The American Journal of Dermatopathology

IMMUNOPHENOTYPIC DIFFERERENCES IN TUMOR-INFILTRATING LYMPHOCYTES AND NEOVASCULARIZATION BETWEEN PRIMARY CUTANEOUS MELANOMA WITH AND WITHOUT METASTASIS: AN IMMUNOHISTOCHEMICAL STUDY OF 80 CASES

--Manuscript Draft--

Manuscript Number:	
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Article Type:	Original Article
Keywords:	Tumor-infiltrating lymphocytes (TILs); immunophenotype; melanoma; metastasis; neovascularization
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Manuscript Region of Origin:	SPAIN
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Title: Importance of the inflammatory infiltrate profile and neovascularization associated with melanoma and the correlation with metastases: an immunohistochemical study of 80 cases.

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No conflict of interest and No source of funding statement

ABSTRACT

The prognostic implications of the immunophenotype of the tumor-infiltrating lymphocytes in primary cutaneous melanoma are well known. In recent years, the study of this immunophenotype has also resulted in immunotherapeutic consequences.
The aims of this study were to characterize the subpopulations of tumor-infiltrating lymphocytes in primary cutaneous melanoma, in cases with and without metastasis, as well as the neovascularization associated with the primary neoplasm, and its influence on the development of metastasis. To this end, the immunophenotype of tumor-infiltrating lymphocytes and the neovascularization of 80 patients with primary cutaneous melanoma (40 each with metastatic and non-metastatic melanoma) were analyzed by immunohistochemistry for CD3, CD4, CD8, FOXP3, PD-1, CD31, and D2-40 antibodies. We found that higher frequencies of tumor-infiltrating lymphocytes with brisk pattern, and CD4+, CD8+, and CD20+ cells in tumor-infiltrating lymphocytes, and a lower frequency of CD31+ vessels were histopathological features associated with better prognosis in primary cutaneous melanoma.

Our results support the notion that immunohistochemical study of tumor-infiltrating lymphocytes and neovascularization in primary cutaneous melanoma may be helpful tools for identifying patients at increased risk of metastasis development.

KEY WORDS

Tumor-infiltrating lymphocytes (TILs), immunophenotype, melanoma, metastasis, neovascularization.

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No conflicts of interest or sources of funding to declare

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INTRODUCTION

The immune response to solid neoplasms, including cutaneous melanoma, has proved to have prognostic consequences and possible implications for their immune-targeted treatments. For those reasons, the immunophenotypic characterization of tumor-infiltrating lymphocytes (TILs) has been extensively investigated in recent years.

In 1863, Virchow believed that neoplasms originated from sites of chronic inflammation and that tumor growth was stimulated by this inflammatory process.[1] However, it was soon realized that the inflammatory infiltrate was not the origin of the neoplasm but, in fact, quite the contrary: the host immune response is a defensive mechanism against the tumor and its presence is associated with better prognosis.[2]

The TIL immunophenotype is known to have prognostic implications in cutaneous melanoma, and, in recent years, its potential as the target for new immunotherapies has been investigated.

In this study, we analyzed the TIL immunophenotype in a series of 80 primary cutaneous melanomas (40 each with and without metastasis). We also studied the neovascularization associated with this infiltrate through the immunohistochemical endothelial markers CD31 and D2-40, because some multivariate analyses in cutaneous melanoma have suggested that tumor neovascularization is the most important independent factor predicting survival, making it an even more relevant factor than tumor thickness.[3] Previous studies have analyzed the immunophenotype of TILs in primary cutaneous melanoma in addition to neovascularization but, to our knowledge, this is the first series from a single institution in which both characteristics have been analyzed simultaneously in the same series of cases.

MATERIAL AND METHODS

Study population

80 patients with primary cutaneous melanoma, treated between 2000 and 2020 in the Department of Dermatology of the Puerta de Hierro University Hospital, Madrid, Spain, were enrolled in this study. The study protocol was approved by the ethics committee of the hospital.

Two series of patients were assembled. The first consisted of 40 patients with primary cutaneous melanomas that had developed metastasis (including cutaneous, visceral and/or lymph node metastasis). The inclusion criteria consisted of:

1) A confirmed histopathological diagnosis of both primary cutaneous melanoma and its cutaneous, lymph node and/or visceral metastases.
2) Available paraffin-embedded material from the primary melanoma and the subsequent metastases.
3) Available clinico-pathological and follow-up information from patient medical records

The second series comprised 40 non-metastatic primary cutaneous melanomas. The inclusion criteria were:

1) A confirmed histopathological diagnosis of primary cutaneous melanoma with a Breslow depth > 1 mm and negative results from a sentinel lymph node (SLN) biopsy.
2) Available paraffin-embedded material from the primary cutaneous melanoma.

Available clinico-pathological information from patient medical records, and no evidence of metastatic disease during the follow-up.

Histopathological study

Hematoxylin and eosin (H&E) stained sections for metastatic and non-metastatic melanomas were independently reviewed by two expert dermatopathologists. In most cases, additional immunohistochemcial studies included immunostaining for S100 protein, Melan A, HMB-45 and SOX-10. The recorded histopathological criteria included: thickness (Breslow depth), presence or absence of ulceration, mitoses count, melanoma subtype according to the classic Clark classification (lentigo maligna melanoma [LMM], superficial spreading melanoma [SSM], acro-lentiginous melanoma [ALM], and nodular melanoma [NM]), presence or absence of histopathological features of regression in more than 25% of the tumor mass, vascular involvement, perineural involvement, and presence or absence of any associated melanocytic nevi. TILs were graded as brisk (present throughout the vertical growth phase [VGP] area, or infiltrating the entire base of the VGP area), non-brisk (present only in focal areas of the VGP), or absent (no TILs in contact with the VGP areas, although possibly present in perivascular or fibrotic areas). Patients' SLN biopsies were recorded as positive or negative.

Immunohistochemical studies

All specimens were immunohistochemically studied to determine the status of the following lymphocytic markers: CD3, CD4, CD8, Granzyme B, FOXP3, CD20, and PD-1. All cases were also immunostained for the endothelial markers CD31 and D2-40. Table 1 summarizes the antibodies, clones, sources, dilutions, and antigenic retrieval techniques used in the study.

Formalin-fixed, paraffin-embedded (FFPE) primary melanomas were deparaffinized, rehydrated, and subjected to heat-induced epitope retrieval in citrate buffer in a microwave oven. Sections were quenched with hydrogen peroxidase, incubated in blocking solution and then in primary antibody, diluted in blocking solution at a

concentration of 1:500 for 1 hour at room temperature and at 4°C overnight. Sections were washed and incubated in a 1:200 dilution of biotinylated antibody solution for 1 hour and incubated with Vectastain ABC reagent. Slides were counterstained with hematoxylin, then dehydrated, before applying mounting medium and coverslips.

The immunohistochemical study was performed on the tissue microarrays (TMAs) obtained from the samples of each patient. For this study, two TMAs were constructed with 160 samples. An MTA-1 Beecher manual tissue microarrayer was used. An arrayer equipped with a micrometric digital measurement of 1.5-mm diameter needles was used, taking the sample from the area in which the H&E stain had identified the densest inflammatory infiltrate at the base of the primary cutaneous melanoma.

A semi-quantitative score for CD3, CD4, CD8, FOXP3, CD20, PD-1, and Granzyme B expression was ascribed to the densest area of the antibody staining of the infiltrate. The percentages of stained cells were scored as: 0 (0-10% of stained cells; +1 (10-25%), +2 (25-50%), and +3 (> 50%). For neovascularization, the number of positive vessels for CD31 and D2-40 immunostains was counted in the densest infiltrate area in 10 high-power fields (x400).

Statistical analysis

The study was conducted in a double-blind manner. The data of patients from each group (metastatic and non-metastatic) were coded. The researchers who evaluated the histopathological images, measured the variables, and undertook the statistical analysis did not know the group to which each patient belonged. Only after completion of the statistical analysis was the coding removed.

The statistical analysis was carried out in R 4.0.1 (R Core Team, 2020). Variables expressed as proportions are reported numerically as percentages. Continuous data are

summarized numerically as the mean and standard deviation, and graphically as the median, interquartile range and extremes. The two groups were compared in univariate analyses, using chi-square tests to compare proportions in the categorical variables, and Student's unpaired samples t tests for continuous variables (Breslow depth and age).

RESULTS

In the metastatic melanoma series, the mean age was 61.5 years, 25 (62.5%) patients were male and 14 (35%) were female. In the non-metastatic melanoma series, the mean age was 63.6 years, 12 (30%) patients were male and 28 (70%) were female. The follow-up for patients of metastatic melanoma series ranged from 6 to 233 months (mean, 110 months), whereas the follow-up for patients of non-metastatic melanoma series ranged from 2 and 120 months (mean, 89 months). These differences between the groups were not statistically significant.

With respect to the location of the primary melanoma in the metastatic series, 16 (40%) patients had melanoma in BANS (*b*ack, *a*rms, *n*eck or *s*calp) areas, while in the series of non-metastatic melanomas, 24 (60%) had melanoma in non-sun-exposed areas. These differences were not statistically significant.

In the series of metastatic melanomas, 32 (80%) patients had lymph node metastases and 24 (60%) had visceral metastases.

Histopathological findings in the metastatic melanoma series were: 2 (5%) patients had LMM, 17 (42.5%) had NM, 11 (27.5%) had SMM, and 6 (15%) had ALM, regression was present in 14 (35%) patients and 8 (20%) had a preexisting melanocytic nevus in the same excised specimen. The median Breslow depth in this group was 5.0 mm. TILs were absent or non-brisk in 31 (77.5%) patients and brisk in 9 (22.5%) patients. In the series of non-metastatic melanomas, 7 (17.5%) patients had LMM, 7 (17.5%) had NM,

24 (60%) had SMM, and 2 (5%) had ALM, regression was present in 20 (50%) patients and 3 (7.5%) patients had an associated melanocytic nevus in the same specimen. The median Breslow depth in this series was 2.2 mm. TILs were absent or non-brisk in 16 (40%) patients and brisk in 24 (60%) patients.

Table 2 summarizes the clinical and histopathological features of the two series and Figures 1 and 2 illustrate some histopathological subtypes with examples from both series of melanomas.

TIL analysis of CD3 in metastatic series showed values of 0 in 19 (24.7%) samples, +1 in 30 (39.0%), +2 in 21 (27.3%), and +3 in 7 (9.1%) samples, while in the non-metastatic series the values were 0 in 11 (14.1%) samples, +1 in 28 (35.9%), +2 in 22 (28.2%), and +3 in 17 (21.8%) samples (Fig. 3). These differences were not statistically significant ($p = 0.094$).

CD4 expression analysis in metastatic series showed values of 0 in 46 (59.7%) samples, +1 in 24 (31.2%), +2 in 7 (9.1%), and +3 in 0 (0%) samples, while in the non-metastatic series the values were 0 in 17 (21.5%) samples, +1 in 34 (43.0%), +2 in 23 (29.1%), and +3 in 5 (6.3%) samples. These differences were statistically significant ($p < 0.001$).

Our results for the immunohistochemical expression of CD8 (Fig. 3), CD20, and FOXP3 are summarized in Table 3. Statistically significant differences were found in the expression of CD8 and CD20 between the two series ($p < 0.05$), but not for the expression of FOXP3.

Very few TIL cells expressed CD20, because no expression was found in 76 (98.7%) metastatic and 64 (81.0%) non-metastatic samples.

TIL expression values for PD1 in the metastatic series were 0 in 19 (25.3%) samples, +1 in 29 (36.7%), +2 in 11 (13.9%), and +3 in 1 (1.3%) sample, while in the non-metastatic

series the values were 0 in 29 (36.7%) samples, +1 in 36 (45.6), +2 in 11 (13.9%), and +3 in 3 (3.8%) samples. None of the differences between the series was statistically significant.

Weak expression (+1) for granzyme B was seen in only 2 (3%) samples of the non-metastatic series.

Neovascularization was studied through the expression of CD31 and D2-40 (Fig. 4). We found a statistically significant difference in the mean of the CD31+ vessels in the metastatic and non-metastatic series (16.9 *vs.* 9.4; $p < 0.001$). Analysis of lymphatic vascularization counting D2-40 expression revealed no statistically significant differences between the series.

DISCUSSION

TILs are frequently seen in neoplastic lesions as the expression of the immunological response to the tumor. Conversely, the TIL immunophenotype is quite closely correlated with the prognosis and biological behavior of tumors, since the different subpopulations of lymphocytes have different functions within the tumor microenvironment.

Initial studies by Clark et al.[4] of TILs in cutaneous melanoma considered only the infiltrate density, classifying it as intense (present throughout the VGP or at the base of the VGP), moderate (inflammatory infiltrate in one or more foci of the VGP) or absent (no infiltrate or TIL outside of the neoplastic aggregates of melanoma). This grading of infiltrate density proved to be an independent prognostic factor for overall survival (OS), with an odds ratio (OR) of 11.3 for an intense infiltrate and 3.5 for a moderate or absent infiltrate.

Some years later, a similar study to that of Clark et al. found a good rate of inter-observer agreement (Kappa > 0.6) between three dermatologists and three dermatopathologists.[5] The authors concluded that the categorization of TILs can be easily taught and applied with an acceptable level of reproducibility in routine diagnostic practice.

With respect to the TIL categories, of the 80 cases in our study, 19 were absent, 33 were non-brisk, and 28 were brisk. Considering the metastatic and non-metastatic melanomas separately revealed that TILs are more frequently absent in metastatic than in non-metastatic cases, and that 60.0% of non-metastatic cases showed brisk TILs compared with 22.5% of cases in the metastatic series. These differences were statistically significant ($p < 0.05$). Our results are similar to others previously published,[6] that supported the notion that higher TIL values and a brisk pattern are directly related to better prognosis.

In a 2019 meta-analysis, Fu et al.[7] found that the presence of CD3+ lymphocytes in TILs was directly related to a better prognosis in patients with cutaneous melanoma. Our separate analysis of metastatic and non-metastatic melanomas showed that metastatic cases showed no expression or very few CD3+ cells in TILs more frequently than did non-metastatic cases, although the differences were not statistically significant. The exact mechanism accounting for why CD3+ TILs behave as a good prognostic factor is not currently known, but could be related to the regulation of cell signaling.

There are several published reports of analyses of various subpopulations of T lymphocytes in TIL of melanoma, including CD4+, CD8+, and Treg (FOXP3+) cells.

CD4+ T-helper lymphocytes are a heterogeneous group of cells. Th1+ cells have an important role in activating cytotoxic lymphocytes, while Th2+ cells help to stimulate

humoral immunity and to activate eosinophils. In terms of antitumor activity, Th2 activation is less effective than Th1 stimulation.[8] Kluger et al.[9], in their series of metastatic melanomas, found that most tumors showed TILs with both CD4+ and CD8+ cells, while only a minority of these neoplasms exclusively expressed CD4+. These authors described that 70% of cases exhibited brisk TILs, with CD4+ and CD8+ cells present in similar proportions. In contrast, in the series published by Kim et al.[10] and Fridman et al.[11], the presence of CD8+, but not of CD4+, was significantly associated with better survival. Both research groups pointed out that it was an expected finding, because the memory and effector role of CD8+ cells in response to different inflammatory cytokines, and their role in preventing not only tumor invasion, but also the development of metastasis, are well known. In our series, we found that metastatic melanomas frequently showed few or no CD4+ and CD8+ cells in TILs, whereas non-metastatic melanoma had a moderate or large number of CD4+ and CD8+ cells. These differences were statistically significant and are consistent with previously published results.

In addition to Th1 and Th2 lymphocytes, a subtype of CD4+ T lymphocytes, the so-called T regulatory (Treg) cells, have a suppressor effect on effector T lymphocytes.[12] Although Treg lymphocytes also co-express CD4 and CD25, FOXP3 is the most specific marker for them.[13] Some studies have shown that FOXP3+ TILs are associated with a negative prognosis in cutaneous melanoma, while other investigators have reported an association with better prognosis, and some have even reported no significant association.[7,14,15] A 2019 meta-analysis[7] concluded a prognostically favorable role for FOXP3+ cells, whereby patients with high frequency of FOXP3+ TILs had a better prognosis. In contrast, a study of two groups of melanoma patients who either showed or did not experience local disease recurrence found that the

percentage of CD4+ T cells expressing double immunostaining for CD25 and FOXP3 was significantly higher in the primary tumors of patients who had recurrent disease.[16] Similarly, Gerber et al.[17] studied the proportion of FOXP3+ cells in TILs of 158 primary melanomas and found that disease-free survival and OS were better in patients with a low frequency of FOXP3+ cells, and that this was a factor independent of tumor thickness as represented by the Breslow depth. In a subsequent study of 90 primary cutaneous melanomas, FOXP3 immunoexpression failed to show any significant associations with patient survival, tumor thickness, or ulceration.[18] Similar results were obtained in another study of metastatic melanoma, comprising 140 patients, in which FOXP3+ cell density was not prognostically associated with survival.[19] Finally, a study of metastatic SLN of melanoma revealed a negative association of high counts of FOXP3+ TIL with recurrence-free survival and OS.[20] These apparently disparate results may be due to FOXP3+ heterogeneity. FOXP3+ cells are currently classified into three subpopulations based on their expression of CD45RA and FOXP3, and include naive Treg ($CD45RA^{+}FOXP3^{lo}CD25^{lo}CD4^{+}$), effector Treg (eTreg, $CD45RA^{-}$ $FOXP3^{hi}CD25^{hi}CD4^{+}$) and non-Treg ($CD45RA^{-}FOXP3^{lo}CD25^{lo}CD4^{+}$).[21] Given that FOXP3+ TIL are a heterogeneous population of T cells, further research is required to differentiate immunosuppressive FOXP3+ Treg from FOXP3+ non-Treg, and to determine their value in histopathological diagnosis, particularly as, in our study, there were no statistically significant differences in FOXP3 immunoexpression between metastatic and non-metastatic melanomas.

The interaction between PD-1 molecules expressed on the cell membrane of effector T lymphocytes and PD-L1 molecules of the antigen-presenting cells (APCs) is one of the immune system checkpoints with which not only is there restriction of the tissue damage secondary to the immune response triggered after an infection or an

inflammatory process, but also inhibition of autoimmune responses.[22] In tumors, PD-1 and PD-L1 are expressed not only by neoplastic cells, but also by most TIL cells, and their expression is reciprocally regulated. Thus, some studies have demonstrated that the PD-L1 expression in neoplastic cells and in cells of the immune system is strongly associated with the expression of PD-1 in TILs.[23] It has been postulated that the expression of PD-L1 by neoplastic cells is one of the mechanisms by which they escape the action of the immune system. The interaction between the PD-L1 molecule of the tumor cells and the PD-1 receptor of T lymphocytes would result in suppression of anti-tumor lymphocyte activity.[23] For this reason, a high level of expression of PD1 is associated with a worse prognosis of melanoma, but also with a better response to the new immune-targeted treatments. Recent studies, such as those of Tumeh et al.[24] and Taube et al.,[25] have noted the great importance of the location of TILs and the melanoma cells that express PD-L1, whereby the presence of CD8+ cells in TILs and the expression of PD-L1 in the invasive margin of melanoma are associated with increased response to immunotherapy.

In our study, we evaluated the expression of PD-1+ cells in TILs, but not of PD-L1 in neoplastic cells of melanoma. We found no differences in PD-1 between metastatic and non-metastatic melanomas. As part of the characterization of TIL immunophenotype in melanoma, the presence of B-lymphocytes was investigated by studying the immunohistochemistry of CD20. However, in our study, most cases (140/156) had no CD20+ TIL cells. There were significantly more cases entirely lacking CD20+ cells in the metastatic melanoma series than in the non-metastatic melanoma ($p = 0.004$). A literature review revealed that most studies reported the frequency of CD20+ TIL cells in melanoma to be directly related to better prognosis, although no prognostic relationship was found in some of the other studies.[7,26]

The ability of melanoma to spread through both blood and lymphatic vessels is well known. Dadras et al.[27] established that the density of both is greater in melanomas than in normal human skin, and were the first to demonstrate both intratumoral and peritumoral lymphangiogenesis in primary cutaneous melanomas. They reported a direct relationship between this lymphangiogenesis and the risk of developing lymph node metastases and lower patient survival. They concluded that the density of peritumoral lymphatic vessels might serve as a marker of risk of lymph node metastases in patients with primary cutaneous melanoma. They also found that bigger vessels covering a significantly larger area were correlated with a shorter disease-free period and poorer survival.

The meta-analysis of Patushenko et al.[28] showed that the density of peritumoral lymphatic vessels, measured by D2-40 immunostaining, was the best predictor of lymphatic involvement and distant metastasis, as well as disease-free time and OS. By contrast, a multivariate analysis published by Barnhill et al., [27] that included the relationship between angiogenesis and clinical evolution of the patients, concluded that there was not any significant association between them.

Our study revealed a higher frequency of blood vessels in metastatic melanoma cases than in non-metastatic ones, although the differences were not statistically significant.

We must acknowledge that our study has some limitations. First, it was not a cohort study, so there may have been a degree of selection bias associated with the inclusion of patients. Furthermore, OS and disease-free time were not analyzed, meaning that the data presented are descriptive; no potential correlations with these variables have been examined.

In conclusion, in this study of 80 patients with primary cutaneous melanoma we found that higher frequencies of TILs with brisk pattern, of CD4+, CD8+ and CD20+ cells in TILs, and lower frequencies of CD31+ vessels were all histopathological features associated with better prognosis. The immunohistochemical study of TILs and neoangiogenesis may be helpful in guiding the identification of patients at higher risk of developing metastases.

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FIGURE LEGENDS

Fig. 1. Histopathological subtypes of metastatic melanomas. **A-D**. Superficial spreading malignant melanoma (hematoxylin-eosin, original magnifications Ax10, B x40, C x100, D x200). **E-H**. Nodular malignant melanoma (hematoxylin-eosin, original magnifications Ax10, B x40, C x100, D x200). **I-L**. Superficial spreading malignant melanoma associated with an intradermal melanocytic nevus (hematoxylin-eosin, original magnifications Ax10, B x40, C x400, D x400).

Fig. 2. Histopathological subtypes in non-metastatic melanomas. A-**D**. Lentigo maligna melanoma (hematoxylin-eosin, original magnifications Ax10, B x40, C x200, D x400). **E-H**. Superficial spreading malignant melanoma (hematoxylin-eosin, original magnifications Ax10, B x10, C x100, D x200). **I-L**. Superficial spreading malignant melanoma (hematoxylin-eosin, original magnifications Ax10, B x40, C x100, D x200).

Fig. 3. Examples of the 0, +1, +2, and +3 expression categories in TMAs for CD3 and CD8 (original magnifications, x20).

Fig. 4. Examples of immunoexpression for CD31 and D2-40 (each immunostaining is illustrated with original magnifications, x20 and x100).

Table 1: Antibodies, clones, sources, dilutions and antigen-retrieval protocols used in the study.

Antibody	Clone	Source	Dilution/ incubation time (min)	Antigen retrieval
CD3	Polyclonal	DAKO (Glostrup, Denmark)	Ready to use; 20	High pH
CD4	4B12	Leica (Buffalo Grove, IL, USA)	1:50; 20	High pH
CD8	C8/144B	DAKO (Glostrup, Denmark)	Ready to use; 20	High pH
CD20	L26	DAKO (Glostrup, Denmark)	Ready to use; 20	Low pH
Granzyme B	GrB-7	DAKO (Glostrup, Denmark)	1:50; 20	Low pH
PD-1	NAT105	Roche (Indianapolis, IN, USA)	Ready to use; 40	CC1
CD 31	JC70A	DAKO	Ready to use; 20	High pH

		(Glostrup, Denmark)		
Podoplanin	D2-40	DAKO (Glostrup, Denmark)	Ready to use; 20	High pH
FOXP3	Clone 236 A7E7	Invitrogene (Carlsbad, CA, USA)	1:25; 20	Low pH

pH low: 10 mM sodium citrate at pH 6-7; pH high: 10 mM sodium bicarbonate at pH 7.

Table 2: Clinical and histopathological characteristics of metastatic and non-metastatic melanomas in this study.

	Categories	Metastatic melanomas n (%)	Non-metastatic melanomas n (%)	*p*
Sex	Male Female	26 (65.0) 14 (35.0)	12 (30) 28 (70)	0.003
Age	Mean (SD)	61.5 (17.2)	63.6 (16.0)	0.565
BANS	No Yes	24 (60) 16 (40)	16 (40) 24 (60)	0.096
Sentinel node biopsy	No Yes	21 (52.5) 19 (47.5)	0 (0) 40 (100)	< 0.001
Positive/negative	- +	6 (31.6) 13 (68.4)	40 (100) 0 (0)	< 0.001
Histopathologic subtype of melanoma	LMM SMM ALM NM Others*	2 (5) 11 (27.5) 6 (15) 17 (42.5) 4 (10)	7 (17) 24 (58) 2 (5) 7 (17) 0 (0)	0.002
Regression	No Yes	26 (65) 14 (35)	20 (50) 20 (50)	0.212

TIL (absent/brisk/non-brisk)	Absent Brisk Non-brisk	11 (27.5) 9 (22.5) 20 (50)	8 (20) 24 (60) 8 (20)	0.011
Lymphatic invasion	No Yes	37 (92.5) 3 (7.5)	38 (95) 2 (5)	0.977
Blood vessel invasion	No Yes	37 (92.5) 3 (7.5)	38 (95) 2 (5)	0.977
Perineural invasion	No Yes	35 (87.5) 5 (12.5)	40 (100) 0 (0)	0.192
Associated melanocytic nevus	No Yes	32 (80) 8 (20)	37 (92) 3 (8)	0.180
Breslow depth	Mean (SD)	5.0 (3.5)	2.2 (1.8)	< 0.001
Lymph node metastases	No Yes	8 (20) 32 (80)	41 (100) 0 (0)	< 0.001
Visceral metastases	No Yes	16 (40) 24 (60)	41 (100) 0 (0)	< 0.001

*Others: epithelioid melanoma (1 case), spitzoid melanoma (2 cases), and nevoid melanoma (1 case).

Table 3. Immunohistochemical results in metastatic and non-metastatic melanomas.

Antibody	Score	MM n (%)	Non-MM n (%)	*p*
CD3	0	19 (24.7)	11 (14.1)	0.094
	1	30 (39.0)	28 (35.9)	
	2	21 (27.3)	22 (28.2)	
	3	7 (9.1)	17 (21.8)	
CD4	0	46 (59.7)	17 (21.5)	< 0.001
	1	24 (31.2)	34 (43.0)	
	2	7 (9.1)	23 (29.1)	
	3	0 (0.0)	5 (6.3)	
CD8	0	33 (43.4)	10 (12.7)	< 0.001
	1	31 (40.8)	24 (30.4)	
	2	8 (10.5)	23 (29.1)	
	3	4 (5.3)	22 (27.8)	
CD20	0	76 (98.7)	64 (81.0)	0.004
	1	1 (1.3)	10 (12.7)	
	2	0 (0.0)	4 (5.1)	
	3	0 (0.0)	1 (1.3)	
FOXP3	0	38 (48.1)	32 (40.0)	0.090
	1	29 (36.7)	43 (53.8)	
	2	11 (13.9)	5 (6.2)	
	3	1 (1.3)	0 (0.0)	

PD-1	0	19 (25.3)	29 (36.7)	0.031
	1	27 (36.0)	36 (45.6)	
	2	20 (26.7)	11 (13.9)	
	3	9 (12.0)	3 (3.8)	
Granzyme B	0	0 (0.0)	77 (97%)	0.830
	1	0 (0.0)	2 (3%)	
	2	0 (0.0)	0 (0.0)	
	3	0 (0.0)	0 (0.0)	
CD31	Mean (SD)	16.9 (7.2)	9.4 (5.2)	< 0.001
D2-40	Mean (SD)	4.4 (2.5)	4.5 (2.1)	0.716

MM: Metastatic melanoma; Non-MM: non-metastatic melanoma.

Figure 1

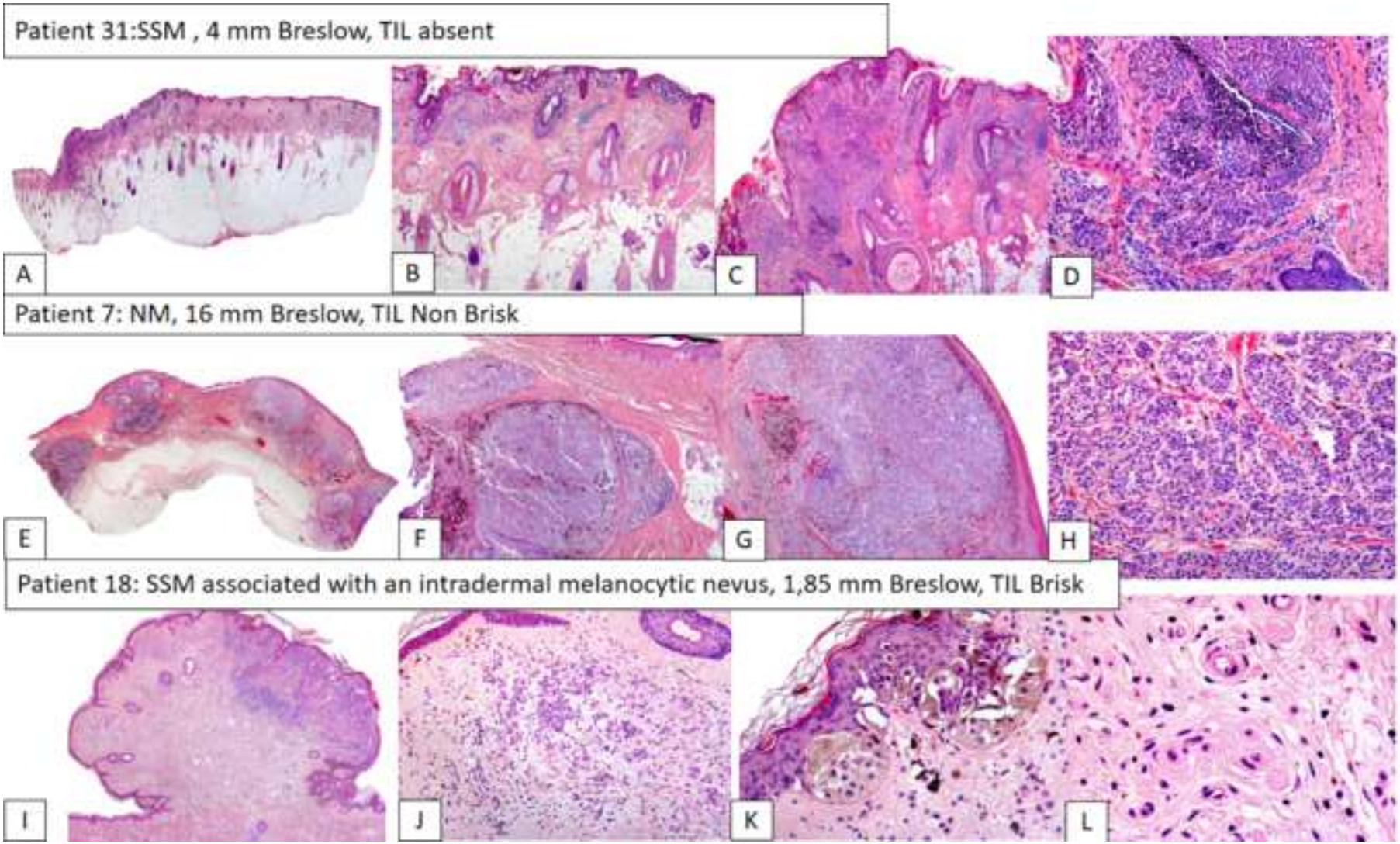

Figure 2

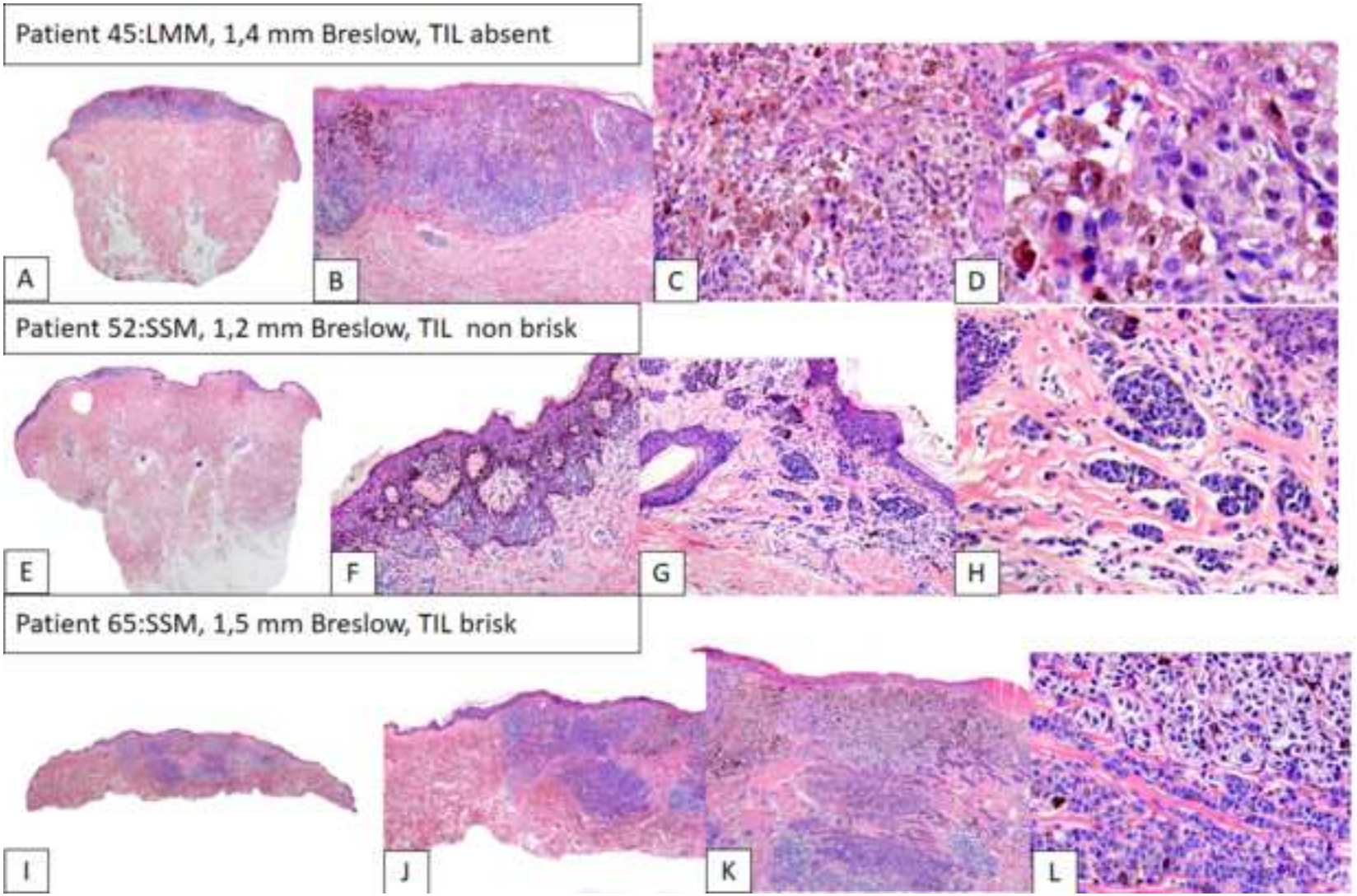

Figure 3

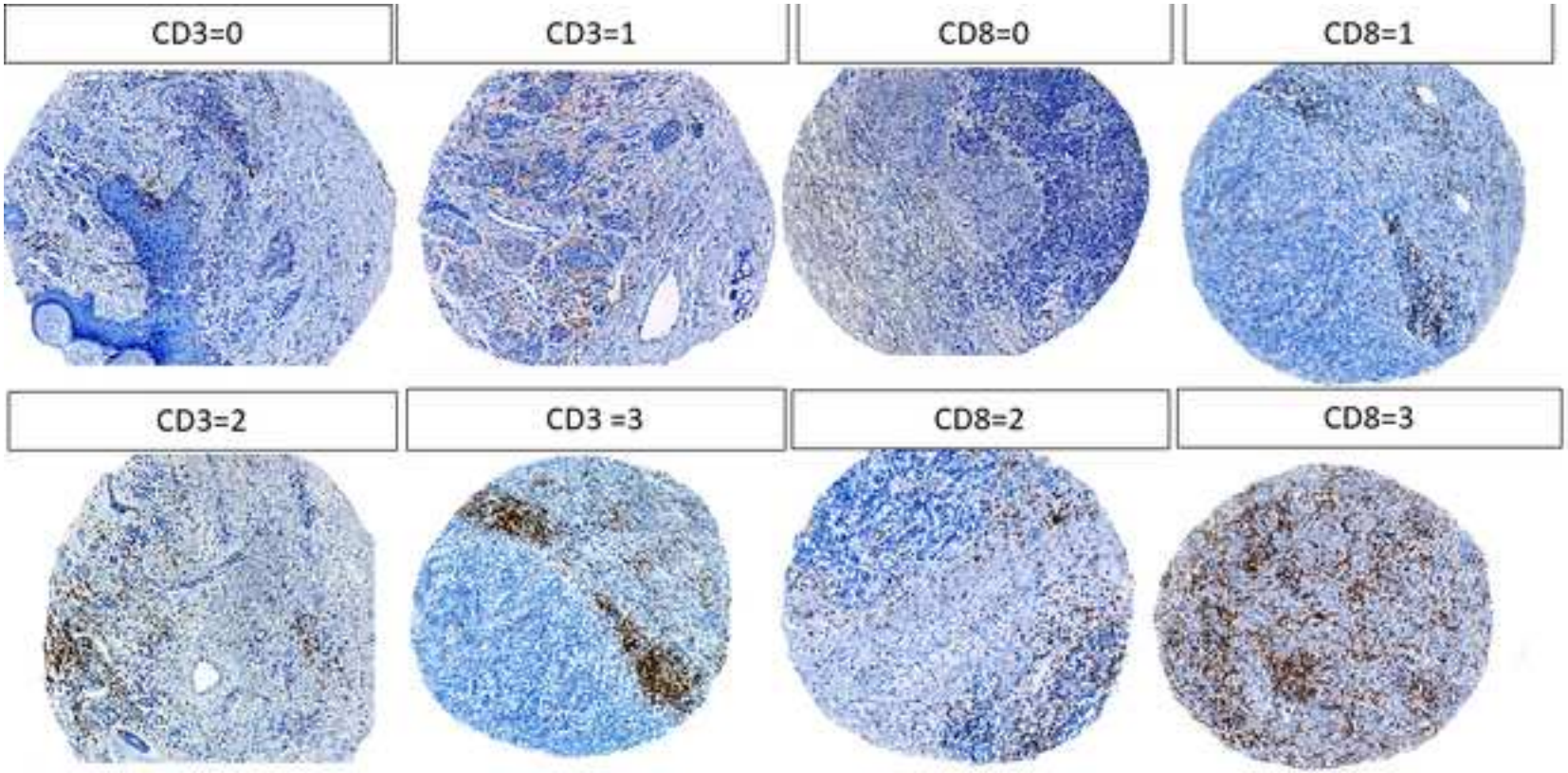

Figure 4

Click here to access/download;Figure;figure 4 CD31d2-40.tif