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ORIGINAL ARTICLE

Malignant pleural mesothelioma immune microenvironment and checkpoint expression: correlation with clinical–pathological features and intratumor heterogeneity over time

G. Pasello[1], G. Zago[1], F. Lunardi[2], L. Urso[3], I. Kern[4], G. Vlacic[4], F. Grosso[5], M. Mencoboni[6], G. L. Ceresoli[7], M. Schiavon[2], F. Pezzuto[2], A. Pavan[1,3], S. E. Vuljan[2], P. Del Bianco[8], P. Conte[1,3], F. Rea[2] & F. Calabrese[2*]

[1]Medical Oncology 2, Istituto Oncologico Veneto IRCCS, Padova; Departments of [2]Cardiac, Thoracic and Vascular Sciences; [3]Surgery, Oncology and Gastroenterology, University of Padova, Padova, Italy; [4]Pathology Laboratory, University Clinic Golnik, Golnik, Slovenia; [5]Mesothelioma Unit, Oncology, SS Antonio e Biagio General Hospital, Alessandria; [6]Oncology Unit, Villa Scassi Hospital, ASL 3 Genovese, Genova; [7]Oncology, Cliniche Humanitas Gavazzeni, Bergamo; [8]Clinical Trials and Biostatistics Unit, Istituto Oncologico Veneto IRCCS, Padova, Italy

Correspondence to: Prof. Fiorella Calabrese, Pathology Unit, Department of Cardiac, Thoracic and Vascular Sciences, Via Gabelli 61, University of Padova, 35121 Padova, Italy. Tel: +39-0498272268; Fax: +39-0498272294; E-mail: fiorella.calabrese@unipd.it

Background: Tumor immune microenvironment (TME) plays a key role in malignant pleural mesothelioma (MPM) pathogenesis and treatment outcome, supporting a role of immune checkpoint inhibitors as anticancer approach. This study retrospectively investigated TME and programmed death ligand 1 (PD-L1) expression in naïve MPM cases and their change under chemotherapy.

Patients and methods: Diagnostic biopsies of MPM patients were collected from four Italian and one Slovenian cancer centers. Pathological assessment of necrosis, inflammation, grading, and mitosis was carried out. Ki-67, PD-L1 expression, and tumor infiltrating lymphocytes were detected by immunohistochemistry. When available, the same paired sample after chemotherapy was analyzed. Pathological features and clinical characteristics were correlated to overall survival.

Results: TME and PD-L1 expression were assessed in 93 and 65 chemonaive MPM samples, respectively. Twenty-eight samples have not sufficient tumor tissue for PD-L1 expression. Sarcomatoid/biphasic samples were characterized by higher CD8+ T lymphocytes and PD-L1 expression on tumor cells, while epithelioid showed higher peritumoral CD4+ T and CD20+ B lymphocytes. Higher CD8+ T lymphocytes, CD68+ macrophages, and PD-L1 expression were associated with pathological features of aggressiveness (necrosis, grading, Ki-67). MPM cases characterized by higher CD8+ T-infiltrate showed lower response to chemotherapy and worse survival at univariate analysis. Patients stratification according to a combined score including CD8+ T lymphocytes, necrosis, mitosis, and proliferation index showed median overall survival of 11.3 months compared with 16.4 months in cases with high versus low combined score ($P < 0.003$). Subgroup exploratory analysis of 15 paired samples before and after chemotherapy showed a significant increase in cytotoxic T lymphocytes in MPM samples and PD-L1 expression in immune cells.

Conclusions: TME enriched with cytotoxic T lymphocytes is associated with higher levels of macrophages and PD-L1 expression on tumor cells and with aggressive histopathological features, lower response to chemotherapy and shorter survival. The role of chemotherapy as a tumor immunogenicity inducer should be confirmed in a larger validation set.

Key words: malignant pleural mesothelioma, tumor immune microenvironment, PD-L1, epithelioid, sarcomatoid

Introduction

Malignant pleural mesothelioma (MPM) is an occupational disease mainly due to asbestos exposure. Inhaled asbestos fibers cannot be broken down by the normal phagocytic process and persist in the pleural cavity, chronically activating macrophages, leading to the increase in the local immune infiltrate and malignant transformation of mesothelial cells [1, 2].

Given the close interaction between the immune infiltrate and mesothelial cells, the prognostic role of the immune microenvironment was investigated, mainly on small and heterogeneous series without conclusive data [3–6].

High tumor-associated macrophages (TAMs) ratio with CD8+ and CD20+ cells were found to be independent predictors of worse overall survival (OS) [6], while low lymphocyte to monocyte ratio in peripheral blood and tissue is reported to have negative prognostic significance [7].

The programmed cell death pathway (PD-1/PD-L1) plays a critical role in tumor immune escape control. PD-1 is mainly expressed on activated CD4 T cells, CD8 T and B cells [8]. PD-L1, the ligand of PD-1, is not only expressed in immune cells but also in some cells including cancer cells helping immune evasion by interacting with PD-1 on T-cells [9, 10].

On the basis of a high proportion of PD-L1 positive MPM, especially in nonepithelioid subgroup [11–13], treatment with immune checkpoint inhibitors is under active investigation also in this disease. Despite the remarkable durability of responses seen in some patients, the overall response rates with immunotherapy remain 10%–20% [14].

To date, PD-L1 expression has not shown a clear relationship with response to treatment in MPM [14, 15]. No evidence is available about the pathological features other than histology of MPM samples associated with different tumor immune microenvironment (TME) and checkpoint expression, and about their integrated prognostic value.

The primary aim of this retrospective study was to assess the TME and PD-L1 expression in epithelioid compared with nonepithelioid samples of a naïve MPM multicenter case series. Secondary end points were firstly to correlate TME and PD-L1 expression with several pathological features and patient outcome, then to develop a new composite prognostic score for patient stratification. TME and PD-L1 expression change after chemotherapy in a small subset of MPM samples was also explored.

Patients and methods

Patient samples and data collection

Starting from 2011, we centrally collected and analyzed epithelioid, biphasic, and sarcomatoid samples from the diagnostic biopsies of MPM patients who were referred to four Italian and one Slovenian cancer centers.

Chemo-naïve patients were considered eligible if they had a histological diagnosis of MPM, and adequate tumor samples for immunohistochemistry (IHC).

Clinical information about patients enrolled in the study was retrospectively collected.

Histology

Tissue samples were processed and classified according to the recent World Health Organization classification as epithelioid, biphasic, and sarcomatoid. For each chemo-naïve sample, necrosis and inflammation were evaluated over the entire tumor surface both in intratumoral and peritumoral areas and were quantified with a score of 0–3 (0: absent; 1: <10%; 2: 10%–20%; 3: >20%). Grading was categorized in three groups (I, II, and III) according to the nuclear grading system. Mitosis was counted in each square millimeter.

Immunohistochemistry

Inflammatory cell characterization was retrospectively carried out by IHC with specific antibodies. Immunoreactivity was expressed as percentage of positive cells on total inflammatory cells.

IHC for PD-L1 was also carried out. The positivity for PD-L1 was evaluated either in neoplastic cells or in the inflammatory background. Tumors with ≥1% of tumor cells were considered positive.

The proliferative index was expressed as number of Ki-67-positive cells on total cell number.

Statistics

The Mann–Whitney Rank Sum test was carried out to evaluate a different expression of TME and PD-L1 expression in the two histological subgroups and according to first-line treatment outcome. Correlation between TME and PD-L1 expression and tumor grading, necrosis, mitosis, and proliferation index were investigated through the Spearman linear correlation analysis.

OS curves were designed according to the Kaplan–Meier method. Univariate and multivariate analyses were carried out to show any possible impact of pathological parameters and clinical features on OS.

We further stratified patients according to a combined score based on the association of peritumoral and intratumoral CD8+ T lymphocytes, necrosis, mitosis, and proliferation index, which was considered in OS analysis. The differences between paired naïve and treated samples were assessed through Wilcoxon Signed Rank Test.

Further details on patients and methods are reported in the supplementary material, available at *Annals of Oncology* online.

Results

Patients

Ninety-three chemonaive MPM patients were enrolled in the study, 57 epithelioid and 36 nonepitheliod (22 biphasic and 14 sarcomatoid). Most patients were male, with ECOG PS 1, and a median age of 71 years. Patient characteristics are described in Table 1.

TME and PD-L1 expression in epithelioid compared with nonepithelioid MPM

TME was evaluable in all 93 chemonaive MPM samples, while PD-L1 expression in 65 (36 epithelioid and 29 sarcomatoid/biphasic); 28 samples showed not sufficient tumor cells to be analyzed for PD-L1 expression.

Peritumoral infiltrates of CD4+ T lymphocytes ($P = 0.03$) and CD20+ B lymphocytes ($P = 0.034$) were statistically higher in epithelioid samples compared with nonepithelioid (Figure 1A and B; supplementary Table S1, available at *Annals of Oncology* online). Peritumoral and intratumoral CD8+ T lymphocytes

Table 1. Patient features

	n = 93 (100%)
Age	Median 71 (range 36–85)
Gender	
Male	76 (82%)
Female	17 (18%)
ECOG PS	
0	18 (19%)
1	60 (65%)
2	14 (15%)
3	1 (1%)
EORTC PrS	
Low	40 (43%)
High	53 (57%)
Histology	
Epithelioid	57 (61%)
Biphasic	22 (24%)
Sarcomatoid	14 (15%)
First-line platinum-pemetrexed	78 (84%)
Stage	
I	7 (7%)
II	18 (19%)
III	34 (37%)
IV	34 (37%)
Response to first-line chemotherapy	
PR/SD	60 (77%)
PD	18 (23%)
Chemotherapy lines	
0–1	69 (74%)
>1	24 (26%)
Surgery	24 (26%)

ECOG PS, Eastern Cooperative Oncology Group Performance Status; EORTC PrS, European Organization for Research and Treatment of Cancer Prognostic Score; PR, partial response; SD, stable disease; PD, progressive disease.

were more represented in sarcomatoid/biphasic tumor samples, although without statistical significance (Figure 1C and D).

PD-L1 was positive in 35 MPM samples (18 epithelioid and 17 sarcomatoid/biphasic) and its expression was significantly higher in sarcomatoid/biphasic compared with epithelioid MPM samples ($P = 0.05$); 17 (59%) of sarcomatoid/biphasic cases were positive, with 20% median percentage of positive cells, while 18 (50%) of epithelioid cases were positive, with 1% median percentage of positive cells (Figure 1E).

No significant difference in terms of PD-L1 expression on immune infiltrate was observed between the two histologic subgroups (Figure 1F).

Correlation of TME and PD-L1 expression with pathological features

MPM samples with higher levels of CD68+ macrophages showed higher levels of CD8+ infiltrates at intratumoral [correlation coefficient (cc): 0.213; $P = 0.046$] and peritumoral levels (cc: 0.234; $P = 0.033$) (data not shown).

PD-L1 expression was significantly correlated with inflammatory cells, mainly in intratumoral areas (Table 2).

When we analyzed TME and pathological features, we observed higher levels of CD8+ lymphocytes and CD68+ macrophages either in intratumoral or peritumoral areas and PD-L1 expression in tumors with higher levels of necrosis.

Higher PD-L1 expression on tumor cells was correlated with tumor grading in epithelioid samples (Table 2 and supplementary Figure S1A and S1B, available at *Annals of Oncology* online). Ki-67 was significantly correlated with peritumoral and intratumoral CD8+ lymphocytes (Table 2 and supplementary Figure S1C and S1D, available at *Annals of Oncology* online).

Correlation of TME and PD-L1 expression with response to systemic treatment and patients outcome

Patients who achieved partial response/stable disease to first-line chemotherapy showed lower peritumoral CD3+ and CD8+ T lymphocytes, lower Ki-67, and mitosis. Even though not statistically significant, intratumoral CD3+ and CD8+ T lymphocytes were also lower in responders compared with progressive patients (supplementary Table S2, available at *Annals of Oncology* online).

Univariate analysis for OS showed a negative prognostic value of high peritumoral and intratumoral CD8+ T-lymphocyte infiltrate, which was confirmed in the epithelioid subgroup (Figure 2A and B and supplementary Figure S2, available at *Annals of Oncology* online). Median OS was 14–16.5 months in MPM patients with lower peritumoral ($P = 0.007$) and intratumoral ($P < 0.001$) CD8+ T lymphocytes, respectively, compared with 7.8 to 9.7 months in cases with higher CD8+ T lymphocytes, respectively.

In nonepithelioid subgroup, CD68+/CD8+ ratio showed a negative prognostic impact, with shorter survival of cases with higher ratio (supplementary Figure S2, available at *Annals of Oncology* online). Higher infiltrate of CD4+ T lymphocytes was associated with longer survival in the whole population ($P = 0.031$) and in the epithelioid subgroup. Moreover, we confirmed a negative prognostic impact of other pathological features such as high necrosis, proliferation index, mitosis, and sarcomatoid/biphasic histologic subtype (Figure 2D–G).

When considering the 'combination score', patients with high levels of peritumoral and intratumoral CD8+ T lymphocytes, necrosis, mitosis, and proliferation index showed shorter OS (Figure 2H). Median OS in cases with a higher combination score was 11.3 months compared with 16.4 months in cases with a lower combination score ($P < 0.003$).

At multivariate analysis, the only significant features associated with OS were surgery and response to first-line chemotherapy (supplementary Table S3, available at *Annals of Oncology* online).

Intratumor heterogeneity over time: TME and PD-L1 expression before and after chemotherapy

Paired MPM specimens (14 epithelioid and 1 biphasic) obtained for diagnostic purposes before platinum-pemetrexed chemotherapy and at the time of resection were analyzed. After

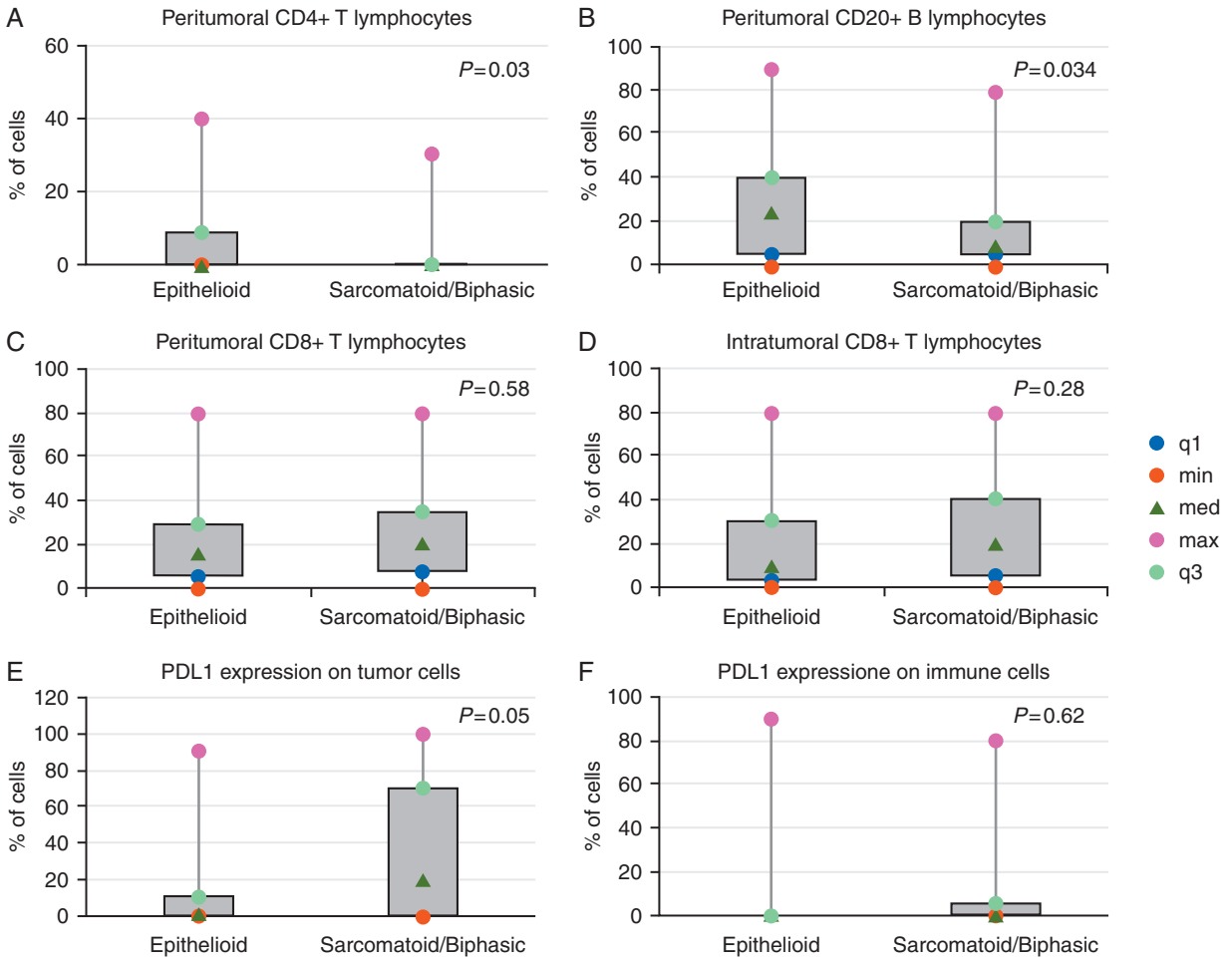

Figure 1. Main differences in B and T lymphocytes distribution (A–D). Different expression of PD-L1 on tumor cells (E) and immune cells (F) between epithelioid and sarcomatoid/biphasic MPM.

Table 2. Correlation among pathological features and tumor immune microenvironment and PD-L1 expression

	Necrosis cc; *P* value	Ki-67 cc; *P* value	Grading cc; *P* value	PD-L1 TC cc; *P* value	PD-L1 IC cc; *P* value
CD8+ P	**0.249; 0.02**	**0.257; 0.02**	0.05; 0.6	0.178; 0.169	0.203; 0.256
CD8+ I	**0.364; <0.001**	**0.312; 0.004**	0.129; 0.23	**0.28; 0.03**	**0.339; 0.05**
CD4+ P	0.05; 0, 624	0.131; 0.232	**–0.2; 0.05**	0.02; 0.89	0.142; 0.412
CD4+ I	0.06; 0.558	–0.109; 0.32	–0.11; 0.298	0; 0.95	0.237; 0.170
CD20+ P	0.127; 0.221	–0.11; 0.3	–0.18; 0.075	0.09; 0.48	0.08; 0.641
CD20+ I	0.06; 0.536	0.02; 0.8	–0.11; 0.269	0.172; 0.178	0.164; 0.34
CD3+ P	**0.246; 0.02**	0.183; 0.089	0; 0.998	0.192; 0.141	0.326; 0.068
CD3+ I	0.186; 0.076	**0.217; 0.04**	–0.1; 0.33	0.113; 0.393	0.297; 0.098
CD68+ P	**0.2; 0.05**	0, 114; 0.287	0.024; 0.818	0.173; 0.182	**0.43; 0.015**
CD68+ I	**0.289; 0.044**	–0.03; 0.77	0; 0.94	**0.39; 0.0014**	**0.49; 0.0028**
PD-L1 + TC	**0.337; 0.006**	0.211; 0.106	**0.256; 0.04**	NA	NA
PD-L1 + IC	**0.373; 0.027**	0.211; 0.251	0.196; 0.256	NA	NA

Statistically significant correlations are indicated in bold.
P, peritumoral; I, intratumoral; TC, tumor cell; IC, immune cell; cc, correlation coefficient; NA, not applicable.

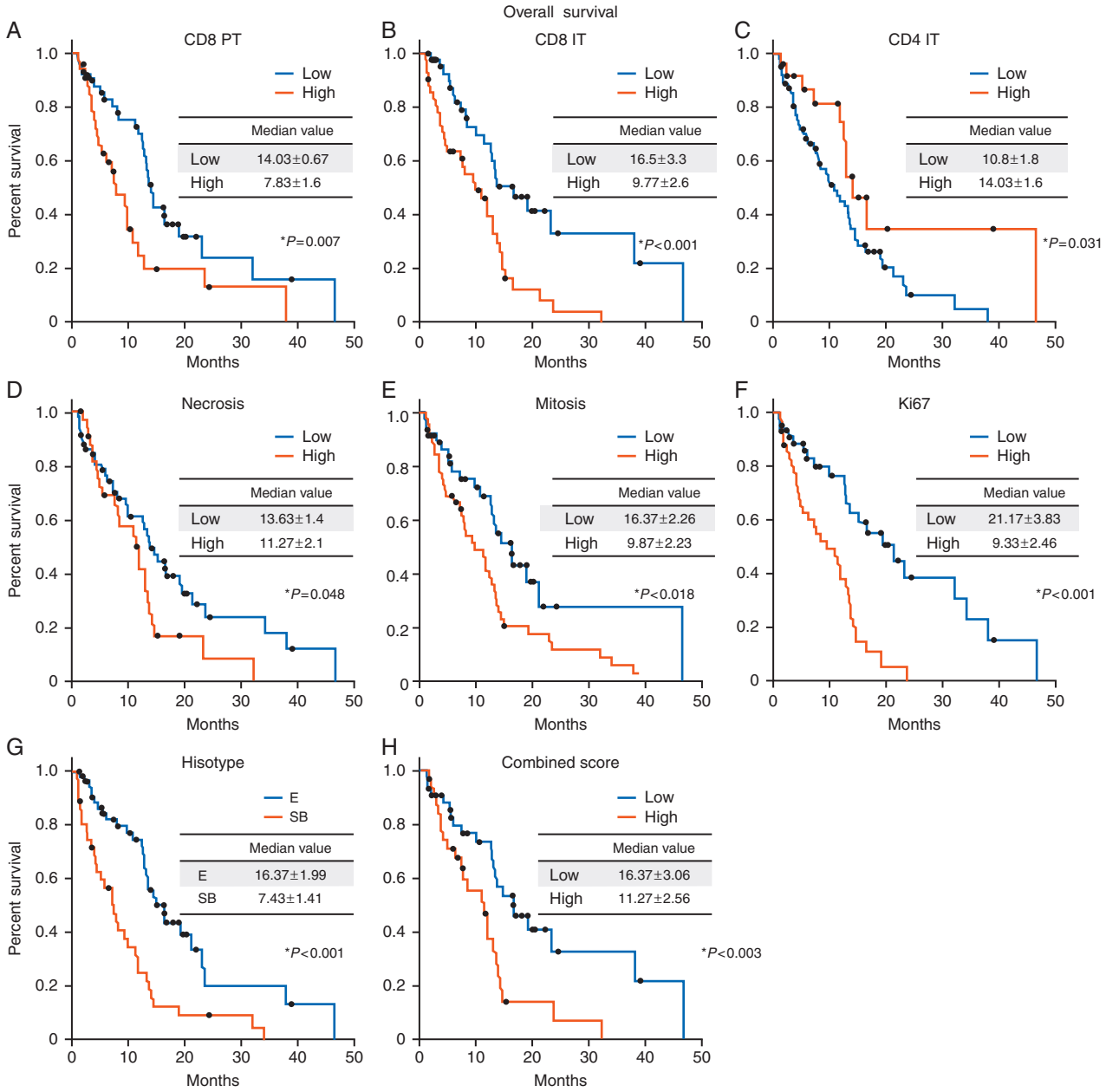

Figure 2. Kaplan–Meier survival curves according to: (A) peritumoral CD8+ T lymphocytes levels; (B) intratumoral CD8+ T lymphocytes levels; (C) intratumoral CD4+ T lymphocytes levels; (D) necrosis; (E) mitosis; (F) Ki-67; (G) histological subtypes; (H) combination score level. PT, peritumoral; IT, intratumoral; E, epithelioid; SB, sarcomatoid/biphasic.

chemotherapy, MPM samples showed peritumoral and intratumoral increase in CD68+ macrophages and CD3+ T lymphocytes, even though only peritumoral CD3+ lymphocytes significantly increased ($P = 0.001$) (supplementary Table S4, available at *Annals of Oncology* online). Low levels of CD4+ and CD8+ lymphocytes were observed in naive samples, while after chemotherapy CD8+ significantly increased both at peritumoral ($P = 0.012$) and intratumoral levels ($P = 0.05$) (Figures 3 and 4 and supplementary Table S4, available at *Annals of Oncology* online).

CD8+/CD68+ ratio increased after chemotherapy, although without statistical significance (data not shown). Chemotherapy-induced PD-L1 expression in tumor cells ($P = 0.02$) and even more in lymphomonocitic infiltrate ($P = 0.004$)

(Figures 3 and 4 and supplementary Table S4, available at *Annals of Oncology* online).

Discussion

Our work showed higher CD8+ T-lymphocyte infiltrate in MPM samples with aggressive biology (sarcomatoid/biphasic histology, higher necrosis, and proliferation index). Higher CD8+ T-lymphocyte infiltrate was associated with higher CD68+

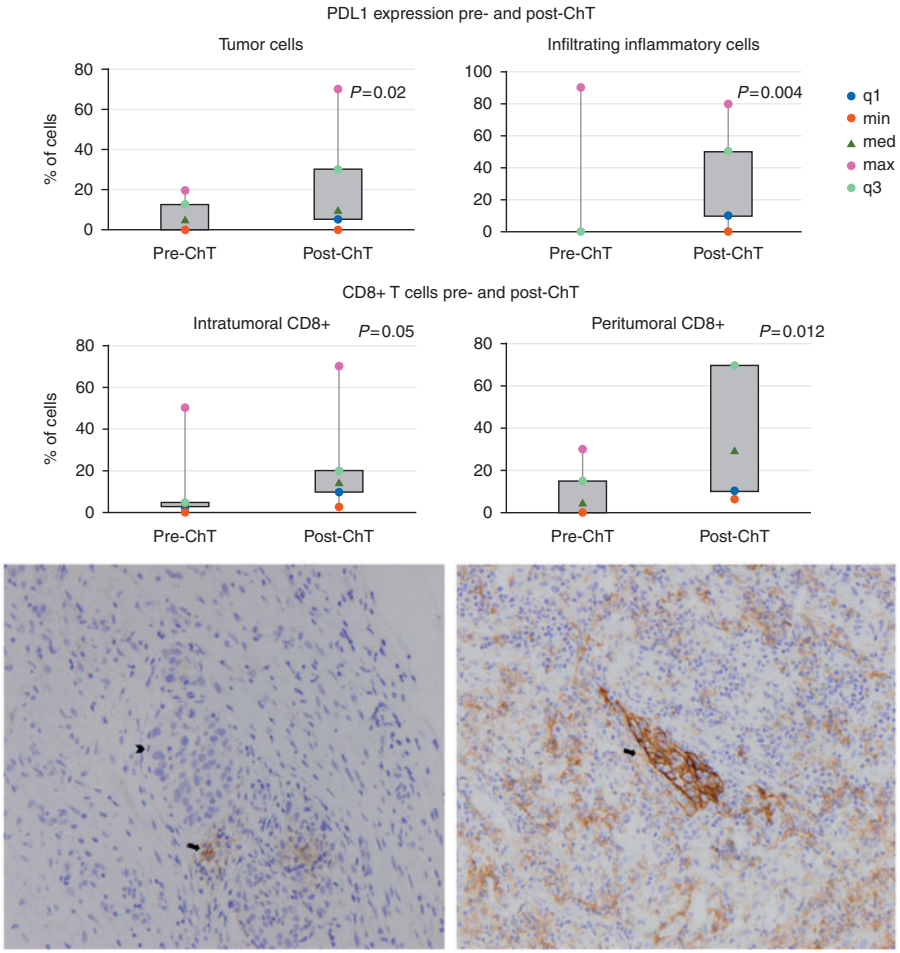

Figure 3. Different PD-L1 expression levels on tumor and immune cells and peritumoral and intratumoral CD8+ T lymphocytes in paired tumor samples from the same patients before and after chemotherapy. PD-L1 immunostained sections showed negative mesothelial cells (arrow head) and only few lymphocytes (arrow) before chemotherapy and strong positivity in many mesothelial cells (arrow on cluster of positive mesothelial cells) and many lymphocytes after chemotherapy; original magnification ×160, respectively. ChT, chemotherapy.

macrophages and PD-L1 expression; moreover, PD-L1 expression was upregulated in sarcomatoid/biphasic and in epithelioid tumors with higher grading.

While CD4+ T and CD20+ B lymphocytes infiltrate was associated with better prognosis, in line with the recent literature data on a wide epithelioid MPM case series [6], CD8+ T lymphocytes and CD68+ macrophages infiltrate was associated with shorter survival, and CD68+/CD8+ ratio evaluated in peritumoral stroma correlated with worse prognosis only in sarcomatoid/biphasic histotype. In contrast, most available literature data reported a favorable prognostic value of high levels of Tumor Infiltrating Lymphocytes in several tumors [16–18] and also in mesothelioma [3, 4]; however, their true biological role in suppressing and promoting tumor growth and metastasis is governed by several positive or negative T-cell factors as the PD/PD-L1 pathway. Tissue expression of PD-L1 has been reported in various tumors, including MPM, often associated with greater tumor aggressiveness and poor clinical outcome [11, 12, 19]. Indeed, PD-L1 expression could trigger a negative feedback loop favoring immune escape [13]. In our case series, high levels of CD8+ T lymphocytes concomitantly associated with high PD-L1 expression might explain our association with worse prognosis. Moreover, available series [3, 6, 7] included mostly epithelioid histotype, which might have its own prognostic value and often is suitable for surgery; on the contrary, our case series included balanced percentage of epithelioid and sarcomatoid/biphasic histologic subtypes, and this probably differentiates this work from other available.

Recent trials with checkpoint inhibitors in MPM showed that PD-L1 expression in tumor cells is not clearly predictive of a higher response rate [14, 15]. Thus, how can we optimize checkpoint inhibitor treatment outcome in MPM? Available evidence suggests that the answer lies in the tumor microenvironment, with particular reference to TAMs and their interconnection within the tumor and T cells [20].

In our analysis, we also observed a considerable variation in the proportion of infiltrating cells and PD-L1 across tumor samples and this could explain why only a minority of PD-L1 positive mesotheliomas responded to pembrolizumab in the KEYNOTE-028 study [14].

We used the cut-off point of 1% to define PD-L1 positive tumor samples; this was derived from clinical trials, even though

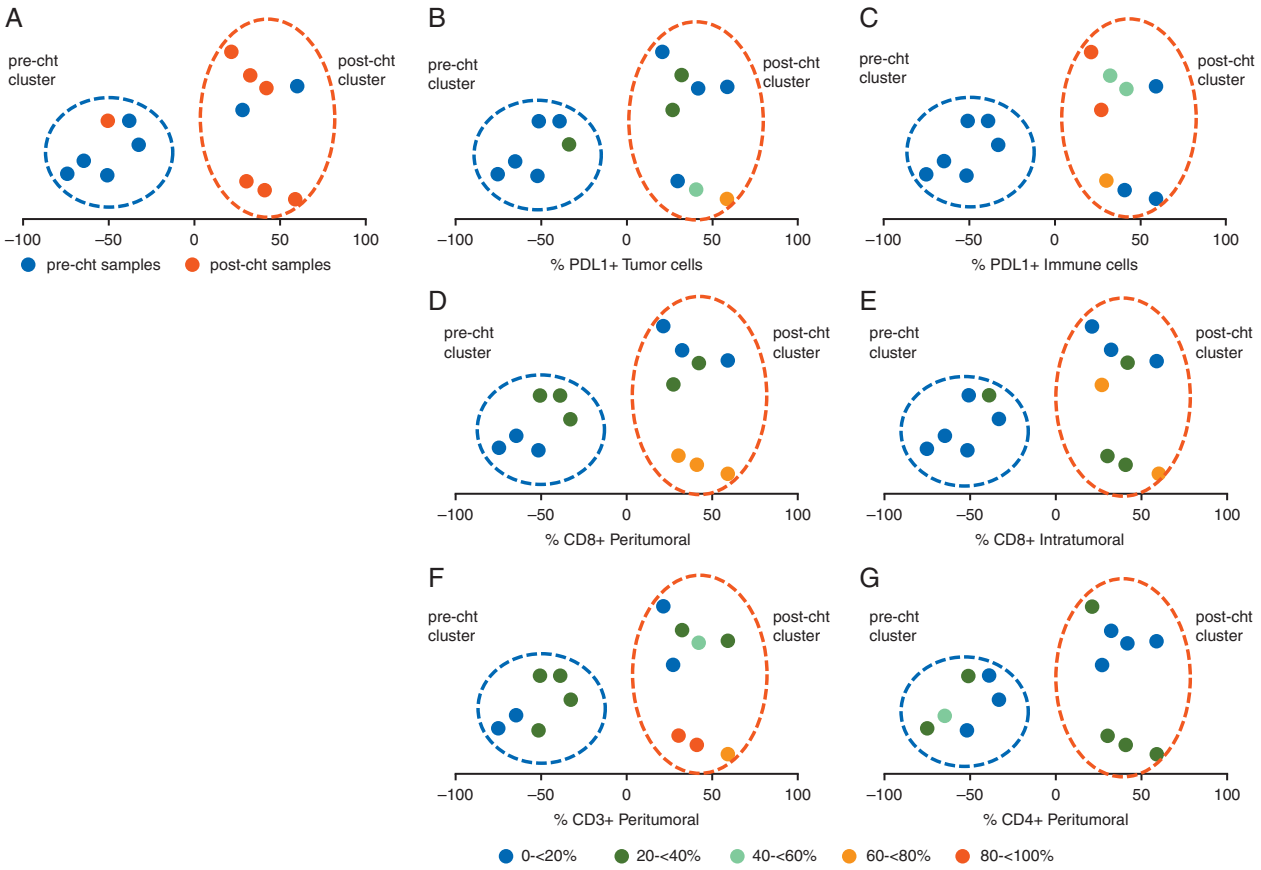

Figure 4. Comparison of samples before versus after chemotherapy, by t-SNE analysis. (A) Samples segregated into two distinct groups, a 'Prechemotherapy Cluster' (blue dotted circle) and a 'Postchemotherapy Cluster' (red dotted circle). The percentage of PD-L1 expression on tumor cells is shown in (B) and on immune cells is shown in (C). T CD8+ lymphocytes at peritumoral level are shown in (D) and at intratumoral level in (E). Peritumoral CD3+ T lymphocytes are shown in (F) and CD4+ T lymphocytes in (G). The percentage of positivity is color coded according to the heatmap provided. ChT, chemotherapy.

other cut-off points would be of interest, the small number of cases assessed for PD-L1 expression limited this evaluation.

All patients who underwent surgery showed lower CD8+ T-lymphocyte infiltrate and PD-L1 expression which increased after chemotherapy. The increase in PD-L1 expression was particularly evident in immune cells. These results could explain the failure of previous immunotherapy clinical trials, and suggest that combination strategies with immunotherapy, e.g. chemotherapy plus or before, could potentiate antitumor effects by promoting a robust T-cell response and favorable microenvironment for tumor antigen presentation and systemic immune response.

Today surgery is a controversial approach to MPM patients, thus the availability of specimens after cytotoxic treatments might be a challenge. However, on the basis of our results, it is tempting to speculate that a rebiopsy before inclusion in clinical trials with immunotherapeutic agents should be useful to estimate TME.

Conclusion

In summary, this is the first report that comprehensively evaluates tumor inflammatory cell distribution and PD-L1 protein expression in fragments from naïve mesothelioma—of all histologic subtypes—and in surgical specimens coming from a subset of the same patients who underwent surgery after systemic chemotherapy. The main limitations of the present work lie in the retrospective nature of our analyses, and in the lack of a validation set.

Recognition of the major actors playing a role in immune modulation, and their dynamicity over time and under chemotherapy pressure, might lead to new associations of immunogenic approaches for MPM treatment. Moreover, the identification of highly immunogenic tumor samples and their pathological features might help in patient selection for future clinical trials with anticancer immunotherapy and optimize the benefit and the cost-effectiveness of these drugs in MPM. A prospective confirmatory study might answer to these unmet needs.

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Disclosure

The authors have declared no conflicts of interest.

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