Journal of Thoracic Oncology

Potential diagnostic and prognostic role of micro-environment in malignant pleural mesothelioma

--Manuscript Draft--

Manuscript Number:	JTO-D-18-01716R1
Full Title:	Potential diagnostic and prognostic role of micro-environment in malignant pleural mesothelioma
Article Type:	Original Article
Keywords:	malignant pleural mesothelioma; Tumor-infiltrating lymphocytes; T-regulatory cells; myeloid-derived suppressor cells; immune checkpoints
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Abstract:	Introduction. A comprehensive analysis of the immune-cell infiltrate collected from pleural fluid and from biopsies of malignant pleural mesothelioma (MPM) may contribute to understand the immune-evasion mechanisms related to tumor progression, aiding in differential diagnosis and potential prognostic stratification. Till now such approach has not routinely been verified. Methods. We enrolled in 275 patients with an initial clinical diagnosis of pleural effusion. Specimens of pleural fluids and pleural biopsies used for the pathological diagnosis and the immune-phenotype analyses were blindly investigated by multi-parametric flow cytometry. The results were analyzed by Kruskal-Wallis test. The Kaplan-Meier and log-rank tests were used to correlate immune-phenotype data with patients' outcome. Results. The cut-offs of intra-tumor T-regulatory (Treg; >1.1%) cells, M2-macrophages (>36%), granulocytic and monocytic myeloid-derived suppressor cells (MDSC; >5.1% and 4.2%, respectively), CD4+PD1+ (>5.2%) and CD8+PD1+ (6.4%) cells, CD4+LAG-3+ (>2.8%) and CD8+LAG-3+ (>2.8%) cells, CD4+TIM-3+ (>2.5%) and CD8+TIM-3+ (>2.6%) cells discriminated MPM from pleuritis with 100% sensitivity and 89% specificity. The presence of intra-tumor MDSC contributed to the anergy of tumor-infiltrating lymphocytes (TILs). The immune-phenotype of pleural fluid cells had no prognostic significance. By contrast, the intra-tumor Treg and MDSC levels significantly correlated with progression-free and overall survival, the PD-1+/LAG-3+/TIM-3+ CD4+TILs correlated with overall survival. Conclusions. A clear immune-signature of pleural fluids and tissues of MPM patients may contribute to better predict patients' outcome.

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Torino, 10/03/2019

To the attention of
Prof. Thomas John
Associate Editor
Journal of Thoracic Oncology

Dear Professor John,
We resubmit our manuscript related to the experimental work entitled "**Potential diagnostic and prognostic role of micro-environment in malignant pleural mesothelioma**" (JTO-D-18-01716).
In accordance with the Reviewers' comments, we have extensively revised the manuscript and figures.
A version with changes highlighted in red and a clean version are included.
We would like to take this opportunity to thank the Reviewers for their valuable comments. The review process has contributed to greatly improve the overall quality of the manuscript.
We hope that the revised version will be suitable for publication in Journal of Thoracic Oncology.
The presented data were obtained as part of an original research. The manuscript has not been published previously and is not being considered concurrently by another journal.

The conflict of interest of each Author has been carefully amended.
Chiara Riganti

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Reply to Reviewer 1

Reviewer #1: I reviewed the manuscript by Salaroglio et al on the "Potential diagnostic and predictive role of micro-environment in malignant pleural mesothelioma". In their work they look at the immunologic micro environment between the pleural effusions or tissue of patients with mesothelioma, other cancers or benign effusions. Their work is strengthened by the functional assays; however, there are limitations that preclude its publication at this time as described below.

1) Why were both parametric and non-parametric statistics used in a small retrospective cohort. Typically, non-parametric statistics are more meaningful in this situation.
Although we checked the normality distribution of each parameter with the D'Agostino and Pearson test, and all parameters had a Gaussian distribution or approximation in pleuritis and pleural malignant mesothelioma (MPM) groups, following the Reviewer's comment, we applied a non-parametric (Kruskal Wallis) test to the comparisons. We re-calculated the statistical significance applying this non-parametric assay (GraphPad PRISM 5.0 software). We modified abstract (page 3, line 4), Materials and Methods (page 11, line 2), Figures and legends, accordingly.
To correlate these values with progression-free survival (PFS) and overall survival (OS), we divided the population according to the median value, applying the log-rank test, because there are not previously established cut-offs dividing patients with high or low immune-parameters reported in the literature and the size of the cohort analyzed was small.

2) *How and why were a sample size calculated? This is typically done for prospective trial design, not correlative analyses from a retrospective cohort. However, the results of the sample size calculations utilized?*
We apologize for not having clearly explained how the sample size was calculated. We modified the Materials and Methods section accordingly (page 10, line 20).
We agree with the Reviewer's comment that sample size is typically calculated for prospective trial design. In our case, sample size was calculated "prospectively" (i.e. before performing the laboratory determinations). In detail, before starting the patient enrollment, we calculated the sample size necessary to achieve significant results in MPM group, setting significance level (α error probability) $\leq$ 0.05, power (1-β error probability) = 0.80, effect size ρ = 0.40, using the G*Power Software (www.gpower.hhu.de). The sample size required was 34±2 for MPM group. We thus analyzed 49 samples from MPM pleural fluid, 33 samples from MPM pleural tissue. The discrepancy between the required minimal number of samples (34) and the analyzed samples (49) in the pleural fluid group was due to the fact that the pathologists performing the diagnosis and the researchers analyzing the immune-infiltrate worked in a blind-manner. The immune-infiltrate analysis was always performed before the pathological diagnosis was known. Given the low frequency of MPM, we had to enrolled 275 patients who received diagnostic thoracoscopies with an initial clinical diagnosis of pleural effusion (page 6, line 24), to achieve the required sample size of patients with a confirmed pathological diagnosis of MPM.

3) Were other grouping strategies explored other than the median cutoff method described?
In order to test the association of the immune-parameters analyzed with patients' outcome (PFS and OS), we did not explore grouping strategies other than the division in 2 categories using the median as cutoff. In fact, in literature there are no previously reported and validated cut-off the for the immune-phenotype parameters analyzed. Grouping the patients according to tertiles or quartiles could be significantly limited by the small sample size (n=49 MPM derived from pleural fluid; n =33 MPM

derived from pleural tissue). Indeed dividing by tertiles and quartiles such small cohort, implies a very low number of patients (e.g. 3-5) attributed to each subgroup.
The definition of the optimal cut-off was beyond the scopes of this analysis. A larger prospective study is ongoing ay our Institution, with the aim of validating the clinical significance of the immune-phenotypic signature found in this work. Different grouping strategies will be applied to define in a more robust way the correlations between immune-phenotypic parameters, patients clinical history, mutational profiling, response to therapy and clinical outcome.
We modified Material and Methods (page 11, line 8) and Discussion (page 15, line 6; page 17, line 16).

4) For Figures 1-3, Supplemental Figure 3, the labeling is not reader friendly. The values of the asterisks, circles or hash symbols change based on the figure, and I cannot always tell what is being compared by looking at the figure.
We reported the statistical significance in uniform way. Asterisks are referred to the comparison of MPM or pleural metastases of malignant tumors (MTS) towards pleuritis; circles are referred to the comparison of MPM towards MTS (Figure 1-5, new Supplementary Figure 4). In Figure 3, panels D-F, hash symbols are referred to the comparison of Gr-MDSC and Mo-MDSC from MPM patients cultured with T-cytotoxic lymphocytes from pleuritis patients towards Gr-MDSC and Mo-MDSC from pleuritis patients cultured with T-cytotoxic lymphocytes from the same MPM patient.
We modified the legends of main figures and supplementary figures accordingly.

5) Also, in regards to the figures, since the authors are claiming cut-offs for groups, it would be useful to display these results in a format other than bar and line graphs as recommended by statisticians: https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.1002128
Without this type of display, I cannot tell if parametric tests are appropriate since the authors do not state that the checked the normality of their data.
We modified all the bar and line graphs as the Reviewer suggested, using GraphPad PRISM 5.0 software. To allow a better visualization of the cut-off mentioned in the text, we added them in Figure 1 (panels D, F), Figure 2 (panels B, D, F, H), new Supplementary Figure 5 (panels A-F).
We checked the normality of our data by applying the D'Agostino and Pearson test. The results are reported in the Table below.

	Normality distribution results					
Figure 1	pleuritis fluid	pleuritis tissue	MPM fluid	MPM tissue	MTS fluid	MTS tissue
A	Yes	Yes	Yes	Yes	Yes	too small
B	Yes	Yes	Yes	Yes	Yes	too small
C	No	Yes	Yes	Yes	Yes	too small
E	Yes	Yes	Yes	Yes	Yes	too small
Figure 2						
A	No	No	Yes	Yes	Yes	too small
C	No	Yes	Yes	Yes	Yes	too small
E	No	Yes	Yes	Yes	Yes	too small
G	No	Yes	Yes	No	No	too small
Figure 3						
A	Yes	Yes	Yes	Yes	too small	too small
B	Yes	Yes	Yes	Yes	too small	too small
C	No	Yes	Yes	Yes	too small	too small

D	Yes	Yes	Yes	Yes	too small	too small
E	Yes	Yes	Yes	Yes	too small	too small
F	Yes	No	Yes	Yes	too small	too small
Figure 4						
A	Yes	No	Yes	No	Yes	No
B	Yes	Yes	Yes	No	too small	too small
C	Yes	No	Yes	Yes	Yes	Yes
D	Yes	No	Yes	No	too small	too small
E	Yes	No	Yes	No	Yes	No
F	Yes	No	Yes	Yes	too small	too small
G	Yes	Yes	Yes	Yes	Yes	No
H	Yes	Yes	Yes	Yes	Yes	No
Figure 5						
A		Yes		Yes		Yes
B		Yes		Yes		Yes
C		No		Yes		Yes
D		No		Yes		No
Figure S1A	No	No	Yes	No	Yes	too small
Figure S1A	No	Yes	No	No	No	too small
Figure S2	Yes	Yes	Yes	Yes	No	too small
Figure S3	Yes	Yes	Yes	Yes	No	too small

We added a statement on the normality test used in the Materials and Methods (page 11, line 1). However, as previously specified, following Reviewer's comment, we conservatively decided to perform non–parametric tests.

6) The conclusion that MDSCs are the primum movens of MPM is far too strong since there are no models to demonstrate that they are necessary and sufficient to drive immunosuppression in this malignancy. Previously published data on PD-L1 expression suggest that this immune checkpoint is also a significant driver with prognostic significance.

We agree with the Reviewer's criticism, since our experiments prove that MDSC play an important role but are not the only necessary and sufficient finding to determine immune-suppression in MPM. We smoothened the sentence commenting our data on MDSC role (Discussion, page 16, line 5). We already mentioned the role of PD-L1 as a mediator of immune-suppression and as a marker of poor prognosis in MPM (Introduction, page 5, line 20; references 11, 12, 31 and 32 in the previous version, references 20, 21, 22, 23 in the revised version).

7) Also, I think it is too strong of the conclusion that the cut offs presented can be used to differentiate the groups of patients included in the study. Without discovery and validation cohorts, these conclusions cannot be made. The concluding paragraph about using these values to differentiate challenging clinical cases cannot stand as written.

We recognize that we cannot propose the cut-off identified in our study as means to differentiate the patients' groups. As specified above, a larger prospective study, including a discovery and validation cohort, is ongoing to evaluate the potential use of the immune-phenotypic signature identified to differentiate the challenging clinical cases.

We rewrote the final paragraph accordingly (page 16, line 17).

8) The authors do not account for multiplicity. So many statistical test were performed yet know adjustments were made for the large number of comparisons performed. There are either needs to be an explanation for why no adjustments were made or a more stringent P value needs to be applied.
Given the exploratory nature of the study, we did not make adjustments.
Following the Reviewer suggestion, we applied a more stringent p value ($p<0.01$ instead of $p<0.05$), to account, at least partially, for the multiplicity of tests. Only the analysis of survival, considering its exploratory nature, was performed with a $p<0.05$ cutoff.
We re-calculated the statistical significance with this cut-off and modified Figures and Materials and Methods (page 11, lines 5 and 14) accordingly.

9) Finally, the use of "predictive" in the title is not appropriate. Predictive markers suggest which patients would benefit from therapy. Whereas prognostic markers are suggestive of survival outcomes.
We modified the title changing "predictive" role into "prognostic role".

Minor
1. The use of some abbreviations is not common, such as PD-1L
We checked the abbreviations. In particular we corrected the abbreviation PD-1L into PD-L1.

2. Copy editing would help with spelling and grammar (page 5, line 44, "maaged")
We checked spelling and grammar throughout the manuscript.

Reply to Reviewer 2

Reviewer #2: In this, the age of immunotherapy a comprehensive knowledge of the immune-evasion mechanisms has the potential to discover parameters related to tumour progression and prognostication. FACS assessment of TILs provides a detailed description of T cell subsets, their differentiation and immune checkpoint expression. In this paper by Prof. Riganti et al, the authors have conducted a broad explorative analysis on the immune microenvironment of pleural fluid and pleural biopsy specimens of patients with suspected mesothelioma. They uncovered some features in the immune profile that may potentially be of use in differentiating mesothelioma from pleuritis and secondary deposits in the pleura They found a prognostic role for intra-tumor Treg and MDSC levels and PD-1+/LAG-3+/TIM-3+ CD4+TILs. Immune-phenotype of pleural fluid cells showed no prognostic significance.

The authors are to be congratulated for their exhaustive effort and a well written manuscript.

I think this paper helps further define the immune evasive environment in mesothelioma and sheds some light on the prime roles of MDSC and tumour associated macrophages. More importantly, it helps delineate the differences in the immune microenvironment in mesothelioma versus pleuritis. This could be helpful in the diagnosis of difficult cases. I do however think that the latter findings need to validated in larger independent cohorts.

Some points to clear out:
1) A flow chart (perhaps in the supplemental data section) showing the number of samples collected, the numbers included and reason/s for exclusion would be very helpful as this information is not clear.

Was the collection of samples prospective? Was there a reason why only a proportion had both pleural fluid and tissue available for analysis?

Following the Reviewer's suggestion, we added a flow chart (new Supplementary Figure 1) and we added the missing information about collecting and processing samples (page 6, line 24), and inclusion criteria (page 8, line 22), in the revised manuscript.

We enrolled 275 patients with an initial clinical diagnosis of pleural effusion of unknown origin. The patients enrollment was terminated when we reach a sample size with an adequate power in the MPM patient cohort (see reply to Reviewer 1, point 2).

Two aliquots of pleural fluid and pleural tissue were collected by thoracic surgeons at the same time for each patient. One aliquot was used for diagnostic purposes according to local pathology guidelines, the second one was used for the research study. Priority was given to the diagnostic workup, the left-over was dedicated to the research study. On the basis of the pathologists' decision, in case of limited volume of pleural fluid or limited dimension of pleural tissue, both aliquots were dedicated to the diagnostic workup. In accordance with the above reported criteria, 183 pleural fluids and 119 pleural biopsies were available for the research study.

The research unit performed a technical feasibility check of the immune-analysis for each sample and established to include in the study only samples with $\geq 1\times10^6$ viable (i.e. Trypan-blue negative) cells in the supernatants or adherent in flask. Preliminary set-up experiments indicated that $\geq 1\times10^6$ viable cells were the minimal number required allowing to acquire 1×10^5 cells/staining, to measure each population or immune-parameter on each sample. Samples with $\leq 1\times10^6$ viable cells were excluded, since they did not allow to acquire 1×10^5 cells/staining. Acquiring $< 1\times10^5$ cells/staining reduced the accuracy of detection of rare immune-populations (i.e. < 10% cells present in the immune-infiltrate) or rare immune-checkpoints (< 10% positive cells).

According to this inclusion criterion, from all the collected samples 63 non-malignant pleuritis, 49 MPM and 32 MTS for pleural fluid; 16 non-malignant pleuritis, 33 MPM and 5 MTS for pleural tissue biopsies were included in the present study. 9 patients with diagnosed pleuritis and 20 patients with diagnosed MPM group had both pleural fluid and pleural tissue available.

2*) Were the arbitrary cut-offs just the medians for each of the parameters?*

The cut-offs indicated in the abstract, in the text and in Figure 1 (panels D, F), Figure 2 (panels B, D, F, H), Supplementary Figure 5 (panels A-F), were fixed in order to discriminate patients with MPM from patients with pleuritis with the highest sensitivity. Using the established cut-offs, we yielded a sensitivity of 100% (i.e. 0% false negative for all the parameters), and a specificity between 89% and 100% (i.e. 11% false positive for Treg and Mo-MDSC; 0% false positive for all the remaining parameters).

We better specified this point in the Abstract (page 4, line 6) and Materials and Methods (page 11, line 6). We reported *in extenso* throughout the Abstract and the Results section all the cut-offs which met the sensitivity and specificity criteria indicated above. We reported the cut-off in the figures and figures legends, along with the values of false negative, false positive, sensitivity and specificity.

In Table 1, Supplementary Figures 6, 7 and 8, we used the medians as cut-offs, to group patients with "high" or "low" immune-parameters and correlate the meaning of the immune-parameters evaluated with PFS and OS, because of the absence of established cut-offs in the literature and the limited sample size of the cohort.

3) We know that there is much inter and intra patient variation in the intensity and composition of TILs, do the authors believe that this may have an effect on the ability to differentiate different mesothelioma samples from pleuritis and secondaries?

The intra-tumor immune-infiltrate analysis has been performed on a small cohort of MPM (27 epithelioid MPM, 3 sarcomatous MPM, 3 biphasic MPM). We did not see correlations between different intensity/compositions of TILs, different anamnestic features (age, sex, smoking status, presence or absence of asbestos exposure, professional or environmental asbestos exposure, Supplementary Table 2), different features of MPM samples (e.g. histological type, Supplementary Table 2; mutational profile of each patient, not reported in the manuscript).
We did not divide further the sample into subgroups according to the qualitative and quantitative differences in TILs, to investigate if such differences may have an impact on the differential diagnosis between each MPM subgroup, pleuritis or MTS. Working with such small number of patients could result in statistical biases due to the limited number of patients in each subgroup.
A larger prospective study is currently ongoing at our Institution. The aims of this prospective study are:
i) to validate the clinical meaning of the immune-phenotypic signature found in this work, defining in a more robust way the correlations between immune-phenotypic parameters, anamnestic information of the patients, MPM histotypes, mutational profiling, response to therapy and clinical outcome;
ii) to evaluate if the analysis of the immune-parameters identified in this work may be routinely applied in the diagnostic workup to refine the differential diagnosis with pleuritis and MTS.
We commented this point in the revised manuscript (Discussion, page 17, line 19; page 18, line 6).

4) The prognostic significance of intra-tumor Treg and MDSC levels and PD-1+/LAG-3+/TIM-3+ CD4+TILs is interesting. However, it appears that a multivariate analysis was not done? Are we reasonably confident that these are independent of factors like tumour histological subtype as we know that the immune-microenvironment varies with histology.
As stated in reply to point 3, we analyzed intra-tumor Treg, MDSC, PD-1^+/LAG-3^+/TIM-3^+ $CD4^+$TILs in a small cohort of MPM. We did not plan a multi-parametric analysis, to avoid possible statistical biases due to the small size of the subgroups analyzed. However, we agree with the Reviewer that multi-parametric analysis will give important information, and we plan to perform such analyses in the ongoing multi-center validation study.

Reply to Reviewer 3
Reviewer #3: Dr. Salaroglio and colleagues have undertaken a thoughtful analysis of the microenvironment in pleural fluid. They have identified features of pleural fluid that are potentially diagnostic of the etiology of the fluid - pleuritis, mesothelioma, or metastatic malignant disease. I do have several constructive comments as below:

1) Please clarify when relative to the clinical course the samples underwent the research testing analyses. The text indicates in parallel. Does that mean that samples were not aggregated for batch testing? Or, rather each and every time there was an effusion this battery of testing was performed?
We apologize for not having detailed the clinical course of sample processing. We added the missing information about collecting and processing samples (page 6, line 24), and inclusion criteria (page 8, line 22) in the revised manuscript.
We specified that we performed the whole set of tests for each sample and each time (Materials and Methods, page 9, line 1).

2) Please include some discussion of the statistical results around the cut-offs described in the results section.

For the detailed explanation on the choice of the cut-offs, please see the replies to Reviewer 2 (point 2) and Reviewer 1 (point 3).
We commented the choice of such cut-offs in the Discussion (page 14, line 23).
We specified that the aim of the study was to maximize the correct identification of MPM patients, accepting the risk to have sporadic cases of pleuritis identified erroneously as MPM. Since all samples were subjected to the usual pathological diagnosis, the rare cases of pleuritis erroneously diagnosed as MPM - on the basis of immune-phenotyping - could be easily re-verified by the pathologist. The analysis of the immune-parameters was not intended to replace the pathological diagnosis. Our aim was to propose a set of new tests supporting the pathological diagnosis of MPM and a set of new prognostic factors.

3) There are some contextualization errors throughout the manuscript but within the discussion. For example, the statement that "ctla-4 is an important therapeutic target" and referencing an older single arm study instead of the large randomized placebo-controlled trial of tremelimumab that was negative, casts a mistaken impression around the efficacy of CTLA-4 inhibition in mesothelioma.
We revised the manuscript to avoid contextualization errors. Specifically, we modified the paragraph on CTLA-4, including the reference on the more recent randomized placebo-controlled trial of tremelimumab (page 5, line 24).

4) The authors suggest use of these immune profiling measures in border line or difficult to analyze cases but provide no information regarding inclusion of those sorts of cases in the development of this tool. How many of the analyzed specimens were complex or difficult? Were any cases included where the standard analysis was inconclusive? To propose using these standards without demonstrating that they were derived from similar cases is not appropriate.
We agree that we cannot state that the immune-profiling can be used in border-line or difficult to analyze cases. Indeed, in our study we had no cases where the standard pathological analysis was inconclusive. Only ampler prospective study, currently ongoing at our Institution, may include border-line or difficult to analyze cases and may allow to compare the accuracy of the standard pathological diagnosis and of the immune-phenotype analysis in such cases.
We modified Abstract (page 3, line 15) and Discussion (page 17, line 24) accordingly.

5) Additionally, the entire cohort is described as having suspected mesothelioma. Please clarify what is meant by this? This certainly limits the generalizability of the results.
We apologize for the sentence reported in the materials and Methods of the previous version:
“From June 2016 through June 2018 we collected 183 consecutive pleural fluids and 119 pleural biopsies from 275 patients with suspected MPM”
that may have induced a misunderstanding.
Patients who received diagnostic thoracoscopy (n=275) had an initial diagnosis of pleural effusion of unknown origin, as reported in the Abstract (page 3, line 1) and Introduction (page 6, line 18) in the previous version. The samples were processed by the Pathology Unit for the diagnostic workup and by the research unit for the immune-parameters analysis, in a blind manner. The association between pathological diagnosis and immune-phenotype analysis was performed during the final data analysis. Only samples with a certain pathological diagnosis of non-malignant pleuritis, MPM or pleural MTS of other tumors were included in the research study. We clarified this point in the Materials and Methods section (page 7, lines 11 and 24).

6) Overall the discussion is too long and includes a great deal of immunology background that should be included preferentially in the introduction as it is not specific to the results herein.

We shortened the Discussion and moved some key immunology background information in the Introduction. The References section was modified accordingly.

Manuscript (All Manuscript Text Pages in MS Word format,
including Title Page, References and Figure Legends)

Potential diagnostic and prognostic role of micro-environment in malignant pleural mesothelioma

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Conflicts of interest statement

Silvia Novello received honoraria for speaker bureau and advisory role from Astrazeneca, BI, Roche, Pfizer, MSD, Eli Lilly, Abbvie, Celgene for activities unrelated to the present study.

Giorgio Scagliotti received honoraria for speaker bureau and advisory role from Astrazeneca, Roche, Pfizer, MSD, Eli Lilly, Abbvie and travel support from MSD and Bayer for activities unrelated to the present study.

The other authors have no conflict of interests

Abstract

Introduction. A comprehensive analysis of the immune-cell infiltrate collected from pleural fluid and from biopsies of malignant pleural mesothelioma (MPM) may contribute to understand the immune-evasion mechanisms related to tumor progression, aiding in differential diagnosis and potential prognostic stratification. Till now such approach has not routinely been verified.

Methods. We enrolled in 275 patients with an initial clinical diagnosis of pleural effusion. Specimens of pleural fluids and pleural biopsies used for the pathological diagnosis and the immune-phenotype analyses were blindly investigated by multi-parametric flow cytometry. The results were analyzed by Kruskal-Wallis test. The Kaplan-Meier and log-rank tests were used to correlate immune-phenotype data with patients' outcome.

Results. The cut-offs of intra-tumor T-regulatory (Treg; >1.1%) cells, M2-macrophages (>36%), granulocytic and monocytic myeloid-derived suppressor cells (MDSC; >5.1% and 4.2%, respectively), $CD4^+PD1^+$ (>5.2%) and $CD8^+PD1^+$ (6.4%) cells, $CD4^+LAG\text{-}3^+$ (>2.8%) and $CD8^+LAG\text{-}3^+$ (>2.8%) cells, $CD4^+TIM\text{-}3^+$ (>2.5%) and $CD8^+TIM\text{-}3^+$ (>2.6%) cells discriminated MPM from pleuritis with 100% sensitivity and 89% specificity. The presence of intra-tumor MDSC contributed to the anergy of tumor-infiltrating lymphocytes (TILs). The immune-phenotype of pleural fluid cells had no prognostic significance. By contrast, the intra-tumor Treg and MDSC levels significantly correlated with progression-free and overall survival, the $PD\text{-}1^+/LAG\text{-}3^+/TIM\text{-}3^+$ $CD4^+$TILs correlated with overall survival.

Conclusions. A clear immune-signature of pleural fluids and tissues of MPM patients may contribute to better predict patients' outcome.

Key words: malignant pleural mesothelioma; tumor-infiltrating lymphocytes; T-regulatory cells; myeloid-derived suppressor cells; immune checkpoints

Abbreviations:

DCFDA-AM: 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate-acetoxymethyl ester; FBS: fetal bovine serum; Gr-MDSC: granulocytic myeloid-derived suppressor cells; HR: hazard ratio; IDO: 2,3-indoleamine dioxygenase; Mo-MDSC: monocytic myeloid-derived suppressor cells; MPM: malignant pleural mesothelioma; MTS: metastases;

OS: overall survival; PFS: progression free survival; NK: natural killer; NO: nitric oxide; ROS: reactive oxygen species; TAM: tumor-associated macrophage; Treg: T-regulatory; TIL: tumor-infiltrating lymphocyte.

Introduction

Malignant pleural mesothelioma (MPM) is an asbestos-related cancer characterized by a long latency.[1] It has a low mutational burden and the tumor micro-environment rather than the genetic abnormalities in mesothelial cells may contribute to MPM development and progression.[2] The MPM micro-environment is rich of immune-suppressive and anergic immune cells,[4-6] such as T-regulatory (Treg),[7-8] granulocytic and monocytic myeloid-derived suppressor cells (Gr-MDSC/Mo-MDSC)[5,7] and M2-polarized tumor associated macrophages (TAMs),[9] that – together with soluble factors, such as cytokines, chemokines[7] and kynurenine, the product of 2,3-indoleamine dioxygenase (IDO) enzyme [8] – lead to a poor response to immune-therapy.[10]

Several cytokines accumulated in the pleural effusion of MPM patients promote the M2 polarization of macrophages.[11,12] M2/M3-macrophages and[11,13] Gr-MDSC[5] of MPM patients, as well as MPM cells[14] reduce the proliferation of heterologous $CD8^+$T-lymphocytes, by producing immune-suppressive mediators such as reactive oxygen species (ROS), nitric oxide (NO) and kynurenine.[5,8,15] MDSC are killed by active $CD8^+$T-lymphocytes,[16,17] while either Treg and MDSC reduce $CD8^+$T-lymphocytes activity[5,7,18] and memory $CD8^+$T-lymphocytes recruitment,[19] inducing a vicious immune-suppressive circle.

The high expression of immune-checkpoints on T-lymphocytes and of their ligands on MPM cell has another crucial role in the MPM-induced anergy of tumor-infiltrating lymphocytes (TILs).[6] The PD-1/PD-L1 axis is the more extensively studied and the most correlated with early progression and shorter survival.[3,4,20,21,22,23] LAG-3 and TIM-3 have been detected on $CD4^+$ and $CD8^+$TILs[24] or in formalin-fixed paraffin embedded samples.[25] Despite the high inter-patient and intra-sample variability,[26] both LAG-3 and TIM-3 contribute to the functional exhaustion of TILs. Furthermore CTLA-4 has been investigated as a potential therapeutic target in MPM, but the CTLA-4 inhibitor Tremelimumab did not show higher efficacy than placebo

in a double blind phase 2 trial, [27] raising concerns about the role of this immune-checkpoint in the MPM-induced immune-suppression.

The dissection of the immune-environment of MPM is quite difficult and has often twisted conflicting findings for different reasons. For instance, the number of T-cells and macrophages detected in pleural effusion not always mirrors the amount of the same cells infiltrating MPM tissue.[28] Some studies reported the same immune-signature in the MPM pleural effusion and in the peripheral blood,[24] while others did not,[5] raising concerns about peripheral blood as a biological surrogate that reliably reproduces the MPM immune-environment. Indeed, immune cells can be continuously exchanged between pleural cavity and tumor tissue, and/or between peripheral blood and pleural environment. This dynamic immune-environment leads to an inhomogeneous distribution of immune-cells within MPM tissue. Moreover, the immune-environment is exposed to time-changes related to the natural tumor progression and/or to chemotherapy-related effects.[28] Also qualitative and quantitative changes in tumor/stroma ratio may produce a dramatic rewiring in the MPM-infiltrating immune cell subsets.[18] The high intra-patient variability, the intra-tumor heterogeneity,[29] the different timing of analysis may partially justify the divergent results.

A simultaneous analysis of all the immune-populations detectable in the pleural effusion and infiltrating the tissue has never been performed. The present study aims at a comprehensive analysis of the immune-infiltrate detected in the pleural fluid and in the biopsies of pleural tissue, collected during routine diagnostic procedures from patients with pleural effusions of unknown origin, in order to identify an immune-phenotype with diagnostic and prognostic value in MPM patients.

Materials and methods

Samples collection. From June 2016 through June 2018 we enrolled 275 patients with an initial clinical diagnosis of pleural effusion of unknown origin. The enrollment of patients was

stopped when we have reached the sample size with an adequate power in the MPM patient cohort (see "Statistical analysis" paragraph). Samples were obtained during diagnostic thoracoscopies, at the Thoracic Surgery Division of San Luigi Gonzaga Hospital, Orbassano, and AOU Città della Salute e della Scienza, Torino, Italy. Two aliquots of pleural fluid and pleural tissue were collected by thoracic surgeons at the same time for each patient. Each sample was managed by the Pathology Unit of the two Institutions: one aliquot was used for diagnostic purposes according to local pathology guidelines, the second one was used for the research study here reported. In case of limited volume of pleural fluid or limited dimension of pleural tissue, both aliquots were dedicated to the diagnostic workup. In accordance with this criterion, 183 pleural fluids and 119 pleural biopsies were available for the research study. Within all these samples, 63 pathologically diagnosed non-malignant pleuritis, 49 MPM and 32 pleural metastases - MTS - of malignant tumors for pleural fluid; 16 non-malignant pleuritis, 33 MPM and 5 MTS for pleural tissue biopsies met the technical inclusion criteria (see "Phenotyping of immune cells" paragraph) and were analyzed in this study. 9 patients with pathologically diagnosed pleuritis and 20 patients with pathologically diagnosed MPM had both pleural fluid and pleural tissue available for the study. The flow chart of sample collection and processing is reported in the **Supplemental Figure 1**. The characteristics of samples used for the immune-phenotyping in the present study are reported in the **Supplemental Table 1.** The anonymized patients' history (asbestos exposure and smoking status, whenever available), the pathological diagnosis and the clinical follow-up (progression free survival, PFS; overall survival, OS) of MPM patients, performed at the Thoracic Oncology Unit, San Luigi Gonzaga Hospital, are reported in the **Supplemental Table 2**. All patients with MPM were in advanced clinical stage and all were treated with standard cytotoxic chemotherapy according to current guidelines.[30] The researchers performing the immune-phenotyping analyses reported below worked in a blind manner, being unaware of the pathological diagnosis at the time of the assays.

The association between pathological diagnosis, immune-phenotype analysis was performed during the final data analysis. The Ethics Committee of San Luigi Gonzaga Hospital, Orbassano, Italy approved the study (#126/2016).

Sample processing and mesothelial/tumor histological analysis. 50 ml of pleural fluid were centrifuged at 1200×g for 5 min, washed in PBS containing 1 mg/ml ciprofloxacin (Sigma Chemicals Co., St. Louis, MO), and re-suspended at 1×10^6 cells/ml in Ham's F10 nutrient mixture medium (Invitrogen Life Technology, Milano, Italy), supplemented with 10% v/v fetal bovine serum (FBS; Sigma Chemicals Co.), 1% v/v penicillin-streptomycin (Sigma Chemicals Co.). Tissues were digested in medium containing 1 mg/ml collagenase and 0.2 mg/ml hyaluronidase (Sigma Chemicals Co.) for 1 h at 37°C. Cells from pleural fluid and digested tissue were seeded in culture flasks (Becton Dickinson, Franklin Lakes, NJ) for 24 h in complete medium. After this period, the cells floating in the supernatant, i.e. immune cells in the pleural fluid or infiltrating the tissue, were collected, counted and analyzed for their immune-phenotype as detailed below. Adherent cells were analyzed for their mesothelial/tumor origin: after detaching by gentle scraping, cells were centrifuged at 1200×g for 5 min, fixed in 4% v/v formalin at 4 °C overnight and stained with the following antibodies: calretinin (Thermo Fisher Scientific, Waltham, MA), Wilms tumor-1 antigen (Thermo Fisher Scientific), cytokeratin 5 (Menarini Diagnostics, Bagno a Ripoli, Italy), podoplanin (Dako, Santa Clara, CA), pancytokeratin (Dako), epithelial membrane antigen (EMA, Dako), carcino-embrionic antigen (CEA, Dako), using an automated immunostainer (Benchmark Ventana Medical Systems, Tucson, AZ).

Phenotyping of immune cells. From all the samples available for research purpose, only samples with $\geq 1\times10^6$ viable (i.e. Trypan-blue negative) cells in the supernatants or adherent in flask were included in the analysis. Preliminary set-up experiments indicated that $\geq 1\times10^6$ viable cells was the minimal number required allowing to acquire 1×10^5 cells/staining, in order

to perform the whole set of the immune-phenotyping tests on each sample. Samples with $\leq$ 1×10^6 viable cells were excluded, since they did not allow to acquire 1×10^5 cells/staining. The supernatants were centrifuged at 1200×g for 5 min, the pellet was washed in PBS and re-suspended in PBS containing 5% v/v FBS. A three- and four-color flow cytometry was performed, with the appropriate combinations of antibodies (all diluted 1:10, from Miltenyi Biotec, Teterow, Germany if not otherwise specified) against: CD3 (mouse clone REA613), CD4 (mouse clone M-T466), CD8 (mouse clone BW135/80) for T-lymphocytes; CD25 (mouse clone 4E3), CD127 (mouse clone MB1518C9) for Treg cells; CD56 (mouse clone AF127H3), CD335/NKp46 (mouse clone 9E2) for natural killer (NK) cells; CD19 (mouse clone REA675) for B-lymphocytes; CD14 (mouse clone TÜK4) and CD68 (mouse clone Y1/82A) for monocytes and macrophages; CD68 (mouse clone Y1/82A), CD208 (mouse clone DCN228) and Arginase-1 (sheep polyclonal, # IC5868A, R&D Biosystems; Minneapolis, MN) for M2-polarized macrophages; CD68 (mouse clone Y1/82A), CD86 (mouse, clone FM95) and iNOS (rabbit polyclonal, #SPC-1325, StressMark Biosciences Inc., Victoria, Canada) for M1-polarized macrophages; CD11b (rat clone M1/70.15.11.5), CD14 (mouse clone TÜK4), CD15 (mouse clone VIMC6) for granulocytic myeloid-derived suppressor cells (Gr-MSDC) and monocytic myeloid-derived suppressor cells (Mo-MDSC). In each combination staining, 1×10^5 cells were analyzed using a Guava® easyCyte flow cytometer (Millipore, Bedford, MA), equipped with the InCyte software.

Expression of immune-checkpoints and immune-checkpoint ligands. $CD3^+$ cells were isolated from 1×10^6 immune cells of the supernatant of each culture with the Pan T Cell Isolation Kit (Miltenyi Biotec.), washed and re-suspended in PBS containing 5% v/v FBS. The detection of immune-checkpoints on $CD3^+$ T-lymphocytes and/or immune-checkpoint ligands on MPM cells were performed using antibodies for CD279/PD-1 (mouse clone PD1.3.1.3), CD223/LAG-3 (clone REA351), CD366/TIM-3 (mouse clone F38-2E2), CD152/CTLA-4

(mouse clone BNI3; all diluted 1:10, Miltenyi Biotec.), CD274/PD-1L (1:100, mouse clone 29E.2A3, BioLegend, San Diego, CA), anti-GAL-9 (mouse, clone 9M1-3, BioLegend). In each combination staining 1×10^5 cells were analyzed using a Guava® EasyCyte flow cytometer (Millipore), equipped with the InCyte software.

MDSC functional properties. ROS were measured using the fluorescent probe 5-(and-6)-chloromethyl-2′ ,7′ -dichlorodihydro-fluorescein diacetate-acetoxymethyl ester (DCFDA-AM), as previously reported.[31] The levels of nitrite, the stable derivative of NO, in cell culture supernatants were measured by the Griess method.[32] The amount of kynurenine was assessed by spectrophotometry.[33] The results were expressed as nmoles/mg cellular proteins.

Proliferation and activation of T-lymphocytes co-cultured with MDSC. 1×10^4 sorted intra-tissue Gr-MDSC and Mo-MDSC were co-cultured for 6 days at a 1:1 ratio with sorted intra-tissue $CD3^+CD8^+$T-cytotoxic lymphocytes, either autologous or heterologous, as detailed in the experimental section, in the presence of anti-CD3/anti-CD28 dynabeads (Invitrogen Life Technologies) to activate lymphocytes. The proliferation of T-lymphocytes was assessed by adding 1 μCi of [^{3}H]thymidine (PerkinElmer, Waltham, MA) 18 h before the end of the co-cultures, then harvesting the plates and counting the radioactivity by liquid scintillation count. The results were expressed as count per minute (cpm). The percentage of $CD8^+CD107a^+$ and the production of IFN-γ, considered indexes of activated cytotoxic T-lymphocytes, were measured by flow cytometry and ELISA, as reported.[14]

Statistical analysis. Before starting the enrollment of patients, the requested number of patients in the MPM cohort was calculated using the G*Power Software (www.gpower.hhu.de) using the following assumptions: significance level (α error probability) $\leq$ 0.05, power (1-β error probability) = 0.80, effect size ρ = 0.40). With these parameters, the sample size required was 34±2 MPM.

The normality distribution of each parameter analyzed was checked with the D'Agostino and Pearson test. All parameters had a Gaussian distribution or approximation. However, the results were conservatively analyzed by the non-parametric Kruskal-Wallis test (GraphPad PRISM 6.0 software). All data in the figures are provided as means±SD. Medians and quartile values are reported in the figure legends. In order to partially correct for the multiplicity of tests performed, a conservative $p<0.01$ was considered statistically significant. For each parameter specific cut-off discriminating MPM from pleuritis were calculated in order to have a 100% sensitivity (0% false negative) and >89% specificity (<11% false positive). To correlate the immune-parameters with PFS and OS, since there are no previously reported and validated cut-off values for the immune-phenotypic parameters analyzed, patients were exploratively divided into "low expressing" and "highly expressing" groups, if their value was below or equal/above the median value. The Kaplan-Meier method was used to calculate PFS and OS. Log rank test was used to compare the outcome of each group as hazard ratio (HR, i.e. risk of patient death). Considering the exploratory nature of the survival analysis, no adjustment for multiplicity was made, and $p<0.05$ was considered statistically significant.

Results

High T-regulatory cells/high myeloid-derived suppressor cells discriminate malignant pleural mesothelioma from inflammatory pleuritis and secondary pleural tumors

$CD3^+$T-lymphocytes represented the prevalent immune population within pleural fluid or pleural tissue, without differences between samples of pleuritis, MPM or MTS (**Figure 1A**). $CD3^+CD4^+$T-helper lymphocytes were up to 60% in all the samples, without differences between patient subgroups (**Figure 1B**). $CD3^+CD8^+$T-cytotoxic lymphocytes, representing approximately 5% of immune cells in pleural fluids from pleuritis, significantly increased in MPM and MTS-pleural fluid samples. By contrast they represented up to 50% of TILs in pleuritis and significantly decreased in MPM and MTS (**Figure 1C**). This trend allowed to

discriminate pleuritis (all with $CD3^+CD8^+$cells <6.3% in pleural fluid) from MPM (all with $CD3^+CD8^+$cells >25% in pleural fluid) (**Figure 1D**). T-reg cells were significantly higher in both pleural fluid and tissue from MPM patients compared to pleuritis and MTS-derived samples (**Figure 1E**). A cut-off of >1.4% in pleural fluid and >1.1% in pleural tissue Treg cells identified 100% (20/20) MPM and excluded 88.8% (8/9) pleuritis (**Figure 1F**). NK and B-lymphocytes were poorly represented and did not show any differences among groups (**Supplemental Figure 2A-B**).

Also monocytes did not differ (**Supplemental Figure 3**), while pleural fluid and tissue macrophages were significantly higher in MPM and MTS-derived samples (**Figure 2A**). All pleuritic samples had <34% macrophages in pleural fluid and <35% in tissue while all MPM samples were above these thresholds (**Figure 2B**). More relevantly the pro-tumorigenic M2-macrophages were higher in MPM and MTS compared to pleuritis (**Figure 2C**). According to these parameters, a clear separation between pleuritis (100% of patients with M2-macrophages <29% in pleural fluid and <36% in pleural tissue) and MPM (100% of patients with M2-macrophages >41% in pleural fluid and >44% in pleural tissue) was clearly identified (**Figure 2D**). Anti-tumorigenic M1-macrophages were lower in both pleural fluid and tissue of MPM and MTS compared with pleuritis (**Supplemental Figure 4**).

A significantly higher number of Gr-MDSC (**Figure 2E**) and Mo-MDSC (**Figure 2G**) in pleural fluid and tissue was detected in MPM compared to pleuritis and MTS. A clear threshold of 5.1% Gr-MDSC in both pleural fluid and tissue discriminated 100% pleuritis from 100% MPM (**Figure 2F**). A cut-off of >3.6% Mo-MDSC in pleural fluid and >4.2% in pleural tissue identified 100% MPM and excluded 88.8% pleuritis (**Figure 2H**).

The intratumor myeloid-derived suppressor cells determine T-lymphocytes anergy

The MDSC isolated from MPM tissue showed increased production of ROS (**Figure 3A**), NO (**Figure 3B**) and kynurenine (**Figure 3C**), compared to those obtained from tissue biopsies of

other subgroups. In co-culture with $CD8^+$T-cytotoxic lymphocytes from the tissue of the same patient, both Gr-MDSC and Mo-MDSC derived from MPM reduced $CD8^+$T-lymphocytes proliferation (**Figure 3D**), CD107a positivity (**Figure 3E**) and IFN-γ production (**Figure 3F**), compared to the autologous $CD8^+$T-lymphocytes/MDSC-co-cultures derived from the pleuritis and MTS subgroups. The immune-suppressive properties of either Gr-MDSC and Mo-MDSC were further demonstrated by co-incubating Gr-MDSC and Mo-MDSC derived from MPM tissue with heterologous T-lymphocytes derived from patients with pleuritis. In this setting, proliferation, CD107a positivity and IFN-γ production of T-lymphocytes derived from pleuritis was reduced to the same level of T-lymphocytes derived from MPM (**Figure 3D-F**).

A high number of intra-tumor $PD\text{-}1^+/LAG\text{-}3^+/TIM\text{-}3^+$ infiltrating lymphocytes is peculiar of malignant pleural mesothelioma

The immune-checkpoints PD-1 (**Figure 4A-B**), LAG-3 (**Figure 4C-D**) and TIM-3 (**Figure 4E-F**) were higher on $CD4^+$T-helper and $CD8^+$T-cytotoxic lymphocytes of MPM compared to the lymphocytes from pleuritis and MTS, either in pleural fluid and in pleural tissue. The cut-off values of 6.7% in pleural fluid, 5.2% in pleural tissue for $CD4^+PD1^+$ (**Supplemental Figure 5A**), 6.3% in pleural fluid, 6.4% in pleural tissue for $CD8^+PD1^+$cells (**Supplemental Figure 5B**), 4.9% in pleural fluid, 2.8% in pleural tissue for $CD4^+LAG\text{-}3^+$ (**Supplemental Figure 5C**), 5.2% in pleural fluid, 2.8% in pleural tissue for $CD8^+LAG\text{-}3^+$cells (**Supplemental Figure 5D**), 1.9% in pleural fluid, 2.5% in pleural tissue for $CD4^+TIM\text{-}3^+$ (**Supplemental Figure 5E**), 2.4% in pleural fluid, 2.6% in pleural for $CD8^+TIM\text{-}3^+$cells tissue (**Supplemental Figure 5F**), discriminated 100% MPM from pleuritis. In our series, the lymphocytic expression of CTLA-4 did not differ between each subgroup (**Figure 4G-H**).

The higher expression of immune-checkpoints on T-lymphocytes was paralleled by the higher expression of PD-L1 (**Figure 5A**), LAG-3 (**Figure 5B**), TIM-3 (**Figure 5C**) and GAL-9

(**Figure 5D**) on MPM cells compared to pleuritis and – for LAG-3 and TIM-3 – compared to MTS.

Specific immune-signatures of intratumor infiltrate have a prognostic value

Furthermore we investigated the potential prognostic role of the immune-phenotypic parameters that significantly differed between MPM and pleuritis.

The amount of $CD3^+CD8^+$ cells, Treg, M2-polarized macrophages, Gr/Mo-MDSC, PD-1^+/LAG-3^+/$TIM3^+$ $CD4^+$ or $CD8^+$ cells present in the pleural fluids did not show any significant correlation with PFS and OS of MPM patients (**Table 1**). As far as the intratumor immune-infiltrate was concerned, the amount of $CD3^+CD8^+$ cells and M2 were not associated with any prognostic significance, while high intratumor Treg, Gr-MDSC and Mo-MDSC significantly correlated with shorter PFS and OS (**Table 1**; **Supplemental Figure 6A-E**). The amount of PD-1^+ and LAG-3^+ $CD4^+$ cells correlated with a lower OS (**Table 1**; **Supplemental Figure 7A-C**). The expression of immune-checkpoints on intratumor $CD8^+$ had no prognostic significance (**Table 1**; **Supplemental Figure 8A-C**).

Discussion

Specific immune-phenotypic markers on tissue microarrays[20] or fine-needle aspirate[24] are currently investigated to better characterize the immune-environment of MPM. The present study for the first time assessed a comprehensive and multi-parametric analysis of the immune-infiltrate of either pleural fluid and pleural tissue, performed parallelly to the routine diagnostic procedures. Based on flow cytometry assays, our experimental model allows to recover viable cells, coupling the phenotypic analysis with functional assays of adaptive immunity. Moreover, we detected a quite robust immune-signature that discriminates MPM from pleuritis and from pleural metastases secondary to other malignancies. By selecting specific cut-offs for each immune-parameter analyzed with 100% sensitivity and $\geq$89% good specificity, we maximized the possibility to identify correctly all the MPM patients, accepting the possibility to have

sporadic cases of pleuritis erroneously identified as MPM. Since all samples were subjected to the usual pathological diagnosis, the rare cases of pleuritic erroneously identified as MPM on the basis of immune-phenotype analysis could be easily re-verified by the pathologist and/or clinically followed-up more strictly . The analysis of the immune-parameters was not intended to replace the pathological diagnosis but simply to complement the pathological diagnosis of MPM with a set of immunological tests and potential new prognostic information. Each parameter was indeed correlated with PFS or OS, exploratively using the median values as cut-off since in literature there are no previously reported and validated cut-offs for the parameters analyzed and the identification of the optimal cutoff was beyond the scope of this analysis.

The first relevant finding discriminating MPM from non-malignant pleuritis was the higher intra-pleural fluid $CD8^+$T-lymphocytes coupled with the lower intra-pleural $CD8^+$TILs. The amount of $CD8^+$TILs has been associated with adverse[18,34] or good [20] prognosis, but in our case series, we did not detect any prognostic significance. It is likely that - more than the absolute number of $CD8^+$T-lymphocytes - their functional exhaustion, due to the presence of Treg cells and MDSC,[7,18] is critical in determining tumor progression and patients' outcome.

We identified high Treg cells within pleural fluid and tissue as specific biomarkers of MPM rather than of benign pleural disease or pleural MTS. The high intra-MPM Treg was a negative prognostic factor, while the amount of Treg in pleural fluid had no clinical significance, as reported previously.[18] This data pointed out that the intratumor immune-infiltrate – more than the immune-population in the pleural effusion – in this case is more reliable in predicting the patients' outcome.

As expected, we did not detect significant differences in overall TAMs between MPM and metastatic patients. Compared to pleuritis, M2-macrophages were increased in both tumor and pleural fluid of MPM. The correlations between the number of TAMs or the ratio M2/TAM and the tumor progression or patients' outcomes [9,18,20,34] are highly discordant. According to

our data M2 percentage is not a significant predictor of patients' outcome but it is an immune-marker highly differentiating MPM from non-malignant conditions, as Gr-MDSC and – to a lesser extent – Mo-MDSC.

In our study, Gr- and Mo-MDSC abrogated proliferation and cytotoxic activity of autologous TILs and of TILs derived from patients with pleuritis. These data suggest that MDSC mediate an important role MPM immune-suppression, likely by the increased production of ROS, NO and kynurenine. Again, only the intratumor-infiltrating MDSC - and not the MDSC of pleural fluid - are significantly associated with poorer PFS and OS. Taking into consideration the dynamic exchange occurring between pleural fluid and tumor,[28] pleural fluid can be considered as a reservoir of immune-suppressive cells: higher is their migration from pleural fluid into the tumor, higher is the chance to characterize an immune-escape status and predict tumor progression.

Immune-checkpoints expression are other key players of immune-suppression in MPM. The immunohistochemical expression of PD-L1 and PD-1 is not always reliable as biomarker in discriminating pleuritis from MPM: PD-L1 expression was absent in benign lesions, but PD-1 resulted more expressed on TILs of non-tumoral samples than in TILs of MPM analyzed by immunohistochemistry,[23] a finding contrasting with the high percentage of PD-1^{+}cells reported in MPM using flow cytometry.[3,4,6] The type of diagnostic antibody and the abundance of the immune-infiltrate in the examined sample may explain this discrepancy. The multi-parametric flow cytometry analysis revealed that the amount of PD-1 expressed in $CD4^{+}$ and $CD8^{+}$T-lymphocytes well discriminates MPM from non-malignant pleuritis, but not from metastatic cancers, in line with the observation that pleural effusion from lung cancer are also rich of $PD1^{+}CD8^{+}$T-lymphocytes and PD-$L1^{+}$-cancer cells.[35]

Our data indicate that the expression of PD-1, LAG-3 and TIM-3 in TILs, but not in T-cells in pleural effusion, correlated with lower OS. The lack of correlation between immune-checkpoint levels and PFS likely suggests that the accumulation of immune-checkpoint-positive, anergic lymphocytes occurs at advanced stages in MPM patients and determines prognosis in the terminal stages only. More interestingly, only PD-1^+/LAG-3^+/TIM-$3^+$$CD4^+$ cells, but not $CD8^+$ cells, were negative prognostic factors. Since $CD4^+$ T-cells are a hub in the engagement of other immune populations, we may hypothesize that a loss of function of $CD4^+$ T-lymphocytes impairs the immune-recognition of MPM cells more than a dysfunction in the effector $CD8^+$ T-cells, leading to faster progression and reduced survival. Among the immune-checkpoints analyzed in MPM patients, CTLA-4 was poorly expressed on the surface of T-lymphocytes. This is likely related to the cytosol localization of CTLA-4 and the high recycling rate.[36] Our data need to be re-assessed when a staining optimization for intracellular CTLA-4 antigen will be achieved.

In conclusion, we propose a comprehensive multi-parametric analysis of several immunological parameters that define a MPM immune-signature, reliably applied to both pleural fluid and tissue routinely collected in standard clinical practice. We are aware that our discriminating cut-off values, although significant, have been obtained in a small patient cohort, and we emphasize that the identification of the optimal cutoff was beyond the scope of this analysis. The parameters are now being validated in a larger prospective study, including discovery and validation cohorts, in order to improve the specificity of the proposed thresholds and evaluate the possible clinical utility of the immune-parameters found. In such ongoing study, refined grouping strategies will be adopted, in order to define more accurately the quantitative values of the immune-phenotypic parameters significantly correlated with patient survival and other clinical parameters. Only if the validation study will confirm this pilot experience, the immune biomarkers identified in the present study may be considered as

additional parameters to be analyzed - beyond the parameters already adopted to perform MPM diagnosis - in challenging clinical cases.

Of note, this study is the first one aimed to systematically characterize the immune-infiltrate of pleural fluid and tumor derived from the same patient. Our results indicated that the analysis of the intratumor immune-infiltrate – better than that of pleural fluid – identifies potential prognostic factors. Given the high inter-patients and intra-patients variability observed in MPM, the larger prospective validation study will also clarify if the qualitative and quantitative differences in the intra-tumor immune-infiltrate may be correlated with specific histological, cyto-genetic and mutational features. This may contribute to a more accurate patients' stratification, for a rationale and personalized immune-therapy of MPM.

Funding

This work was supported by Italian Association for Cancer Research (IG21408 to CR); Italian Ministry of University and Research (Future in Research program RBFR12SOQ1 to CR; Basic Research Funding Program, FABR2017 to LR and CR); Italian Ministry of Health (GR-2011-02348356 to LR; Italian Mesothelioma Network-CCM2012 to GVS), Azienda Ospedaliera "SS. Antonio e Biagio e Cesare Arrigo" to GVS for the research activity "MESOLINE", University of Torino (Intramural Grant 2015 to SN and CR; Intramural Grant 2016 to LR; Intramural Grant 2017 to JK); Fondazione Cassa di Risparmio di Torino (2016-2443 to CR). ICS is a post-doctoral research fellow supported by the Fondazione Franco e Marilisa Caligara for Multidisciplinary Sciences, Torino, Italy. VM is a PhD fellow of Erasmus Mundus-ERAWEB Action 2 program. FN is a student of the Biomedical Sciences and Oncology PhD program.

The funding institutions had no role in the study design, data collection and analysis, or in writing the manuscript.

Acknowledgments

We are grateful to Mr. Costanzo Costamagna, Dept of Oncology, University of Torino for the technical assistance.

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Figure legends

Figure 1. Lymphocyte subtypes present in pleural fluid and tissue of malignant pleural mesothelioma

Cells collected from pleural fluid (PF) of patients with pleuritis (n=63), malignant pleural mesothelioma (MPM; n=49) and other tumors metastatizing to pleura (MTS; n=32), and from digested pleural tissue (PT) of patients with pleuritis (n=16), MPM (n=33) and MTS (n=5) were analyzed by flow cytometry. In n=9 pleuritis and n=20 MPM, PF and PT from the same patients were analyzed in parallel. **A-C.** Percentage of total ($CD3^+$), T-helper ($CD3^+CD4^+$) and T-cytotoxic ($CD3^+CD8^+$) lymphocytes. Data are presented as means±SD. Values of 25^{th} percentile, median, 75^{th} percentile: pleuritis PF: 52.00, 61.00, 66.00; pleuritis PT: 15.00, 21.00, 26.75; MPM PF: 52.00, 62.00, 71.00; MPM PT: 37.75, 47.00, 58.75; MTS PF: 51.00, 55.00, 64.00; MTS PT: 36.50, 41.00, 51.50 (panel **A**); pleuritis PF: 39.25, 52.00, 60.50; pleuritis PT: 51.00, 61.00, 67.00; MPM PF: 48.25, 61.00, 70.25; MPM PT: 51.00, 56.00, 71.00; MTS PF: 45.10, 51.50, 60.50; MTS PT: 54.00, 61.00, 67.50 (panel **B**); pleuritis PF: 2.90, 3.75, 5.98; pleuritis PT: 30.00, 41.00, 49.00; MPM PF: 25.00, 29.00, 33.00; MPM PT: 12.00, 15.00, 19.00; MTS PF: 14.50, 20.00, 26.00; MTS PT: 10.50, 11.00, 15.50 (panel **C**). ***$p<0.001$: MPM/MTS vs pleuritis; not significant: MPM vs MTS. **D.** Disaggregated data of T-cytotoxic cells percentage in PF and PT from the same patient. Dotted line: 6.3% cut-off value in PF (false negative: 0%; false positive: 0%; sensitivity: 100%; specificity: 100%). **E.** Percentage of T-regulatory (Treg; $CD4^+$ $CD25^+CD127^{low}$) cells. Data are presented as means±SD. Values of 25^{th} percentile, median, 75^{th} percentile: pleuritis PF: 0.90, 1.20, 1.50; pleuritis PT: 0.30, 0.50, 0.90; MPM PF: 2.73, 2.95, 3.48; MPM PT: 2.10, 2.80, 3.25; MTS PF: 1.10, 1.25, 1.50; MTS

PT: 0.75, 1.10, 1.25. ***p<0.001: MPM vs pleuritis; °°p<0.005, °°°p<0.001: MPM vs MTS. **F.** Disaggregated data of Treg cells percentage in PF and PT from the same patient. Dotted line: 1.4% cut-off value in PF; dashed line: 1.1% cut-off value in PT (false negative: 0%; false positive: 11%; sensitivity: 100%; specificity: 89%)

Figure 2. Macrophage and myeloid-derived suppressor cell subtypes present in pleural fluid and pleural tissue of malignant pleural mesothelioma

Cells collected from pleural fluid (PF) of patients with pleuritis (n=63), malignant pleural mesothelioma (MPM; n=49) and other tumors metastatizing to pleura (MTS; n=32), and from digested pleural tissue (PT) of patients with pleuritis (n=16), MPM (n=33) and MTS (n=5) were analyzed by flow cytometry. In n=9 pleuritis and n=20 MPM, PF and PT from the same patients were analyzed in parallel. **A**. Percentage of macrophages ($CD14^+CD68^+$ cells). Data are presented as means±SD. Values of 25th percentile, median, 75th percentile: pleuritis PF: 15.00, 19.00, 31.00; pleuritis PT: 21.00, 25.00, 32.00; MPM PF: 49.25, 61.00, 71.00; MPM PT: 41.75, 55.00, 62.25; MTS PF: 48.25, 54.00, 66.25; MTS PT: 48.50, 65.00, 69.50. *p<0.01, ***p<0.001: MPM/MTS vs pleuritis. **B.** Disaggregated data of macrophage percentage in PF and PT from the same patient. Dotted line: 34% cut-off value in PF, dashed line: 35% cut-off value in PT (false negative: 0%; false positive: 0%; sensitivity: 100%; specificity: 100%). **C**. Percentage of M2-macrophages ($CD68^+CD206^+Arg1^+$cells). Data are presented as means±SD. Values of 25th percentile, median, 75th percentile: pleuritis PF: 17.25, 22.00, 28.00; pleuritis PT: 23.00, 28.00, 34.50; MPM PF: 45.00, 55.00, 62.25; MPM PT: 56.00, 60.00, 68.25; MTS PF: 41.25, 44.00, 57.00; MTS PT: 53.00, 64.00, 65.00. ***p<0.001: MPM/MTS vs pleuritis. **D.** Disaggregated data of M2-macrophage percentage in PF and PT from the same patient. Dotted line: 29% cut-off value in PF, dashed line: 36% cut-off value in PT (false negative: 0%; false positive: 0%; sensitivity: 100%; specificity: 100%). **E.** Percentage of granulocytic myeloid-derived suppressor cells (Gr-MDSC; $CD11b^+CD14^-CD15^+$cells). Data are presented

as means±SD. Values of 25th percentile, median, 75th percentile: pleuritis PF: 2.20, 2.80, 3.68; pleuritis PT: 2.10, 2.60, 3.10; MPM PF: 11.23, 13.05, 17.73; MPM PT: 11.80, 13.15, 17.03; MTS PF: 3.28, 5.15, 6.30; MTS PT: 4.80, 5.10, 7.70. ***p<0.001: MPM vs pleuritis; °°°p<0.001: MPM vs MTS. **F.** Disaggregated data of Gr-MDSC percentage in PF and PT from the same patient. Dashed line: 5.1% cut-off value in PF and PT (false negative: 0%; false positive: 0%; sensitivity: 100%; specificity: 100%). **G.** Percentage of monocytic myeloid derived suppressor cells (Mo-MDSC; CD11b^{+}CD14^{+}CD15^{low}cells). Data are presented as means±SD. ***p<0.001: MPM vs pleuritis; °°°p<0.001: MPM vs MTS. Values of 25th percentile, median, 75th percentile: pleuritis PF: 2.10, 2.40, 3.45; pleuritis PT: 2.13, 2.75, 3.18; MPM PF: 5.90, 8.20, 10.20; MPM PT: 8.10, 9.75, 11.28; MTS PF: 1.30, 2.30, 2.90; MTS PT: 1.90, 2.35, 2.80. **H.** Disaggregated data of Mo-MDSC percentage in PF and PT from the same patient. Dotted line: 3.6% cut-off value in PF, dashed line: 4.2% cut-off value in PT (false negative: 0%; false positive: 11%; sensitivity: 100%; specificity: 89%).

Figure 3. Intratumor myeloid-derived suppressor cells determines CD8^{+} T-lymphocytes anergy

1×10^{4} sorted intra-tissue Gr-MDSC and Mo-MDSC derived from pleuritis (n=10), MPM (n=12) and other tumors metastatizing to pleura (MTS; n=4) were seeded and analyzed after 24 h (panels **A**-**C**), or co-cultured (panels **D**-**F**) for 6 days with the sorted intra-tissue CD3^{+}CD8^{+}T-cytotoxic lymphocytes of the corresponding patient (autologous setting). When indicated, Gr-MDSC and Mo-MDSC from MPM patients were cultured with T-cytotoxic lymphocytes from pleuritis patients (pleu/MPM setting; n=8). **A-C.** Intracellular ROS (**panel A**) were measured fluorimetrically, nitrite (**panel B**) and kynurenine (**panel C**) released in the supernatants were measured spectrophotometrically. Data are presented as means±SD. Values of 25th percentile, median, 75th percentile: pleuritis Gr-MDSC: 0.59 0.79, 0.94; pleuritis Mo-MDSC: 0.43, 0.65, 0.80; MPM Gr-MDSC: 2.08, 2.40, 2.78; MPM Mo-MDSC: 1.78, 2.20,

2.68; MTS Gr-MDSC: 0.61, 0.72, 0.81; MTS Mo-MDSC: 0.43, 0.66, 0.82 (panel **A**); pleuritis Gr-MDSC: 1.58, 2.00, 2.45; pleuritis Mo-MDSC: 1.60, 2.10, 2.95; MPM Gr-MDSC: 8.20, 9.75, 12.25; MPM Mo-MDSC: 6.05, 6.85, 8.55; MTS Gr-MDSC: 1.30, 1.75, 2.65; MTS Mo-MDSC: 1.63, 2.15, 2.45 (panel **B**); pleuritis Gr-MDSC: 0.55, 0.72, 0.82; pleuritis Mo-MDSC: 0.72, 0.87, 1.16; MPM Gr-MDSC: 3.73, 4.14, 4.85; MPM Mo-MDSC: 4.25, 5.26, 5.35; MTS Gr-MDSC: 0.59, 0.89, 1.19; MTS Mo-MDSC: 0.78, 1.00, 1.24 (panel **C**). *p<0.01, **p<0.005, ***p<0.001: MPM vs pleuritis; °p<0.01, °°p<0.005, °°°p<0.001: MPM vs MTS. **D-F.** The proliferation of T-cytotoxic lymphocytes (**panel D**) was measured radiometrically, the percentage of $CD8^+CD107a^+$lymphocytes (**panel E**) was measured by flow cytometry, the amount of IFN-γ (**panel F**) in the supernatants was measured by ELISA. The proliferation of T-lymphocytes in the absence of anti-CD3/anti-CD28 dynabeads, used as negative control, was <3500 cpm for all experimental conditions. Data are presented as means±SD. Values of 25^{th} percentile, median, 75^{th} percentile: pleuritis Gr-MDSC: 18.09, 23.12, 26.03; pleuritis Mo-MDSC: 27.53, 30.4, 34.62; MPM Gr-MDSC:2.42, 4.04, 5.00; MPM Mo-MDSC: 4.03, 5.71, 6.84; MTS Gr-MDSC: 16.66, 22.69, 28.46; MTS Mo-MDSC: 23.27, 31.72, 33.96; pleu/MPM Gr-MDSC: 5.33, 5.80, 7.19; pleu/MPM Mo-MDSC: 6.44, 8.25, 11.70 (panel **D**); pleuritis Gr-MDSC:2.08, 2.60, 2.83; pleuritis Mo-MDSC: 2.48, 3.00, 3.25; MPM Gr-MDSC: 0.40, 0.60, 0.80; MPM Mo-MDSC: 0.45, 0.80, 1.05; MTS Gr-MDSC: 1.95, 2.65, 3.05; MTS Mo-MDSC: 2.83, 3.05, 3.65; pleu/MPM Gr-MDSC: 0.53, 0.75, 0.80; pleu/MPM Mo-MDSC: 0.40, 0.65, 0.88 (panel **E**); pleuritis Gr-MDSC: 409.50, 441, 636.50; pleuritis Mo-MDSC: 459.80, 497.50, 521.00; MPM Gr-MDSC: 111.80, 151.00, 203.30; MPM Mo-MDSC: 147.00, 177.00, 236.00; MTS Gr-MDSC: 322.00, 463.50, 506.80; MTS Mo-MDSC: 373.80, 480.00, 585.50; pleu/MPM Gr-MDSC: 168.80, 204.50, 263.80; pleu/MPM Mo-MDSC: 209.80, 298.00, 335.80 (panel **F**).**p<0.005, ***p<0.001: MPM vs pleuritis; °p<0.01, °°p<0.005, °°°p<0.001: MPM

vs MTS in autologous settings; $^{\#}p<0.01$, $^{\#\#\#}p<0.001$: pleu/MPM setting vs pleuritis autologous setting.

Figure 4. Immune-checkpoint expression in T-lymphocytes contained in pleural fluid and pleural tissue

Cells collected from pleural fluid of patients with pleuritis (n=63), malignant pleural mesothelioma (MPM; n=49) and other tumors metastatizing to pleura (MTS; n=32), and from digested pleural tissue of patients with pleuritis (n=16), MPM (n=33) and MTS (n=5) were analyzed by flow cytometry. Data are presented as means±SD. **A**-**B**. Percentage of PD-1^{+}T-helper ($CD3^{+}CD4^{+}$) and T-cytotoxic ($CD3^{+}CD8^{+}$) lymphocytes. Values of 25^{th} percentile, median, 75^{th} percentile: pleuritis $CD3^{+}CD4^{+}$: 2.45, 4.60, 5.60; pleuritis $CD3^{+}CD8^{+}$: 2.50, 3.90, 5.50; MPM $CD3^{+}CD4^{+}$: 15.53, 18.30, 22.65; MPM $CD3^{+}CD8^{+}$: 14.83, 17.65, 21.38; MTS $CD3^{+}CD4^{+}$: 7.95, 10.20, 12.30; MTS $CD3^{+}CD8^{+}$: 7.03, 9.00, 11.33 (panel **A**); pleuritis $CD3^{+}CD4^{+}$: 2.10, 3.15, 4.10; pleuritis $CD3^{+}CD8^{+}$: 2.03, 2.90, 4.18; MPM $CD3^{+}CD4^{+}$: 10.20, 12.50, 16.15; MPM $CD3^{+}CD8^{+}$: 11.28, 15.20, 18.05; MTS $CD3^{+}CD4^{+}$: 3.60, 4.30, 6.50; MTS $CD3^{+}CD8^{+}$: 3.10, 6.20, 8.15 (panel **B**). ***p<0.001: MPM vs pleuritis; °p<0.01, °°°p<0.001: MPM vs MTS. **C**-**D**. Percentage of LAG-3^{+}T-helper ($CD3^{+}CD4^{+}$) and T-cytotoxic ($CD3^{+}CD8^{+}$) lymphocytes. Values of 25^{th} percentile, median, 75^{th} percentile: pleuritis $CD3^{+}CD4^{+}$: 1.90, 2.80, 4.15; pleuritis $CD3^{+}CD8^{+}$: 1.90, 2.80, 4.15; MPM $CD3^{+}CD4^{+}$: 10.83, 13.35, 16.25; MPM $CD3^{+}CD8^{+}$: 8.83, 10.60, 14.05; MTS $CD3^{+}CD4^{+}$: 3.88, 7.20, 8.40; MTS $CD3^{+}CD8^{+}$: 3.75, 7.15, 8.60 (panel **C**); pleuritis $CD3^{+}CD4^{+}$: 1.50, 1.85, 2.10; pleuritis $CD3^{+}CD8^{+}$: 1.60, 1.80, 2.18; MPM $CD3^{+}CD4^{+}$: 8.38, 10.50, 14.28; MPM $CD3^{+}CD8^{+}$: 8.10, 10.50, 14.10; MTS $CD3^{+}CD4^{+}$: 3.40, 4.60, 5.40; MTS $CD3^{+}CD8^{+}$: 2.95, 4.10, 4.65 (panel **D**). ***p<0.001: MPM vs pleuritis; °p<0.01, °°p<0.005, °°°p<0.001: MPM vs MTS. **E**-**F**. Percentage of TIM-3^{+}T-helper ($CD3^{+}CD4^{+}$) and T-cytotoxic ($CD3^{+}CD8^{+}$) lymphocytes. Values of 25^{th} percentile, median, 75^{th} percentile: pleuritis $CD3^{+}CD4^{+}$:1, 1.80, 2.48; pleuritis

$CD3^+CD8^+$: 0.90, 1.35, 1.70; MPM $CD3^+CD4^+$: 6.10, 7.30, 8.40; MPM $CD3^+CD8^+$: 6.65, 8.30, 10.70; MTS $CD3^+CD4^+$: 2.18, 3.85, 4.43; MTS $CD3^+CD8^+$: 1.98, 3.15, 4.13 (panel **E**); pleuritis $CD3^+CD4^+$: 1.13, 1.50, 2.05; pleuritis $CD3^+CD8^+$: 1.20, 1.35, 1.58; MPM $CD3^+CD4^+$: 6.18, 8.10, 8.53; MPM $CD3^+CD8^+$: 6.78, 9.10, 10.20; MTS $CD3^+CD4^+$: 1.25, 1.60, 2.20; MTS $CD3^+CD8^+$: 1.15, 1.40, 1.85 (panel **F**). ***p<0.001: MPM vs pleuritis; °°p<0.005, °°°p<0.001: MPM vs MTS. **G-H**. Percentage of CTLA-4^+T-helper ($CD3^+CD4^+$) and T-cytotoxic ($CD3^+CD8^+$) lymphocytes. Values of 25^{th} percentile, median, 75^{th} percentile: pleuritis $CD3^+CD4^+$: 1.20, 1.40, 1.90; pleuritis $CD3^+CD8^+$: 0.90, 1.30, 1.58; MPM $CD3^+CD4^+$: 1.10, 1.60, 1.90; MPM $CD3^+CD8^+$: 1.10, 1.20, 1.50; MTS $CD3^+CD4^+$: 1.18, 1.50, 2.10; MTS $CD3^+CD8^+$: 1.20, 1.30, 1.80 (panel **G**); pleuritis $CD3^+CD4^+$: 1.10, 1.20, 2.05; pleuritis $CD3^+CD8^+$: 1.25, 1.40, 1.70; MPM $CD3^+CD4^+$: 1.30, 1.80, 2.10; MPM $CD3^+CD8^+$: 1.20, 1.50, 1.90; MTS $CD3^+CD4^+$: 1.45, 1.70, 2.38; MTS $CD3^+CD8^+$: 1.13, 1.40, 1.73 (panel **H**).

Figure 5. Immune-checkpoint ligands expressed in malignant pleural mesothelioma cells

Mesothelial cells collected from patients with pleuritis (n=24), malignant pleural mesothelioma cells (MPM; n=33) or cells from different tumors metastatizing to pleura (MTS; n=16), were analyzed by flow cytometry for the expression of PD-L1 (panel **A**), LAG-3 (panel **B**), TIM-3 (panel **C**), GAL-9 (panel **D**). Data are presented as means±SD. Values of 25^{th} percentile, median, 75^{th} percentile: pleuritis: 2.55, 3.60, 4.35; MPM: 15.05, 17.25, 22.95; MTS: 5.60, 9.30, 12.70 (panel **A**); pleuritis: 1.43, 1.95, 2.83; MPM: 7.40, 9.10, 11.25; MTS: 2.40, 4.10, 5.10 (panel **B**); pleuritis: 1.20, 1.90, 2.73; MPM: 7.35, 8.70, 10.25; MTS: 1.65, 2.40, 5.35 (panel **C**); pleuritis: 1.30, 2.40, 6.23; MPM: 13.40, 17.20, 21.50; MTS: 4.70, 7.80, 9.25 (panel **D**).***p<0.001: MPM vs pleuritis; °°p<0.005, °°°p<0.001: MPM vs MTS.

Table 1. Survival analysis according to the immune-phenotypic parameter characterizing mesothelioma patients

Immune-population	Sample	PFS (months) (95% CI)	p value	OS (months) (95% CI)	p value
$CD3^+CD8^+$ low vs $CD3^+CD8^+$ high	PF	6.3±1.6 vs 6.3±1.0 (5.0-8.3)	0.913	10.0±2.2 vs 10.8±1.1 (8.2-12.7)	0.6927
Treg low vs Treg high	PF	6.0±1.1 vs 7.0±1.6 (4.7-8.1)	0.496	10.8±1.6 vs 9.7±1.4 (8.2-12.7)	0.4347
M2 low vs M2 high	PF	7.0±1.3 vs 5.7±1.0 (4.7-7.8)	0.2851	10.9±1.5 vs 9.8±1.9 (8.2-12.7)	0.7424
Gr-MDSC low vs Gr-MDSC high	PF	7.1±1.1 vs 5.6±1.3 (4.6-8.1)	0.3351	10.6±1.4 vs 10.8±2.2 (8.2.9-13.1)	0.4522
Mo-MDSC low vs Mo-MDSC high	PF	6.8±1.2 vs 5.9±1.4 (4.4-8.1)	0.8755	10.8±1.6 vs 9.4±1.7 (7.9-12.5)	0.5498
$CD4^+PD\text{-}1^+$ low vs $CD4^+PD\text{-}1^+$ high	PF	5.7±1.3 vs 7.3±1.1 (4.7-8.1)	0.713	8.6±1.5 vs 12.4±1.6 (8.2-12.7)	0.1126
$CD4^+LAG\text{-}3^+$ low vs $CD4^+LAG\text{-}3^+$ high	PF	7.2±1.4 vs 7.3±1.1 (5.0-8.4)	0.4585	9.6±2.5 vs 10.9±1.1 (8.2-12.7)	0.7886
$CD4^+TIM\text{-}3^+$ low vs $CD4^+TIM\text{-}3^+$ high	PF	8.1±1.1 vs 5.0±1.3 (4.9-8.4)	0.1047	11.9±1.2 vs 9.1±2.1 (8.4-12.9)	0.5417
$CD8^+PD\text{-}1^+$ low vs $CD8^+PD\text{-}1^+$ high	PF	7.2±1.3 vs 6.8±1.2 (5.3-8.8)	0.6309	9.4±2.0 vs 10.0±1.4 (7.6-11.9)	0.9571
$CD8^+LAG\text{-}3^+$ low vs $CD8^+LAG\text{-}3^+$ high	PF	6.5±1.4 vs 4.8±0.8 (4.1-7.2)	0.2127	11.0±1.8 vs 10.1±1.9 (8.1-13.0)	0.8569
$CD8^+TIM\text{-}3^+$ low vs $CD8^+TIM\text{-}3^+$ high	PF	7.7±1.1 vs 4.3±1.2 (4.31-7.6)	0.0956	11.0±1.6 vs 9.7±1.6 (8.2-12.3)	0.5441
$CD3^+CD8^+$ low vs $CD3^+CD8^+$ high	PT	7.4±1.0 vs 8.0±1.3 (6.1-9.2)	0.8624	9.6±1.4 vs 10.3±1.3 (8.3-11.9)	0.9594
Treg low vs Treg high	PT	9.1±1.8 vs 4.7±1.4 (6.1-9.3)	**0.0172**	12.1±1.2 vs 7.6±1.0 (8.3-11.9)	**0.0046**
M2 low vs M2 high	PT	7.6±1.7 vs 8.8±1.1 (6.4-10.2)	0.8228	10.4±1.4 vs 9.3±1.2 (8.1-11.7)	0.4016
Gr-MDSC low vs Gr-MDSC high	PT	9.1±1.1 vs 5.6±1.0 (5.9-9.2)	**0.0427**	11.3±1.3 vs 7.5±1.1 (7.6-11.2)	**0.037**
Mo-MDSC low vs Mo-MDSC high	PT	11.2±1.2 vs 7.3±1.4 (7.7-11.5)	**0.0178**	11.1±1.7 vs 8.0±1.0 (8.1-11.4)	**0.026**
$CD4^+PD\text{-}1^+$ low vs $CD4^+PD\text{-}1^+$ high	PT	7.9±1.0 vs 7.9±1.7 (6.2-9.5)	0.6616	11.4±1.1 vs 8.3±1.1 (8.5-11.8)	**0.043**
$CD4^+LAG\text{-}3^+$ low vs $CD4^+LAG\text{-}3^+$ high	PT	8.3±1.1 vs 7.0±1.3 (6.1-9.3)	0.364	11.5±1.4 vs 8.0±0.9 (8.0-11.5)	**0.0077**
$CD4^+TIM\text{-}3^+$ low vs $CD4^+TIM\text{-}3^+$ high	PT	6.8±1.6 vs 8.4±0.9 (6.1-9.4)	0.7595	11.7±1.2 vs 7.8±1.2 (8.0-11.7)	**0.044**
$CD8^+PD\text{-}1^+$ low vs $CD8^+PD\text{-}1^+$ high	PT	7.8±1.3 vs 7.9±1.2 (6.2-9.5)	0.9333	10.3±1.3 vs 10.0±1.2 (8.4-11.8)	0.9315
$CD8^+LAG\text{-}3^+$ low vs $CD8^+LAG\text{-}3^+$ high	PT	6.7±1.3 vs 8.0±1.1 (5.7-9.0)	0.6298	9.7±1.5 vs 10.6±1.1 (8.3-11.9)	0.9219
$CD8^+TIM\text{-}3^+$ low vs $CD8^+TIM\text{-}3^+$ high	PT	6.8±1.7 vs 8.4±0.8 (6.1-9.3)	0.7423	8.5±1.6 vs 10.6±1.2 (8.0-11.6)	0.574

The median values of $CD3^+CD8^+$ lymphocytes, Treg, M2-macrophages, Gr-MDSC, Mo-MDSC, $CD4^+PD\text{-}1^+$, $CD4^+LAG\text{-}3^+$, $CD4^+TIM\text{-}3^+$, $CD8^+PD\text{-}1^+$, $CD8^+LAG\text{-}3^+$, $CD8^+TIM\text{-}3^+$ cells was calculated in pleural fluid (PF; n=49) and pleural tissue (PT; n=33). Patients were classified as "low" or "high" if the percentage of each population was low or equal/higher than the median value. Progression free survival (PFS) and overall survival (OS) probability were calculated using the Kaplan-Meier method, and expressed as measn±SD (months). CI: confidence interval. Significant values are indicated by bold characters.

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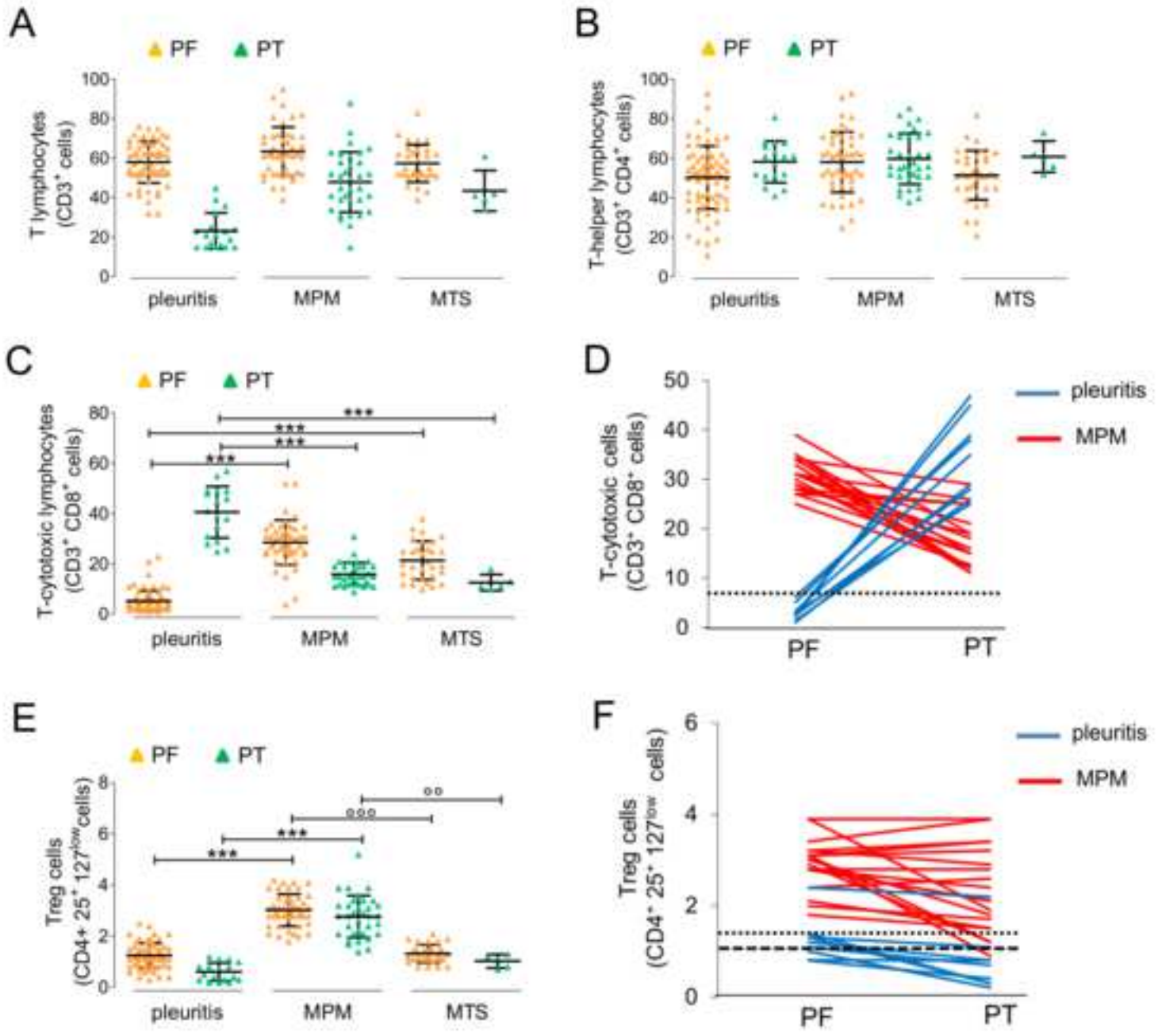

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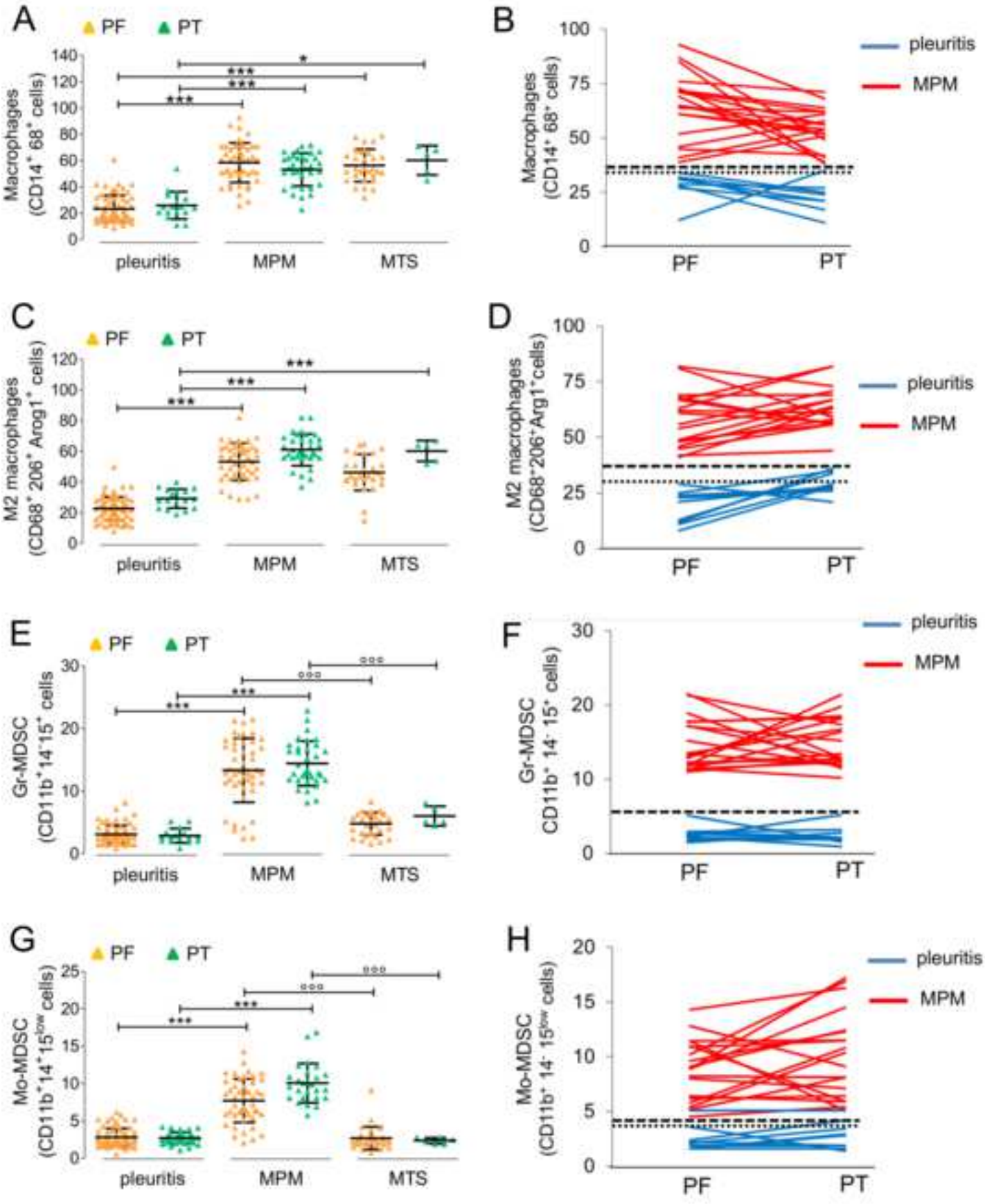

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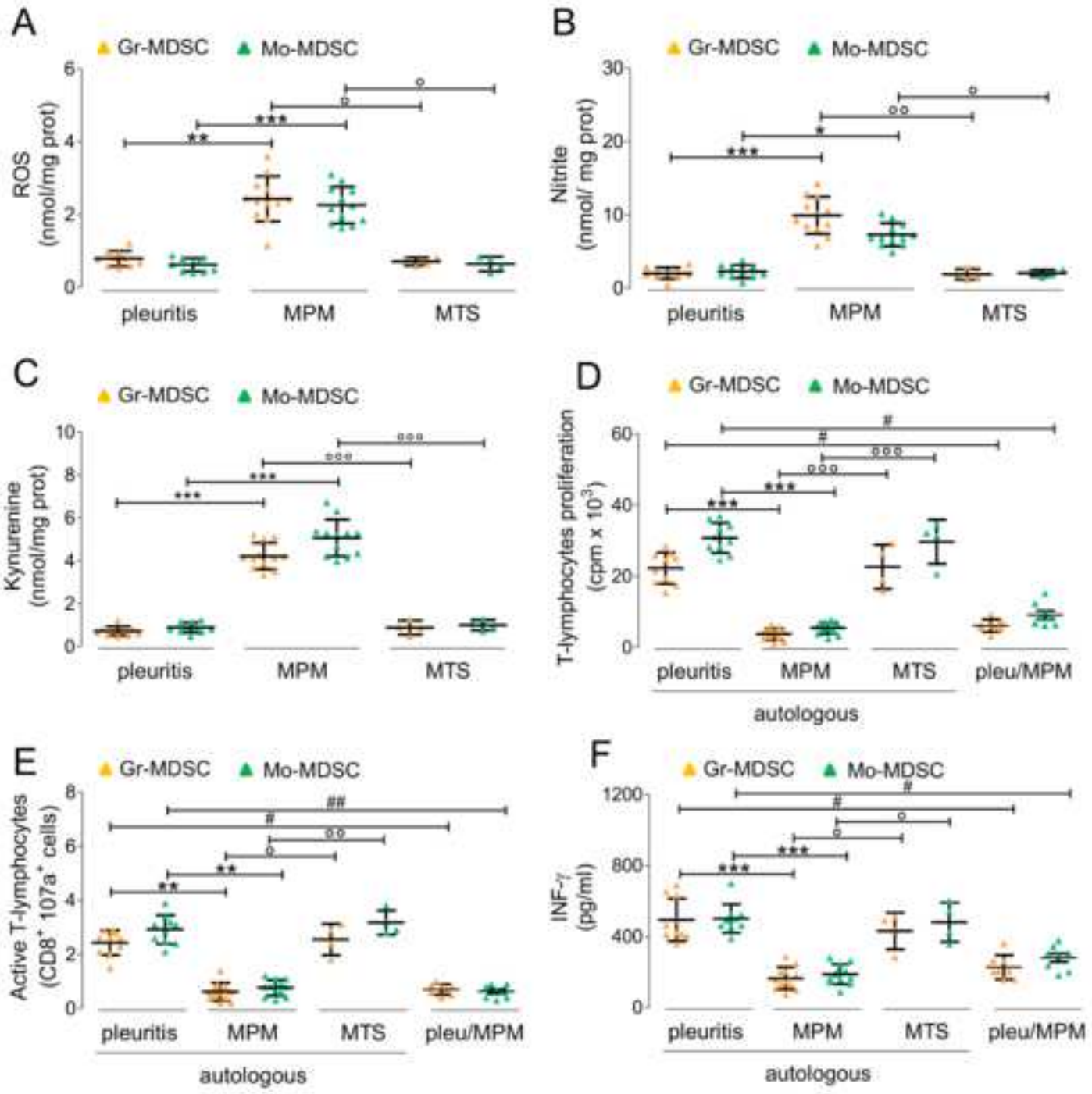

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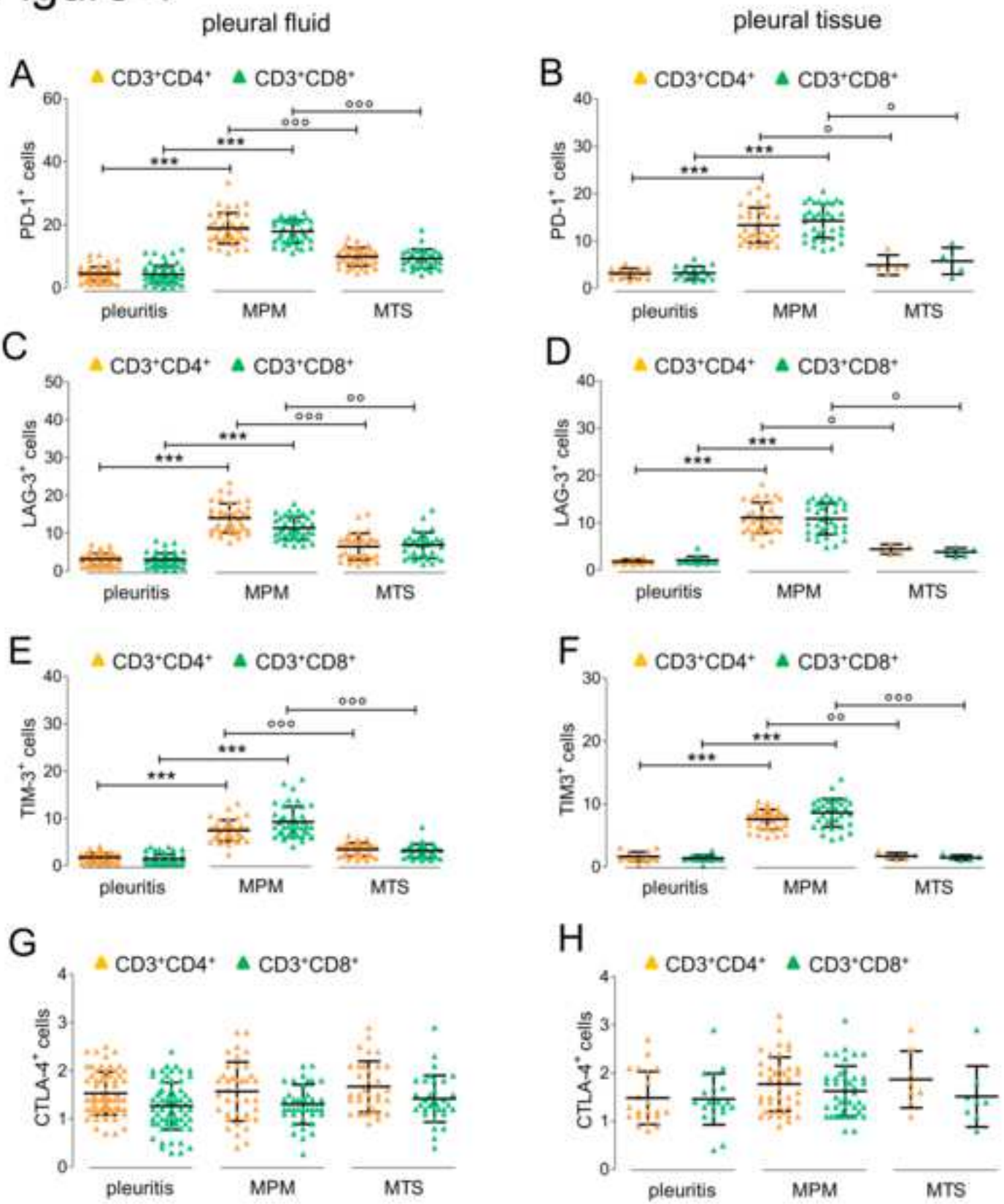

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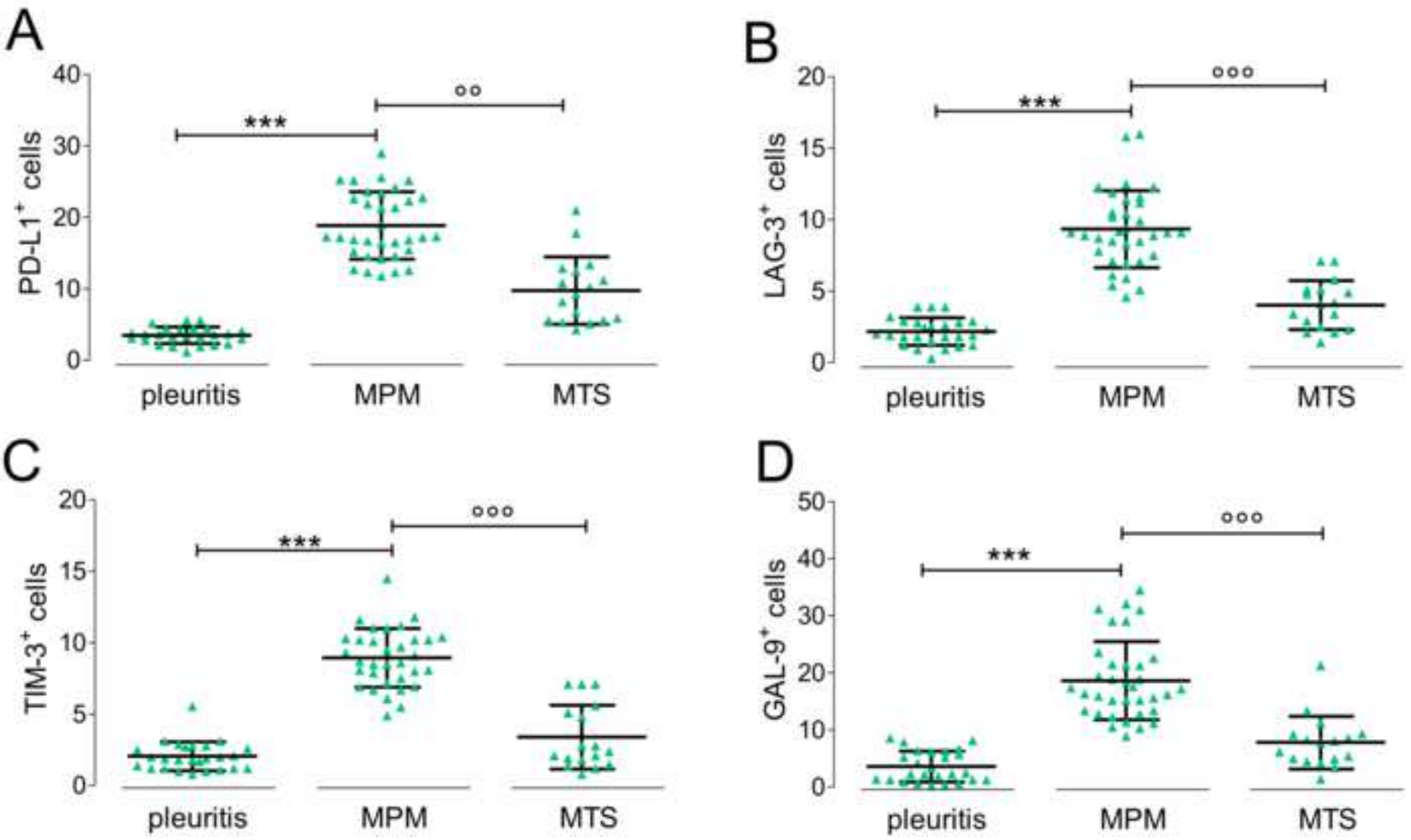

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Potential diagnostic and prognostic role of micro-environment in malignant pleural mesothelioma

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Conflicts of interest statement

Silvia Novello received honoraria for speaker bureau and advisory role from Astrazeneca, BI, Roche, Pfizer, MSD, Eli Lilly, Abbvie, Celgene for activities unrelated to the present study. Giorgio Scagliotti received honoraria for speaker bureau and advisory role from Astrazeneca, Roche, Pfizer, MSD, Eli Lilly, Abbvie and travel support from MSD and Bayer for activities unrelated to the present study.

The other authors have no conflict of interests

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Abstract

Introduction. A comprehensive analysis of the immune-cell infiltrate collected from pleural fluid and from biopsies of malignant pleural mesothelioma (MPM) may contribute to understand the immune-evasion mechanisms related to tumor progression, aiding in differential diagnosis and potential prognostic stratification. Till now such approach has not routinely been verified.

Methods. We enrolled in 275 patients with an initial clinical diagnosis of pleural effusion. Specimens of pleural fluids and pleural biopsies used for the pathological diagnosis and the immune-phenotype analyses were blindly investigated by multi-parametric flow cytometry. The results were analyzed by Kruskal-Wallis test. The Kaplan-Meier and log-rank tests were used to correlate immune-phenotype data with patients' outcome.

Results. The cut-offs of intra-tumor T-regulatory (Treg; >1.1%) cells, M2-macrophages (>36%), granulocytic and monocytic myeloid-derived suppressor cells (MDSC; >5.1% and 4.2%, respectively), $CD4^+PD1^+$ (>5.2%) and $CD8^+PD1^+$ (6.4%) cells, $CD4^+LAG\text{-}3^+$ (>2.8%) and $CD8^+LAG\text{-}3^+$ (>2.8%) cells, $CD4^+TIM\text{-}3^+$ (>2.5%) and $CD8^+TIM\text{-}3^+$ (>2.6%) cells discriminated MPM from pleuritis with 100% sensitivity and 89% specificity. The presence of intra-tumor MDSC contributed to the anergy of tumor-infiltrating lymphocytes (TILs). The immune-phenotype of pleural fluid cells had no prognostic significance. By contrast, the intra-tumor Treg and MDSC levels significantly correlated with progression-free and overall survival, the $PD\text{-}1^+/LAG\text{-}3^+/TIM\text{-}3^+$ $CD4^+$TILs correlated with overall survival.

Conclusions. A clear immune-signature of pleural fluids and tissues of MPM patients may contribute to better predict patients' outcome.

Key words: malignant pleural mesothelioma; tumor-infiltrating lymphocytes; T-regulatory cells; myeloid-derived suppressor cells; immune checkpoints

Abbreviations:

DCFDA-AM: 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate-acetoxymethyl ester; FBS: fetal bovine serum; Gr-MDSC: granulocytic myeloid-derived suppressor cells; HR: hazard ratio; IDO: 2,3-indoleamine dioxygenase; Mo-MDSC: monocytic myeloid-derived suppressor cells; MPM: malignant pleural mesothelioma; MTS: metastases;

OS: overall survival; PFS: progression free survival; NK: natural killer; NO: nitric oxide; ROS: reactive oxygen species; TAM: tumor-associated macrophage; Treg: T-regulatory; TIL: tumor-infiltrating lymphocyte.

Introduction

Malignant pleural mesothelioma (MPM) is an asbestos-related cancer characterized by a long latency.[1] It has a low mutational burden and the tumor micro-environment rather than the genetic abnormalities in mesothelial cells may contribute to MPM development and progression.[2] The MPM micro-environment is rich of immune-suppressive and anergic immune cells,[4-6] such as T-regulatory (Treg),[7-8] granulocytic and monocytic myeloid-derived suppressor cells (Gr-MDSC/Mo-MDSC)[5,7] and M2-polarized tumor associated macrophages (TAMs),[9] that – together with soluble factors, such as cytokines, chemokines[7] and kynurenine, the product of 2,3-indoleamine dioxygenase (IDO) enzyme [8] – lead to a poor response to immune-therapy.[10]

Several cytokines accumulated in the pleural effusion of MPM patients promote the M2 polarization of macrophages.[11,12] M2/M3-macrophages and[11,13] Gr-MDSC[5] of MPM patients, as well as MPM cells[14] reduce the proliferation of heterologous $CD8^+$T-lymphocytes, by producing immune-suppressive mediators such as reactive oxygen species (ROS), nitric oxide (NO) and kynurenine.[5,8,15] MDSC are killed by active $CD8^+$T-lymphocytes,[16,17] while either Treg and MDSC reduce $CD8^+$T-lymphocytes activity[5,7,18] and memory $CD8^+$T-lymphocytes recruitment,[19] inducing a vicious immune-suppressive circle.

The high expression of immune-checkpoints on T-lymphocytes and of their ligands on MPM cell has another crucial role in the MPM-induced anergy of tumor-infiltrating lymphocytes (TILs).[6] The PD-1/PD-L1 axis is the more extensively studied and the most correlated with early progression and shorter survival.[3,4,20,21,22,23] LAG-3 and TIM-3 have been detected on $CD4^+$ and $CD8^+$TILs[24] or in formalin-fixed paraffin embedded samples.[25] Despite the high inter-patient and intra-sample variability,[26] both LAG-3 and TIM-3 contribute to the functional exhaustion of TILs. Furthermore CTLA-4 has been investigated as a potential therapeutic target in MPM, but the CTLA-4 inhibitor Tremelimumab did not show higher efficacy than placebo

in a double blind phase 2 trial, [27] raising concerns about the role of this immune-checkpoint in the MPM-induced immune-suppression.

The dissection of the immune-environment of MPM is quite difficult and has often twisted conflicting findings for different reasons. For instance, the number of T-cells and macrophages detected in pleural effusion not always mirrors the amount of the same cells infiltrating MPM tissue.[28] Some studies reported the same immune-signature in the MPM pleural effusion and in the peripheral blood,[24] while others did not,[5] raising concerns about peripheral blood as a biological surrogate that reliably reproduces the MPM immune-environment. Indeed, immune cells can be continuously exchanged between pleural cavity and tumor tissue, and/or between peripheral blood and pleural environment. This dynamic immune-environment leads to an inhomogeneous distribution of immune-cells within MPM tissue. Moreover, the immune-environment is exposed to time-changes related to the natural tumor progression and/or to chemotherapy-related effects.[28] Also qualitative and quantitative changes in tumor/stroma ratio may produce a dramatic rewiring in the MPM-infiltrating immune cell subsets.[18] The high intra-patient variability, the intra-tumor heterogeneity,[29] the different timing of analysis may partially justify the divergent results.

A simultaneous analysis of all the immune-populations detectable in the pleural effusion and infiltrating the tissue has never been performed. The present study aims at a comprehensive analysis of the immune-infiltrate detected in the pleural fluid and in the biopsies of pleural tissue, collected during routine diagnostic procedures from patients with pleural effusions of unknown origin, in order to identify an immune-phenotype with diagnostic and prognostic value in MPM patients.

Materials and methods

Samples collection. From June 2016 through June 2018 we enrolled 275 patients with an initial clinical diagnosis of pleural effusion of unknown origin. The enrollment of patients was

stopped when we have reached the sample size with an adequate power in the MPM patient cohort (see "Statistical analysis" paragraph). Samples were obtained during diagnostic thoracoscopies, at the Thoracic Surgery Division of San Luigi Gonzaga Hospital, Orbassano, and AOU Città della Salute e della Scienza, Torino, Italy. Two aliquots of pleural fluid and pleural tissue were collected by thoracic surgeons at the same time for each patient. Each sample was managed by the Pathology Unit of the two Institutions: one aliquot was used for diagnostic purposes according to local pathology guidelines, the second one was used for the research study here reported. In case of limited volume of pleural fluid or limited dimension of pleural tissue, both aliquots were dedicated to the diagnostic workup. In accordance with this criterion, 183 pleural fluids and 119 pleural biopsies were available for the research study. Within all these samples, 63 pathologically diagnosed non-malignant pleuritis, 49 MPM and 32 pleural metastases - MTS - of malignant tumors for pleural fluid; 16 non-malignant pleuritis, 33 MPM and 5 MTS for pleural tissue biopsies met the technical inclusion criteria (see "Phenotyping of immune cells" paragraph) and were analyzed in this study. 9 patients with pathologically diagnosed pleuritis and 20 patients with pathologically diagnosed MPM had both pleural fluid and pleural tissue available for the study. The flow chart of sample collection and processing is reported in the **Supplemental Figure 1**. The characteristics of samples used for the immune-phenotyping in the present study are reported in the **Supplemental Table 1.** The anonymized patients' history (asbestos exposure and smoking status, whenever available), the pathological diagnosis and the clinical follow-up (progression free survival, PFS; overall survival, OS) of MPM patients, performed at the Thoracic Oncology Unit, San Luigi Gonzaga Hospital, are reported in the **Supplemental Table 2**. All patients with MPM were in advanced clinical stage and all were treated with standard cytotoxic chemotherapy according to current guidelines.[30] The researchers performing the immune-phenotyping analyses reported below worked in a blind manner, being unaware of the pathological diagnosis at the time of the assays.

The association between pathological diagnosis, immune-phenotype analysis was performed during the final data analysis. The Ethics Committee of San Luigi Gonzaga Hospital, Orbassano, Italy approved the study (#126/2016).

Sample processing and mesothelial/tumor histological analysis. 50 ml of pleural fluid were centrifuged at 1200×g for 5 min, washed in PBS containing 1 mg/ml ciprofloxacin (Sigma Chemicals Co., St. Louis, MO), and re-suspended at 1×10^6 cells/ml in Ham's F10 nutrient mixture medium (Invitrogen Life Technology, Milano, Italy), supplemented with 10% v/v fetal bovine serum (FBS; Sigma Chemicals Co.), 1% v/v penicillin-streptomycin (Sigma Chemicals Co.). Tissues were digested in medium containing 1 mg/ml collagenase and 0.2 mg/ml hyaluronidase (Sigma Chemicals Co.) for 1 h at 37°C. Cells from pleural fluid and digested tissue were seeded in culture flasks (Becton Dickinson, Franklin Lakes, NJ) for 24 h in complete medium. After this period, the cells floating in the supernatant, i.e. immune cells in the pleural fluid or infiltrating the tissue, were collected, counted and analyzed for their immune-phenotype as detailed below. Adherent cells were analyzed for their mesothelial/tumor origin: after detaching by gentle scraping, cells were centrifuged at 1200×g for 5 min, fixed in 4% v/v formalin at 4 °C overnight and stained with the following antibodies: calretinin (Thermo Fisher Scientific, Waltham, MA), Wilms tumor-1 antigen (Thermo Fisher Scientific), cytokeratin 5 (Menarini Diagnostics, Bagno a Ripoli, Italy), podoplanin (Dako, Santa Clara, CA), pancytokeratin (Dako), epithelial membrane antigen (EMA, Dako), carcino-embrionic antigen (CEA, Dako), using an automated immunostainer (Benchmark Ventana Medical Systems, Tucson, AZ).

Phenotyping of immune cells. From all the samples available for research purpose, only samples with $\geq 1\times10^6$ viable (i.e. Trypan-blue negative) cells in the supernatants or adherent in flask were included in the analysis. Preliminary set-up experiments indicated that $\geq 1\times10^6$ viable cells was the minimal number required allowing to acquire 1×10^5 cells/staining, in order

to perform the whole set of the immune-phenotyping tests on each sample. Samples with ≤ 1×10^6 viable cells were excluded, since they did not allow to acquire 1×10^5 cells/staining. The supernatants were centrifuged at 1200×g for 5 min, the pellet was washed in PBS and re-suspended in PBS containing 5% v/v FBS. A three- and four-color flow cytometry was performed, with the appropriate combinations of antibodies (all diluted 1:10, from Miltenyi Biotec, Teterow, Germany if not otherwise specified) against: CD3 (mouse clone REA613), CD4 (mouse clone M-T466), CD8 (mouse clone BW135/80) for T-lymphocytes; CD25 (mouse clone 4E3), CD127 (mouse clone MB1518C9) for Treg cells; CD56 (mouse clone AF127H3), CD335/NKp46 (mouse clone 9E2) for natural killer (NK) cells; CD19 (mouse clone REA675) for B-lymphocytes; CD14 (mouse clone TÜK4) and CD68 (mouse clone Y1/82A) for monocytes and macrophages; CD68 (mouse clone Y1/82A), CD208 (mouse clone DCN228) and Arginase-1 (sheep polyclonal, # IC5868A, R&D Biosystems; Minneapolis, MN) for M2-polarized macrophages; CD68 (mouse clone Y1/82A), CD86 (mouse, clone FM95) and iNOS (rabbit polyclonal, #SPC-1325, StressMark Biosciences Inc., Victoria, Canada) for M1-polarized macrophages; CD11b (rat clone M1/70.15.11.5), CD14 (mouse clone TÜK4), CD15 (mouse clone VIMC6) for granulocytic myeloid-derived suppressor cells (Gr-MSDC) and monocytic myeloid-derived suppressor cells (Mo-MDSC). In each combination staining, 1×10^5 cells were analyzed using a Guava® easyCyte flow cytometer (Millipore, Bedford, MA), equipped with the InCyte software.

Expression of immune-checkpoints and immune-checkpoint ligands. $CD3^+$ cells were isolated from 1×10^6 immune cells of the supernatant of each culture with the Pan T Cell Isolation Kit (Miltenyi Biotec.), washed and re-suspended in PBS containing 5% v/v FBS. The detection of immune-checkpoints on $CD3^+$ T-lymphocytes and/or immune-checkpoint ligands on MPM cells were performed using antibodies for CD279/PD-1 (mouse clone PD1.3.1.3), CD223/LAG-3 (clone REA351), CD366/TIM-3 (mouse clone F38-2E2), CD152/CTLA-4

(mouse clone BNI3; all diluted 1:10, Miltenyi Biotec.), CD274/PD-1L (1:100, mouse clone 29E.2A3, BioLegend, San Diego, CA), anti-GAL-9 (mouse, clone 9M1-3, BioLegend). In each combination staining 1×10^5 cells were analyzed using a Guava® EasyCyte flow cytometer (Millipore), equipped with the InCyte software.

MDSC functional properties. ROS were measured using the fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate-acetoxymethyl ester (DCFDA-AM), as previously reported.[31] The levels of nitrite, the stable derivative of NO, in cell culture supernatants were measured by the Griess method.[32] The amount of kynurenine was assessed by spectrophotometry.[33] The results were expressed as nmoles/mg cellular proteins.

Proliferation and activation of T-lymphocytes co-cultured with MDSC. 1×10^4 sorted intra-tissue Gr-MDSC and Mo-MDSC were co-cultured for 6 days at a 1:1 ratio with sorted intra-tissue $CD3^+CD8^+$T-cytotoxic lymphocytes, either autologous or heterologous, as detailed in the experimental section, in the presence of anti-CD3/anti-CD28 dynabeads (Invitrogen Life Technologies) to activate lymphocytes. The proliferation of T-lymphocytes was assessed by adding 1 µCi of [^{3}H]thymidine (PerkinElmer, Waltham, MA) 18 h before the end of the co-cultures, then harvesting the plates and counting the radioactivity by liquid scintillation count. The results were expressed as count per minute (cpm). The percentage of $CD8^+CD107a^+$ and the production of IFN-γ, considered indexes of activated cytotoxic T-lymphocytes, were measured by flow cytometry and ELISA, as reported.[14]

Statistical analysis. Before starting the enrollment of patients, the requested number of patients in the MPM cohort was calculated using the G*Power Software (www.gpower.hhu.de) using the following assumptions: significance level (α error probability) ≤ 0.05, power (1-β error probability) = 0.80, effect size ρ = 0.40). With these parameters, the sample size required was 34±2 MPM.

The normality distribution of each parameter analyzed was checked with the D'Agostino and Pearson test. All parameters had a Gaussian distribution or approximation. However, the results were conservatively analyzed by the non-parametric Kruskal-Wallis test (GraphPad PRISM 6.0 software). All data in the figures are provided as means±SD. Medians and quartile values are reported in the figure legends. In order to partially correct for the multiplicity of tests performed, a conservative $p<0.01$ was considered statistically significant. For each parameter specific cut-off discriminating MPM from pleuritis were calculated in order to have a 100% sensitivity (0% false negative) and >89% specificity (<11% false positive). To correlate the immune-parameters with PFS and OS, since there are no previously reported and validated cut-off values for the immune-phenotypic parameters analyzed, patients were exploratively divided into "low expressing" and "highly expressing" groups, if their value was below or equal/above the median value. The Kaplan-Meier method was used to calculate PFS and OS. Log rank test was used to compare the outcome of each group as hazard ratio (HR, i.e. risk of patient death). Considering the exploratory nature of the survival analysis, no adjustment for multiplicity was made, and $p<0.05$ was considered statistically significant.

Results

High T-regulatory cells/high myeloid-derived suppressor cells discriminate malignant pleural mesothelioma from inflammatory pleuritis and secondary pleural tumors

$CD3^+$T-lymphocytes represented the prevalent immune population within pleural fluid or pleural tissue, without differences between samples of pleuritis, MPM or MTS (**Figure 1A**). $CD3^+CD4^+$T-helper lymphocytes were up to 60% in all the samples, without differences between patient subgroups (**Figure 1B**). $CD3^+CD8^+$T-cytotoxic lymphocytes, representing approximately 5% of immune cells in pleural fluids from pleuritis, significantly increased in MPM and MTS-pleural fluid samples. By contrast they represented up to 50% of TILs in pleuritis and significantly decreased in MPM and MTS (**Figure 1C**). This trend allowed to

discriminate pleuritis (all with $CD3^+CD8^+$cells <6.3% in pleural fluid) from MPM (all with $CD3^+CD8^+$cells >25% in pleural fluid) (**Figure 1D**). T-reg cells were significantly higher in both pleural fluid and tissue from MPM patients compared to pleuritis and MTS-derived samples (**Figure 1E**). A cut-off of >1.4% in pleural fluid and >1.1% in pleural tissue Treg cells identified 100% (20/20) MPM and excluded 88.8% (8/9) pleuritis (**Figure 1F**). NK and B-lymphocytes were poorly represented and did not show any differences among groups (**Supplemental Figure 2A-B**).

Also monocytes did not differ (**Supplemental Figure 3**), while pleural fluid and tissue macrophages were significantly higher in MPM and MTS-derived samples (**Figure 2A**). All pleuritic samples had <34% macrophages in pleural fluid and <35% in tissue while all MPM samples were above these thresholds (**Figure 2B**). More relevantly the pro-tumorigenic M2-macrophages were higher in MPM and MTS compared to pleuritis (**Figure 2C**). According to these parameters, a clear separation between pleuritis (100% of patients with M2-macrophages <29% in pleural fluid and <36% in pleural tissue) and MPM (100% of patients with M2-macrophages >41% in pleural fluid and >44% in pleural tissue) was clearly identified (**Figure 2D**). Anti-tumorigenic M1-macrophages were lower in both pleural fluid and tissue of MPM and MTS compared with pleuritis (**Supplemental Figure 4**).

A significantly higher number of Gr-MDSC (**Figure 2E**) and Mo-MDSC (**Figure 2G**) in pleural fluid and tissue was detected in MPM compared to pleuritis and MTS. A clear threshold of 5.1% Gr-MDSC in both pleural fluid and tissue discriminated 100% pleuritis from 100% MPM (**Figure 2F**). A cut-off of >3.6% Mo-MDSC in pleural fluid and >4.2% in pleural tissue identified 100% MPM and excluded 88.8% pleuritis (**Figure 2H**).

The intratumor myeloid-derived suppressor cells determine T-lymphocytes anergy

The MDSC isolated from MPM tissue showed increased production of ROS (**Figure 3A**), NO (**Figure 3B**) and kynurenine (**Figure 3C**), compared to those obtained from tissue biopsies of

other subgroups. In co-culture with $CD8^+$T-cytotoxic lymphocytes from the tissue of the same patient, both Gr-MDSC and Mo-MDSC derived from MPM reduced $CD8^+$T-lymphocytes proliferation (**Figure 3D**), CD107a positivity (**Figure 3E**) and IFN-γ production (**Figure 3F**), compared to the autologous $CD8^+$T-lymphocytes/MDSC-co-cultures derived from the pleuritis and MTS subgroups. The immune-suppressive properties of either Gr-MDSC and Mo-MDSC were further demonstrated by co-incubating Gr-MDSC and Mo-MDSC derived from MPM tissue with heterologous T-lymphocytes derived from patients with pleuritis. In this setting, proliferation, CD107a positivity and IFN-γ production of T-lymphocytes derived from pleuritis was reduced to the same level of T-lymphocytes derived from MPM (**Figure 3D-F**).

A high number of intra-tumor $PD\text{-}1^+/LAG\text{-}3^+/TIM\text{-}3^+$ infiltrating lymphocytes is peculiar of malignant pleural mesothelioma

The immune-checkpoints PD-1 (**Figure 4A-B**), LAG-3 (**Figure 4C-D**) and TIM-3 (**Figure 4E-F**) were higher on $CD4^+$T-helper and $CD8^+$T-cytotoxic lymphocytes of MPM compared to the lymphocytes from pleuritis and MTS, either in pleural fluid and in pleural tissue. The cut-off values of 6.7% in pleural fluid, 5.2% in pleural tissue for $CD4^+PD1^+$ (**Supplemental Figure 5A**), 6.3% in pleural fluid, 6.4% in pleural tissue for $CD8^+PD1^+$cells (**Supplemental Figure 5B**), 4.9% in pleural fluid, 2.8% in pleural tissue for $CD4^+LAG\text{-}3^+$ (**Supplemental Figure 5C**), 5.2% in pleural fluid, 2.8% in pleural tissue for $CD8^+LAG\text{-}3^+$cells (**Supplemental Figure 5D**), 1.9% in pleural fluid, 2.5% in pleural tissue for $CD4^+TIM\text{-}3^+$ (**Supplemental Figure 5E**), 2.4% in pleural fluid, 2.6% in pleural for $CD8^+TIM\text{-}3^+$cells tissue (**Supplemental Figure 5F**), discriminated 100% MPM from pleuritis. In our series, the lymphocytic expression of CTLA-4 did not differ between each subgroup (**Figure 4G-H**).

The higher expression of immune-checkpoints on T-lymphocytes was paralleled by the higher expression of PD-L1 (**Figure 5A**), LAG-3 (**Figure 5B**), TIM-3 (**Figure 5C**) and GAL-9

(**Figure 5D**) on MPM cells compared to pleuritis and – for LAG-3 and TIM-3 – compared to MTS.

Specific immune-signatures of intratumor infiltrate have a prognostic value

Furthermore we investigated the potential prognostic role of the immune-phenotypic parameters that significantly differed between MPM and pleuritis.

The amount of $CD3^+CD8^+$ cells, Treg, M2-polarized macrophages, Gr/Mo-MDSC, PD-1^+/LAG-3^+/$TIM3^+$ $CD4^+$ or $CD8^+$ cells present in the pleural fluids did not show any significant correlation with PFS and OS of MPM patients (**Table 1**). As far as the intratumor immune-infiltrate was concerned, the amount of $CD3^+CD8^+$ cells and M2 were not associated with any prognostic significance, while high intratumor Treg, Gr-MDSC and Mo-MDSC significantly correlated with shorter PFS and OS (**Table 1**; **Supplemental Figure 6A-E**). The amount of PD-1^+ and LAG-3^+ $CD4^+$ cells correlated with a lower OS (**Table 1**; **Supplemental Figure 7A-C**). The expression of immune-checkpoints on intratumor $CD8^+$ had no prognostic significance (**Table 1**; **Supplemental Figure 8A-C**).

Discussion

Specific immune-phenotypic markers on tissue microarrays[20] or fine-needle aspirate[24] are currently investigated to better characterize the immune-environment of MPM. The present study for the first time assessed a comprehensive and multi-parametric analysis of the immune-infiltrate of either pleural fluid and pleural tissue, performed parallelly to the routine diagnostic procedures. Based on flow cytometry assays, our experimental model allows to recover viable cells, coupling the phenotypic analysis with functional assays of adaptive immunity. Moreover, we detected a quite robust immune-signature that discriminates MPM from pleuritis and from pleural metastases secondary to other malignancies. By selecting specific cut-offs for each immune-parameter analyzed with 100% sensitivity and ≥89% good specificity, we maximized the possibility to identify correctly all the MPM patients, accepting the possibility to have

sporadic cases of pleuritis erroneously identified as MPM. Since all samples were subjected to the usual pathological diagnosis, the rare cases of pleuritic erroneously identified as MPM on the basis of immune-phenotype analysis could be easily re-verified by the pathologist and/or clinically followed-up more strictly . The analysis of the immune-parameters was not intended to replace the pathological diagnosis but simply to complement the pathological diagnosis of MPM with a set of immunological tests and potential new prognostic information. Each parameter was indeed correlated with PFS or OS, exploratively using the median values as cut-off since in literature there are no previously reported and validated cut-offs for the parameters analyzed and the identification of the optimal cutoff was beyond the scope of this analysis.

The first relevant finding discriminating MPM from non-malignant pleuritis was the higher intra-pleural fluid $CD8^+$T-lymphocytes coupled with the lower intra-pleural $CD8^+$TILs. The amount of $CD8^+$TILs has been associated with adverse[18,34] or good [20] prognosis, but in our case series, we did not detect any prognostic significance. It is likely that - more than the absolute number of $CD8^+$T-lymphocytes - their functional exhaustion, due to the presence of Treg cells and MDSC,[7,18] is critical in determining tumor progression and patients' outcome.

We identified high Treg cells within pleural fluid and tissue as specific biomarkers of MPM rather than of benign pleural disease or pleural MTS. The high intra-MPM Treg was a negative prognostic factor, while the amount of Treg in pleural fluid had no clinical significance, as reported previously.[18] This data pointed out that the intratumor immune-infiltrate – more than the immune-population in the pleural effusion – in this case is more reliable in predicting the patients' outcome.

As expected, we did not detect significant differences in overall TAMs between MPM and metastatic patients. Compared to pleuritis, M2-macrophages were increased in both tumor and pleural fluid of MPM. The correlations between the number of TAMs or the ratio M2/TAM and the tumor progression or patients' outcomes [9,18,20,34] are highly discordant. According to

our data M2 percentage is not a significant predictor of patients' outcome but it is an immune-marker highly differentiating MPM from non-malignant conditions, as Gr-MDSC and – to a lesser extent – Mo-MDSC.

In our study, Gr- and Mo-MDSC abrogated proliferation and cytotoxic activity of autologous TILs and of TILs derived from patients with pleuritis. These data suggest that MDSC mediate an important role MPM immune-suppression, likely by the increased production of ROS, NO and kynurenine. Again, only the intratumor-infiltrating MDSC - and not the MDSC of pleural fluid - are significantly associated with poorer PFS and OS. Taking into consideration the dynamic exchange occurring between pleural fluid and tumor,[28] pleural fluid can be considered as a reservoir of immune-suppressive cells: higher is their migration from pleural fluid into the tumor, higher is the chance to characterize an immune-escape status and predict tumor progression.

Immune-checkpoints expression are other key players of immune-suppression in MPM. The immunohistochemical expression of PD-L1 and PD-1 is not always reliable as biomarker in discriminating pleuritis from MPM: PD-L1 expression was absent in benign lesions, but PD-1 resulted more expressed on TILs of non-tumoral samples than in TILs of MPM analyzed by immunohistochemistry,[23] a finding contrasting with the high percentage of PD-1^{+}cells reported in MPM using flow cytometry.[3,4,6] The type of diagnostic antibody and the abundance of the immune-infiltrate in the examined sample may explain this discrepancy. The multi-parametric flow cytometry analysis revealed that the amount of PD-1 expressed in $CD4^{+}$ and $CD8^{+}$T-lymphocytes well discriminates MPM from non-malignant pleuritis, but not from metastatic cancers, in line with the observation that pleural effusion from lung cancer are also rich of $PD1^{+}CD8^{+}$T-lymphocytes and PD-$L1^{+}$-cancer cells.[35]

Our data indicate that the expression of PD-1, LAG-3 and TIM-3 in TILs, but not in T-cells in pleural effusion, correlated with lower OS. The lack of correlation between immune-checkpoint levels and PFS likely suggests that the accumulation of immune-checkpoint-positive, anergic lymphocytes occurs at advanced stages in MPM patients and determines prognosis in the terminal stages only. More interestingly, only PD-1$^+$/LAG-3$^+$/TIM-3$^+$CD4$^+$ cells, but not CD8$^+$ cells, were negative prognostic factors. Since CD4$^+$ T-cells are a hub in the engagement of other immune populations, we may hypothesize that a loss of function of CD4$^+$ T-lymphocytes impairs the immune-recognition of MPM cells more than a dysfunction in the effector CD8$^+$ T-cells, leading to faster progression and reduced survival. Among the immune-checkpoints analyzed in MPM patients, CTLA-4 was poorly expressed on the surface of T-lymphocytes. This is likely related to the cytosol localization of CTLA-4 and the high recycling rate.[36] Our data need to be re-assessed when a staining optimization for intracellular CTLA-4 antigen will be achieved.

In conclusion, we propose a comprehensive multi-parametric analysis of several immunological parameters that define a MPM immune-signature, reliably applied to both pleural fluid and tissue routinely collected in standard clinical practice. We are aware that our discriminating cut-off values, although significant, have been obtained in a small patient cohort, and we emphasize that the identification of the optimal cutoff was beyond the scope of this analysis. The parameters are now being validated in a larger prospective study, including discovery and validation cohorts, in order to improve the specificity of the proposed thresholds and evaluate the possible clinical utility of the immune-parameters found. In such ongoing study, refined grouping strategies will be adopted, in order to define more accurately the quantitative values of the immune-phenotypic parameters significantly correlated with patient survival and other clinical parameters. Only if the validation study will confirm this pilot experience, the immune biomarkers identified in the present study may be considered as

additional parameters to be analyzed - beyond the parameters already adopted to perform MPM diagnosis - in challenging clinical cases.

Of note, this study is the first one aimed to systematically characterize the immune-infiltrate of pleural fluid and tumor derived from the same patient. Our results indicated that the analysis of the intratumor immune-infiltrate – better than that of pleural fluid – identifies potential prognostic factors. Given the high inter-patients and intra-patients variability observed in MPM, the larger prospective validation study will also clarify if the qualitative and quantitative differences in the intra-tumor immune-infiltrate may be correlated with specific histological, cyto-genetic and mutational features. This may contribute to a more accurate patients' stratification, for a rationale and personalized immune-therapy of MPM.

Funding

This work was supported by Italian Association for Cancer Research (IG21408 to CR); Italian Ministry of University and Research (Future in Research program RBFR12SOQ1 to CR; Basic Research Funding Program, FABR2017 to LR and CR); Italian Ministry of Health (GR-2011-02348356 to LR; Italian Mesothelioma Network-CCM2012 to GVS), Azienda Ospedaliera "SS. Antonio e Biagio e Cesare Arrigo" to GVS for the research activity "MESOLINE", University of Torino (Intramural Grant 2015 to SN and CR; Intramural Grant 2016 to LR; Intramural Grant 2017 to JK); Fondazione Cassa di Risparmio di Torino (2016-2443 to CR). ICS is a post-doctoral research fellow supported by the Fondazione Franco e Marilisa Caligara for Multidisciplinary Sciences, Torino, Italy. VM is a PhD fellow of Erasmus Mundus-ERAWEB Action 2 program. FN is a student of the Biomedical Sciences and Oncology PhD program. The funding institutions had no role in the study design, data collection and analysis, or in writing the manuscript.

Acknowledgments

We are grateful to Mr. Costanzo Costamagna, Dept of Oncology, University of Torino for the technical assistance.

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Figure legends

Figure 1. Lymphocyte subtypes present in pleural fluid and tissue of malignant pleural mesothelioma

Cells collected from pleural fluid (PF) of patients with pleuritis (n=63), malignant pleural mesothelioma (MPM; n=49) and other tumors metastatizing to pleura (MTS; n=32), and from digested pleural tissue (PT) of patients with pleuritis (n=16), MPM (n=33) and MTS (n=5) were analyzed by flow cytometry. In n=9 pleuritis and n=20 MPM, PF and PT from the same patients were analyzed in parallel. **A-C.** Percentage of total ($CD3^+$), T-helper ($CD3^+CD4^+$) and T-cytotoxic ($CD3^+CD8^+$) lymphocytes. Data are presented as means±SD. Values of 25^{th} percentile, median, 75^{th} percentile: pleuritis PF: 52.00, 61.00, 66.00; pleuritis PT: 15.00, 21.00, 26.75; MPM PF: 52.00, 62.00, 71.00; MPM PT: 37.75, 47.00, 58.75; MTS PF: 51.00, 55.00, 64.00; MTS PT: 36.50, 41.00, 51.50 (panel **A**); pleuritis PF: 39.25, 52.00, 60.50; pleuritis PT: 51.00, 61.00, 67.00; MPM PF: 48.25, 61.00, 70.25; MPM PT: 51.00, 56.00, 71.00; MTS PF: 45.10, 51.50, 60.50; MTS PT: 54.00, 61.00, 67.50 (panel **B**); pleuritis PF: 2.90, 3.75, 5.98; pleuritis PT: 30.00, 41.00, 49.00; MPM PF: 25.00, 29.00, 33.00; MPM PT: 12.00, 15.00, 19.00; MTS PF: 14.50, 20.00, 26.00; MTS PT: 10.50, 11.00, 15.50 (panel **C**). ***p<0.001: MPM/MTS vs pleuritis; not significant: MPM vs MTS. **D.** Disaggregated data of T-cytotoxic cells percentage in PF and PT from the same patient. Dotted line: 6.3% cut-off value in PF (false negative: 0%; false positive: 0%; sensitivity: 100%; specificity: 100%). **E.** Percentage of T-regulatory (Treg; $CD4^+$ $CD25^+CD127^{low}$) cells. Data are presented as means±SD. Values of 25^{th} percentile, median, 75^{th} percentile: pleuritis PF: 0.90, 1.20, 1.50; pleuritis PT: 0.30, 0.50, 0.90; MPM PF: 2.73, 2.95, 3.48; MPM PT: 2.10, 2.80, 3.25; MTS PF: 1.10, 1.25, 1.50; MTS

PT: 0.75, 1.10, 1.25. ***p<0.001: MPM vs pleuritis; °°p<0.005, °°°p<0.001: MPM vs MTS. **F.** Disaggregated data of Treg cells percentage in PF and PT from the same patient. Dotted line: 1.4% cut-off value in PF; dashed line: 1.1% cut-off value in PT (false negative: 0%; false positive: 11%; sensitivity: 100%; specificity: 89%)

Figure 2. Macrophage and myeloid-derived suppressor cell subtypes present in pleural fluid and pleural tissue of malignant pleural mesothelioma

Cells collected from pleural fluid (PF) of patients with pleuritis (n=63), malignant pleural mesothelioma (MPM; n=49) and other tumors metastatizing to pleura (MTS; n=32), and from digested pleural tissue (PT) of patients with pleuritis (n=16), MPM (n=33) and MTS (n=5) were analyzed by flow cytometry. In n=9 pleuritis and n=20 MPM, PF and PT from the same patients were analyzed in parallel. **A**. Percentage of macrophages ($CD14^+CD68^+$ cells). Data are presented as means±SD. Values of 25th percentile, median, 75th percentile: pleuritis PF: 15.00, 19.00, 31.00; pleuritis PT: 21.00, 25.00, 32.00; MPM PF: 49.25, 61.00, 71.00; MPM PT: 41.75, 55.00, 62.25; MTS PF: 48.25, 54.00, 66.25; MTS PT: 48.50, 65.00, 69.50. *p<0.01, ***p<0.001: MPM/MTS vs pleuritis. **B.** Disaggregated data of macrophage percentage in PF and PT from the same patient. Dotted line: 34% cut-off value in PF, dashed line: 35% cut-off value in PT (false negative: 0%; false positive: 0%; sensitivity: 100%; specificity: 100%). **C**. Percentage of M2-macrophages ($CD68^+CD206^+Arg1^+$cells). Data are presented as means±SD. Values of 25th percentile, median, 75th percentile: pleuritis PF: 17.25, 22.00, 28.00; pleuritis PT: 23.00, 28.00, 34.50; MPM PF: 45.00, 55.00, 62.25; MPM PT: 56.00, 60.00, 68.25; MTS PF: 41.25, 44.00, 57.00; MTS PT: 53.00, 64.00, 65.00. ***p<0.001: MPM/MTS vs pleuritis. **D.** Disaggregated data of M2-macrophage percentage in PF and PT from the same patient. Dotted line: 29% cut-off value in PF, dashed line: 36% cut-off value in PT (false negative: 0%; false positive: 0%; sensitivity: 100%; specificity: 100%). **E.** Percentage of granulocytic myeloid-derived suppressor cells (Gr-MDSC; $CD11b^+CD14^-CD15^+$cells). Data are presented

as means±SD. Values of 25th percentile, median, 75th percentile: pleuritis PF: 2.20, 2.80, 3.68; pleuritis PT: 2.10, 2.60, 3.10; MPM PF: 11.23, 13.05, 17.73; MPM PT: 11.80, 13.15, 17.03; MTS PF: 3.28, 5.15, 6.30; MTS PT: 4.80, 5.10, 7.70. ***p<0.001: MPM vs pleuritis; °°°p<0.001: MPM vs MTS. **F.** Disaggregated data of Gr-MDSC percentage in PF and PT from the same patient. Dashed line: 5.1% cut-off value in PF and PT (false negative: 0%; false positive: 0%; sensitivity: 100%; specificity: 100%). **G.** Percentage of monocytic myeloid derived suppressor cells (Mo-MDSC; CD11b^{+}CD14^{+}CD15^{low}cells). Data are presented as means±SD. ***p<0.001: MPM vs pleuritis; °°°p<0.001: MPM vs MTS. Values of 25th percentile, median, 75th percentile: pleuritis PF: 2.10, 2.40, 3.45; pleuritis PT: 2.13, 2.75, 3.18; MPM PF: 5.90, 8.20, 10.20; MPM PT: 8.10, 9.75, 11.28; MTS PF: 1.30, 2.30, 2.90; MTS PT: 1.90, 2.35, 2.80. **H.** Disaggregated data of Mo-MDSC percentage in PF and PT from the same patient. Dotted line: 3.6% cut-off value in PF, dashed line: 4.2% cut-off value in PT (false negative: 0%; false positive: 11%; sensitivity: 100%; specificity: 89%).

Figure 3. Intratumor myeloid-derived suppressor cells determines CD8^{+} T-lymphocytes anergy

1×10^{4} sorted intra-tissue Gr-MDSC and Mo-MDSC derived from pleuritis (n=10), MPM (n=12) and other tumors metastatizing to pleura (MTS; n=4) were seeded and analyzed after 24 h (panels **A**-**C**), or co-cultured (panels **D**-**F**) for 6 days with the sorted intra-tissue CD3^{+}CD8^{+}T-cytotoxic lymphocytes of the corresponding patient (autologous setting). When indicated, Gr-MDSC and Mo-MDSC from MPM patients were cultured with T-cytotoxic lymphocytes from pleuritis patients (pleu/MPM setting; n=8). **A-C.** Intracellular ROS (**panel A**) were measured fluorimetrically, nitrite (**panel B**) and kynurenine (**panel C**) released in the supernatants were measured spectrophotometrically. Data are presented as means±SD. Values of 25th percentile, median, 75th percentile: pleuritis Gr-MDSC: 0.59 0.79, 0.94; pleuritis Mo-MDSC: 0.43, 0.65, 0.80; MPM Gr-MDSC: 2.08, 2.40, 2.78; MPM Mo-MDSC: 1.78, 2.20,

2.68; MTS Gr-MDSC: 0.61, 0.72, 0.81; MTS Mo-MDSC: 0.43, 0.66, 0.82 (panel **A**); pleuritis Gr-MDSC: 1.58, 2.00, 2.45; pleuritis Mo-MDSC: 1.60, 2.10, 2.95; MPM Gr-MDSC: 8.20, 9.75, 12.25; MPM Mo-MDSC: 6.05, 6.85, 8.55; MTS Gr-MDSC: 1.30, 1.75, 2.65; MTS Mo-MDSC: 1.63, 2.15, 2.45 (panel **B**); pleuritis Gr-MDSC: 0.55, 0.72, 0.82; pleuritis Mo-MDSC: 0.72, 0.87, 1.16; MPM Gr-MDSC: 3.73, 4.14, 4.85; MPM Mo-MDSC: 4.25, 5.26, 5.35; MTS Gr-MDSC: 0.59, 0.89, 1.19; MTS Mo-MDSC: 0.78, 1.00, 1.24 (panel **C**). *p<0.01, **p<0.005, ***p<0.001: MPM vs pleuritis; °p<0.01, °°p<0.005, °°°p<0.001: MPM vs MTS. **D-F.** The proliferation of T-cytotoxic lymphocytes (**panel D**) was measured radiometrically, the percentage of $CD8^+CD107a^+$lymphocytes (**panel E**) was measured by flow cytometry, the amount of IFN-γ (**panel F**) in the supernatants was measured by ELISA. The proliferation of T-lymphocytes in the absence of anti-CD3/anti-CD28 dynabeads, used as negative control, was <3500 cpm for all experimental conditions. Data are presented as means±SD. Values of 25th percentile, median, 75th percentile: pleuritis Gr-MDSC: 18.09, 23.12, 26.03; pleuritis Mo-MDSC: 27.53, 30.4, 34.62; MPM Gr-MDSC:2.42, 4.04, 5.00; MPM Mo-MDSC: 4.03, 5.71, 6.84; MTS Gr-MDSC: 16.66, 22.69, 28.46; MTS Mo-MDSC: 23.27, 31.72, 33.96; pleu/MPM Gr-MDSC: 5.33, 5.80, 7.19; pleu/MPM Mo-MDSC: 6.44, 8.25, 11.70 (panel **D**); pleuritis Gr-MDSC:2.08, 2.60, 2.83; pleuritis Mo-MDSC: 2.48, 3.00, 3.25; MPM Gr-MDSC: 0.40, 0.60, 0.80; MPM Mo-MDSC: 0.45, 0.80, 1.05; MTS Gr-MDSC: 1.95, 2.65, 3.05; MTS Mo-MDSC: 2.83, 3.05, 3.65; pleu/MPM Gr-MDSC: 0.53, 0.75, 0.80; pleu/MPM Mo-MDSC: 0.40, 0.65, 0.88 (panel **E**); pleuritis Gr-MDSC: 409.50, 441, 636.50; pleuritis Mo-MDSC: 459.80, 497.50, 521.00; MPM Gr-MDSC: 111.80, 151.00, 203.30; MPM Mo-MDSC: 147.00, 177.00, 236.00; MTS Gr-MDSC: 322.00, 463.50, 506.80; MTS Mo-MDSC: 373.80, 480.00, 585.50; pleu/MPM Gr-MDSC: 168.80, 204.50, 263.80; pleu/MPM Mo-MDSC: 209.80, 298.00, 335.80 (panel **F**).**p<0.005, ***p<0.001: MPM vs pleuritis; °p<0.01, °°p<0.005, °°°p<0.001: MPM

vs MTS in autologous settings; $^{\#}p<0.01$, $^{\#\#\#}p<0.001$: pleu/MPM setting vs pleuritis autologous setting.

Figure 4. Immune-checkpoint expression in T-lymphocytes contained in pleural fluid and pleural tissue

Cells collected from pleural fluid of patients with pleuritis (n=63), malignant pleural mesothelioma (MPM; n=49) and other tumors metastatizing to pleura (MTS; n=32), and from digested pleural tissue of patients with pleuritis (n=16), MPM (n=33) and MTS (n=5) were analyzed by flow cytometry. Data are presented as means±SD. **A-B**. Percentage of PD-1^{+}T-helper ($CD3^{+}CD4^{+}$) and T-cytotoxic ($CD3^{+}CD8^{+}$) lymphocytes. Values of 25^{th} percentile, median, 75^{th} percentile: pleuritis $CD3^{+}CD4^{+}$: 2.45, 4.60, 5.60; pleuritis $CD3^{+}CD8^{+}$: 2.50, 3.90, 5.50; MPM $CD3^{+}CD4^{+}$: 15.53, 18.30, 22.65; MPM $CD3^{+}CD8^{+}$: 14.83, 17.65, 21.38; MTS $CD3^{+}CD4^{+}$: 7.95, 10.20, 12.30; MTS $CD3^{+}CD8^{+}$: 7.03, 9.00, 11.33 (panel **A**); pleuritis $CD3^{+}CD4^{+}$: 2.10, 3.15, 4.10; pleuritis $CD3^{+}CD8^{+}$: 2.03, 2.90, 4.18; MPM $CD3^{+}CD4^{+}$: 10.20, 12.50, 16.15; MPM $CD3^{+}CD8^{+}$: 11.28, 15.20, 18.05; MTS $CD3^{+}CD4^{+}$: 3.60, 4.30, 6.50; MTS $CD3^{+}CD8^{+}$: 3.10, 6.20, 8.15 (panel **B**). $^{***}p<0.001$: MPM vs pleuritis; $^{\circ}p<0.01$, $^{\circ\circ\circ}p<0.001$: MPM vs MTS. **C-D**. Percentage of LAG-3^{+}T-helper ($CD3^{+}CD4^{+}$) and T-cytotoxic ($CD3^{+}CD8^{+}$) lymphocytes. Values of 25^{th} percentile, median, 75^{th} percentile: pleuritis $CD3^{+}CD4^{+}$: 1.90, 2.80, 4.15; pleuritis $CD3^{+}CD8^{+}$: 1.90, 2.80, 4.15; MPM $CD3^{+}CD4^{+}$: 10.83, 13.35, 16.25; MPM $CD3^{+}CD8^{+}$: 8.83, 10.60, 14.05; MTS $CD3^{+}CD4^{+}$: 3.88, 7.20, 8.40; MTS $CD3^{+}CD8^{+}$: 3.75, 7.15, 8.60 (panel **C**); pleuritis $CD3^{+}CD4^{+}$: 1.50, 1.85, 2.10; pleuritis $CD3^{+}CD8^{+}$: 1.60, 1.80, 2.18; MPM $CD3^{+}CD4^{+}$: 8.38, 10.50, 14.28; MPM $CD3^{+}CD8^{+}$: 8.10, 10.50, 14.10; MTS $CD3^{+}CD4^{+}$: 3.40, 4.60, 5.40; MTS $CD3^{+}CD8^{+}$: 2.95, 4.10, 4.65 (panel **D**). $^{***}p<0.001$: MPM vs pleuritis; $^{\circ}p<0.01$, $^{\circ\circ}p<0.005$, $^{\circ\circ\circ}p<0.001$: MPM vs MTS. **E-F**. Percentage of TIM-3^{+}T-helper ($CD3^{+}CD4^{+}$) and T-cytotoxic ($CD3^{+}CD8^{+}$) lymphocytes. Values of 25^{th} percentile, median, 75^{th} percentile: pleuritis $CD3^{+}CD4^{+}$:1, 1.80, 2.48; pleuritis

$CD3^+CD8^+$: 0.90, 1.35, 1.70; MPM $CD3^+CD4^+$: 6.10, 7.30, 8.40; MPM $CD3^+CD8^+$: 6.65, 8.30, 10.70; MTS $CD3^+CD4^+$: 2.18, 3.85, 4.43; MTS $CD3^+CD8^+$: 1.98, 3.15, 4.13 (panel **E**); pleuritis $CD3^+CD4^+$: 1.13, 1.50, 2.05; pleuritis $CD3^+CD8^+$: 1.20, 1.35, 1.58; MPM $CD3^+CD4^+$: 6.18, 8.10, 8.53; MPM $CD3^+CD8^+$: 6.78, 9.10, 10.20; MTS $CD3^+CD4^+$: 1.25, 1.60, 2.20; MTS $CD3^+CD8^+$: 1.15, 1.40, 1.85 (panel **F**). ***p<0.001: MPM vs pleuritis; °°p<0.005, °°°p<0.001: MPM vs MTS. **G-H**. Percentage of CTLA-4^+T-helper ($CD3^+CD4^+$) and T-cytotoxic ($CD3^+CD8^+$) lymphocytes. Values of 25^{th} percentile, median, 75^{th} percentile: pleuritis $CD3^+CD4^+$: 1.20, 1.40, 1.90; pleuritis $CD3^+CD8^+$: 0.90, 1.30, 1.58; MPM $CD3^+CD4^+$: 1.10, 1.60, 1.90; MPM $CD3^+CD8^+$: 1.10, 1.20, 1.50; MTS $CD3^+CD4^+$: 1.18, 1.50, 2.10; MTS $CD3^+CD8^+$: 1.20, 1.30, 1.80 (panel **G**); pleuritis $CD3^+CD4^+$: 1.10, 1.20, 2.05; pleuritis $CD3^+CD8^+$: 1.25, 1.40, 1.70; MPM $CD3^+CD4^+$: 1.30, 1.80, 2.10; MPM $CD3^+CD8^+$: 1.20, 1.50, 1.90; MTS $CD3^+CD4^+$: 1.45, 1.70, 2.38; MTS $CD3^+CD8^+$: 1.13, 1.40, 1.73 (panel **H**).

Figure 5. Immune-checkpoint ligands expressed in malignant pleural mesothelioma cells

Mesothelial cells collected from patients with pleuritis (n=24), malignant pleural mesothelioma cells (MPM; n=33) or cells from different tumors metastatizing to pleura (MTS; n=16), were analyzed by flow cytometry for the expression of PD-L1 (panel **A**), LAG-3 (panel **B**), TIM-3 (panel **C**), GAL-9 (panel **D**). Data are presented as means±SD. Values of 25^{th} percentile, median, 75^{th} percentile: pleuritis: 2.55, 3.60, 4.35; MPM: 15.05, 17.25, 22.95; MTS: 5.60, 9.30, 12.70 (panel **A**); pleuritis: 1.43, 1.95, 2.83; MPM: 7.40, 9.10, 11.25; MTS: 2.40, 4.10, 5.10 (panel **B**); pleuritis: 1.20, 1.90, 2.73; MPM: 7.35, 8.70, 10.25; MTS: 1.65, 2.40, 5.35 (panel **C**); pleuritis: 1.30, 2.40, 6.23; MPM: 13.40, 17.20, 21.50; MTS: 4.70, 7.80, 9.25 (panel **D**).***p<0.001: MPM vs pleuritis; °°p<0.005, °°°p<0.001: MPM vs MTS.

Table 1. Survival analysis according to the immune-phenotypic parameter characterizing mesothelioma patients

Immune-population	Sample	PFS (months) (95% CI)	p value	OS (months) (95% CI)	p value
$CD3^+CD8^+$ low vs $CD3^+CD8^+$ high	PF	6.3±1.6 vs 6.3±1.0 (5.0-8.3)	0.913	10.0±2.2 vs 10.8±1.1 (8.2-12.7)	0.6927
Treg low vs Treg high	PF	6.0±1.1 vs 7.0±1.6 (4.7-8.1)	0.496	10.8±1.6 vs 9.7±1.4 (8.2-12.7)	0.4347
M2 low vs M2 high	PF	7.0±1.3 vs 5.7±1.0 (4.7-7.8)	0.2851	10.9±1.5 vs 9.8±1.9 (8.2-12.7)	0.7424
Gr-MDSC low vs Gr-MDSC high	PF	7.1±1.1 vs 5.6±1.3 (4.6-8.1)	0.3351	10.6±1.4 vs 10.8±2.2 (8.2.9-13.1)	0.4522
Mo-MDSC low vs Mo-MDSC high	PF	6.8±1.2 vs 5.9±1.4 (4.4-8.1)	0.8755	10.8±1.6 vs 9.4±1.7 (7.9-12.5)	0.5498
$CD4^+PD\text{-}1^+$ low vs $CD4^+PD\text{-}1^+$ high	PF	5.7±1.3 vs 7.3±1.1 (4.7-8.1)	0.713	8.6±1.5 vs 12.4±1.6 (8.2-12.7)	0.1126
$CD4^+LAG\text{-}3^+$ low vs $CD4^+LAG\text{-}3^+$ high	PF	7.2±1.4 vs 7.3±1.1 (5.0-8.4)	0.4585	9.6±2.5 vs 10.9±1.1 (8.2-12.7)	0.7886
$CD4^+TIM\text{-}3^+$ low vs $CD4^+TIM\text{-}3^+$ high	PF	8.1±1.1 vs 5.0±1.3 (4.9-8.4)	0.1047	11.9±1.2 vs 9.1±2.1 (8.4-12.9)	0.5417
$CD8^+PD\text{-}1^+$ low vs $CD8^+PD\text{-}1^+$ high	PF	7.2±1.3 vs 6.8±1.2 (5.3-8.8)	0.6309	9.4±2.0 vs 10.0±1.4 (7.6-11.9)	0.9571
$CD8^+LAG\text{-}3^+$ low vs $CD8^+LAG\text{-}3^+$ high	PF	6.5±1.4 vs 4.8±0.8 (4.1-7.2)	0.2127	11.0±1.8 vs 10.1±1.9 (8.1-13.0)	0.8569
$CD8^+TIM\text{-}3^+$ low vs $CD8^+TIM\text{-}3^+$ high	PF	7.7±1.1 vs 4.3±1.2 (4.31-7.6)	0.0956	11.0±1.6 vs 9.7±1.6 (8.2-12.3)	0.5441
$CD3^+CD8^+$ low vs $CD3^+CD8^+$ high	PT	7.4±1.0 vs 8.0±1.3 (6.1-9.2)	0.8624	9.6±1.4 vs 10.3±1.3 (8.3-11.9)	0.9594
Treg low vs Treg high	PT	9.1±1.8 vs 4.7±1.4 (6.1-9.3)	**0.0172**	12.1±1.2 vs 7.6±1.0 (8.3-11.9)	**0.0046**
M2 low vs M2 high	PT	7.6±1.7 vs 8.8±1.1 (6.4-10.2)	0.8228	10.4±1.4 vs 9.3±1.2 (8.1-11.7)	0.4016
Gr-MDSC low vs Gr-MDSC high	PT	9.1±1.1 vs 5.6±1.0 (5.9-9.2)	**0.0427**	11.3±1.3 vs 7.5±1.1 (7.6-11.2)	**0.037**
Mo-MDSC low vs Mo-MDSC high	PT	11.2±1.2 vs 7.3±1.4 (7.7-11.5)	**0.0178**	11.1±1.7 vs 8.0±1.0 (8.1-11.4)	**0.026**
$CD4^+PD\text{-}1^+$ low vs $CD4^+PD\text{-}1^+$ high	PT	7.9±1.0 vs 7.9±1.7 (6.2-9.5)	0.6616	11.4±1.1 vs 8.3±1.1 (8.5-11.8)	**0.043**
$CD4^+LAG\text{-}3^+$ low vs $CD4^+LAG\text{-}3^+$ high	PT	8.3±1.1 vs 7.0±1.3 (6.1-9.3)	0.364	11.5±1.4 vs 8.0±0.9 (8.0-11.5)	**0.0077**
$CD4^+TIM\text{-}3^+$ low vs $CD4^+TIM\text{-}3^+$ high	PT	6.8±1.6 vs 8.4±0.9 (6.1-9.4)	0.7595	11.7±1.2 vs 7.8±1.2 (8.0-11.7)	**0.044**
$CD8^+PD\text{-}1^+$ low vs $CD8^+PD\text{-}1^+$ high	PT	7.8±1.3 vs 7.9±1.2 (6.2-9.5)	0.9333	10.3±1.3 vs 10.0±1.2 (8.4-11.8)	0.9315
$CD8^+LAG\text{-}3^+$ low vs $CD8^+LAG\text{-}3^+$ high	PT	6.7±1.3 vs 8.0±1.1 (5.7-9.0)	0.6298	9.7±1.5 vs 10.6±1.1 (8.3-11.9)	0.9219
$CD8^+TIM\text{-}3^+$ low vs $CD8^+TIM\text{-}3^+$ high	PT	6.8±1.7 vs 8.4±0.8 (6.1-9.3)	0.7423	8.5±1.6 vs 10.6±1.2 (8.0-11.6)	0.574

The median values of $CD3^+CD8^+$ lymphocytes, Treg, M2-macrophages, Gr-MDSC, Mo-MDSC, $CD4^+PD\text{-}1^+$, $CD4^+LAG\text{-}3^+$, $CD4^+TIM\text{-}3^+$, $CD8^+PD\text{-}1^+$, $CD8^+LAG\text{-}3^+$, $CD8^+TIM\text{-}3^+$ cells was calculated in pleural fluid (PF; n=49) and pleural tissue (PT; n=33). Patients were classified as "low" or "high" if the percentage of each population was low or equal/higher than the median value. Progression free survival (PFS) and overall survival (OS) probability were calculated using the Kaplan-Meier method, and expressed as measn±SD (months). CI: confidence interval. Significant values are indicated by bold characters.

Supplemental materials

Supplemental data 1. Supplemental Figure 1.tif

Supplemental data 2. Supplemental Table 1.docx

Supplemental data 3. Supplemental Table 2.docx

Supplemental data 4. Supplemental Figure 2.tif

Supplemental data 5. Supplemental Figure 3.tif

Supplemental data 6. Supplemental Figure 4.tif

Supplemental data 7. Supplemental Figure 5.tif

Supplemental data 8. Supplemental Figure 6.tif

Supplemental data 9. Supplemental Figure 7.tif

Supplemental data 10. Supplemental Figure 8.tif