.88) between cases and controls, sensitivity and specificity were quite low. Our findings are consistent with previous case-control studies comparing MPM with PAE subjects [30-32].

We also investigated Fibulin-3, a secreted glycoprotein able to promote tumor growth by modulation of the AKT signaling pathway [33]. Plasmatic Fibulin-3 levels were higher in MPM than in PAE subjects, and concentrations were comparable to those previously reported by Pass and colleagues [34]. However, Fibulin-3 concentrations reported in the literature are inconsistent [32, 35], probably due to the lack of standardization of sample storage and different Fibulin-3 detection methods [36]. The diagnostic ability to circulate Fibulin-3 is also controversial, as studies conducted in North American, Chinese, Turkish, and Egyptian populations were strongly supportive [34, 37-39], while other conducted in European and Australian populations reported low diagnostic performance and discrimination ability, in particular with non-MPM malignancies [40, 41]. In the present study, Fibulin-3 showed the highest discriminating ability as a single biomarker, and our results are coherent with a previous study conducted by Pass and colleagues on 92 MPM and 136 PAE subjects (AUC 0.98) [34]. However, a recent meta-analysis conducted by Pei and colleagues reported lower pooled sensitivity (62%) and specificity (82%) than those observed in the present study [36]. Further validation studies in large populations, including groups with non-MPM malignancies, and standardization of detection methods are needed to overcome the inconsistencies and evaluate the actual reliability of circulating Fibulin-3 as a diagnostic biomarker for MPM.

HMGB1 protein is a damage-associated molecular pattern protein released in the extracellular space during necrosis, leading to chronic inflammation [42-44]. This circulating protein is involved in tumorigenesis and is known to be actively secreted by transformed MPM cells [42, 45, 46]. We observed a higher concentration of circulating HMGB1 in MPM and a good discriminating ability with an AUC of 0.92 (0.86, 0.97), a sensitivity of 92%, and a specificity of 80%. To our knowledge, we were the first to investigate HMGB1 in plasma samples [15, 32] The results obtained are similar to what was reported in the literature for HMGB1 serum concentrations [42, 47, 48], thus indicating plasma as a suitable biological fluid for the detection of this biomarker. In particular, Napolitano and colleagues reported an AUC of 0.83 with very high specificity (100%) and a sensitivity of 72% [42] and suggested that the hyper-acetylated form of HMGB1 had higher performance. However, a recent concern cast doubt on the validity of data on the hyper-acetylated form of the protein [49].

On the other hand, the study of Ying and colleagues reported an AUC of 0.94 with a lower specificity of 57% and the highest sensitivity of 100% [47]. The inconsistencies observed might be due to the different detection methods applied. Indeed, while the group of Napolitano and colleagues took advantage of the mass spectrometry technique, the group of Ying and colleagues and ours used the ELISA detection method. Moreover, some studies

reported no significant differences between patients with MPM and patients with other non-MPM malignancies or asbestosis [47, 48]. Further studies with particular attention to the standardization of analysis methods are needed to define the actual discriminating ability to circulate HMGB1.

None of the tested biomarkers showed significant differences when we considered the TNM stages and histotype. However, the small number of subjects in each category prevents any firm conclusion.

In recent years, the use of circulating miRNAs for MPM diagnosis has been widely explored, and several studies reported specific miRNA signatures related to MPM diagnosis and prognosis [12, 16, 50, 51]. miRNAs are small noncoding RNAs involved in regulating gene expression and modulation of various cellular functions, such as proliferation, differentiation, and invasion [52, 53]. In the present and our previous study, we specifically focused on circulating EV-associated miRNA expression, as they are emerging as an active mechanism of communication between cells. Thus, we hypothesized they might reflect the active crosstalk between cancer and the immune system rather than a passive release in the extracellular environment. We previously identified the specific two-EV-miRNA signature miR-103a-3p and miR-30e-3p that was able to discriminate between patients with MPM and PAE subjects, with an AUC of 0.94 (95%CI 0.87±1.00), a sensitivity of 96% and a specificity of 80% [20]. In the current study, the two-EV-miRNA signature generated an AUC of 0.90, but the sensitivity and specificity were lower than those observed in our previous study.

Since combining different biomarkers has been encouraged to set diagnostic tools with higher accuracy [12, 15, 42, 49], we tested different combinations of the biomarkers examined in the present study. As the discrimination ability of the two EV-miRNA-panel resulted lower than that of Fibulin-3 and HMGB1 even taken alone, we considered only Mesothelin, Fibulin-3, and HMGB1 and observed that in combination, they showed the highest discriminating ability (AUC of 0.99), sensitivity (96%), and specificity (93%). The present study is the first one that evaluated the combination of plasmatic Mesothelin, Fibulin-3, and HMGB1 biomarkers together. In a previous study, plasmatic Mesothelin, Calretinin, and the Megakaryocyte potentiating factor generated an AUC of 0.94 in a population of 128 males (sensitivity 82%, specificity 95%), and AUC of 0.94 in a population of 38 females compared to healthy controls (sensitivity 87%, specificity 95%) [54], thus showing a lower discriminating potential than that of the panel tested in the current study. When we evaluated the combination only of Mesothelin and Fibulin-3, the AUC did not differ, but a lower sensitivity was detected. Thus, the actual improvement conferred by HMGB1 to the Mesothelin and Fibulin-3 panel needs to be confirmed in a larger study. In their recent meta-analysis, Schillebeeckx et al. encourage to focus on external validation of already identified biomarkers and biomarker panels, indicating Mesothelin, Fibulin-3, and HMGB1 as the most promising biomarkers for MPM detection with translational potential to the routine clinical practice [15].

Other studies have explored using plasmatic biomarker panels, in particular, combining Mesothelin with different markers to improve the discriminating potential in a diagnostic setting. In particular, Weber and colleagues combined Mesothelin with mir103a-3p in a study including 43 MPM and 52 PAE and observed an increased diagnostic performance (AUC 0.90, sensitivity 95%, specificity 81%) compared to the single biomarkers (mesothelin: AUC 0.81, sensitivity 74%, specificity 85%; miR-103-ap AUC 0.76, sensitivity 89%, specificity 81%).

We acknowledge some limitations of the present study. First, the small number of subjects and the lack of subjects with other respiratory diseases (e.g., lung cancer, benign pleural effusion, asbestosis) prevent definite conclusions. Second, we acknowledge that MPM patients showed a slightly unhealthy metabolic profile (more smokers, higher BMI, and older age). However, we considered these variables in the statistical analyses to assess the independent role of the tested biomarkers on the odds of being a patient with MPM. Moreover, as an explorative study, we did not perform an independent validation for the combined biomarkers. Thus, validation studies are needed in larger populations to confirm the results described.

5. Conclusions

The combination of the three plasmatic Mesothelin, Fibulin-3, and HMGB1 biomarkers showed the highest discrimination ability between MPM patients and PAE subjects and appears to be a promising high-specificity biomarker panel for MPM diagnosis.

Despite the high number of studies that evaluated previously identified and new potential biomarkers, none of them can be considered sufficiently reliable to be used in the surveillance of asbestos-exposed subjects or the early diagnosis of MPM. As underlined by the recent ERS/ESTS/EACTSD/ESTR European guidelines for the management of MPM: "routine determination of previously proposed biomarkers in MPM has no current validated role in diagnosis, prognosis or clinical follow-up (disease monitoring)" [1]. The rarity of the disease, even in cohorts of asbestos-exposed subjects, and the lack of clear evidence of effective treatments leading to survival improvements (reduction of mortality) in early-diagnosed cases are other factors hampering the use of the biomarkers described so far. Even their possible application as diagnostic biomarkers to guide the selection of those subjects needing further investigations for early detection of the disease remains controversial.

However, several authors, including Scherpereel et al., in the ERS/ESTS/EACTSD/ESTR guidelines [1], encouraged further research into the role of biomarkers. Further case-control validation studies have to be conducted in larger populations, including subjects with other non-MPM malignancies, and prospective longitudinal studies to confirm the evidence observed. The results obtained in the present study contribute to identifying new potential non-invasive biomarkers for future validation studies.

Supplementary materials: Supplementary Table S1: Geometric means of biomarkers in the four TNM stages; Supplementary Table S2: Geometric means of biomarkers in the three histotypes; Supplementary Table S3: Univariate logistic regression of protein, miRNA, and covariates estimating the odds of MPM; Supplementary Table S4: multiple logistic regression estimating the odds of MPM; Supplementary Table S5: ROC comparisons.

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Informed consent statement: All participants provided signed written informed consent. The study design, research aims, and measurements were approved by the Ethics Committee "Comitato Etico - Milano Area 2" of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, 20122 Milan, Italy (approval number #2423), in agreement with principles of the Helsinki Declaration. MPM patients were followed-up in June 2021 to ascertain their vital status.

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Declaration of interest: Carolina Mensi served as a consultant for the court and patients in litigations concerning asbestos-related diseases. The other authors declare no conflicts of interest.

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Appendix: Supplementary material

Supplementary Table S1. Geometric means of biomarkers in the four TNM stages.

Biomarker	TNM stage	Geometric Mean	LCI	UCI	p-value of comparisons	Overall p-value
HMGB1	I	11.9	7.3	19.3	Reference	0.317
	II	9.5	5.4	16.7	0.542	
	III	6.5	3.8	10.8	0.090	
	IV	11.6	6.3	21.5	0.957	
Fibulin-3	I	53.9	44.8	64.9	Reference	0.854
	II	48.5	39.2	60.1	0.446	
	III	53.9	44.2	65.7	0.997	
	IV	51.2	40.5	64.7	0.722	
Mesothelin	I	2.0	0.9	4.7	Reference	0.974
	II	2.2	0.8	5.8	0.893	
	III	2.2	0.9	5.3	0.911	
	IV	1.6	0.6	4.8	0.754	
hsa-miR-103a-3p	I	435.6	87.2	2176.8	Reference	0.745
	II	217.1	38.2	1234.1	0.547	
	III	191.7	38.4	958.1	0.462	
	IV	120.3	17.9	807.5	0.295	
hsa-miR-30e-3p	I	294.8	63.0	1379.8	Reference	0.620
	II	125.0	23.6	662.3	0.441	
	III	92.6	19.8	433.2	0.282	
	IV	75.5	12.2	469.0	0.250	

LCI; lower 95% confidence interval; UCI: upper 95% confidence interval; tissue specimens were classified according to the TNM staging system established by IMIG and IASLC.

Supplementary Table S2. Geometric means of biomarkers in the three histotypes.

Biomarker	Histology	Geometric Mean	LCI	UCI	p-value of comparisons	Overall p-value
HMGB1	Epithelioid	10.6	6.7	16.7	Reference	0.815
	Biphasic	9.7	3.5	26.9	0.528	
	Sarcomatoid	8.8	6.0	12.9	0.851	
Fibulin-3	Epithelioid	52.1	44.2	61.4	Reference	0.995
	Biphasic	53.0	36.7	76.5	0.988	
	Sarcomatoid	52.0	45.2	59.7	0.921	
Mesothelin	Epithelioid	2.2	1.1	4.6	Reference	0.830
	Biphasic	1.3	0.3	6.7	0.843	
	Sarcomatoid	2.0	1.1	3.7	0.609	
hsa-miR-103a-3p	Epithelioid	214.3	57.5	798.5	Reference	0.569
	Biphasic	964.4	50.9	18269.7	0.884	
	Sarcomatoid	189.1	59.7	599.5	0.297	
hsa-miR-30e-3p	Epithelioid	154.1	42.6	557.9	Reference	0.661
	Biphasic	363.1	20.4	6449.8	0.610	
	Sarcomatoid	100.5	32.5	310.6	0.398	

LCI; lower 95% Confidence Interval; UCI: upper 95% Confidence Interval.

Supplementary Table S3. Univariate logistic regression of protein, miRNA, and covariates estimating the odds of MPM.

Variable	Estimate	SE	p-value	OR	LCI	UCI
hsa-miR-103a-3p	0.0000	0.0001	0.726	0.99996	0.99970	1.000
hsa-miR-30e-3p	0.0000	0.0003	0.873	1.00004	0.99950	1.001
Mesothelin	1.3458	0.4244	0.002	3.841	1.672	8.826
Fibulin-3	0.2607	0.0733	0.000	1.298	1.124	1.498
HMGB1	0.1749	0.0582	0.003	1.191	1.063	1.335
Age	0.144	0.042	0.001	1.155	1.064	1.253
BMI	-0.132	0.065	0.044	0.876	0.771	0.996
Gender M vs F	0.350	0.309	0.256	2.014	0.601	6.752
Smoking habits YES vs NO	0.196	0.517	0.705	1.875	0.364	9.643
Smoking habits Former vs Never	0.237	0.355	0.504	1.953	0.701	5.440

SE: standard error; OR: Odds Ratio; LCI; lower 95% Confidence Interval; UCI: upper 95% Confidence interval.

Supplementary Table S4. Multiple logistic regression estimating the odds of MPM.

Outcome: MPM	Estimate	SE	p-value	OR	LCI	UCI
Mesothelin	1.14	0.60	0.058	3.13	0.96	10.16
Fibulin-3	0.69	0.29	0.017	2.00	1.14	3.53
HMGB1	0.24	0.13	0.055	1.27	1.00	1.63
Smoking habits YES vs No	-4.03	2.44	0.099	0.02	<0.001	2.13
Smoking habits Former vs No	-3.10	3.02	0.304	0.05	<0.001	16.69
Age	0.45	0.22	0.040	1.57	1.02	2.41
BMI	-0.28	0.15	0.070	0.76	0.56	1.02
gender M vs F	-5.94	3.60	0.099	0.00	<0.001	3.03

OR: Odds ratio; lower 95% Confidence Interval; UCI: upper 95% confidence interval. Models adjusted for gender, age, smoking habits, and BMI.

Supplementary Table S5. ROC comparisons.

ROC comparisons	Estimate ROC difference	SE	LCI	UCI	p-value
Fibulin-3 Mesothelin HMGB1 Vs Fibulin-3 Mesothelin	0.003	0.008	-0.012	0.018	0.674
hsa-miR-30e-3p hsa-miR-103 Vs Fibulin-3 Mesothelin	-0.086	0.036	-0.156	-0.017	0.015

SE: standard error; LCI: lower 95% Confidence Interval; UCI: upper 95% Confidence Interval. Comparison of models adjusted for gender, age, smoking habits, and BMI.