

## *Review*

# **Lymphovascular invasion: assessment and prognostic impact in melanoma and breast cancer**

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**Summary.** The presence of lymphovascular invasion is a recognised poor prognostic factor in a wide range of tumour types. Vascular invasion was historically identified through haematoxylin and eosin staining, however this technique is non-specific and differentiates poorly between blood and lymphatic vessels. Newer techniques using immunohistochemistry allow more sensitive and specific identification of lymphovascular invasion and are able to accurately differentiate between lymphatic and blood vessels.

This review will discuss the current methods available for the assessment of lymphovascular invasion. Additionally, it will focus on the role of lymphovascular invasion in breast cancer and melanoma, discussing the relative importance of lymphatic and blood vessel invasion in each tumour type.

**Key words:** Breast cancer, Melanoma, Lymphovascular invasion

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## **Introduction**

Cancer metastasis is the leading cause of cancer mortality, accounting for approximately 90% of cancer related deaths (Gupta and Massague, 2006). It has additional significance as the presence of metastatic disease often defines the point at which the malignant

disease becomes incurable. In order to disseminate throughout the body and seed at distant sites, malignant cells must gain access to the circulatory system, and spread either haematogenously, or through the lymphatics. It is well recognised histologically that the presence of tumour cells within lymphovascularity is a poor prognostic sign and associated with distant metastasis and increased mortality in a wide range of tumour types (Ku et al., 2013; Elder, 2014; Gujam et al., 2014; Mollberg et al., 2014)

The mechanism through which tumour cells gain access to the lymphovascular system is complex and requires a series of steps, the so called “metastatic cascade” (Valastyan and Weinberg, 2011). The first of which is loss of cellular attachment to both the surrounding extra-cellular matrix and neighbouring cells. In order for local invasion to occur, a change in gene expression profile is often seen, resulting in down regulation of adhesion related proteins such as E cadherin, cytoskeletal reorganisation and up-regulation of mesenchymal markers such as vimentin (Sarrío et al., 2008; Lamouille et al., 2014). This change in gene expression profile is termed epithelial-mesenchymal transition, and confers increased migratory and invasive properties. Following this the cell must breach the basement membrane, which provides a physical boundary to metastasis. This is often achieved through secretion of matrix metalloproteinases, a family of extracellular proteases, which cleave components of the ECM (Davies, 2014). It must then migrate towards blood or lymphatic vessels, either through mesenchymal or amoeboid patterns of migration (Pankova et al., 2010), before crossing of the pericyte or endothelial barrier of the vessel. This process of invasion is a complex active

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process, modulated by numerous cytokines and growth factors thought to induce migration via chemical concentration gradients towards the blood and/or lymphatic vasculature (Muller et al., 2001). Once in the circulatory system, cancer cells are able to travel to distant sites, providing them with the opportunity for metastatic seeding.

Lymphovascular invasion (LVI) has been shown to be an independent predictor of distant disease relapse in a wide range of tumour types, including breast, colorectal, lung and melanoma (Kashani-Sabet et al., 2001; Schnitt, 2010; Al-Alao et al., 2014). It is defined as the presence of tumour emboli within a definite endothelial lined space, whether that be blood or lymphatic vessels (Song et al., 2011). Whilst macrometastatic spread within local lymph nodes has long been part of the staging system for a wide range of cancers, more recently the importance of the histological presence of lymphovascular invasion is being taken into account for patient risk stratification and treatment decisions in the adjuvant setting (Washington et al., 2009; Song et al., 2011).

Interestingly, different tumour types have varying preferences for utilising blood and lymphatic vessels as their initial route of spread, with tumours such as breast and melanoma exhibiting a strong preference for lymphatics (Xu et al., 2014), whereas tumours such as renal and colorectal cancer are more likely to utilise haematogenous routes (Messenger et al., 2012; Santiago-Agredano et al., 2013). The reasons for such differences are currently unknown and has only become apparent over recent years as methods for differentiating blood and lymphatic vessels have improved. This review will focus methods of assessment of lymphovascular invasion and concentrate on the role of lymphovascular invasion in breast cancer and malignant melanoma.

### **Methods of assessment**

The traditional method of assessing lymphovascular invasion is through haematoxylin and eosin (H&E) staining, an established technique in use for over a century (Fischer et al., 2008). This technique stains nucleic acids a deep purple colour whilst with haematoxylin, non-specific proteins are stained pink, allowing identification of a wide range of cytoplasmic and nuclear detail. Whilst this method of assessment is able to give a great deal of diagnostic information, it is difficult to distinguish between lymphatic and blood vessels, often leading to the histological reporting of both lymphatic and blood vessel invasion being combined and classified under the umbrella term of lymphovascular invasion (Washington et al., 2009). Although there are some characteristics which may aid in vessel differentiation, such as erythrocytes or fibrin clots in blood vessels, and the lack of a basement membrane in lymphatic vessels, these methods are unreliable, leading to misidentification in up to 25% of cases (Schoppmann, 2005). This is of additional

significance due to the recent finding that lymphatic and blood vessel invasion can confer differing prognostic impact dependent on tumour type, underscoring the importance of precise vessel identification (Liang et al., 2007).

H&E staining has a number of limitations in addition to the difficulty in differentiating between blood and lymphatic vessels. False positive results may be obtained due to retraction artefacts around tumour sheets caused by the fixation process (Tsuda, 2008). Additionally, difficulties in identifying tumour cells filling small vessels may lead to false negative results. To avoid these pitfalls, some pathologists choose to only include vessels with a “clear-cut” endothelium without considering the small, collapsed intra-tumour vessels or vessels with tumour cells completely filled inside (Van den Eynden et al., 2006).

In view of the limitations of H&E staining, newer techniques utilising immunohistochemistry (IHC) have been developed for more accurate identification of lymphovascular invasion. There are a number of immunohistochemical markers which have been shown to be able to differentiate between blood and lymphatic vessels to varying degrees of specificity (Table 1). Despite the improved differentiation between blood and lymphatic invasion, some of these markers may not be ideal for routine histopathological assessment of LVI where it is preferable to use markers which identify the cell as a whole rather than specific subcellular locations such as the nucleus. Of the markers available, podoplanin/D2-40 and CD31/CD34 are becoming increasingly recognised and adopted as robust markers to differentiate lymphatic from blood endothelium respectively, as well as allowing assessment of additional parameters such as lymphatic and blood microvessel density as a surrogate for angiogenesis and lymph-angiogenesis. Although use of IHC greatly assists the differentiation of blood from lymphatic vessels, there is varying specificity between markers, with some also expressed by other cell types. Additional details regarding the specificity of such markers are beyond the scope of the current review, but are reviewed elsewhere (Mohammed et al., 2009). Despite its limitations, in a systematic review of the methods assessment of lymphovascular invasion in breast cancer, immunohistochemistry was shown to be more reliable and specific in assessing LVI than traditional H&E (Gujam et al., 2014).

### **Lymphovascular invasion in breast cancer**

As indicated above, the role of lymphovascular invasion in breast cancer as assessed by H&E has been well studied and is of established clinical relevance, being shown to be linked to disease free and overall survival in a number of studies (McCready et al., 2000; Jmor et al., 2002; Woo et al., 2002; Dekker et al., 2013; Gujam et al., 2014). Whilst patients who have macroscopic lymph node spread are recognised to have a

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poorer prognosis, patients with lymph node negative disease represent a heterogeneous group, and in this cohort lymphovascular invasion has been shown to be an adverse prognostic marker, being associated with decreased survival in addition to a range of other unfavourable histopathological characteristics such as greater size, high Ki-67 expression and HER2 overexpression (Dekker et al., 2013).

The presence of LVI, as assessed by both IHC and H&E has been shown to be associated with lymph node positivity in a number of studies (Rakha et al., 2012; Yoshihara et al., 2013), but it has also been shown to be an independent prognostic marker in its own right (Mohammed et al., 2011a,b; Rakha et al., 2012). In one study of 716 node negative breast cancer patients, LVI as assessed by H&E was an independent predictor of tumour recurrence (Lin et al., 2013). In another larger study looking at the role of assessment of LVI in breast cancer in 4482 patients, LVI was an adverse prognostic feature in all subgroups, regardless of tumour size, lymph node status, ER/HER2 status, and remained an independent predictor of survival when adjusted for lymph node status, size and grade. It had particular importance in the LN negative/ER negative subgroup and triple negative subgroups, where multivariate analysis showed it was the only significant predictor of

breast cancer specific survival. Interestingly, the study showed LVI positivity in node negative tumours had prognostic value equal to that provided by the presence of 1-3 positive lymph nodes in LVI negative tumours (Rakha et al., 2012). It is important to note that the prognostic impact of LVI is not confined to node negative tumours, as demonstrated by a study of 552 node positive breast cancer specimens, where IHC assessed LVI was shown to be an independent poor prognostic marker in patients with a single positive lymph node (Mohammed et al., 2014).

Although a number of studies have found LVI to have prognostic value, this finding is not universal. In a study of 1478 patients treated with radiotherapy and surgery, H&E assessed LVI was shown to be associated with other poor prognostic features but not to be an independent predictor of local-regional control or survival upon multivariate analysis (Freedman et al., 2012). In a large Danish population based study of 16,172 patients with operable early stage breast cancer, there was no association between LVI and DFS in patients with low risk disease, whereas LVI positive high risk patients showed significantly poorer DFS and OS, implying that LVI was not sufficient to elevate patients from a lower risk group to a high risk group (Ejlertsen et al., 2009). This study was, however, criticised due to the

**Table 1.** Specific markers for lymphatic and blood vessels.

Marker	Function	LEC	BEC	References
Prox 1	Transcription factor	++	-	Wigle and Oliver, 1999; Gröger et al., 2004
von Willebrand factor (vWF)	Coagulation factor	+	++(+)	Pusztaszeri et al., 2006
Podoplanin	Transmembrane glycoprotein	++	-	Breiteneder-Geleff et al., 1999
CD44	Hyaluronan receptor	-	+	Kriehuber et al., 2001
CD31/PECAM	Adhesion molecule	+	++	Podgrabinska et al., 2002
CD34	Adhesion molecule, L-selectin receptor	+(-)	++	Breiteneder-Geleff et al., 1999; Podgrabinska et al., 2002
ICAM-1/CD54	Adhesion molecule	-	+	Erhard et al., 1996; Petrova et al., 2002
N-cadherin	Adhesion molecule	-	+	Petrova et al., 2002; Hirakawa et al., 2003
VEGF-C	Growth factor	-	+	Kriehuber et al., 2001
VEGFR-1/Flt1	Growth factor receptor	-	+	Hirakawa et al., 2003
VEGFR-3/Flt4	Receptor tyrosine kinase	++	-(+)	Kaipainen et al., 1995; Hirakawa et al., 2003
LYVE-1	Hyaluronan receptor for extracellular matrix	++	-	Banerji et al., 1999; Jackson, 2003
β-chemokine receptor D6	Chemokine receptor	+	-	Schoppmann, 2005
Mannose receptor	L-selectin receptor	+	-	Irjala et al., 2001
Integrin α5	Adhesion molecule, subunit of fibronectin receptor	-	+	Petrova et al., 2002
Integrin α9	Adhesion molecule, subunit of osteopontin and tenascin receptors, VEGFR-3 coreceptor	+	-	Petrova et al., 2002
VE-cadherin	Adhesion molecule	+	++	Kriehuber et al., 2001
Neuropilin-1	growth factor receptor	-	+	Hong et al., 2002; Petrova et al., 2002
Endoglin/CD105	Low-affinity receptor for TGF-β	-/+	++	Hirakawa et al., 2003
Desmoplakin	Anchoring protein of adhering junctions	+(-)	-	Ebata et al., 2001; Fedele et al., 2004
Plakoglobin	Connect cadherins to cytoskeleton in cell-cell junctions	+	-(+)	Petrova et al., 2002; Hirakawa et al., 2003; Fedele et al., 2004
CCL21/SLC	CC-chemokine/secondary lymphoid-tissue chemokine	+	-	Gunn et al., 1998; Kriehuber et al., 2001
IL-8	CXC-chemokine	-	++	Petrova et al., 2002
Versican	Chondroitin sulphate proteoglycan	-	++	Bode-Lesniewska et al. 1996; Petrova et al., 2002; Hirakawa et al., 2003
Laminin	Basement membrane molecule	-/(+)	++	Barsky et al., 1983; Ebata et al., 2001; Petrova et al., 2002
PAL-E	Antibody recognizing Neuropilin-1	-	++	Schlingemann et al., 1985; Podgrabinska et al. 2002; Schoppmann, 2005; Niemela et al., 2005

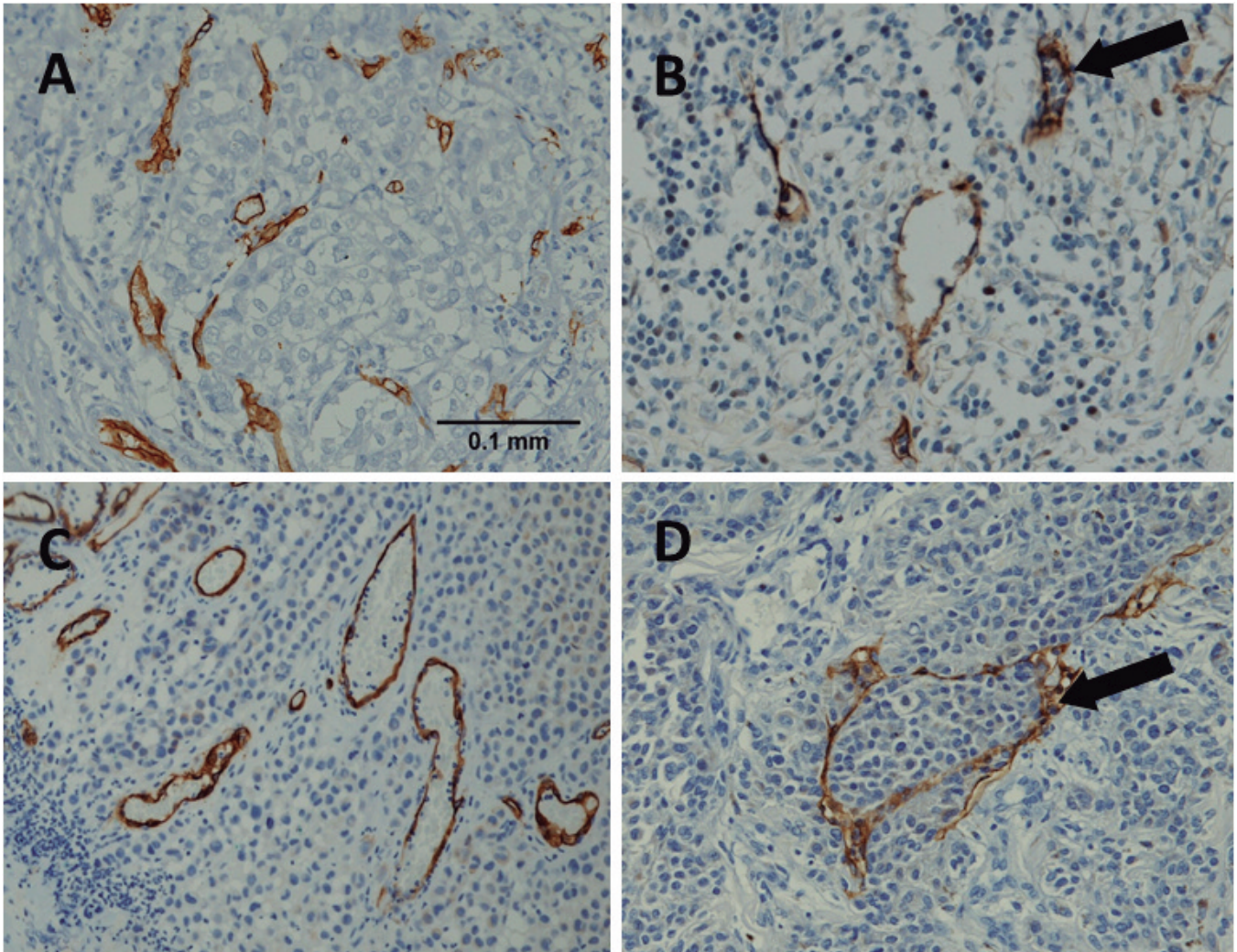
1, VEGFR-3 has been observed expressed on tumour-associated blood vessels. 2, Large collecting lymphatic vessels have complete basement membrane. Peripheral lymphatic vessels may have an incomplete basement membrane (Cueni and Detmar, 2006).

lower than expected number of patients in the study classified as being LVI positive, the large number of laboratories involved in LVI assessment with no central control and its short term follow up (Debled et al., 2010).

The IHC assessment of LVI in breast cancer has been shown to have increased sensitivity of detection in comparison to H&E staining (35% vs 24%) (Gujam et al., 2014). In a review of published studies, there was less inter-study variability between the presence of LVI when assessed by IHC in comparison to H&E, implying a greater degree of reliability when assessed by IHC (Gujam et al., 2014). Improved IHC differentiation between blood and lymphatic vessels has demonstrated that in breast cancer the primary route of vascular invasion seems to be through lymphatics rather than

haematogenously, with BVI being an uncommon phenomenon. A study of over one thousand patients with node-negative breast cancer, reported blood vessel invasion (BVI) in less than 1% of cases in comparison to LVI in 21% of cases (Mohammed et al., 2011a,b). A recent review of published studies on vascular invasion in breast cancer found the rate of IHC detected BVI to be in the region of 1-29% in comparison to an LVI rate of 22-42% (Gujam et al., 2014). The prognostic role of BVI in breast cancer is less established than LVI, with conflicting findings as to its significance with some studies demonstrating a poorer outcome whilst others show it has no prognostic impact (Kato et al., 2003; Mohammed et al., 2011a,b).

The relative importance of intra-tumoral and peritumoral LVI is also slightly unclear. Whilst some studies



**Fig. 1.** Photomicrographs of tissue stained with anti-CD34 (pan-vascular marker), D2-40 (lymphatic marker) and haematoxylin and eosin (H&E) from melanoma and breast tumours (A). Breast tissue stained with anti-CD-34 (B). Breast tissue stained with D2-40 (C). Melanoma stained with anti-CD34 (D). melanoma stained with D2-40. Arrows indicate presence of tumour emboli within lymphovasculture. x 20

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have demonstrated the presence of either to be a poor prognostic marker (Yamauchi et al., 2007; Mohammed et al., 2009), other studies have shown peri-tumoral but not intratumoral LVI to be of significance. In invasive ductal carcinomas, peri-tumoral LVI and lymphatic vessel density (LVD) was associated with a number of adverse clinicopathological parameters such as size, nodal status (Kandemir et al., 2012), and in sentinel node positive invasive breast cancers, peri-tumoral LVI was an independent risk factor for the involvement of additional non-sentinel lymph nodes (Kwon et al., 2011). In summary, whilst the majority of studies looking at LVI in breast cancer have been performed using H&E, the use of IHC seems to be a more sensitive and specific marker. Although beyond the scope of the current review, being able to objectively discriminate between lymphatic and blood vessel invasion is allowing mechanistic and regulatory studies to progress, providing more information about the early steps in the metastatic cascade (Schoppmann et al., 2006; Naoi et al., 2007; Storr et al., 2011). IHC has created a paradigm shift in breast cancer, showing the relative importance of lymphatics over blood vessels in the initial steps of metastasis. The presence of LVI has been shown to be an independent poor prognostic marker in multiple studies. Whilst this finding is more widely recognised in node negative disease, it has also been shown to be a prognostic marker in patients with lymph node involvement, and there is a strong argument for including IHC assessed LVI for risk stratifying patients in the adjuvant setting (Kato et al., 2003; Gujam et al., 2014).

### **Lymphovascular invasion in melanoma**

There is a large volume of research describing the role of lymphovascular invasion in melanoma, with some discordance as to its clinical relevance. Low lymphatic density, as determined by IHC has been associated with both improved and adverse survival, with some studies showing no association with survival at all (Straume et al., 2003; Valencak et al., 2004; Massi et al., 2006; Storr et al., 2012). Similar results are described for IHC determined lymphatic vessel invasion, where some studies show an association with survival and some show no link with altered clinical outcome (Xu et al., 2008; Doeden et al., 2009; Petersson et al., 2009; Storr et al., 2012). Whilst one study of 94 patients showed LVI to have a strong correlation with stage, but not SLN, OS or DM on multivariate analysis, another study of 106 patients showed that in addition to being associated with a range of adverse histopathological features, LVI was also associated with shorter DFS and increased melanoma specific death (Xu et al., 2008; Doeden et al., 2009). The reported detection rates for lymphatic vessel invasion in melanoma range between 16 to 63% (Shields et al., 2004; Niakosari et al., 2005, 2008; Sahni et al., 2005; Xu et al., 2008; Doeden et al., 2009; Petersson et al., 2009; Pettitt et al., 2009; Emmett

et al., 2010; Fohn et al., 2011; Storr et al., 2012) and, as with breast cancer, lymphatic invasion seems to occur more frequently than blood vessel invasion (Storr et al., 2012; Pasquali et al., 2015). As above, the use of IHC, rather than morphometric analysis on H&E stained sections, to assess lymphovascular invasion in melanoma has been shown to improve the detection rate from 8% to 30%, 17% to 40% and 3% to 18% in the same cohorts of patients (Rose et al., 2011; Storr et al., 2012; Pasquali et al., 2015).

Some large studies describing morphometrically determined lymphovascular invasion on H&E stained sections have been published. A recent study contains 2,243 patients with melanomas with a Breslow thickness of 1mm or less; and describes an association between lymphovascular invasion and disease relapse, with a detection rate of 31% (Maurichi et al., 2014). A study of 1,643 patients with superficial spreading melanoma has shown that lymphovascular invasion was detected in 6% of cases; the presence of lymphovascular invasion was associated with disease-free and overall survival (Egger et al., 2013). In 1,621 melanoma patients who underwent a sentinel node biopsy the presence of morphometrically determined lymphovascular invasion was associated with microsatellitosis, and in those patients with microsatellites lymphovascular invasion was associated with sentinel lymph node positivity and melanoma-specific survival (Bartlett et al., 2014).

A number of studies have assessed melanoma patients using IHC determined vascular invasion, however these are typically smaller than their morphometrically determined lymphovascular invasion counterparts. A study investigating 202 cutaneous melanoma patients has shown that any lymphatic invasion is significantly associated with tumour staging, histological subtype, Breslow thickness, ulceration, mitotic rate and microsatellites (Storr et al., 2012). This was in contrast to blood vessel invasion which was also assessed in the same study. Whilst 27% of cases demonstrated lymphatic invasion, blood vessel invasion was only observed in 4% of cases and this was not significantly associated with any of the clinicopathological criteria assessed. An interesting determinant of this study was the comparison between morphometrically assessed and IHC determined vessel invasion; IHC determined vessel invasion was significantly associated with tumour stage, histological subtype, Breslow thickness, ulceration, mitotic rate, and microsatellites, in comparison to no associations with clinicopathological criteria with morphometrically determined vessel invasion. Although this study demonstrated a number of associations with clinicopathological criteria, no association was observed with relapse-free survival or overall survival. 256 primary melanomas assessed for lymphovascular invasion demonstrated an association with tumour thickness, ulceration, mitotic figures and histological subtype (Rose et al., 2011). In contrast with previous IHC studies, this study demonstrated an association with

disease-free and overall survival. A study of 156 scalp/neck melanomas have shown similar results, with lymphatic vessel invasion identified in 35% of cases, with the presence of lymphovascular invasion associated with tumour thickness, ulceration, mitotic rate and tumour histotype (Pasquali et al., 2015). IHC detected lymphovascular invasion was associated with disease-free survival, but not melanoma-specific survival in this study.

A recent meta-analysis looked at some of the studies available on lymphatic invasion, in addition to both blood and lymphatic microvessel density. The meta-analysis pooled studies with IHC determined lymphatic vessel invasion, microvessel density or lymphatic vessel density (Pastushenko et al., 2014). The analysis concludes that lymphatic vessel density in the peritumoral area and lymphatic vessel invasion can provide useful information on the development of metastasis in patients with malignant melanoma. When lymphovascular invasion is determined morphometrically in H&E stained sections studies suggest a link between its presence and adverse clinicopathological factors and in some studies, clinical outcome. In addition to improving differentiation between LVI and BVI in melanoma, IHC is also allowing additional mechanistic studies to progress (Jewell et al., 2015) In summary, the link between lymphovascular invasion and malignant melanoma is not as clear as that observed in breast cancer, however it may provide prognostic information for some patient subgroups.

## Conclusion

Tumour cell invasion into the lymphatic vasculature is a vital step in metastatic seeding. The recent development in IHC has allowed for more sensitive and specific identification of this phenomenon over traditional H&E staining, with better differentiation between lymphatic and vascular invasion (Jewell et al., 2015) The presence of LVI has been demonstrated to have an adverse prognostic impact in a range of tumour types, although some controversy remains regarding the relative prognostic impact between tumour types. To date, the majority of studies on lymphovascular invasion have been performed with H&E staining and further exploration on the impact of LVI using IHC is required. This may allow incorporation of LVI status into clinical practice in order to improve risk stratification of patients, aiding in clinical decision making in the adjuvant setting.

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