Received: 5 March 2023 | Revised: 29 September 2023 | Accepted: 30 October 2023

DOI: 10.1111/cup.14561

ORIGINAL ARTICLE

JOURNAL OF CUTANEOUS PATHOLOGY WILEY

Can angiotropism and lymphovascular invasion refine the current cutaneous melanoma staging system?

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Abstract

Background: Several prognostic factors for primary cutaneous melanoma (PCM) have been identified, and these predict metastasis and survival, to a certain extent. We sought to determine the frequency of angiotropism (AT) and lymphovascular invasion (LVI) in PCM and the relationship between AT, LVI, and other clinicopathological parameters and patient's prognosis.

Methods: This study included 538 cases of PCM diagnosed between 2003 and 2016. It comprised 246 females and 292 males whose clinicopathological variables were evaluated with respect to LVI and AT using univariate and multivariate analyses. Overall survival (OS) was assessed by Kaplan–Meier (KM) analysis and Cox regression multivariate analysis.

Results: AT occurred more frequently than LVI. Ulceration, mitotic rate, and Breslow thickness were found to be highly associated with both LVI and AT ($p < 0.01$). All LVI + cases had AT, with a significant positive correlation ($p < 0.01$). Both AT and LVI predicted lymph node (LN) metastasis (odds ratio [OR] = 1.47, 1.12, respectively). Multivariate analysis showed LN metastasis, Breslow thickness, LVI, and AT as predictors of OS. LVI and AT independently predicted adverse OS by Cox regression analysis (hazard ratio [HR] = 1.66, 1.49, respectively) and with KM survival analysis.

Conclusion: AT is a marker for angiotropic extravascular migratory tumor spread (angiotropic EVMM), and LVI is a marker for intra-lymphovascular tumor spread. Both predict poor prognosis. Given its ease of detection, AT could be adopted as a histologpathological feature in the routine assessment of primary cutaneous malignant melanoma cases.

KEYWORDS
angiotropism, lymphovascular invasion, primary cutaneous melanoma

1 | INTRODUCTION

Malignant melanoma is known to metastasize aggressively at an early stage and to virtually any tissue.[1] Various mechanisms encourage metastasis, such as intravascular spread of tumor cells in lymphatic and blood vessels leading to lymph node (LN) and distant organ metastasis. In addition, extravascular migratory tumor spread (angiotropic extravascular migratory metastasis [angiotropic EVMM]) is now

accepted as an additional mechanism of cancer spread and metastasis, for example, in melanoma, pancreatic cancer, glioblastoma, and other solid tumors.[2] Angiotropic cellular migration has a basis in embryological development and thus represents an advantageous means of metastasis.[3] In contrast, intravascular circulation is not necessarily an advantageous route for metastasis, as the vast majority of circulating tumor cells are destroyed.[4] Both types of metastasis are important prognostic markers.[1,5,6] Indeed, distant organ metastasis is the main cause of death in patients with malignant melanoma.[1] Lymphovascular spread can be due to lymphangiogenesis or angiogenesis, where new vessels grow within the tumor or within the tumor microenvironment.[7,8] However, angiogenesis or lymphangiogenesis is not a requisite for cancer growth and metastasis since the recognition of vascular co-option (the absence of angiogenesis) and angiotropic EVMM as legitimate mechanisms of tumor growth and tumor spread.[9]

Revising the current prognostic factors and staging of cutaneous melanoma to reflect the best current knowledge of malignant melanoma biology is crucial for better risk stratification of patients. It is also important in the development of surveillance strategies to reduce the hazard of subsequent metastasis. Several prognostic factors for primary cutaneous melanoma (PCM) have been identified, and these predict metastasis and survival, to a certain extent.[10] Breslow thickness and ulceration are considered the only histopathological parameters that determine tumor stage in the eighth edition of the American Joint Committee on Cancer (AJCC) melanoma staging system.[11] Lymphovascular invasion (LVI) is defined histopathologically as the presence of tumor cells within the lymphatic and blood vessels[8,9] and is believed to be a mechanism by which melanoma cells can disseminate to regional LNs and to distant sites.[12] Despite the difficulty that can sometimes affect the frequency of detection of LVI on routine histology slides, such as retraction artifacts and obliteration of the whole vascular lumen by tumor[13] as well as infolding and simple folding of the vascular or lymphatic wall in the presence of angiotropism (AT),[3] there is a wealth of evidence showing the link between true LVI and adverse prognosis.[14–16] However, it is important to note that because of this ambiguity, LVI may in fact be AT in some percentage of cases, as apparent "intravascular" tumor may not be intravascular but rather abluminal. Strategies such as the use of histochemical or immunohistochemical markers to show vascular structures has improved the rate of detection in equivocal cases in melanoma and other cancers.[17] Additional meticulous studies of "LVI" are needed in order to better sort out the exact location of tumor cells in relation to lymphatic and microvascular lumina (intravascular vs. extravascular) and the various types of artifacts confounding the recognition of true versus false LVI.

In addition, malignant melanocytes have the ability to migrate along the external surfaces of vessels, forming what is known as the angio-tumoral complex (ATC).[18] In this complex, melanocytes become attached to an amorphous matrix containing laminin.[19] This phenomenon is known as angiotropic extravascular migratory metastasis (EVMM).[18] AT is the histopathological marker of EVMM and is defined as cuffing of the vascular channels at the advancing front of the tumor by melanoma cells in a pericyte-like fashion, also known as pericytic mimicry.[20] There is still relatively limited data regarding the frequency of AT in cutaneous melanoma, its relation to other histopathological parameters including LVI, and its association with patients' prognosis. Studies have revealed overlapping genes expressed in both migrating cancer cells and embryonic stem cells during neural crest migration.[21] The clinical relevance of lymphatic invasion and AT in melanoma prognosis is still controversial and they are not included as part of the tumor staging system; however, increasing evidence has indicated that AT has important prognostic value in melanoma.[3,22–25] Therefore, it is imperative to study these parameters in large cohorts of patients with primary melanoma to determine the prognostic significance of LVI and AT.

The aims of this study were (i) to determine the frequency of AT and LVI in routine histopathological slides of 538 primary melanoma cases; (ii) to investigate the relationship between AT and LVI and known histopathological prognostic parameters; and (iii) to establish the independent prognostic significance of AT and LVI.

2 | MATERIALS AND METHODS

2.1 | Patient characteristics and data

Five-hundred and thirty-eight cases of PCM diagnosed between 2003 and 2016 were used as a random study cohort. Archived routine Haematoxylin and Eosin (H&E) sections of each case (average of four sections/case) were retrieved. The melanoma cohort used was linked to annotated clinicopathological data such as age, sex, tumor site, Breslow thickness (mm), ulceration, mitotic number (per mm^2). Clark level, lymph node (LN) metastasis, growth phase (vertical/radial), and histopathological subtypes in addition to patient survival (Table 1). Melanoma-specific survival (MSS) was defined as the duration between the date of initial diagnosis to the date of melanoma-related death or the census date (if still alive) in months. Tumor staging was done according to the eighth edition of the AJCC staging.[4] Ethical approval of the study was given by the Nottingham Health Science Biobank Access Committee.

2.2 | Immunohistochemistry

Immunohistochemistry (IHC) was used to detect AT and LVI in equivocal cases using lymphatic endothelial cell markers D2-40 and CD34. IHC was performed on formalin-fixed, paraffin-embedded sections using commercially available Roche Ventana Ultraview detection kits (Cat 05269806001), which use an indirect biotin-free system for mouse monoclonal anti-human CD34 (IgG1, Ventana CONFIRM, CAT 7902927) and mouse monoclonal anti-human D2-40 (IgG, CAT 7804305), and OptiView HQ linker according to manufacturer's recommended standard operating procedures. Detection was achieved with horseradish peroxidase Multimer and 3,3′ diaminobenzidine tetrahydrochloride (DAB) with hematoxylin counterstaining. Positive and negative controls were included for validation. Full details of the IHC techniques are described in previous publications.[5,6]

TABLE 1 Summary of the clinicopathological data for melanoma patients.

Factor	Frequency *n* (%)
Gender	
Male	292 (54)
Female	246 (46)
Age at primary diagnosis (years)	
<66	262 (49)
>66	276 (51)
Histological subtype[a]	
Unknown	37 (6.8)
SSMM	327 (60.7)
NM	95 (17.6)
ALM	27 (5)
LMM	52 (9.6)
Breslow thickness (mm)	
T2 > 1–2	133 (24.7)
T3 > 2–4	156 (28.9)
T4 > 4	148 (27.5)
Site of tumor	
Head and neck	111 (20.6)
Trunk	88 (16.4)
Upper limb	107 (19.9)
Lower limb	135 (25.1)
Pelvic region	18 (3.3)
Back	79 (14.7)
Ulceration	
Absent	388 (72.1)
Present	150 (28.4)
Growth phase	
Vertical	493 (91.6)
Radial	43 (7.9)
Equivocal	2 (0.4)
Mitosis	
Absent	110 (20.4)
Non-brisk (<4/mm^2)	281 (52.2)
Brisk (>4/mm^2)	147 (27.3)
Clark levels	
1	3 (0.6)
2	52 (9.7)
3	68 (12.6)
4	364 (67.6)
5	51 (9.5)
Lymph node metastasis	
Absent	457 (84.9)
Present	81 (15.1)
Mortality	
Alive	300 (55.8)
Death due to melanoma	108 (20)
Death due to other causes	130 (24.2)

[a]Main histological subtypes are SSMM (superficial spreading malignant melanoma), NM (nodular melanoma), ALM (acral lentiginous melanoma), and LMM (lentigo maligna melanoma).

2.3 | Detection of LVI and AT

Routine H&E sections were evaluated independently by two pathologists. Each case had a minimum of two slides (average of four slides per case). LVI was defined as tumor emboli present within the vessel lumen, and this was applied to both blood and lymphatic vessels. The cases were grouped into positive, negative, and equivocal. IHC was performed on 12 equivocal cases using CD34 and D2-40 monoclonal antibodies for further discrimination. Scenarios of equivocal cases include presence of tumor emboli partially obliterating the majority of the lymphatic vessel lumen, the presence of stromal retraction artifact adjacent to tumor nests, and tumor carryover into lymphatic spaces that can mimic LVI.

AT was defined as melanoma cells cuffing the external surfaces of vessels in a pericytic location without intravascular invasion.[7–9] These vessels are exclusively identified at the invasive front margin of the tumor. Cases were divided into positive and negative. There were 12 equivocal cases that needed IHC staining. Histopathological assessment of AT and LVI was done using an ordinary microscope. IHC-stained sections were scanned using a NanoZoomer C9600-01 scanner (Hamamatsu Photonics, Welwyn Garden City, UK) and images were edited/annotated using the NDP view 2 software version 2.6.13. IHC slides were interpreted as positive or negative based on the presence or absence of LVI and AT.

2.4 | Statistical analysis

IBM SPSS software version 24 (Chicago, IL, USA) was used to analyze the relation between AT and LVI in relation to clinicopathological parameters and survival. Kappa (κ) coefficient was used to measure inter-observer agreement. Any cases where the two pathologists disagreed were reviewed at a double-headed microscope and a consensus decision was made. The association of AT and LVI with clinicopathological parameters was assessed using the chi-squared test or Fisher's exact test. Metastasis-free survival and melanoma-specific survival were assessed using the Kaplan–Meier (KM) log-rank test. Cox hazard regression analysis was performed to quantify the risk of clinicopathological parameters including AT and LVI on survival, and 95% confidence interval (CI) was estimated. Where the 95% CI did not include the null value 1, the hazard ratio (HR) was considered significant at the $p < 0.05$ level. A p-value of <0.05 (two-tailed) was considered to denote statistical significance.

3 | RESULTS

3.1 | Frequency of AT and LVI

Routine H&E slides for 538 cases of PCM were examined to determine the frequency of LVI and AT. The analysis was carried out on a thick melanoma cohort with deeper invasion. Overall, AT was more common in PCM than LVI. The frequency of AT was 283 (53%) positive, 255 (47%) negative, and 12 (2%) equivocal. The frequency of LVI was 91 (17%) positive, 477 (83%) negative, and 12 (2%) equivocal. Figure 1 shows representative photomicrographs of positive LVI and AT cases.

3.2 | LVI is associated with poor prognostic parameters

The association between LVI and other clinicopathological factors is summarized in Table 2. There was significant association between LVI and Breslow thickness, Clark level, ulceration and mitotic rate, LN metastasis, and growth phase ($p < 0.05$). Cases with LVI were twice as likely to have a Breslow thickness >4 (45/91, 50%) or ulceration (45/91, 49%) than those without LVI (Breslow thickness >4: 103/447, 23%; ulceration: 105/447, 23%). Age and tumor site did not have any significant association with LVI. The majority of the LVI+ cases had a vertical growth phase (89/91, 98%). The four cases confirmed with D2-40 IHC had a mean thickness of 4.3 mm. Three of them had ulceration and mitosis with a Clark level of 4. Two had LN metastasis.

3.3 | AT is associated with poor prognostic parameters

There was significant association between AT and ulceration, mitotic rate, Clark level, Breslow thickness, and LN metastasis ($p > 0.05$). Cases with AT were 10% more likely to have a Clark level of 4 (205/283, 72%) and a Breslow thickness >4.00 (93/283, 33%) than those without AT (Clark level of 4,159/255, 62%; Breslow thickness >4.00, 55/255, 22%). Most AT+ cases had a vertical phase growth (266/283, 94%) as seen with LVI+ cases. Age and tumor site did not have any significant association with AT as well. The AT cases confirmed with IHC had the same characteristics as the LVI IHC-confirmed cases. A summary of this association is shown in Table 3.

3.4 | AT and LVI are predictors of LN metastasis

LN metastasis was more frequent with cases positive for AT (53/283, 19%) and LVI (21/91, 23%) than with the negative ones (AT 28/255, 11%, LVI 66/447, 13%) (Tables 2 and 3). There was a significant association between LVI, AT, and LN metastasis ($p < 0.05$). Logistic regression analysis showed that patients with LVI and AT had an increased risk of LN metastasis with an odds ratio (OR) of 1.12 and 1.47, respectively. Ulceration had the highest OR (1.69), with increased Breslow thickness ($p < 0.05$) being associated with an increased likelihood of getting LN metastasis (Table 3). LN metastasis significantly predicted poor survival with HR = 2.46 ($p < 0.05$) compared to other variables (Tables 4 and 5).

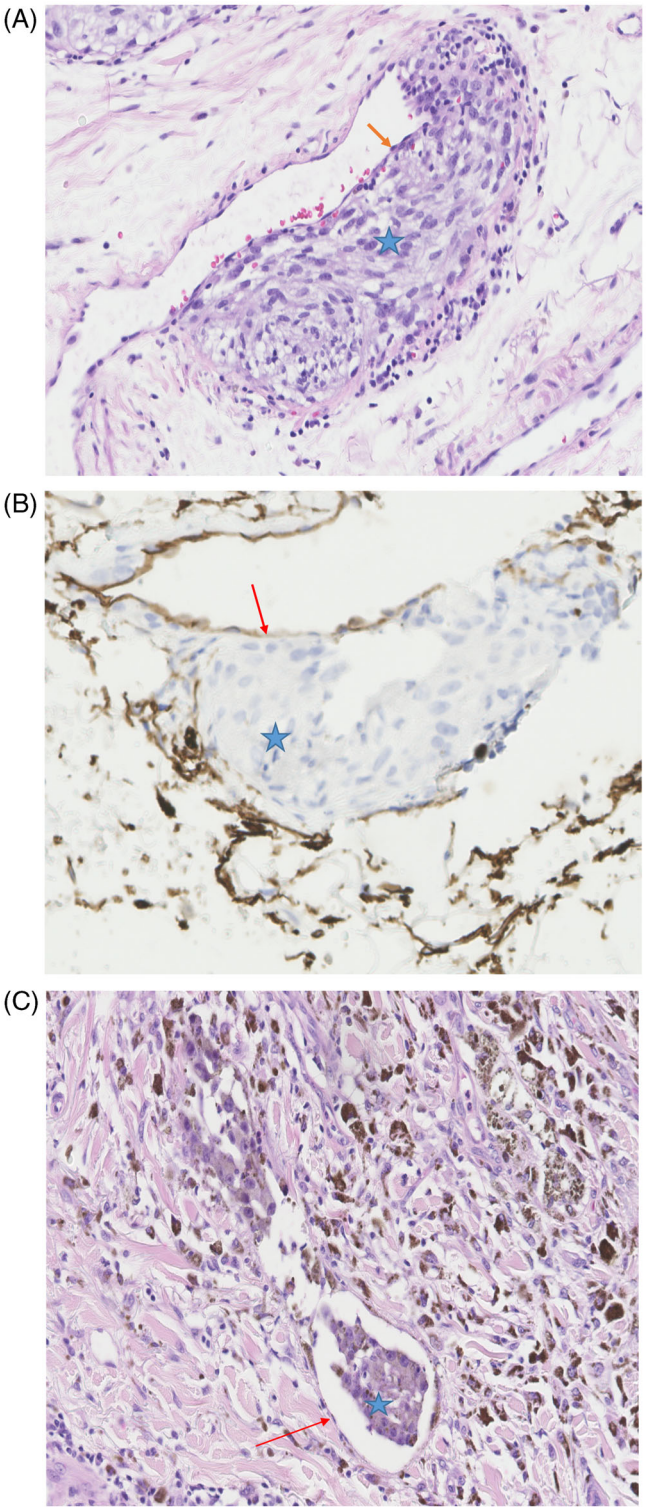

FIGURE 1 Legend on next page.

3.5 | LVI and AT have a negative effect on melanoma-specific survival

The mean survival for the cohort was 49.8 months (283 at risk). There was a significant difference between the KM curves for those with and without LVI (Figure 2A, Table 5). Positive cases had a mean survival of 43.4 months (95% CI: 38.9–47.9 months) as compared to those with negative LVI tumors (51.3 months; 95% CI: 49.6–52.9 months) ($p > 0.05$).

The presence of AP was significantly associated with reduced overall survival ($p < 0.05$) on univariate KM analysis (Figure 2B, Table 5). Cases with AT had a mean survival of 48.3 months (95% CI: 46–50.7 months) as compared to patients without AT, with a mean of 51.5 months (95% CI: 49.3–53.8 months).

On multivariate Cox regression, LVI was a significant predictor of worse survival with HR = 1.57 (95% CI: 1.09–2.50) as compared to AT with HR = 0.92 (95% CI: 0.59–1.42; Table 6). LN metastasis, age, and Breslow thickness were also significant predictive factors for adverse survival outcome. Sex and Clark level were eliminated because there was no evidence that these factors significantly modified the predictive effect of LVI.

4 | DISCUSSION

This study showed that AT and LVI can predict adverse prognosis and poor survival. Their association with known negative clinicopathological parameters supports the validity of our findings. AT was found to occur more frequently with a positive correlation to LVI in PCM in this study. The importance of IHC as an ancillary test in equivocal cases of LVI was shown in 4 out of 12 cases.

Although LVI is not a part of the AJCC staging system, we show that detecting LVI in routine histology tests predicts poor prognosis. LVI showed a significant association with known poor prognostic markers such as Breslow thickness, mitotic rate, ulceration, and LN metastasis. A retrospective study on 2183 melanoma cases by Egger et al. found an association between increasing Breslow thickness and ulceration with LVI.[26] Hypoxia due to ulceration releases vascular endothelial growth factor, increasing lymphangiogenesis. These new vessels may be pathways for disseminating tumor cells.[27] In addition, AT has also been associated with ulceration in other studies as ours, and this may be based on the inflammatory neutrophil-rich infiltrates in ulceration that may stimulate AT.[22,28] Prognostic factors were also significantly associated with AT in our univariate analysis. Mitotic rate and Breslow thickness were reported to be associated with AT in a previous study on 20 primary melanomas.[29] AT was also significantly associated with LN metastatic cases in this study. Barnhill et al. reported definite AT in 40% primary metastasizing melanomas.[30] Increased Breslow thickness, mitotic rate, and presence of ulceration were also significantly associated with AT in another study.[31] AT and LVI had similar associations with the same poor prognostic factors in this study. Considering that LVI cases were angiotropic in our study, there is a plausible pathological mechanism linking AT to LVI. This suggests that AT (HR = 0.92) could possibly be a precursor to invasion (HR = 1.57). However, because of the difficulty of identifying LVI using routine histology tests, some LVI cases may be "false positive" when in fact they are angiotropic tumor aggregates that are in reality external to the vascular lumina appearing to be intraluminal as a result of sectioning, folding, or infolding or disruption of vascular walls, which strengthens the need for IHC for confirmation in such instances.

AT occurs more frequently than LVI on routine histology tests, as supported by a number of studies. For example, Barnhill et al. reported only one case with LVI among 21 positive angiotropic metastasizing melanoma cases.[30] Our study detected 3 times more AT+ (53%) cases than LVI+ (17%) cases, with 4 of 17 confirmed by D2-40 immunostaining. A retrospective study by Hung et al. established AT in 70% of the primary lesions.[29] This study confirmed that AT is more frequent than LVI in PCM. This is because tumor cells attach to vascular surfaces following a chemotaxis attraction toward vessels as the tumor advances.[32] A histopathological search of melanomas for AT is a highly pertinent in its own right and not simply as screening for LVI per se because AT is now recognized as an independent marker of extravascular migratory tumor spread and metastasis and as a prognostic factor.[3] Further, with application of specific criteria, AT is easy to identify in H&E-stained slides. Likewise, AT was found in most of our cases. We believe this is for two reasons. First, our cohort consisted of thick melanomas, thus representing an advanced stage of melanoma. Second, angiotropic EVMM can be conceived as a multistep process whereby tumor cells (i) approach vascular channels, (ii) adhere to the abluminal surface in a pericytic location along microvascular channels or an adventitial cell location in larger vessels, (iii) progressively migrate along these vascular channels without intravasation, and (iv) arrest migration in order to form local, regional, or distant metastases. Documenting these stages would require extensive tissue sampling/leveling, which would have made the clinical translation of this morphological biomarker unfeasible. In this study, our goal was to utilize a simple, previously described method for scoring AT that pathologists can implement into routine practice.[2,12] Specific molecular biomarkers are currently being sought to more precisely understand and detect angiotropic EVMM and thus increase its specificity as a prognostic biomarker.[3] AT was significantly

FIGURE 1 (A) Angiotropism showing the intact endothelial lining (arrow) cuffed by melanoma cells (star). Hematoxylin and eosin stain (H&E) ×200 magnification. (B) CD34 staining highlighting the endothelial lining of the blood vessel (arrow), which is cuffed by the melanoma cells (star). Immunohistochemistry staining ×200 magnification. (C) Lymphovascular invasion (LVI) showing melanoma cells (star) within the vascular lumen (arrow) detected by routine histology. H&E stain ×200 magnification. This figure may be interpreted as LVI. However, in addition, in the upper part of the figure, extravascular tumor (outside the lymphatic wall) is seen, suggesting disruption or sheering of the lymphatic endothelial lining and displacement of tumor. Possible angiotropism thus may be present as well, and possibly could be the principal finding. This illustrates the difficulty of interpreting some specimens.

TABLE 2 Association between clinicopathological variables and LVI in primary cutaneous melanoma detected using routine histology, 538 cases.

	LVI with clinicalpathological factors (total = 538 cases)		
	LVI+ (*n* = 91)	LVI− (*n* = 447)	*p*-value
Sex			
Male (*n* = 292)	58 (58)	239 (53)	0.535
Female (*n* = 246)	38 (42)	208 (47)	
Site			
Head and neck (*n* = 111)	25 (27)	86 (20)	0.798
Trunk (*n* = 88)	9 (10)	79 (18)	
Upper limb (*n* = 107)	17 (19)	90 (20)	
Lower limb (*n* = 135)	19 (21)	116 (26)	
Pelvic region (*n* = 18)	3 (3)	15 (3)	
Back (*n* = 79)	18 (20)	61 (14)	
Histological subtype			
SSM (*n* = 327)	46 (50)	281 (63)	0.169
NM (*n* = 95)	23 (25)	72 (16)	
LMM (*n* = 52)	7 (8)	45 (10)	
ALM (*n* = 27)	6 (7)	21 (5)	
Others (*n* = 37)	9 (10)	28 (6)	
Growth phase			
Vertical (*n* = 490)	89 (98)	404 (90.4)	<0.05
Radial (*n* = 43)	2 (2)	41 (9.2)	
Equivocal (*n* = 2)	-	2 (0.4)	
Ulceration			<0.05
Absent (*n* = 388)	46 (51)	342 (77)	
Present (*n* = 150)	45 (4)	105 (23)	
Mitotic rate			
Absent (*n* = 110)	5 (6)	105 (23)	<0.05
Brisk (*n* = 147)	41 (45)	106 (24)	
Non-brisk (*n* = 281)	45 (49)	236 (53)	
Clark level			
1 (*n* = 3)	1 (1)	2 (1)	<0.05
2 (*n* = 52	2 (2)	51 (11)	
3 (*n* = 68)	8 (9)	60 (13)	
4 (*n* = 364)	70 (77)	294 (66)	
5 (*n* = 51)	11 (12)	40 (9)	
Breslow thickness			
≤1.00 (*n* = 101)	4 (4)	98 (22)	<0.05
1.01–2.00 (*n* = 133)	12 (13)	121 (27)	
2.01–4.00 (*n* = 156)	30 (33)	125 (28)	
>4.00 (*n* = 148)	45 (50)	103 (23)	
Age at diagnosis			
Median: 66 years, range: 74 years			
<66 years (*n* = 262)	42 (46)	220 (49)	0.736
≥66 years (276)	49 (54)	227 (51)	
LN metastasis			
Present (*n* = 81)	21 (23)	60 (13)	<0.05
Absent (*n* = 457)	70 (77)	387 (87)	

Abbreviations: ALM, acral lentiginous melanoma; LMM, lentigo maligna melanoma; LN, lymph node; LVI, lymphovascular invasion; NM, nodular melanoma; SSM, spreading malignant melanoma.

TABLE 3 Association between clinicopathological variables and angiotropism in primary cutaneous melanoma detected using routine histology, 538 cases.

	Angiotropism with clinicopathological factors (total = 538 cases)		
	Angiotropism positive ($n = 283$)	Angiotropism negative ($n = 255$)	*p*-value
Sex			<0.05
Male ($n = 292$)	169 (60)	123 (48)	
Female ($n = 246$)	114 (40)	132 (52)	
Site			0.559
Head and neck ($n = 111$)	64 (23)	47 (18)	
Trunk ($n = 88$)	40 (14)	48 (18)	
Upper limb ($n = 107$)	56 (20)	51 (20)	
Lower limb ($n = 135$)	62 (22)	73 (28)	
Pelvic region ($n = 18$)	5 (2)	13 (5)	
Back ($n = 79$)	48 (17)	31 (12)	
Histological subtype			0.285
SSM ($n = 327$)	162 (57)	165 (65)	
NM ($n = 95$)	60 (21)	35 (13)	
LMM ($n = 52$)	27 (10)	25 (10)	
ALM ($n = 27$)	13 (5)	14 (5)	
Others ($n = 37$)	21 (7)	16 (6)	
Growth phase			0.092
Vertical ($n = 493$)	266 (94)	227 (89)	
Radial ($n = 43$)	15 (5.3)	28 (11)	
Equivocal ($n = 2$)	2 (0.7)		
Ulceration			<0.01
Absent ($n = 388$)	186 (66)	202 (79)	
Present ($n = 150$)	97 (34)	53 (21)	
Mitotic rate			<0.01
Absent ($n = 110$)	38 (14)	72 (28)	
Brisk ($n = 147$)	95 (34)	52 (20)	
Non-brisk ($n = 281$	150 (53)	131 (52)	
Clark levels			<0.05
1 ($n = 3$)	2 (1)	1 (0.3)	
2 ($n = 52$	19 (7)	33 (13)	
3 ($n = 68$)	29 (10)	39 (15)	
4 ($n = 364$)	205 (72)	159 (62)	
5 ($n = 51$)	28 (10)	23 (9)	
Breslow thickness			<0.05
≤1.00 ($n = 101$)	38 (13)	63 (25)	
1.01–2.00 ($n = 133$)	61 (22)	72 (28)	
2.01–4.00 ($n = 155$)	91 (32)	64 (25)	
>4.00 ($n = 148$)	93 (33)	55 (22)	
Age at diagnosis			
Median: 66 years, range: 74 years			0.459
<66 years ($n = 262$)	133 (47)	129 (51)	
≥66 years (276)	150 (53)	126 (49)	
LN metastasis			<0.05
Present ($n = 81$)	53 (19)	28 (11)	
Absent ($n = 457$)	230 (81)	227 (89)	

Abbreviations: ALM, acral lentiginous melanoma; LMM, lentigo maligna melanoma; LN, lymph node; LVI, lymphovascular invasion; NM, nodular melanoma; SSM, spreading malignant melanoma.

associated with LVI+ cases because some percentage of LVI represents true AT, misclassified because of various tissue alterations falsely suggesting LVI (false LVI), as mentioned above. Nonetheless, true LVI may occasionally be present.[32] More definitive study is needed to establish the relationship between AT, EVMM, and true LVI.

LVI is difficult to detect using routine histopathology because melanoma cells obscure the endothelial lining; hence its low frequency.[13] Our study suggests that it is not worthwhile to histopathologically search for LVI in the absence of AT, but this requires further study. Importantly, as mentioned above, apparent LVI may be a morphologically altered presentation of AT or artifact in some cases. We detected more LVI+ cases (17%) compared to 7.8% in a study by Egger et al.[26] This could be because the majority of our cases were thick melanomas >2.0 mm, with 82% of the LVI+ cases being thick. Increased Breslow thickness has been shown to be correlated with the presence of LVI.[26] Blood vessel invasion was reported to be uncommon (1%–3%) in some studies[33] but because there was no distinction between blood vessels and lymphatics in our study, the percentage was high.

In a multivariate model, LVI and AT strongly predicted LN metastasis besides mitotic rate and ulceration. Egger et al. established that LVI predicted a greater risk of LN metastasis.[26] However, Breslow thickness had the highest significance in this study ($p < 0.05$) and is considered the most important factor in PCM. Ulceration was a stronger predictor of LN metastasis (OR = 1.69), and literature shows the importance of ulceration when selecting patients for sentinel LN biopsy, especially in thin melanoma.[34] Correlation between LN metastasis and LVI has consensus in some studies confirming the relationship,[35] while other studies have found no supportive evidence.[36,37] In this study, we found some evidence for a significant positive correlation between LN metastasis and LVI (OR = 1.12) detected by routine histology tests. Fohn et al. reported four cases with LVI on histology and D2-40 IHC as positive for LN metastasis ($p < 0.0001$).[38] Our study showed that two cases detected by D2-40 IHC had LN metastasis. A study with 52.5% angiotropic positive metastasizing melanomas[30] supports that AT strongly predicts LN metastasis. Despite the lack of significant association between AT in primary melanomas and corresponding brain metastatic lesions, Hung et al. reported a trend that suggested AT as a cause for these metastases.[29] This implies that the spread of tumor cells through and alongside lymphatics or blood vessels leads to tumor in LN and other organs.

LVI and AT have adverse effects in PCM. Our study found a significant association between AT and LVI detected by routine histology and reduced survival. LVI and AT independently predicted survival in our Cox regression multivariate model when compared with other covariates. Despite a small cohort showing no survival difference with KM analysis, Hung et al. reported that there was a tendency for significance between the angiotropic and non-angiotropic group.[29] However, one study did not observe any effect of AT on survival but had few cases.[30] Egger at al. showed LVI to be associated with reduced survival ($p = 0.0009$) as well as age and thickness.[26] LVI and AT are negative prognostic variables and should be considered as indicators of disease progression and patient outcomes.

This study was retrospectively designed without bias to any parameters involved. Examination of routine diagnostic H&E stained histology sections was used to detect AT and LVI with clear and distinct definitions by two pathologists. This is a strength of our study: we were able to demonstrate these findings in routinely stained slides, and therefore our results should be valid in clinical histological assessment of AT and LVI in PCM. In addition, our findings are supported by those of other published studies. However, some results lacked significance when interrogated with interaction terms with other covariates

TABLE 4 Multivariate logistic regression analysis of clinicopathological variables predictive of lymph node metastasis.

OR for LN	
LVI	1.12 (95% CI: 0.58–2.17) $p < 0.1$
Angiotropism	1.47 (95% CI: 0.81–2.66) $p < 0.1$
Ulceration	1.69 (95% CI: 0.98–2.95) $p < 0.1$
Breslow thickness	1.11* (95% CI: 1.03–1.19)
Mitosis	1.36 (95% CI: 0.88–2.08) $p < 0.1$
Age	0.99 (95% CI: 0.98–1.01) $p < 0.1$

Abbreviations: CI, confidence interval; LN, lymph node; LVI, lymphovascular invasion; OR, odds ratio.
*$p < 0.05$.

TABLE 5 Survival statistics for LVI, angiotropism and LN metastasis.

LVI absent	At risk censored events	447	LVI present	At risk censored events	91
		362			57
		85			34
Angiotropism absent	At risk censored events	255	Angiotropism present	At risk censored events	283
		210			209
		45			74
LN metastasis absent	At risk censored events	457	LN metastasis present	At risk censored events	81
		378			41
		79			40

Abbreviations: LN, lymph node; LVI, lymphovascular invasion.

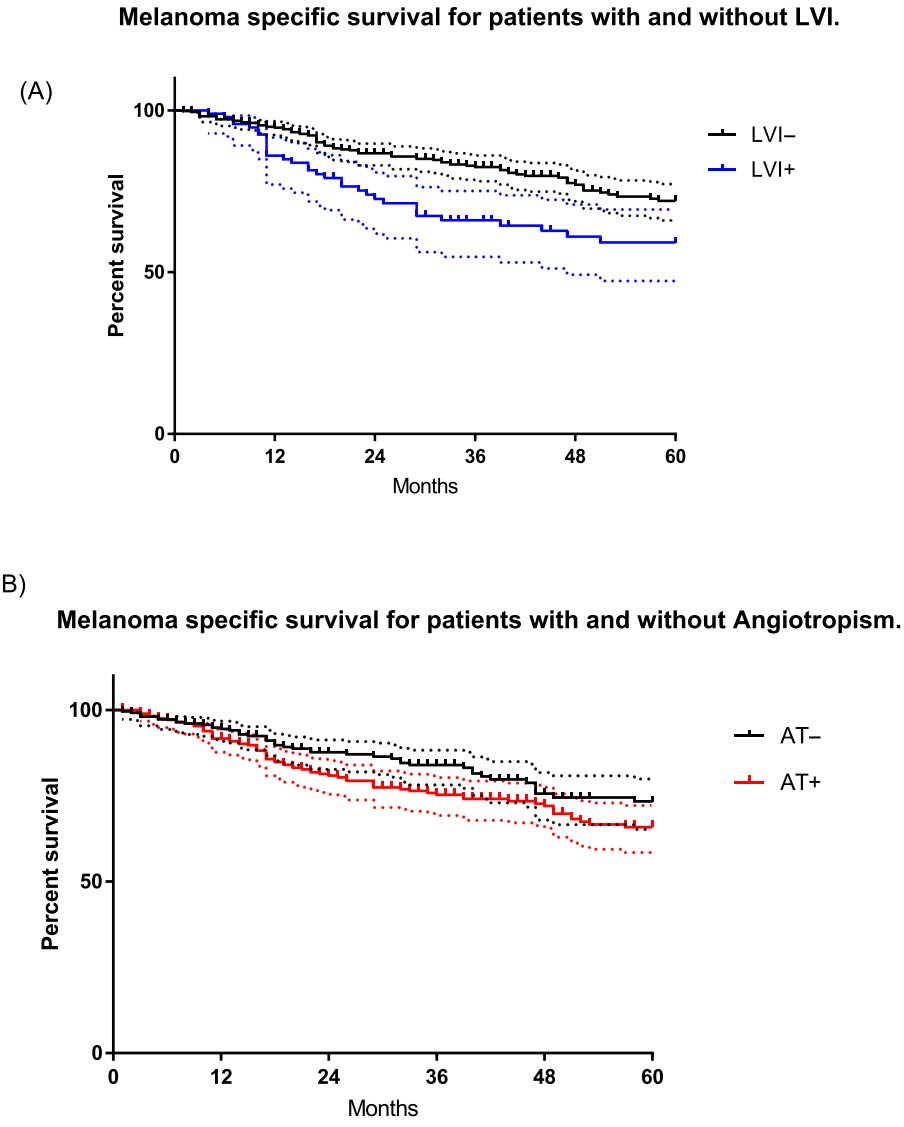

FIGURE 2 (A) Positive, $N = 91$, mean overall survival (OS) = 43.4 months (95% confidence interval [CI]: 38.9–47.9 months). Negative, $N = 447$, mean OS = 51.3 months (95% CI: 49.6–52.9 months) KM survival curves for lymphovascular invasion (LVI), (black, negative; blue, positive). Solid lines with ticks represent mean survival, dotted lines represent 95% CIs. (B) Positive, $N = 283$, mean OS = 48.3 months (95% CI: 46–50.7 months). Negative, $N = 255$, Mean OS = 51.5 months (95% CI: 49.3–53.8 months). KM survival curves for angiotropism (black, negative; red, positive). Solid lines with ticks represent mean survival, dotted lines represent 95% CIs.

TABLE 6 Cox regression for survival analysis showing hazard ratios (HR) of LVI and angiotropism among selected clinicopathological variables.

	HR
Lymphovascular invasion	1.57* (95% CI: 0.98–2.50)
Angiotropism	0.92 (95% CI: 0.59–1.42)
Age of diagnosis	1.04* (95% CI: 1.02–1.05)
Breslow thickness (mm)	1.11* (95% CI: 1.05–1.16)
Ulceration	1.36 (95% CI: 0.91–2.02)
Mitosis	0.99 (95% CI: 0.70–1.41)
LN metastasis	2.46* (95% CI: 1.66–3.66)

Abbreviations: CI, confidence interval; LN, lymph node; LVI, lymphovascular invasion.
*$p < 0.05$.

in the multivariate analyses. Investigation of these effects in a larger cohort would be helpful to ascertain the validity of the effects and eliminate the possibility of a type 2 statistical error. The use of archived material, which may have faded staining, could affect interpretation of the slides and clarity of the histological biomarkers. In cases of poor section quality, re-cuts were performed as would be requested in routine practice. In addition, in any equivocal cases, IHC was performed to aid in discrimination. Finally, we found no evidence that the distribution of the biomarker incidence varied with the date of original diagnosis. In summary, this study provides compelling evidence for the prognostic value of AT and LVI by routine histology tests.

In conclusion, we show that AT and LVI both have significant associations with poor prognostic factors and adverse survival outcomes. It is of interest that AT significantly correlates with LVI, and this relationship merits further analysis. Our results provide additional proof that, as shown in previous studies, AT is much easier to detect on routine histology than LVI and serves as an important marker of angiotropic EVMM. Morphological biomarkers such as LVI and AT are of increasing interest, as the advent of digital pathology and artificial intelligence could aid pathologists in identifying these features in large tissue sections. Independently of its relationship with LVI, AT can be

considered as part of the prognostic factors for PCM, as we have demonstrated in its predictive value for an adverse overall and melanoma-specific survival outcome. Therefore, the value of AT and LVI as prognostic biomarkers is validated in this histological review study of PCM. In further studies, it would be worthwhile investigating the prognostic relevance of identifying AT and LVI in thin melanomas with routine histology in large cohorts. With further investigation, AT and LVI could represent important staging parameters for inclusion in AJCC malignant melanoma staging recommendations. In addition, understanding the biological mechanisms of AT in the overall pathogenesis of malignant melanoma may lead to therapeutic discoveries.

ACKNOWLEDGMENTS

Special thanks go to the Histopathology Department at Queens Medical Centre for the support rendered during the course of the project.

FUNDING INFORMATION

The University of Nottingham sponsored the study.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

ETHICS STATEMENT

Ethical approval for the study was given by the Nottingham Health Science Biobank Access Committee, University of Nottingham.

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How to cite this article: Namubiru P, Dalleywater W, Lashin S, Elsheikh S. Can angiotropism and lymphovascular invasion refine the current cutaneous melanoma staging system? *J Cutan Pathol*. 2023;1-11. doi:10.1111/cup.14561