

Association of combined PD-L1 expression and tumor-infiltrating lymphocytes features with survival and treatment outcomes in patients with metastatic melanoma

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ABSTRACT

Background: Recent advances obtained with immune checkpoint inhibitors (ICIs) targeting the Programmed cell death-1 (PD-1) protein have significantly improved the outcome of patients with metastatic melanoma. The PD-L1 expression in tumor cells as detected by immunohistochemistry is a predictive biomarker in some solid tumors, but appears insufficient as prognostic or predictive factor of response to ICIs in metastatic melanomas.

Objectives: We investigated whether the presence and the features of pretreatment CD8+ tumor-infiltrating T lymphocytes (TILs) could be a complementary prognostic or predictive biomarker in patients with metastatic melanoma.

Methods: In this retrospective study, we evaluated the association of PD-L1 expression $\geq 5\%$ of tumor cells combined with TILs features (CD8, CD28, Ki67) with the overall survival (OS) among 51 patients treated with ICIs and 54 patients treated with other treatment options (non-ICIs).

Results: PD-L1 positivity was observed in 33% and 39% of primary melanomas and matched metastases, respectively, with, however, poor concordance between the primary and the matched metastatic site ($\kappa=0.283$). No significant association was noted between PD-L1 expression and CD8+TILs profile analyzed as single markers and OS or response to immunotherapy. Instead, their combined analysis in primary melanoma samples showed that the PD-L1-/CD8+ status was significantly associated with prolonged OS in the whole population ($P=0.04$) and in the subgroup treated with non-

ICIs ($P=0.009$). Conversely, the PD-L1+/CD8+ status was a good prognostic factor in patients treated with ICIs ($P=0.022$), whereas was significantly associated with poor prognosis in patients treated with non-ICIs ($P=0.014$). While the expression of CD28 was not related to outcome, the Ki67 expression was significantly associated to poor OS in the subgroup CD8+TIL+/PD-L1- ($P=0.02$).

Conclusions: The pre-treatment combination of PD-L1 expression with the level of CD8+TILs could better assess OS and predict therapeutic response of patients with metastatic melanoma treated either by immunotherapy or other treatments regimens.

Keywords: metastatic melanoma, immunotherapy, chemotherapy, PD-L1, CD8 TILs, CD28, Ki67, outcome.

INTRODUCTION

Malignant melanoma is among the types of cancer whose incidence and mortality significantly increased in the last decades. A total of 232,000 new cases are diagnosed and more than 55,000 patients die from a metastatic melanoma each year across the world [1]. Metastatic melanoma represents a highly aggressive form of skin cancer, with an overall 5-year survival rate of less than 20% and a median survival time of approximately 9 months for stage IV disease [2].

The advent of the immunotherapy strategies, in particular the immune checkpoint inhibitors targeting Programmed Death-1 (PD-1) and its ligand PD-L1, represented a true paradigm shift with an impressive 58% increase in the 3-year median survival [3-5]. Following the first phase I study evaluating nivolumab [5], several clinical trials have investigated the use of immunohistochemically (IHC) expression of PD-L1 in tumor cells as a biomarker to predict response to anti-PD-1/PD-L1 immune checkpoint inhibitors (ICIs). PD-L1 expression in tumor cells was found to predict a good response to immunotherapy; however, a significant clinical benefit was also observed in patients whose tumor did not express PD-L1 [6, 7]. Durable response to ICIs is limited to a subset of melanoma patients, while 40% of patients do not respond to anti-PD-1 inhibitors in monotherapy. In most clinical trials, the expression of PD-L1 IHC alone did not allow optimal selection of responding patients [4]. This biomarker currently appears

insufficient to predict a therapeutic response to ICIs in patients with metastatic melanoma, paving the way for further research to optimize predictive tests.

Moreover, the prognostic value of the PD-L1 expression in patients with metastatic melanoma remains controversial, reportedly being associated with either poor or good prognosis according to various studies [8-11]. Therefore, PD-L1 expression does not appear to be a reliable prognostic biomarker for routine practice.

While PD-L1 alone is currently inadequate, as prognostic and predictive marker in metastatic melanoma, other potential biomarkers are currently emerging. The presence of intra-tumor CD8+ tumor-infiltrating lymphocytes (CD8+TIL) could prove to be an important prognostic and predictive marker, particularly in association with the expression of PD-L1 in tumor cells [11]. Some studies have shown the importance of the microenvironment including the analysis of CD8+TIL and the potential correlation with a good response to anti-PD-1 inhibitors in some solid tumors, including metastatic melanoma [7, 11-13].

The classification of tumors into 4 groups, based on the presence or absence of CD8+TILs and the expression of PD-L1 in tumor cells, has recently been proposed to predict the response to immunotherapy [14]. However, studies evaluating the impact of such classification for the stratification of melanoma patients treated with immunotherapy are limited [15, 16].

Recent studies demonstrated that PD-1 suppresses T cell function primarily by inactivating CD28 signaling, while the rescue of exhausted CD8+ TILs by PD-1 targeted therapies is CD28-dependent, suggesting that T cell costimulatory receptor CD28 could be a primary target for PD-1-mediated inhibition [17, 18].

The objective of our study was to correlate the expression of PD-L1 in tumor cells, combined with the quantification of CD8+TILs and their activation status (CD28, Ki67), to overall survival (OS) and response to treatment in order to determine whether this combination could be a more effective prognostic and predictive biomarker than IHC PD-L1 alone.

PATIENTS AND METHODS

Study population

This retrospective cohort included patients with primary cutaneous metastatic malignant melanoma (stage III/IV) diagnosed between July 2013 and February 2017 and treated at the Department of Dermatology, University of Nice, Archet 2 Hospital (Nice, France). The formalin-fixed paraffin-embedded (FFPE) tumor samples were retrieved from different laboratories: Laboratory of Clinical and Experimental Pathology (Pasteur Hospital, Nice, France), Medipath (Mougins, France), DIAG (Nice, France) and CAP (Nice, France). The availability of histological material from the primary tumor and metastasis was a required criterion to include a case in the study.

A total of 202 patients with metastatic melanoma were initially included in the study. Out of these 97 (48%) were excluded for various reasons (primary melanoma not available, small size of the sample, regressed primary melanoma, mucosal melanoma) leaving 105 patients in which the primary tumor and the first metastasis were available. Out of 105 patients 91 presented with regional metastases (35 in transit and 56 lymph node metastases) and 14 with distant metastases (8 lung, and 6 subcutaneous).

The LDH levels at baseline were measured before initiation of the systemic treatment. The assay was performed in 76 patients.

Two groups of patients were distinguished for this study: a group of 51 patients (48.5%) who received at least one treatment of immunotherapy (anti-PD-1 inhibitors-pembrolizumab/nivolumab and/or anti-CTLA4) and a group of 54 patients (51.5%) who have not had immunotherapy treatment, albeit some had other treatments (chemotherapy or targeted therapies with anti-BRAF and anti-MEK agents). Among the 51 patients of the group treated with immunotherapy, 29 (57%) have had exclusively immunotherapy while 22 (43%) have received an immunotherapy treatment before or after having other treatments (chemotherapy or targeted therapies).

All tumor specimens were used with the informed consent from the patients (Hospital-Integrated Biobank BB-0033-00025, Pasteur Hospital, Nice, France).

Histology analysis

Specimens were fixed in 10% formalin, embedded in paraffin, and 4- μ m thick serial tissue sections obtained and stained with hematoxylin and eosin (H&E) for histologic

evaluation. The H&E stained sections were independently assessed by 2 pathologists (CBE and VH) and the histopathological data including histologic subtype, Breslow thickness, ulceration and TNM stage were recorded. The primary malignant melanomas were reclassified with the 8th edition of the AJCC (American Joint Committee Cancer) tumor, node, and metastasis classification. The density of the inflammatory infiltrate (none, mild, moderate, marked) and its location were assessed to determine the presence or the absence of TILs.

Immunohistochemical analysis

Formalin-fixed paraffin-embedded (FFPE) freshly cut serial 4µm thick tissue sections, mounted on positively charged slides were stained for PD-L1 with the anti-human PD-L1 rabbit monoclonal antibody, clone 28-8 (kit PD-L1 PharmDx, Dako). PD-L1 IHC was performed using a Autostainer Link 48 Dako automated staining instrument, according to the manufacturer's recommendations. Staining was performed within 2 steps: a pre-treatment phase (PT Link Dako and EnVision FLEX Target Retrieval low pH solution) during 53 minutes and then the tissue sections were incubated with PD-L1 antibody during 30 minutes with the other components of the PD-L1 28-8 pharmDx kit, followed with a wash buffer (EnVision FLEX) and a 7 minutes counter-stained with hematoxylin (EnVision FLEX).

In addition, CD8, CD28 and Ki67 IHC assays were performed using a BenchMark ULTRA automated staining instrument (Ventana Medical Systems, Tucson, AZ, USA). FFPE freshly cut serial tissue sections were stained with a rabbit monoclonal anti-CD8 antibody (clone SP57; Ventana), a rabbit polyclonal anti-CD28 antibody (SIGMA Life Science) and a rabbit monoclonal anti-Ki67 antibody (clone 30-9; Ventana).

Each IHC run contained a positive control (tonsil) and a negative Ab control (buffer, no primary Ab).

Staining evaluation

The IHC staining was independently assessed by 2 pathologists (CBE, VH). When a discrepancy between the pathologists was noted, the slides were jointly reviewed on a multi-head microscope with a third pathologist (MI or PH) to obtain a consensus.

PD-L1 staining was assessed on tumor cells and was considered positive if at least 5% of the tumor cells exhibited membranous PD-L1 staining of any intensity in a tissue section containing at least 100 cells that could be evaluated, as previously described in clinical trials using the corresponding anti-PD1/PD-L1 inhibitors [4, 19, 20].

Assessment of CD8⁺TILs was performed by using Clark's modified grading system, as recommended by the International Immuno-Oncology Biomarkers Working Group [21, 22]. This grading system is based on the density (absent/mild/moderate/marked, based on H&E staining, score 0–3) and distribution (absent/focal/multifocal/diffuse, score 0–3) of TILs expressing CD8 at a threshold of 10% [21, 22]. TILs were defined as lymphocytes infiltrating and disrupting tumor nests and/or in direct contact with tumor cells. The possible combinations were collapsed into four TILs grades as follows: grade 0 = absent; grade 1 = mild or moderate focal infiltrate, or mild multifocal infiltrate; grade 2 = marked focal, moderate or marked multifocal or mild diffuse infiltrate; grade 3 = moderate or marked diffuse infiltrate. Intense infiltrate referred to a strong heavy lymphocytic infiltrate of a density equivalent to that seen in a lymph node with metastasis [23]. Intratumoral TILs were defined as lymphocytes infiltrating and disrupting tumor nests and/or in direct contact with the invasive tumor area as observed by hematoxylin and eosin staining. Peritumoral TILs were defined as lymphocytes located at distance from the tumor area, perivascular and inside the stromal fibrosis. The grades 1 to 3 TILs expressing CD8 at a threshold of 10% defined the CD8⁺TILs positive group.

Primary tumors and the matched metastases were classified in 4 groups on the basis of their PD-L1 IHC status and presence (grade 1 to 3) or absence of CD8⁺TILs: 1) CD8⁺TIL⁺/PD-L1⁺, 2) CD8⁺TIL⁻/PD-L1⁻, 3) CD8⁺TIL⁺/PD-L1⁻ and 4) CD8⁺TIL⁻/PD-L1⁺. In order to better define the activated T lymphocytes, CD28 and Ki67 were analyzed only in the CD8⁺TIL positive group (n=64).

CD28 staining was assessed considering the percentage of lymphocytes staining for CD28 compared to the lymphocytes staining for CD8. Similarly, the assessment of Ki67 was performed considering the percentage of lymphocytes stained for Ki67 compared to the lymphocytes staining for CD8.

***BRAF* molecular analysis**

The *BRAF* mutational status was determined on tumor DNA isolated from FFPE tissue samples of melanoma metastases using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Pyrosequencing of *BRAF* exon 15 using the Therascreen *BRAF* Pyro Kit (Qiagen), was performed as previously described [24].

Statistical analysis

All statistical analyzes were performed at alpha risk = 5% under bilateral assumption using R.3.2.3 software on Windows. Qualitative data were presented as absolute frequencies, percentages, 95% confidence intervals, missing data percentages. These data were compared using the Chi2 test or the Fisher test in case of non-compliance with Chi2 application conditions. Quantitative data were described by medians, extremes, means, standard deviations and percentages of missing data. These data were compared using the Student's T-test or the Mann-Whitney test in case of non-compliance with the Student test conditions. Overall survival (OS) since primary was defined as the interval between the date of diagnosis of the primary tumor and the date of death of the patient or of the last follow up. OS since the first metastasis was defined as the interval between the date of diagnosis of the first metastasis and the date of death of the patient or of the last follow up. Patients lost to follow-up were censored on the date of last contact. These data were described by survival rates, survival medians and 95% confidence intervals. The survival curves were compared by the Log-Rank test. Kaplan-Meier survival curves were determined to assess the prognostic significance of PD-L1 expression and CD8+TILs (grade 1 to 3) on OS. The cut-off predicting OS for CD28 expression level and Ki-67 index was evaluated graphically by using inflection points of the smoothing spline curve fit for both analytes and confirmed by using statistical rules assessed using R function "bestcut2" for survival data model. Thus, the cutoff was defined at 20 for both analytes. Multivariate analyzes were performed using Cox regression models with corresponding adjusted Hazard Ratio (HR) calculations. For the whole study, maximum patient follow-up was limited to 60 months. p-values <0.05 indicated statistical significance.

RESULTS

PD-L1 expression patterns

The main clinical and histo-molecular characteristics of this cohort are shown in **Table 1**. The PD-L1 expression $\geq 5\%$ tumor cells was observed in 35 out of 105 (33.3%) primary melanomas (range, 5%-50%) and in 41 out of 105 (39%) first metastasis of the matched primary melanoma (range, 5%-100%). There was a poor intra-patient concordance between PD-L1 expression status of the primary melanoma and the first metastatic site ($\kappa=0.283$; 95%CI, 0.072-0.494).

PD-L1 expression was membranous, essentially heterogeneous and focal, most often at the periphery of the tumor, located at the tumor invasion front (**Figure 1**). The mononuclear cells of the tumor microenvironment were mostly lymphocytes with occasional plasma cells or macrophages. This infiltrate was most often located at the border of the tumor areas, as aggregates, and/or more rarely intra-tumoral as isolated cells. The topography and density of this infiltrate was clearly visible on the IHC CD8 staining (**Figure 2**).

Correlations with the clinicopathological characteristics

There was a significant association between the PD-L1 expression status ($\geq 5\%$ tumor cells) and the presence of CD8+TILs ($p=0.008$, **Table 2**).

More than half of primary melanomas were infiltrated with CD8+TILs (64/105; 60.9%; grade 1 to 3). The classification into four subgroups based on the PD-L1 status and the presence or absence of CD8+TILs (grade 1 to 3) [14], showed that the subgroups PD-L1-/TILs+ (34%) and PD-L1-/TILs- (32%) were the most represented, followed by the subgroup PD-L1+/TILs+ (27%) while the subgroup PD-L1+/TILs- (7%) was poorly represented (**Table 2, Figure 3**).

In the metastatic lesions, CD8+TILs positive status was observed in 46.6% (49/105) of cases and the distribution of groups showed a predominance of the PD-L1-/TILs group (45%) followed by the PD-L1+/TILs+ group (30%) and a significant decrease in the PD-L1-/TILs+ infiltrates (16%). The PD-L1+/TILs- group was poorly represented (9%) ($p < 0.001$, **Table 2**).

There was no significant correlation between the CD28 and Ki67 expression in the 64 primary melanomas ($p=0.70$). Moreover, there was no significant association between the PD-L1 status and gender ($p=0.46$), histological subtype ($p=0.23$), ulceration ($p=0.37$), AJCC-T subgrouping ($p=0.54$) or stage ($p=0.19$), *BRAF* status ($p=0.39$) and LDH level at baseline ($p=0.15$).

No significant difference was observed for all of these data in the two groups of patients receiving or not immunotherapy treatment, except for age. Patients treated with immunotherapy were slightly younger than patients not receiving immunotherapy (median, 60 years *versus* 66 years, $p=0.031$).

Overall survival analysis in the whole population

The pTNM stage, high baseline LDH levels and the presence of ulceration were significantly associated to poor OS in our population ($p=0.044$, $p=0.043$, and $p=0.00068$, respectively). Conversely, independently adjusted PD-L1 expression and CD8+TILs status were not significantly correlated with OS ($p=0.50$ and $p=0.27$, respectively).

The OS analysis according to the CD8+TILs/PD-L1 status showed that the PD-L1-/TILs+ subgroup compared to the other groups analyzed together, demonstrated better OS ($p=0.041$, **Figure 4B**). The percentage of patients alive at 60 months was 72% (26/36) for the PD-L1-/TILs+ subgroup compared to 50% (35/69) for all patients in the other 3 groups combined.

Among the 64 primary melanomas tested, the CD28 expression was not a significant prognostic factor for OS, independently of the cutoff (20, $p=0.253$; and 70, $p=0.343$ respectively; data not shown). Similarly, when the CD28 expression was adjusted according to the subgroups PD-L1+/TILs+ and PD-L1-/TILs+, no significant difference was observed, regardless of the cutoff ($p=0.06$, $p=0.936$, $p=0.297$, $p=0.319$; data not shown).

In contrast, the Ki67 expression analyzed alone and in the PD-L1-/CD8+TIL+ subgroup was a significant prognostic factor for poor OS ($p=0.02$; data not shown).

The multivariate analysis, adjusted according to age, histological type, presence of ulceration and pTNM stage, showed that the PD-L1-/TILs+ subgroup was a significant independent prognostic factor for better OS (HR, 0.41; 95% CI, 0.17-0.98; $p=0.04$).

Finally, the univariate OS analyses described for the primary melanomas were also performed according to the expression levels on the first metastasis, regardless of the metastatic site. The expression of PD-L1, the status CD8+TIL and CD8+TIL/PD-L1 subgroups were not significantly related to OS (data not shown).

Overall survival analysis in primary melanoma not treated with immunotherapy

In the population of patients who had never received immunotherapy, PD-L1 expression and CD8+TILs status analyzed alone were not significant prognostic factors ($p=0.11$ and $p=0.61$, respectively); data not shown). Conversely, the PD-L1/CD8+TILs status was significantly associated with favorable OS ($p=0.015$; **Figure 5A**).

The PD-L1-/CD8+TILs+ subgroup compared to the other 3 groups was significantly correlated with better OS ($p=0.009$; **Figure 5B**), with a percentage of patients alive at 60 months of 83% (15/18) versus 47% for the other 3 groups combined (17/36). These results were confirmed in a multivariate analysis that showed that PD-L1-/TILs+ status was a good independent prognostic factor (HR, 0.05; 95% CI, 0.007-0.36; $p=0.002$).

In contrast, the PD-L1+/CD8+TILs+ status, compared to the other 3 groups, was a poor prognostic factor ($p=0.014$; **Figure 5C**). The percentage of patients alive at 60 months was 37% (6/16) compared to 68% for the 3 combined groups (26/38). The PD-L1+/CD8+TILs+ status was confirmed as a significant and independent prognostic factor associated with poor OS by multivariate analysis (HR, 6.7; 95% CI, 1.63-27.93; $p=0.007$).

Overall survival analysis in primary melanoma treated with immunotherapy

In the population of patients treated by immunotherapy, the PD-L1 expression and the CD8+TILs status analyzed alone were not significant prognostic factors ($p=0.50$ and $p=0.30$, respectively; data not shown).

There was no significant difference in OS when analyzing the PD-L1/CD8+TILs groups individually ($p=0.63$). Moreover, the PD-L1-/CD8+TILs status compared to the other 3 groups was not a significant prognostic factor for OS ($p=0.86$; data not shown). As opposed to that, the PD-L1+/CD8+TILs subgroup was correlated to better OS with an increased percentage of patients alive at 60 months compared to the other 3 groups

(66%, 8/12 of patients alive *versus* 53%, 21/39; $p=0.034$; **Figure 5D**).

In the multivariate analysis, including age (HR, 2.277; 95% CI, 0.356-8.02, $p=0.168$), histology (HR, 1.689; 95% CI, 0.689-7.531; $p=0.501$), stage (HR, 3.695; 95% CI, 1.213-11.257; $p=0.018$), ulceration (HR, 2.983; 95% CI, 1.298-6.855; $p=0.008$) and baseline LDH levels (HR, 0.815; 95% CI, 0.294-2.259; $p=0.687$), the stage and PD-L1+/TILs+ status were significant and independent prognostic factors associated with OS compared to the other groups (PD-L1+/TILs+ status; HR, 0.138; 95% CI, 0.024-0.779; $p=0.022$).

DISCUSSION

Treatment with immune checkpoint inhibitors in patients with metastatic melanoma demonstrates impressive response rates. However, although the benefit is restricted to approximately 40% of patients treated with anti-PD-1 therapy, there are no approved stratification strategies for immunotherapy in melanoma [25]. Thus, there is an acute need for robust predictive biomarkers to guide the clinical decision-making [26].

Melanoma is one of the tumors with the highest somatic mutation rates among solid tumors, which is thought to be related to their high immunogenicity (i.e. their ability to induce an adaptive immune response specifically directed against tumor antigens) [27]. In melanoma, the PD-L1 expression in tumor cells is in most cases adaptive, being rarely related to constitutive the activation of mitogen-activated protein kinase pathways [7, 28].

In agreement with previous studies, we found that the adaptive PD-L1 response is constituted by focal expression in tumor cells, often at the periphery of the tumor, at the tumor invasion front, and close to an inflammatory CD8+TIL, [15].

The PD-L1 status is notoriously difficult to estimate due to the variability of methods used across studies but also related to the intrinsic intra- and inter-tumor heterogeneity.

The IHC tests used in the majority of clinical trials employed two different clones (e.g., 28-8 and 22C3) and the positivity thresholds vary from 1% to 5% of tumor cells. Moreover, in some studies the PD-L1 positive immune cells were also taken into account in evaluating the levels of expression and calculating the positivity threshold

(25). In clinical trials, the most widely used positivity threshold is $\geq 5\%$ of tumor cells when using the pharmDx 28-8 kit [4, 5, 20, 29].

Second, there are significant variations in PD-L1 expression within the same tumor and between samples from different tumor sites in the same patient [30, 31]. Given the intra-tumor heterogeneity and in order to analyze the largest tumor area, we evaluated the expression of PD-L1 on whole tissue sections, which can better reflect the distribution and type of cells expressing PD-L1 and also allows assessing the relationship between PD-L1 expression on tumor cells and on immune cells.

As a consequence of the adaptive immune resistance mechanism and the existence of immunogenic or non-immunogenic tumors, the infiltration of immune cells into the tumor, particularly T cells, associated with the expression of PD-L1 could be an important predictive biomarker for PD-1/PD-L1 checkpoint inhibitors [32].

In our study, the proportion of primary melanomas expressing PD-L1 on tumor cells was 33%, similar to some previous reports [15], while other studies reported higher rates. One study observed PD-L1 positivity in 51% of cases, but with a positivity threshold of 1% [30], whereas another study using a 5% threshold reported 53% PD-L1 positive rate; however, the site of sampling, primary or metastatic, was not specified [7].

Most studies that have investigated the predictive value of PD-L1 expression for the response to anti-PD-1/PD-L1 therapy in metastatic melanomas have not distinguished the primary or metastatic site [7, 13, 19]. In our study, the PD-L1 expression was more frequent in metastases (39% versus 33%). Our results are similar to a study by Taube *et al.* (43% versus 35%) [15], while conflicting results have been reported by Madore *et al.* (51% for primary, 57% for regional metastases, and 42% for distant metastases) [30].

We did not find any significant association between PD-L1 expression on tumor cells of primary melanoma and overall survival, as previously shown [15, 33]. In melanoma, the prognostic significance of PD-L1 expression remains controversial, being associated with either a poor [8-10], or with a better prognosis [11].

In our cohort, PD-L1 expression was not significantly associated to the response of PD-1/PD-L1 agents. Whereas the expression of PD-L1 by tumor cells may be predictive of a good response to immunotherapy, nevertheless, a significant clinical benefit can also be observed in most studies in the negative PD-L1 group, even if response rates remain

lower [6, 7, 34]. Overall, the variability of the methods used, the absence of a standardized positivity threshold, the spatio-temporal heterogeneity and the presence of an immunotherapy response in PD-L1 negative patients are such that PD-L1 does not appear to be a good standalone biomarker for response to immunotherapy in melanoma.

The presence of intra-tumoral CD8+TILs is frequently observed in melanomas [15]. In our study 60.9% of primary melanomas had a positive CD8+TILs status with a lower rate in the metastatic samples, for any site combined (46.6%).

In the whole population, we have not found a significant association between the presence of CD8+TILs in primary melanoma or metastases and overall survival, as previously observed in other series [32, 35]. Most studies have shown that the presence of a high density of TILs in primary melanomas was significantly associated with better outcome [36, 37], suggesting that a functional lymphocyte infiltration is necessary for the effectiveness of PD-1/PD-L1 blockage. Moreover, the CD8+TILs status, studied alone in our series, was not predictive of response, unlike previous reports [13, 34].

However, the presence of CD8+TILs infiltrate in the tumor could be an important clinical biomarker when combined to PD-L1 expression. Two studies suggested that the expression of PD-L1 should be interpreted taking into account the tumor microenvironment. The tumors were categorized into 4 different types of tumor microenvironment based on the presence or absence of TILs and PD-L1 expression, suggesting that this classification could be more effective to predict the response to immunotherapy [14, 15].

In our study, the association between the PD-L1 expression and the presence of CD8+TILs was found in 26.6% of the primary melanomas, the majority groups being the CD8+TIL-/PD-L1- and CD8+TIL+/PD-L1- groups. PD-L1 expression without associated CD8+TILs was rare.

These observations were different on metastatic samples. The CD8+TIL-/PD-L1- group became the majority while the CD8+TIL+/PD-L1- group was half as high as in primary melanomas. It is interesting to note that approximately the same distribution of these different groups was found by Taube *et al.*, on both primary and metastatic melanomas [15]. A review of the literature, reports a different distribution showing a predominance of

CD8+TIL+/PD-L1+ (38%) and CD8+TIL-/PD-L1- (41%) status but without distinction between primary and metastatic melanomas [14].

Certain CD8+TIL/PD-L1 subgroups demonstrated prognostic and predictive value in the 105 primary melanomas, depending on the patient group studied, general population, patients treated or not by immunotherapy.

Thus, the group of patients with PD-L1-/CD8+TILs+ status is a group with a good independent prognosis in the general population and even more significantly in the group of patients not treated with immunotherapy. Conversely, in the group of patients treated with immunotherapy, this profile was not a significant prognostic factor for overall survival. It would appear that patients with PD-L1-/CD8+TILs+ tumors are not good responders to immunotherapy. Indeed, immunotherapy does not seem to improve their survival.

Interestingly, our results suggest that for the choice of first line treatment of mutated *BRAF* patients, the PD-L1-/CD8+TILs+ - subgroup could benefit from a combination of targeted therapy (anti-BRAF and anti-MEK). On the other hand, the PD-L1+/CD8+TILs+ subgroup, which is correlated with a good prognosis when treated with immunotherapy, could benefit from first line immunotherapy, despite the mutated *BRAF* status. There was no significant correlation between PD-L1 expression and *BRAF* mutation status, as previously showed in the literature [38].

Furthermore, we showed that the PD-L1+/CD8+TILs+ profile was an independent factor of poor prognosis, significantly observed in the group of patients not treated with immunotherapy, with only 37% of patients alive at 60 months. Conversely, in patients treated by immunotherapy, this profile was an independent factor associated with better outcome, with 66% of patients still alive at 60 months, as previously reported in cutaneous melanomas [13, 39]. In the whole study population and without distinguishing therapeutic management, these results were not significantly discriminated. The tendency of the PD-L1+/CD8+TILs+ subgroup to have a poor prognosis appears to be masked in the general population by patients who have received immunotherapy and whose prognosis has been improved.

With regard to the PD-L1-/CD8+TILs- profile, we could not show a prognostic or predictive significance for response to immunotherapy. However, the percentage of

patients alive at 60 months in the general population and in the groups of patients treated or not with immunotherapy remained stable around 47%. This suggests that the absence of CD8+TILs associated with the absence of PD-L1 expression may be a poor prognostic factor, associated to the absence of response to immunotherapy.

Teng *et al.* suggests that, given the absence of pre-existing functional T lymphocytes, the PD-L1-/CD8+TILs- profile would be associated with poor prognosis and patients would then likely not respond to anti-PD1 monotherapy, whereas the combination of anti-CTLA4 and anti-PD1 agents would be beneficial for these patients [14]. Indeed, anti-CTLA4 would allow early activation of T cells that would be recruited into the tumor and induce adaptive expression of PD-L1, which would then be the target of anti-PD1 [14]. The immunotherapy combinatorial strategies were not in the scope of our study; however, such strategies are of high importance for tumours that do not express PD-L1, and it would therefore be interesting to study the efficacy based on the four PD-L1/CD8+TILs patterns [29, 40].

Finally, the PD-L1+/CD8+TILs- status was not found to be a prognostic nor predictive factor for a response to immunotherapy in any of our patient groups. However, this status, reflecting a constitutive expression of PD-L1, is very poorly represented in our series, as in the various series described in the literature [15]. Finally, while the CD28 expression was not significantly associated to outcome, the Ki67 expression was significantly associated with poor survival in the PD-L1-/CD8+TILs+, suggesting that the prognostic value may be driven by activated CD8+ T cells in the absence of PD-L1 expression.

There are a number of limitations in our study. This is a single-institutional retrospective study with a modest sample size, even though all available cases at Nice University Hospital were collected at the time of the study. Although the hypothesis of this study is well-substantiated in the literature, an independent validation cohort would be optimal.

Another limitation is related to the heterogeneity of the 51 patients treated with immunotherapy. Although the majority of patients received anti-PD-1 monotherapy, few patients had two different lines of immunotherapy (anti-CTLA4 followed by anti-PD-1) and more rarely other types of associated treatments (chemotherapy or targeted therapy) during the follow-up.

In summary, our study shows that PD-L1 expression alone is not a robust prognostic factor in patients with metastatic malignant melanoma, whether this status is assessed on the primary tumor or on the matched metastasis. In exchange, the additional assessment of the CD8+TILs infiltrates could better assess the overall survival of subgroups of patients and predict the therapeutic response of patients with metastatic melanoma treated either by immunotherapy or other treatments regimens. Moreover, this IHC assay can be easily applied to current conventional routine testing and may offer valuable clinical information when considering different treatment options in the absence of established methods for patient stratification.

Table 1. Clinical and histomolecular characteristics of the metastatic melanoma cohorts treated by chemotherapy or immunotherapy. * χ^2 -test or Student's t-test were used to investigate difference between groups.

| Characteristics | Immunotherapy-naive patients (n=54), % | Patients treated by immunotherapy (n=51), % | Total (n=105), % | p-value* |
|-----------------------------------|--|---|------------------|----------|
| Gender | | | | 0.30 |
| Female | 21 (60.00) | 14 (40.00) | 35 (33.33) | |
| Male | 33 (47.14) | 37 (52.86) | 70 (66.67) | |
| Age (y) | | | | |
| Mean | 66.59 | 60.55 | 63.66 | 0.031 |
| Range | 23-92 | 26-83 | 23-92 | |
| LDH baseline | | | | 0.99 |
| Normal | 26 (40.00%) | 39 (60.00%) | 65 | |
| High | 5 (45.45%) | 6 (54.55%) | 11 | |
| Histological subtype | | | | 0.83 |
| Superficial spreading melanoma | 30 (53.57) | 26 (46.43) | 56 (53.33) | |
| Nodular melanoma | 15 (46.88) | 17 (53.12) | 32 (30.48) | |
| Acral lentiginous melanoma | 3 (50.00) | 3 (50.00) | 6 (5.71) | |
| Invasive lentigo maligna melanoma | 3 (75.00) | 1 (25.00) | 4 (3.81) | |
| Not classified | 3 (42.85) | 4 (57.14) | 7 (6.67) | |
| Ulceration | | | | 0.77 |
| Absent | 25 (49.02) | 26 (50.98) | 51 (48.57) | |

| | | | | |
|---------------------------|------------|------------|------------|-------|
| Present | 29 (53.70) | 25 (46.30) | 54 (51.43) | |
| pT stage | | | | 0.84 |
| T1 | 7 (58.33) | 5 (41.67) | 12 (11.43) | |
| T2 | 10 (55.56) | 8 (44.44) | 18 (17.14) | |
| T3 | 20 (52.63) | 18 (47.37) | 38 (36.19) | |
| T4 | 17 (45.95) | 20 (54.05) | 37 (35.24) | |
| pN stage | | | | 0.58 |
| N0 | 47 (52.22) | 43 (47.78) | 90 (85.71) | |
| N1a | 0 (0.00) | 1 (100.00) | 1 (0.95) | |
| N1b | 0 (0.00) | 1 (100.00) | 1 (0.95) | |
| N1c | 6 (60.00) | 4 (40.00) | 10 (9.52) | |
| N3 | 1 (33.33) | 2 (66.67) | 3 (2.86) | |
| Stage at diagnosis | | | | 0.53 |
| I | 13 (61.90) | 8 (38.10) | 21 (20.00) | |
| II | 34 (50.00) | 34 (50.00) | 68 (64.76) | |
| III | 7 (46.67) | 8 (53.33) | 15 (14.28) | |
| IV | 0 (0.00) | 1 (100.00) | 1 (0.95) | |
| BRAF status | | | | 0.071 |
| Mutation | 25 (62.50) | 15 (37.50) | 40 (38.10) | |
| Wild-type | 29 (44.61) | 36 (55.38) | 65 (61.90) | |

Table 2. PD-L1 expression and distribution of the CD8⁺TILs and PD-L1/CD8⁺TILs subgroups in primary melanomas and the paired metastases.

| | Status | Primary melanoma (n=105) | Metastases (n=105) |
|----------------------------|----------|-----------------------------|-----------------------|
| PD-L1 expression | | | |
| | Negative | 70 (67%) | 64 (61%) |
| | Positive | 35 (33%) | 41 (39%) |
| CD8⁺TILs | | | |
| | Negative | 41 (39%) | 56 (53%) |

| | | | |
|--|--------------|----------|----------|
| | Positive | 64 (61%) | 49 (47%) |
| PD-L1/CD8⁺TILs subgroups | | | |
| | PD-L1-/TILs- | 34 (32%) | 47 (45%) |
| | PD-L1+/TILs- | 7 (7%) | 9 (9%) |
| | PD-L1-/TILs+ | 36 (34%) | 17 (16%) |
| | PD-L1+/TILs+ | 28 (27%) | 32 (30%) |

Legend to Figures

Figure 1. Various patterns of PD-L1 expression in melanocytic tumor cells. (A) PD-L1 expressed in 20% of tumor cells at the periphery of tumor areas (original magnification, x100). **(B)** Heterogeneous PD-L1 expression in 50% of tumor cells (original magnification, x100). **(C)** Strong membranous expression (original magnification, x400). **(D)** Low to moderate membranous expression (original magnification, x400).

Figure 2. Distribution patterns of tumor infiltrating lymphocytes (TILs). (A) HES slide (original magnification, x100). **(B)** HES slide (original magnification, x200). **(C)** Periphery excluded infiltrates of CD8⁺TILs (original magnification, x100). **(D)** Periphery and intratumoral infiltrates of CD8⁺TILs (original magnification, x100). **(E)** Immune-desert with absence of CD8⁺TILs (original magnification, x100). **(F)** Peritumoral CD8⁺TILs infiltrates (original magnification, x100).

Figure 3. Patterns observed in the PD-L1+/CD8⁺TILs⁺ and PD-L1-/CD8⁺TILs⁺ subgroups.

Melanoma with inflammatory infiltrate **(A, HES)**, demonstrating presence of CD8⁺TILs **(B)**, associated with the expression of PD-L1 in tumor cells **(C)**. Original magnification, x100.

Melanoma with inflammatory infiltrate (**D**, HES), demonstrating presence of CD8+TILs (**E**), but no expression of PD-L1 in tumor cells (**F**). Original magnification, x100.

Figure 4. Kaplan-Meier overall survival curves according to PD-L1/CD8+TILs status in the 105 primary melanomas. (A) Analysis of the four groups PD-L1/CD8+TILs. **(B)** Analysis of the PD-L1-/CD8+TILs group alone compared to the other 3 groups. The p-values were calculated using the Log-rank test.

Figure 5. Kaplan-Meier overall survival curves according to PD-L1/CD8+TILs status in the primary melanomas non treated by immunotherapy (n=54) or treated by immunotherapy (n=51).

(A) The four groups PD-L1/CD8+TILs in patients non treated by immunotherapy. **(B)** PD-L1-/CD8+TILs+ group alone compared to the other 3 groups, in patients non treated by immunotherapy. **(C)** PD-L1+/CD8+TILs+ group alone compared to the other 3 groups, in patients non treated by immunotherapy. **(D)** PD-L1+/CD8+TILs+ group alone compared to the other 3 groups, in patients treated by immunotherapy. The p-values were calculated using the Log-rank test.

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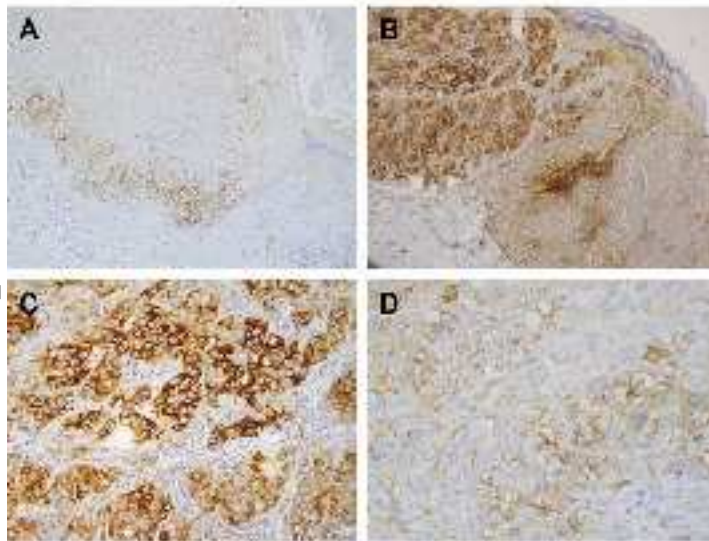
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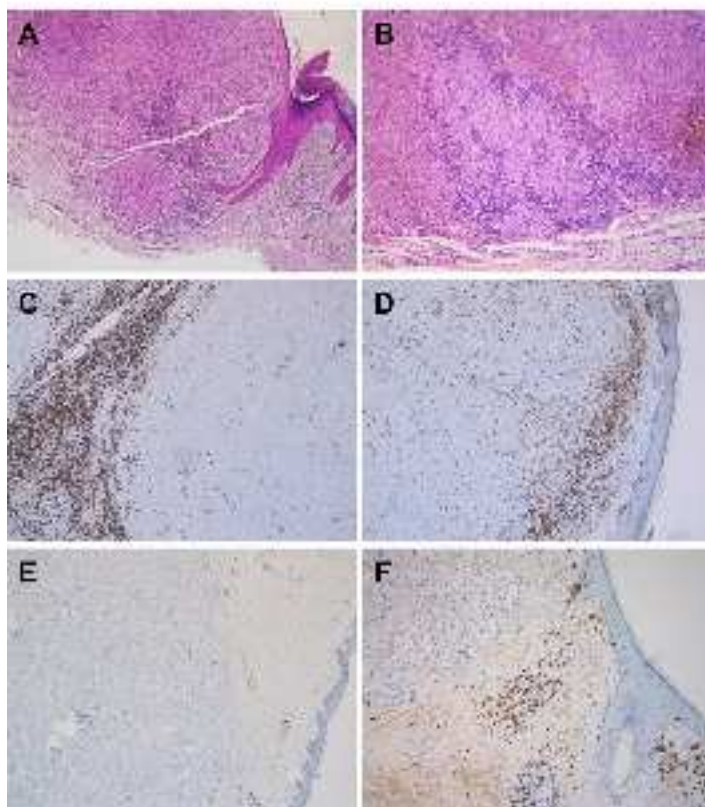
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Figure 1



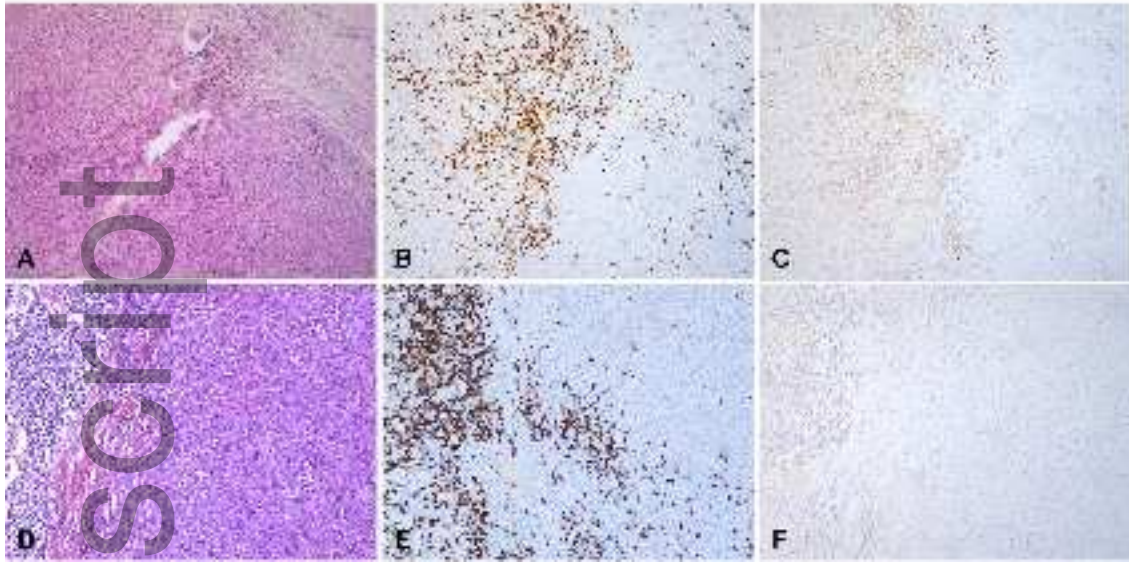
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Figure 2



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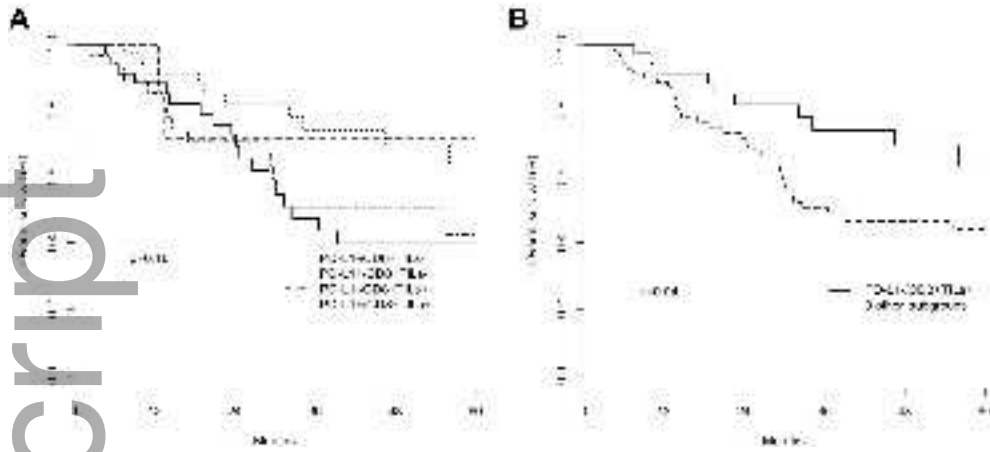
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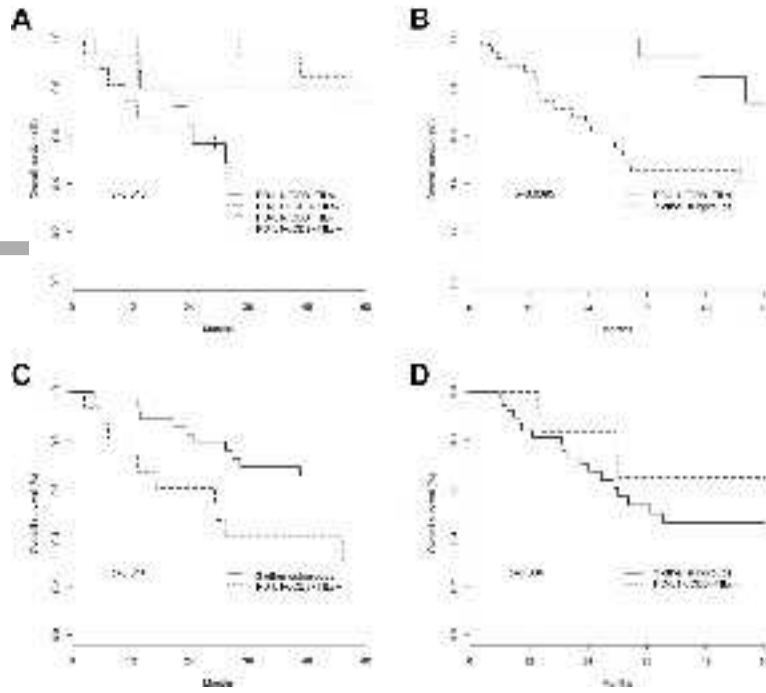
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Figure 5



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