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HEALTH SCIENCES

Density of high endothelial venules and PDL-1 expression: relationship with tumor-infiltrating lymphocytes in primary cutaneous melanomas

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Abstract: Studies have highlighted melanoma immunogenicity, and the prognostic importance of tumor infiltrating lymphocytes (TILs) and mechanisms of tumor immune evasion, such as hyperexpression of programmed cell death ligand 1 (PDL-1). High endothelial venules (HEV) are specialized blood vessels that can facilitate the lymphocytes migration to the tumor. Here we evaluate the association of HEV density and PDL-1 expression in primary cutaneous melanomas with the presence and degree of TILs and with other clinicopathological variables (age, sex, tumor location, melanoma histological type, Breslow thickness, ulceration, regression signs, mitotic index). HEV density and PDL-1 expression were assessed immunohistochemically in 78 melanoma cases, using a specific antibody, and were detected in 59% and 76% of these, respectively. Positive associations were identified between HEV density and PDL-1 expression with the presence and degree of lymphocytic infiltration, melanoma histological type and ulceration presence. No correlation was found between HEV density and PDL-1 expression. Our findings confirm the HEV role in the recruitment and facilitation of lymphocyte transport in cutaneous melanomas, where HEV density is strongly associated with the degree of TILs. Additionally, PDL-1 hyperexpression suggests a possible mechanism of tumor immune evasion, which may lead to inactivation and reduction of the tumor lymphocytes number.

Key words: MECA-79, Melanoma, PDL-1M, tumor infiltrating lymphocytes, tumor microenvironment.

INTRODUCTION

Cutaneous melanomas are aggressive neoplasms that derive from melanocytes and have a high metastatic potential. Mortality rates are high and have increased worldwide in recent decades (Taylor et al. 2007, Fortes et al. 2015, Keun Park et al. 2017).

Several studies have highlighted the immunogenic capacity of melanoma and the importance of tumor infiltrating lymphocytes (TILs) as a manifestation of host immune response (Taylor et al. 2007, Azimi et al. 2012, Thomas et al. 2013, Duprat et al. 2016, Maibach et al. 2020). There is evidence that the presence and degree of TILs, in both primary and metastatic cutaneous melanomas, has a favorable impact on melanoma overall survival, with a lower chance of developing regional and distant lymph node metastases, and is a strong marker of immune response (Clemente et al. 1996, Taylor et al. 2007, Azimi et al. 2012, Thomas et al. 2013, Fortes et al. 2015, Duprat et al. 2016, Keun Park et al. 2017). In this context, the possible role of high endothelial venules (HEV) in melanomas and other solid tumors has been highlighted (Hayasaka et al. 2010, Martinet et al. 2011, 2012a, Avram et al. 2013, Gallimore et al. 2013, Perivoliotis et al. 2017, Asrir et al. 2022).

Investigations into lymphocyte recirculation led to the discovery of L-selectin as a receptor on lymphocytes, involved in their interaction with HEV in lymph nodes. Further studies have shown that L-selectin mediates the interaction and circulation of lymphocytes through HEVs, as the first step in the adhesion signaling cascade that culminates in the recruitment of lymphocytes to lymph nodes (Hemmerich et al. 1994, Drayton et al. 2003, Uchimura & Rosen 2006, Weinstein & Storkus 2016). The presence and density of these venules can be assessed by the expression of the specific marker MECA-79 (Michie et al. 1993, Miyasaka & Tanaka 2004, Middleton et al. 2005, Sinha et al. 2006, Martinet et al. 2012b, Avram et al. 2013). The discovery of MECA-79, a monoclonal antibody (mAb) that reacts with the family of sialomucins collectively known as Peripheral Node Addressin (PNAd), gave rise to a powerful tool for the study of HEVs that express ligands for L-selectin (Michie et al. 1993, Middleton et al. 2005, Martinet et al. 2012a).

Given the diversity of tumor microenvironments, several mechanisms promote tumor immune evasion, resulting in tumor progression (Sapoznik et al. 2012, Quail & Joyce 2013, Taube et al. 2013). One such mechanism is the overexpression of programmed cell death ligand 1 (PDL-1). The binding of PDL-1 to its programmed cell death protein 1 (PD-1) receptor in immune cells inactivates the TILs in the tumor microenvironment. This suggests that PDL-1 expression in tumor cells, evaluated by immunohistochemistry (IHC), has an adverse prognostic value, and reinforces its association with TILs in a variety of solid tumors, including melanoma (Hino et al. 2010, Kashani-Sabet 2010, Leite et al. 2015, Massi et al. 2014, Muenst et al. 2014, Schalper 2014, Velcheti et al. 2014, Jin et al. 2015, Wimberly et al. 2015, Wu et al. 2015, Xu et al. 2015, Beckers et al. 2016, Zeng et al. 2016, Juneja et al. 2017). In this context, immunotherapy studies have shown that blocking the PD-1/PDL-1 axis can reactivate the antitumor immune response, with clinical benefits (Hasan et al. 2011, Sapoznik et al. 2012, Sanlorenzo et al. 2014, Ascierto & Marincola 2015, Philips & Atkins 2015, Chen et al. 2016, Daud et al. 2016, Carbognin et al. 2015).

The aim of this study was to determine not only HEV density and PDL-1 expression in primary cutaneous melanomas, but also the association of these markers with the presence and degree of TILs and with other clinicopathological variables such as age, sex, tumor location, melanoma histological type, Breslow thickness, ulceration, regression signs and mitotic index. To the best of our knowledge, no studies to date have focused on establishing the association of HEV density with PDL-1 expression in primary cutaneous melanomas.

MATERIALS AND METHODS

Case Selection

A retrospective study was conducted to analyze 78 samples from 76 patients with primary cutaneous melanoma, stored in a pathology laboratory in southern Brazil. This study was approved by the Ethics Committee for Research with Human Beings of the University of Passo Fundo (CEP: 78759317.9.0000.5342). The cases were selected from specimens taken only from the complete resection of lesions, which consisted of superficial spreading melanoma (SSM), nodular melanoma (NM), lentigo maligna melanoma (LMM) or acral lentiginous melanoma (ALM). Samples from incisional biopsy, *in situ* melanomas, or melanomas not classified in the four above described histological types, or samples contained within little paraffin block material, were excluded from the study. The clinical characteristics analyzed here included the patient's age and sex and anatomical site of the lesion.

Histopathological Analysis

Melanoma was diagnosed by analyzing tumor sections stained by hematoxylin and eosin (H&E), under an optical microscope, in samples fixed in 10% buffered formalin and embedded in paraffin. Histopathological characteristics such as: histological type (SSM, NM, LMM and ALM), Clark level, Breslow thickness, presence and degree of lymphocytic infiltration, ulceration, mitosis and signs of tumor regression were analyzed. Breslow thickness was assessed according to the criteria of the American Joint Committee on Cancer (Amin et al. 2017). Tumor regression was evaluated according to the following criteria of the College of American Pathologists (Shon et al. 2021, 2022): replacement of tumor cells by lymphocytic inflammation, as well as attenuation of epidermal and dermal fibrosis with inflammatory cells, melanophagocytosis and telangiectasia. The intensity of lymphocytic infiltration was assessed semi-quantitatively, and varied from 0 to 3, as follows: 0 = absent (no lymphocytic infiltration); 1 = mild (lymphocytes focally present in the peritumoral area without intratumoral extension); 2 = moderate (presence of lymphocytes in the peritumoral area with prominent intratumoral extension); 3 = marked (marked lymphocytic infiltration in the intra- and peritumoral areas) (Taube et al. 2014).

Immunohistochemical (IHC) Analysis

The previously analyzed paraffin blocks of the H&E sections were selected in 3µm sections for the IHC analysis.

The monoclonal antibodies Peripheral Node Addressin (clone MECA-79 0.5mg, Novus Biological®) and anti-PDL-1 (clone E1L3N 0.1mg, Cell Signaling®) were used for the detection of high endothelial venules (HEV) and of PDL-1 expression. The slides were incubated overnight with the primary antibodies at room temperature in dilutions of 1:150 and 1:300, respectively. Controls positive for MECA-79 (palatine tonsils) and for PDL-1 (placenta) were included on all the slides. Immunostaining was processed using the chromogen diaminobenzidine (DAB) and all the slides were counterstained with Giemsa.

The IHC analysis was performed by two pathologists participating in this study, using a Zeiss Axio Scope.A1 optical microscope, and the photomicrographs were taken with a Zeiss AxioCam 5.0 camera.

Quantification of High Endothelial Venule Density

The absolute number of MECA-79-positive venules was quantified in the largest area of each tumor section. Venule density, expressed as the number of positive venules per mm^2, was calculated semi-quantitatively for each case, based on previous studies (Martinet et al. 2012a, Avram et al. 2013).

Evaluation of Pdl-1 Expression

PDL-1 expression was analyzed semi-quantitatively, based on the percentage (%) of labeled tumor cells (TC). Samples were considered positive when complete or incomplete cell membrane immunostaining of any intensity was observed in ≥ 1% of the TC. The following immunohistochemical scores (IHCS) were used for each immunohistochemical section: IHCS 0: < de 1% IHCS 1: ≥1% < 5% IHCS 2: ≥ 5% < 10%; IHCS 3: ≥ 10% (52). The intensity of membrane staining in TC was evaluated as weak (1), moderate (2) and intense (3). The location of PDL-1 positive TCs in relation to TILs was also analyzed.

Statistical analysis

The statistical analysis was performed using the SPSS 2.0 software package. The absolute and relative frequencies of the tumor's clinicopathological characteristics were listed.

The association of HEV density (MECA79 +) and PDL-1 expression with the clinicopathological variables was determined using the Chi-square test and the non-parametric Mann-Whitney and Kruskal-Wallis tests. The association between HEV density and PDL-1 expression was determined via the Kruskal-Wallis test. A p value of <0.05 was considered statistically significant.

RESULTS

The average age of the patients (60.3% women and 39.7% men) was 59.8 years. The most common site of primary melanoma was on the trunk (28.2%), followed by the lower limbs (24.4%), head and neck (20.5%) and upper limbs (20.5%). The predominant histological type was SSM (60.3%), followed by NM (17.9%), LMM (14.1%) and ALM (7.7%). Melanomas of the trunk were more frequent among males (63.6%), while melanomas of the lower limbs were more frequent among females (84.2%), with a statistical difference (p = 0.016). A comparison of the patient's sex and type of melanoma revealed no statistical difference (p = 0.2). A higher rate of SSM (57.8%) was found among individuals younger than 55 years old. Most of the patients older than 55 had LMM (55.4%) and ALM (66.7%). Most of the tumors presented Clark levels II (30.8%) and III (38.5%), and Breslow thickness of up to 1mm (47.4%). Tumor infiltrating lymphocytes (TILs) were found in 94.9%, ulceration in 29.5% and regression signs in 19.2% of the patients.

MECA-79-positive venules were found in 69.2% (54/78) of the melanoma samples. The mean density of all MECA-79-positive venules per mm^2 of tumor area was 2.38. Areas with higher HEV density also showed more prominent lymphocytic infiltrate (Fig. 1a,b).

A positive association was found between HEV density and lymphocytic infiltration. This association was stronger with moderate (2) and severe (3) infiltrates, with significant differences in relation to absent or mild infiltrates (p <0.001) (Fig. 2). Higher HEV density was also associated with melanoma histological type, observed in SSM (2.59) and LMM (4.74), with a statistical difference (p <0.025). Melanomas with Breslow thickness of up to 1mm presented higher HEV density (3.7), with a statistical difference (p <0.004). Ulceration was observed in melanomas with a lower density of MECA-79-positive venules (p <0.003). No significant association was found between HEV density and Clark level, anatomical site of the lesion, presence of mitosis and regression signs. Table I summarizes the clinicopathological characteristics of melanomas and their association with HEV density.

PDL-1 expression on TC was observed in 59% (46/78) of the specimens examined. When present, it was predominantly observed in association with TILs (Fig. 3a-d). This pattern was more conspicuous when the infiltrate intensity was moderate to severe than when it was mild or absent, and showed a significant association (p <0.007). Moreover, PDL-1 expression levels were higher the greater the intensity of lymphocytic infiltrate (Fig. 4). No significant association was found between PDL-1 expression levels and histological type, lesion site, Clark level, Breslow thickness, ulceration, regression signs and mitoses. It should be noted that, although there was no significant difference, thicker melanomas showed higher levels of PDL-1 expression and most of the acral melanomas were negative. Table II summarizes the association between PDL-1 expression and the pathological features of melanomas.

No association was found between PDL-1 expression and MECA-79-positive venule density (Fig. 5).

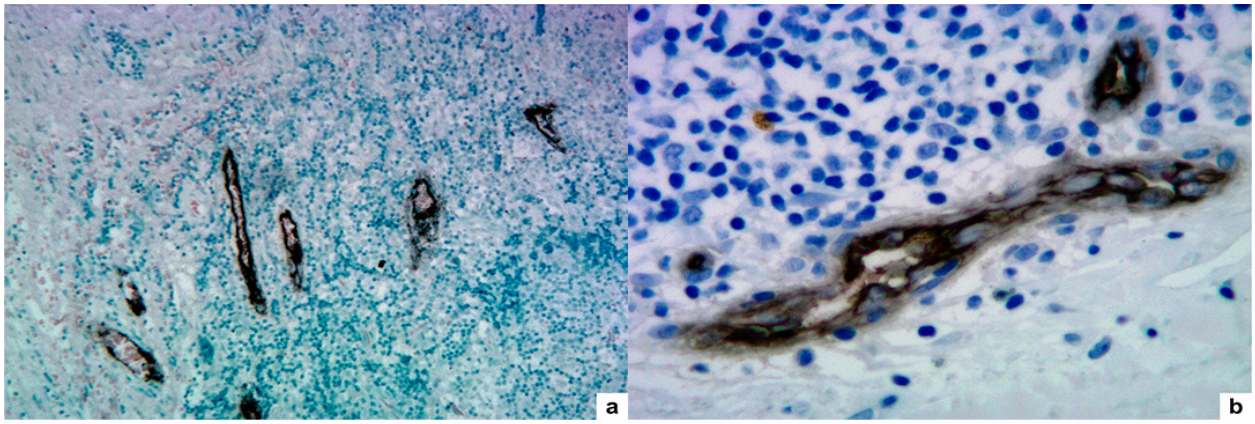

Figure 1. IHC with MECA-79. a) Positive HEVs in the area of the tumor (100x magnification). b) Positive HEVs among TILs (400x magnification).

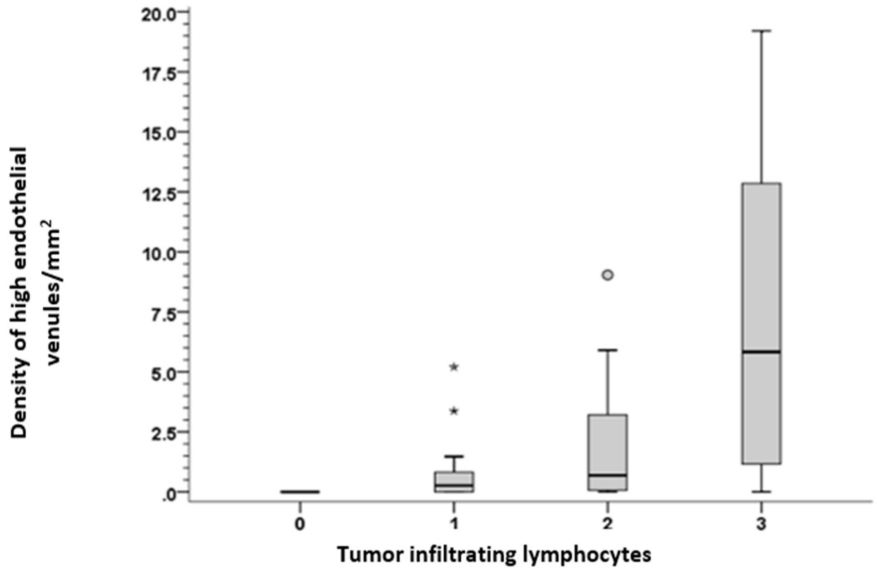

Figure 2. Density of HEVs and TILs in primary cutaneous melanomas.

DISCUSSION

In Brazil, cutaneous melanoma occurs homogeneously in both sexes, but slightly more frequently among women, albeit without significant differences (Matheus & Verri 2015), a fact that was confirmed in this study. Moreover, individuals younger than 40 years of age rarely suffer from cutaneous melanoma. After the fourth decade of life, age-specific incidence rates increase, peaking in the seventh and eighth decades. As for primary tumor location, the preferential location among men is on the trunk and among women on the lower limbs (McCourt et al. 2014, Matheus & Verri 2015). It is also known that age is associated with tumor location, with melanomas on the trunk occurring between the 5th and 6th decades, and head and neck melanomas occurring in patients in their 60s and 70s (Matheus & Verri 2015). In our study, the higher frequency of melanoma among women and of the SSM and NM types is consistent with previously published data, as is the finding that these tumors occur more commonly on the trunk, among men, and on the

Table I. Association between clinicopathological characteristics and HEV density in primary cutaneous melanomas.

	N	HEV/mm²	p	Test
Sex			0.973	MW
Male	31	2.38		
Female	27	2.52		
Age			0.187	MW
< 55	33	2.07		
> 55	41	2.32		
Anatomical site			0.181	KW
Head and neck	16	4.45		
Trunk	22	3.30		
Lower extremities	19	0.16		
Upper extremities	16	2.91		
Histological type			0.096	KW
SSM	47	2.59		
NM	14	0.79		
LMM	11	4.74		
ALM	6	0.17		
Breslow thickness			0.004	KW
Up to 1mm	37	3.74		
1.01 to 2.0 mm	13	2.57		
2.01 to 4.0mm	16	0.71		
> 4.0 mm	12	0.24		
Ulceration			0.003	MW
Present	23	0.57		
Absent	55	3.14		
Regression signs			0.148	MW
Present	15	4.54		
Absent	63	1.87		
Lymphocytic infiltration			<0.001	KW
Negative	4	0		
Mild	30	0.63		
Moderate	28	1.86		
Marked	16	7.18		
Mitosis				KW
Absent	12	2.03	0.562	
1/mm2	17	5.55		
2-4/mm2	24	2.04		
>5/mm2	25	0.73		

lower limbs, among women (McCourt et al. 2014, Matheus & Verri 2015, Vazquez et al. 2015).

The presence of TILs represents the host's immune response against tumor cells. Numerous studies have drawn attention to a favorable prognosis of TILs in many solid tumors (Clemente et al. 1996, Taylor et al. 2007, Oble et al. 2009, Thomas et al. 2013, Fortes et al. 2015, Santoiemma & Powell 2015, Keun Park et al. 2017, Shen et al. 2018). HEVs are specialized venules that facilitate lymphocyte recruitment and migration into melanomas and other tumors (Kiss et al. 2007, Martinet et al. 2012a, b, Avram et al. 2013, Blanchard & Girard 2021, Asrir et al. 2022). Higher densities of these venules are linked with thinner melanomas, lower degrees of invasion and regression signs (Kiss et al. 2007, Martinet et al. 2012a, b, Avram et al. 2013).

Our findings confirm the hypothesis that HEVs are present in most cutaneous melanomas, and that their presence and density may predict a more effective immune response (Blanchard & Girard 2021, Asrir et al. 2022), given the positive association between histopathological variables with a better prognosis and the presence and degree of TILs in tumors with a higher density of these venules. Moreover, it seems reasonable to propose the idea that HEVs can recruit TILs and facilitate their migration into these tumors. A previous study found no significant association between HEV density and the anatomical location of the tumor, a finding that was confirmed in our study (Avram et al. 2013). The higher HEV

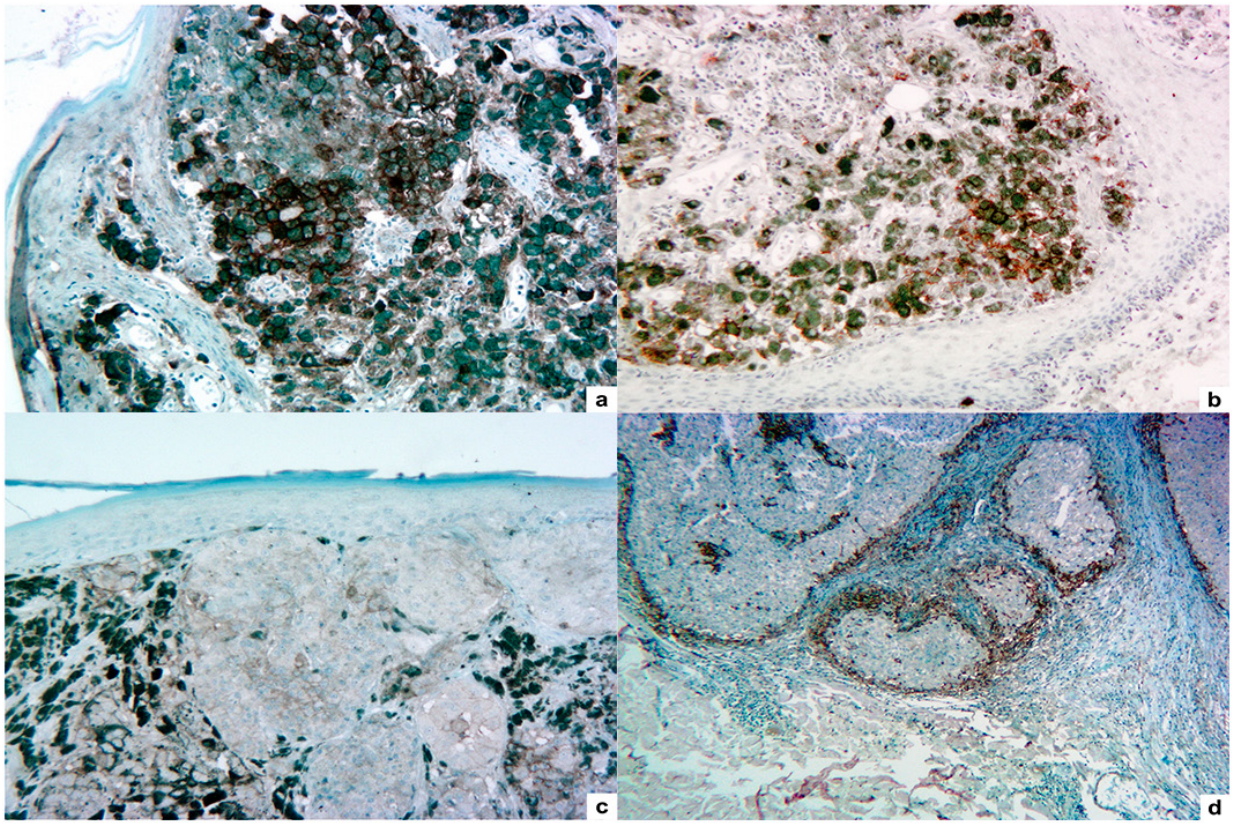

Figure 3. IHC with anti-PDL-1 antibody. a) Membrane positivity of PDL-1 in malignant melanocytes (3+ strong intensity), 40x magnification. b) Membrane positivity of PDL-1 in malignant melanocytes (2+ moderate intensity), 40x magnification. c) Membrane positivity of PDL-1 in malignant melanocytes (1+ weak intensity), 100x magnification. d) Colocation of PDL-1 positive melanocytes in geographic relation to TILs, 40x magnification.

density found in some histological subtypes in this study (higher in LMM and lower in ALM) is in agreement with previously described findings (Weinstein & Storkus 2016). Therefore, the higher HEV density found in LMMs and in thinner melanomas (<1mm) with a higher degree of TILs corroborates our proposition that these vessels constitute a good prognostic biomarker.

There are also mechanisms of tumor immune evasion, an important feature of some tumors, which affect tumor progression and survival. Thus, PDL-1 expression on tumor cells and consequent binding to the PD-1 receptor in lymphocytes leads to their inactivation, reducing the chance of antitumor immune response. Melanomas that express PDL-1 present shorter survival and cells that express this marker are geographically located adjacent to tumor infiltrating lymphocytes (Kashani-Sabet 2010, Taube et al. 2013, Massi et al. 2014, Jin et al. 2015, Madore et al. 2015). Presumably, therefore, treatments aimed at blocking this PD-1/PDL-1 axis may offer clinical benefits by reactivating the antitumor immune response and preventing tumor progression, reducing the likelihood of metastasis. Studies involving the use of antibodies that block the interaction of PD-1 with its ligands in patients with advanced melanomas have proved to be clinically beneficial (Sapoznik et al. 2012, Tsai et al. 2014, Ascierto & Marincola 2015, Carbognin et al. 2015, Philips & Atkins 2015).

Our findings demonstrate that the expression of PDL-1, in most cases found to occur adjacent to TILs, may be a mechanism of tumor immune evasion, since the hyperexpression of this ligand and consequent binding to its PD-1 receptor on lymphocytes leads to their inactivation. Melanomas with greater PDL-1 expression are more heterogeneous and belong to a group of diseases with shorter survival rates, with greater chances of recurrence and metastases (Gadiot et al. 2011, Massi et al. 2014, Muenst et al. 2014, Xu et al. 2015). Our findings also indicate that tumors that express PDL-1 are thicker melanomas with a higher degree of TILs, confirming that PDL-1 expression in geographical relation to TILs may be important mechanism of attempted immune evasion. Although studies have described the negative impact on tumor progression and survival of melanomas with higher PDL-1 expression, patients with this profile are candidates for immunotherapy aimed at blocking this axis, with good response rates to these treatments and with clinical benefits (McCourt et al. 2014, Velcheti et al. 2014, Herbst et al. 2014, Matheus & Verri 2015). In addition, the combination of angiogenic and

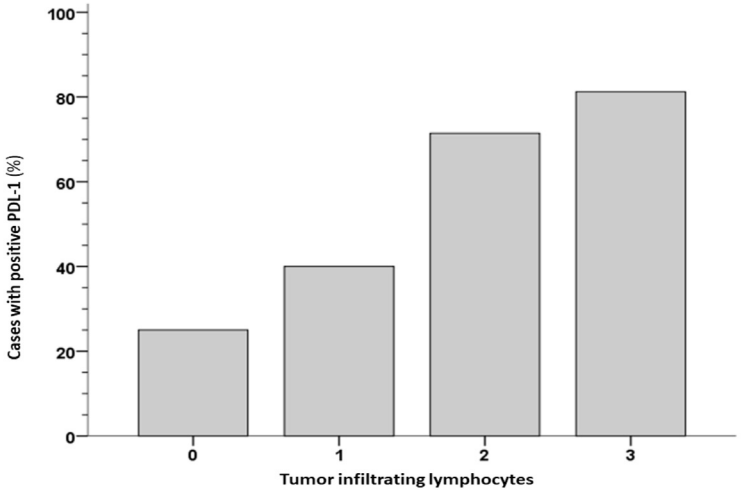

Figure 4. PDL-1 and TILs in primary cutaneous melanomas.

anti-PDL-1 therapies can stimulate antitumor immunity through the formation of HEVs, making the study of these two biomarkers of tumor immune response and their correlation particularly interesting (Hayasaka et al. 2010, Allen et al. 2017).

In conclusion, the identification and quantification of HEVs, the determination of PDL-1 expression and its association with the presence and degree of TILs provides more information about the histopathological characteristics and prognosis of primary cutaneous melanomas. The findings garnered in this study are of great interest, since they may underpin further studies aimed at developing new therapeutic strategies combining the stimulation of HEV formation with blockage of the PD-1/PDL-1 axis. This may boost the antitumor immune response by facilitating the migration of TILs and the deactivation of the tumor immune evasion mechanism.

Table II. Association between pathological characteristics and PDL-1 expression in primary cutaneous melanomas.

	N	% PDL-1 +	% PDL-1 -	p	Test
Histological type				0.169	KW
SSM	47	57.4	42.6		
NM	14	78.6	21.4		
LMM	11	54.5	45.5		
ALM	6	33.3	66.7		
Breslow thickness				0.091	KW
Up to 1mm	37	54.1	45.9		
1.01 to 2.0 mm	13	30.8	69.2		
2.01 to 4.0mm	16	75	25		
> 4.0 mm	12	83.3	16.7		
Ulceration				0.006	CS
Present	23	82.6	17.4		
Absent	55	49.1	50.9		
Regression signs				0.065	CS
Present	15	80	20		
Absent	63	54	46		
Lymphocytic infiltration				0.011	KW
Negative	4	25	75		
Mild	30	40	60		
Moderate	28	71.4	28.6		
Marked	16	81.2	18.8		
Mitosis					KW
Absent	12	52.1	47.9	0.494	
1/mm2	17	67	33		
2-4/mm2	24	64	46		
>5/mm2	25	52.4	47.6		

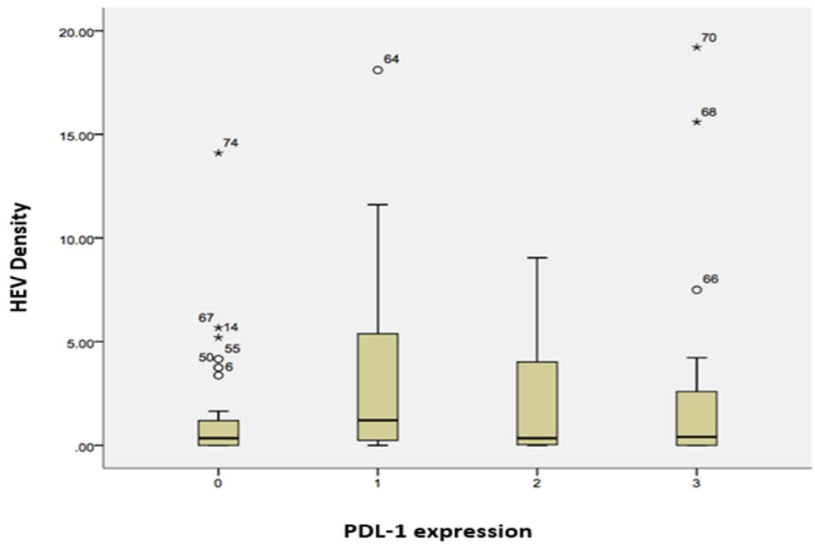

Figure 5. Association between HEV density and PDL-1 expression in primary cutaneous melanomas.

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Conceived and designed the analysis: JBS, ACM. Performed the analysis: JBS, ACM. Analyzed the data: JBS, ACM. Contributed reagents/materials/analysis tools: JBS. Wrote the paper: JBS, ACM.