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Original Article

Dermal vasculature and melanocytic proliferation index in photodamaged skin in the assessment of lateral margins of lentigo maligna and lentigo maligna melanoma

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Abstract: Lentigo maligna (LM) is the most common subtype of melanoma on the face. When it invades the dermis it is called lentigo maligna melanoma (LMM). Its histological delimitation is controversial due to subjectivity. Analysis of peritumoral vasculature and proliferation index of melanocytes may help to differentiate tumor areas from tumor-free areas, as neoplasia-induced angiogenesis in such scenarios, as well as the higher proliferation index of melanocytes in melanomas, are well established. This work compares the peritumoral vasculature and melanocyte proliferation index of LM and LMM with that of adjacent non-neoplastic skin and sun-damaged skin (control). Forty-three resection cases of LM and LMM were selected retrospectively. Immunohistochemistry was performed for anti-CD31 and anti-CD105 to assess vascularization. Melanocyte proliferation index double labeling was performed using the anti-Melan-A and anti-Ki-67. The Chalkley optical grid was used to quantify blood vessel hotspots. Doubly labeled cells with anti-Melan-A and anti-Ki-67 were counted at tumor, free margin, and control skin. Microvasculature quantification under the melanomas, for both CD31 and CD105, was greater than at the margins of the same specimens ($P < 0.0001$; $P = 0.0001$) and greater than control skin ($P = 0.0016$; $P = 0.0027$), with higher density for CD31 than CD105. The mean number of double-labeled proliferating melanocytes at the melanoma periphery was greater than at the adjacent free skin and control skin ($P = 0.0011$). The control skin samples showed the highest CD31-positive vasculature in the head and neck region, with a positive correlation between melanocytic proliferation index and vasculature. The presence of neovascularization (CD105) and proliferating melanocytes (Ki67+/Melan-A+) are suspicious findings for LM/LMM, helping to outline, diagnose, and evaluate tumor margins.

Keywords: Lentigo maligna, melanoma, surgical margin, vasculature, proliferation index

Introduction

Lentigo maligna (LM), or melanoma (MM) *in situ* of photo exposed areas, is a cutaneous lesion with a flat surface and color variations from brown to black. It occurs in individuals with light-colored skin, almost exclusively in Caucasians and the elderly. It has a predilection for the face, where it is the most common subtype of melanoma. It is generally a gradual slow-growing lesion with irregular edges. When LM invades the dermis, it is also known as lentigo maligna melanoma (LMM) [1]. The treatment of choice is surgical excision, which results in the lowest rate of recurrence [1, 2]. Identifying the clinical margins for LM and LMM is intricate because, in addition to being poorly defined, such lesions may be masked by ephelides, pigmented actinic keratoses, lentigo, nevi and seborrheic keratoses [3]. Neoplastic melanocytes often extend beyond the clinical delimitation of the pigmented area histologically [1].

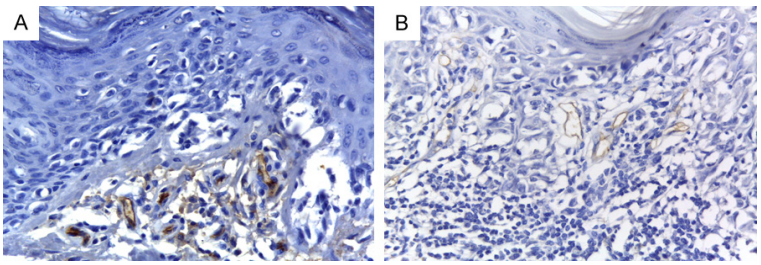

Figure 1. Immunohistochemistry revealing superficial dermal vessels under melanoma cases (400×). A. Vascular endothelial cells positive for CD31 ("pan-endothelial" marker) under an LMM. B. CD105-positive endothelial cells from dermal newly formed blood vessels under an LM.

Histopathologic features include atypical proliferation of uni/multinucleated melanocytes along the basal layer of the epidermis and adjacent structures, arranged in isolated units or in nests, dermal solar elastosis and atrophic epidermis [1].

The histological delimitation of LMs/LMMs, however, is a source of debate, since it is a relatively subjective and difficult task. This is mainly due to the difficulty in differentiating malignant melanocytes at the periphery of MMs from hyperplastic melanocytes, usually found in the chronically sun-exposed skin, where these MMs occur. Melanocytic hyperplasia (MH) secondary to solar actinic damage is often associated with histological atypia such as nucleomegaly, hyperchromatic nuclei, multinucleation, confluence of melanocytes, and migration to suprabasal strata, which are also present in LMs/LMMs [1, 4]. The difficulty in differentiating benign melanocytes from the malignant phenotype increases the difficulty in determining the measurement of the surgical margins, as this is mandatory in the pathology reports for MMs. Moreover, in the face region, it is imperative to preserve vital anatomical structures for both function and aesthetic reasons, which demands precision in delimiting the borders of the lesion.

Analysis of peritumoral vasculature could help to differentiate tumor areas from tumor-free areas, as neoplasia-induced angiogenesis in such scenarios is a well-established event [5]. Another possible strategy would be the comparison of the proliferation index of malignant melanocytes with that of hyperplastic melanocytes, since it is known that malignant neoplastic cells tend to grow rapidly by escaping local control mechanisms [6]. Anti-CD31, a pan-endothelial marker (blood and lymphatic vessels), and anti-CD105, an endothelial marker of neoformed blood vessels, were used for the quantification of microvessels in LM or LMM excision specimens and in sun-damaged skin without melanocytic lesions. In these same specimens, the melanocytic proliferation index was also evaluated by double labeling with anti-Melan-A and anti-Ki-67, melanocyte and dividing cell markers, respectively. The aim of this study was to evaluate whether the peritumoral microvasculature and melanocytic proliferation index could help in the delimitation and diagnosis of LM/LMM.

Materials and methods

Subjects

A total of 43 resection cases specimens of LM and LMM (33 and 10, respectively) were selected retrospectively from the Laboratory of Pathological Anatomy at the *Clinical Hospital* of the State University of Campinas. Case selection was based on free surgical margins in the histopathologic evaluation and no history of recurrence (mean duration of post-surgical follow-up in years: mean 7.7, median 6.0), and 37 cases of control skin (CS) samples, with diagnoses other than melanocytic lesions and with histological signs of chronic sun exposure. CS samples were intentionally similar to those of LMs/LMMs with respect to skin color, gender and age as well as topography of lesions.

All sections from all cases of LM, LMM and CSs were reviewed under light microscopy. Slides were selected from cases that showed tumor sections containing adjacent non-neoplastic skin with free lateral surgical margins. CSs containing non-neoplastic skin with histological signs of chronic exposure to the sun were selected. The following clinical variables were recorded from the medical records for each patient: date of birth, date of diagnosis, skin color, gender, anatomical site of the tumor and, for patients with LM/LMM, the date of the last visit. Cases of LM/LMM that were suspected of

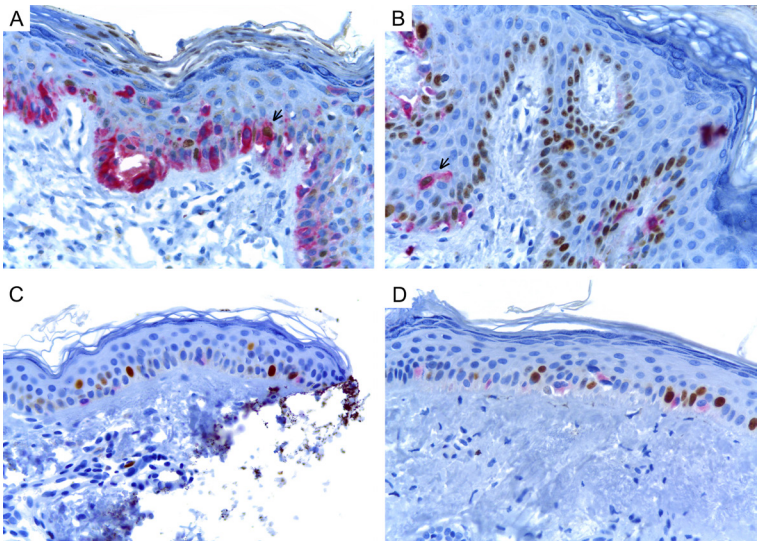

Figure 2. Immunohistochemistry double stain exhibiting melanocytes with red to pinkish cytoplasm (Melan A-positive) and proliferation cells with brown nuclei (Ki67-positive) (400×). (A) Central area and (B) periphery of LM with proliferating double-stained melanocyte (arrow). (C) Free margin skin and (D) control skin with red to pinkish melanocytes and sparse proliferative keratinocytes with brown nuclei. (D) Identified solar elastosis and hyperplastic melanocytes with some atypia and binucleation on control skin.

local recurrence, confirmed or not, were excluded.

Immunohistochemistry

Immunohistochemical staining was performed on the paraffin blocks for the selected slides containing skin samples fixed in 10% buffered formaldehyde solution, from which 4 µm sections were taken and mounted on silanized slides. For the quantification of microvessels, the monoclonal primary mouse anti-human anti-CD31 (clone JC70A; dilution 1:30) and anti-CD105 (clone SN6h; dilution 1:10) were used. Visualization was achieved using the Advance system with diaminobenzidine (DAB). Endothelial cells showing membrane and/or cytoplasm staining were considered positive (**Figure 1**). For the proliferation index of melanocytes, double labeling was performed using the mouse anti-human anti-Melan-A (clone A103; dilution 1:800) and anti-Ki-67 (clone MIB-1; dilution 1:100) monoclonal antibodies. The double labeling distinguishes Ki-67 immunoexpression of melanocytes from keratinocytes. Visualization was achieved using the EnVision G2 system, where red (permanent red) represented anti-Melan-A (cytoplasm) and brown (DAB) represented the anti-Ki-67 complex (nucleus) (**Figure 2**). Appropriate negative and positive controls were present in each assay.

Evaluation of dermal vasculature

Each tumor slide immunolabelled with anti-CD31 and anti-CD105 was thoroughly examined at low magnification (40×) to identify three areas containing highest density of microvessels (hotspots), being three within each LM/LMM and three along the lesion-free margins (FM), with the latter respecting a distance of up to 2 mm from the edge of the fragment. Each CS slide was thoroughly examined at low magnification (40×) and the three areas with the highest density of micro-vessels (hotspots) were identified. In each hotspot (320× magnification), the Chalkley optical grid was applied with 25 intersecting points corresponding to an area of 0.071 mm^2 (grid coupled to a Carl Zeiss Kpl eyepiece at 8×). The grid was positioned so that the marked vessels reached the maximum number of points. Each point of the grid reaching a CD31- or CD105-positive micro-vessel was counted regardless of the presence of visible vascular lumen so that the smallest vessels in formation were not excluded. Only vessels located superficially to the superficial cutaneous vascular plexus (included) were considered. For statistical purposes, the average of the three highest Chalkley scores for each region was calculated. Data for CD105 were also categorized as $a = 0$ and $b \geq 1$. The Chalkley counting method is recognized as a gold standard for quantification of angiogenesis in solid tumors [7].

Proliferation index evaluation

Doubly labeled cells with anti-Melan-A and anti-Ki-67, i.e. proliferating melanocytes, were counted as described below. For the LM and LMM slides: 1. “Tumor periphery”: the section was examined from the tumor-free surgical

Table 1. Clinical data and homogeneity of groups

	Skin color	Gender	Age (years)	Site
LM/LMM n = 43	White 100%	F: 62.79% M: 37.21%	Mean: 63.19 Minimum: 31 Maximum: 86	Head & Neck: 72.09% (face: 55.81%) Trunk: 16.28% Limbs: 11.63%
Control skin n = 37	White 100%	F: 59.46% M: 40.54%	Mean: 68.89 Minimum: 49 Maximum: 92	Head & Neck: 64.86% (face: 51.35%) Trunk: 18.92% Limbs: 16.22%
p-value	-	0.7604*	0.0967**	0.7654*

*Chi-square; **Mann-Whitney.

Table 2. Microvascular quantification using the Chalkley method

Chalkly Score		n	Mean	Median	Standard deviation	Minimum	Maximum	*p*-value
CD31	Under melanoma (UM)	39	6.46	6.67	1.16	3.67	8.67	UM > FM < 0.0001*
	Free margin (FM)	39	5.06	5.00	1.22	3.00	8.00	UM > CS = 0.0016**
	Control skin (CS)	31	5.63	5.67	0.93	4.00	7.67	FM < CS = 0.0244**
CD105	Under melanoma (UM)	38	1.40	0	1.88	0	5.67	UM > FM = 0.0001+
	Free margin (FM)	38	0.04	0	0.27	0	1.67	UM > CS = 0.0027++
	Control skin (CS)	31	0.15	0	0.52	0	2.33	FM < CS = 0.3194+++

*Paired Wilcoxon; **Mann-Whitney; +McNemar; ++Chi-square; +++Fisher's exact.

margin (ink stained) towards the tumor, so that the first field with morphological characteristics compatible with neoplasia was used for counting in a segment of epidermis of 0.5 mm. For the slides containing more than one section presenting an area meeting the criteria of "periphery", cell counts were performed in all such areas, though only the area with the highest count was considered. 2. "Non-peripheral tumor": this refers to any area with morphological characteristics compatible with neoplasia, not fitting in the criteria of "tumor periphery". Each section was evaluated and the area with the highest density of double-labeled intraepidermal cells was identified along a 0.5 mm segment of epidermis. 3. "Free lateral margin" (FM): the free margin of each section was evaluated in a segment of epidermis of 0.5 mm from the surgical margin and considered the highest count. For the CS slides: each slide was fully examined and the area with the highest density of double-labeled intraepidermal cells was identified, with subsequent counting in a 0.5 mm segment of epidermis. For statistical analysis, data were also categorized into a = 0 and b ≥ 1.

Statistical method

The chi-square test was used to compare the categorical variables and, whenever necessary, Fisher's or McNemar's exact test was applied. Mann-Whitney and Kruskal-Wallis tests were used to compare numerical variables. The Spearman correlation coefficient was used to study the relationship between numerical variables. The level of significance was set at 0.05.

Results

All patients were white, had a mean age of 63 years and lesions were localized mainly in areas exposed to sunlight, mostly in the head and neck region. Details on the clinical profiles of the studied groups and CSs are shown in **Table 1**. The clinical profile of the patients studied was similar to that described in the literature for LMs/LMMs.

Microvasculature quantification under the melanomas, for both CD31 and CD105, was greater than at the margins of the same specimens (P < 0.0001 and P = 0.0001) and greater than control skin (P = 0.0016 and P = 0.0027). For all quantified sites, CD31-positive vascular density was higher than CD105. The findings on microvasculature quantification for the two antibodies tested are shown in **Table 2**.

As for proliferating melanocytes, the mean number of double-labeled cells at the center of the LMs/LMMs was greater than at the periph-

Table 3. Melanocyte proliferation index

Proliferating melanocytes (Ki67+/Melan-A+)/0.5 mm of the epidermis		n	Mean	Median	Standard deviation	Minimum	Maximum	% Cases with zero count
Tumor (intraepidermal melanocytes)	Center	32	2.00	1.00	2.64	0	13	28%
	Periphery	35	0.37	0	0.65	0	2	71%
Free margin skin		35	0.03	0	0.17	0	1	97%
Control skin		35	0.09	0	0.28	0	1	91%

ery. In turn, the mean value in the periphery was greater than in FM and CS. The melanocyte proliferation index of both groups is shown in **Table 3**. Comparing the peripheral proliferation index of LMs/LMMs with that of skin without MM, that is, PCs and FMs as a unique group, we found a higher proliferation index in MMs ($P = 0.0011$; Mann-Whitney). When we categorized the proliferation indexes at $a = 0$ and $b \geq 1$ and compared the peripheral findings of LMs/LMMs to those without MMs, that is, PCs and FMs as a unique group, we found a higher relative incidence of "b" events in the MMs ($P = 0.0042$; Fisher's exact).

Regarding the CSs, the sites with the highest CD31-positve vasculature were the head and neck, especially the face, followed by the chest, and finally the limbs. The highest microvasculature values in the face compared to other sites were statistically significant ($P = 0.0385$; Dunn`s post-hoc). Also, in CSs, there was a positive correlation between melanocytic proliferation index and vasculature by CD31 ($P = 0.0335$; Mann-Whitney).

Discussion

Histological delimitation of LM/LMMs is necessary to determine whether the surgical margins are free, as well as to measure the smallest margins on excised specimens; these data are paramount to determine the next therapeutic course, such as the need for extending surgical margins. This, however, remains a challenging task in dermatopathology, especially due to the overlap of histological findings between the intraepidermal component of melanomas and MH. Photodamaged skin without melanocytic neoplasia may exhibit most, if not all, of the suspected histopathologic findings described for LMs or for the intraepidermal component of LMMs, as evaluated by routine staining or immunohistochemistry such as increased melanocyte density, confluence of melanocytes, nests/theque formation, migration to suprabasal strata, adnexal extension, irregular distribution of melanocytes or melanin pigmentation, pleomorphism, and nuclear atypia [4, 8].

Bowen et al. (2011) demonstrated that the number of suspicious findings in melanomas is greater than in MH [4]. The larger the area of tumor for microscopic evaluation, the greater the chances of meeting the histological criteria for diagnosis, which may be relatively easy on most surgical excision specimens of LM/LMM. In some areas of the skin, however, the differential diagnosis with MH is difficult, such as in the periphery of the tumor, for tumor delimitation, or in incisional biopsies. Studies have shown that the positive predictive value of small LM biopsies varied from 20% to 40%, with a false negative rate of 60% to 80% [3]. Furthermore, it is known that in the periphery of these melanomas, suspicious histological findings gradually decrease in intensity and/or frequency as one moves away from the center of the tumor.

Melanocyte proliferation index as well as peri- and intratumoral vasculature have been studied as prognostic factors for MMs [9]. Other previous studies have investigated the proliferation index as a tool for distinguishing between melanocytic nevi and MM and it has also been shown that melanocytes from MMs in general present an increased proliferation index compared to non-neoplastic skin [10, 11] in addition to the presence of proliferating melanocytes in non-melanocytic neoplastic skin [12, 13]. Nonetheless, such parameters have hardly been studied for assessing margins in LMs/LMMs.

Trotter and Tron (1994) studied the dermal vasculature in LMs ($n = 11$) and LMMs ($n = 15$) using the immunohistochemical vascular marker Ulex europaeus agglutinin I (UEA) and reported greater vascularization in the dermis of

tumors when compared to tumor-free adjacent skin. The difference was significant comparing the intraepidermal component of LMMs and non-neoplastic skin. They further described that vasculature under LMs and under LMMs in distant areas of invasive lesions increased at the expense of hypervascularization foci [5]. In the present study, using CD31, greater vascularization was found under LMs/LMMs compared to adjacent normal skin, especially along the free surgical margin ($P < 0.0001$); still, higher vascularity was found when compared to CSs ($P = 0.0016$). Despite the significant difference between the means, however, the variation of the measurements was broad, with significant overlap between different regions (**Table 2**).

Comparing the Chalkley score of CSs to that of the skin along the free margin, the former showed higher values (CS = 5.63; FM = 5.06), which can be explained by the greater skin area evaluated as hotspots in the CS group count compared to the restricted counting area along the surgical margins of LM/LMM excision specimens. The greater the area analyzed, besides increasing the chance of finding hotspots with positive vessels, the higher the chance of identifying incipient lesions that stimulate angiogenesis, such as actinic keratoses and solar lentigos [14].

Unlike CD31 and UEA, CD105 is expressed by the endothelium of newly formed blood vessels [14]. Neoangiogenesis appears to occur in the early stages of MMs progression, including those restricted to the epidermis, increasing as the tumor progresses [5]. This study corroborates this rationale. CD105 positivity was significantly higher in the dermis under LMMs compared to LMs ($P = 0.0227$). This comparison was not significant for CD31, a "pan-endothelial" marker (LMM > LM, $P = 0.8110$). Compared to FM and CS, greater neovascularization was found under the LMs/LMMs ($P = 0.0001$ and $P = 0.0027$, respectively). There was no significant difference when comparing the FM and CS groups ($P = 0.3194$). Despite the difference between groups using CD105, no neoformed vessels were detected in a large percentage of events, limiting the use of this tool for individual cases: the Chalkley score was zero in 57.89% of tumors, 97.37% of FMs and 90.32% of CSs. In FM, only one case was positive to CD105, coincidentally under a focus of actinic keratosis. In CS, only three CD105-positive cases were found, from which one showed positive vessels within a background of nonspecific chronic inflammation; in the remaining two cases, no relation to any lesion was found in the examined sections. The findings of this study support the hypotheses that, regarding the differential diagnosis between MH and LM/LMM, in the surgical margin of an excision specimen (for decision between free or compromised margin), the transition between melanoma and adjacent skin (when delimiting MM) or in an incisional biopsy, the presence of CD105-labeled vessels without any other lesion to justify it, should raise the suspicion of LM/LMM.

The vasculature of the dermis is denser in the deep and superficial plexuses as well as around the appendages, which may justify finding a higher mean vascular density with CD31 positivity in samples from the face compared to other sites ($P = 0.0385$), since the face has a high density of cutaneous appendages. This difference was not observed in FM of the excision specimens, however, since the head and neck samples (including face) showed the highest mean values. This may have occurred because the cell count was confined to a restricted area along the margin, which may not have been fully representative of the vasculature of the region as for the counting on the CSs, for which a larger area of skin was analyzed. The authors of this study are unaware of any previous studies comparing the microvascular density of non-neoplastic skin from different topographies using the Chalkley grid in this scenario. Regarding the proliferation index of melanocytes in CSs, all cases in this study exhibiting double-labeled cells (Ki67+/Melan-A+) were located in the face. There was a correlation between the Chalkley score for CD31 and the number of proliferating melanocytes in the CS group ($P = 0.0335$), probably influenced by the fact that the face is susceptible to sun exposure, with consequent stimulation of melanocytic proliferation.

In the evaluation of proliferating melanocytes, unlike previous studies, counts were limited to a segment 0.5 mm from the lateral margin of the excised specimens and in the periphery of such tumors. The low count of proliferating melanocytes in the periphery of the tumor and the long time elapsed between the patient's

discovery of the tumor and its surgical removal in several cases (median of 5.77 years, maximum of 22 years) are in agreement with the literature regarding the behavior of such tumors: insidious growth with more favorable biological behavior than other types of MM [1]. Furthermore, a correlation was not found between intraepidermal (*in situ*) melanocyte counts in peripheral proliferation LMMs with Clark and Breslow, corroborating previous reports suggesting that the invasive phenotype of this type of MM has different characteristics from the lesion *in situ*, possibly conferring a more aggressive behavior to the former, while the intraepidermal lesion continues its insidious lateral growth [1]. In the present study, proliferating melanocytes were counted in the invasive lesions of some LMMs, using the same magnification field used to count intraepidermal melanocytes (400×); the mean count was 32.5, much higher than the maximum value recorded for *in situ* lesions, which was 13.

Regarding tumor delimitation and assessment of margins, despite a difference in mean proliferation index of melanocytes between the periphery of the tumor and those from the tumor-free epidermis in both FM and CS, 71.43% of the LMs/LMMs evaluated did not show any proliferating melanocytes in their periphery (**Table 3**). Thus, when evaluating individual cases, this parameter was not decisive for precise outlining of such tumors. However, since double labeling of melanocytes in non-tumor areas (1/35 in FM and 3/35 in the CS) was uncommon, the presence of Ki-67 positive melanocytes along the surgical margin of an alleged tumor excision may signify a possible extended lesion.

At the center of the LMs/LMMs studied, the mean proliferation index was higher than the periphery, possibly due to the higher density of neoplastic cells in the central areas. In the group of cases studied, it was found that the proliferation index of the central regions of the tumors was significantly higher in younger individuals (proliferation index = 0: mean age 71.78 years; proliferation index ≥ 1: mean age 59.70 years, P = 0.0292; Mann-Whitney).

Weyers et al. (1996) reported on Ki-67 double-labeled (Ki-67 + S100) melanocyte fraction in ten cases of MM *in situ* from sun-damaged skin (but not necessarily LM), ten skin samples adjacent to basal cell carcinoma (BCC) and ten with MH not related to tumors or inflammation. They found a greater fraction of proliferating melanocytes in the areas of MH adjacent to BCC compared to MMs. Interestingly, although in the present study a higher proliferation index was found in LMs/LMMs compared to CSs, from the three cases of CSs that exhibited double labeling, such proliferation was located in the epidermis adjacent to a BCC in two cases. This study corroborates the conclusion of Weyers et al. (1996) regarding caution in interpreting the presence of proliferating melanocytes in sun-damaged skin [8].

Conclusion

The presence of neovascularization demonstrated by CD105 as well as proliferating melanocytes (Ki67+/Melan-A+) may be considered suspicious findings for LM/LMM, helping to outline, diagnose and evaluate the margins of these tumors. The definitive diagnosis between MH and MM in the establishment and evaluation of LM/LMM margins should take into account all suspicious findings. Whenever possible, the lesion-free sun-damaged skin sample of the same individual should be compared as an attempt to identify MH patterns in the lesion, thus minimizing the effect of the wide overlap of suspicious LM findings previously reported, as well as those studied herein, with those of MH on sun-damaged skin.

Disclosure of conflict of interest

None.

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