Lymphatic and angiogenic characteristics in breast cancer: morphometric analysis and prognostic implications

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PRECLINICAL STUDY

Lymphatic and angiogenic characteristics in breast cancer: morphometric analysis and prognostic implications

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Abstract Controversy exists regarding the topography of lymph vessels in breast cancer, their usefulness as prognostic factors, relationship with angiogenesis and whether active lymphangiogenesis occurs within the tumour. A series of 177 well-characterized breast cancers, with long term follow up, were stained with D2-40, CD31 and CD34. Distribution of lymphatics and lymph vessel density (LVD) were assessed in three areas, intratumoural, peripheral and peritumoural and correlated with clinicopathological criteria and patient prognosis. Microvessel density (MVD) was assessed and correlated with LVD. Double immunohistochemical staining with D2-40 and MIB-1 was carried out to assess the proliferative status of lymphatics and of the tumour emboli within. Peritumoural lymphatics were detected in all tumours whereas peripheral and intratumoural lymphatics were detected in 86 and 41% of specimens, respectively. Tumours with higher total LVD were significantly associated with the presence of lymph node (LN) metastasis and shorter overall survival (OS). In multivariate analysis, tumour grade, LN status and the presence of lymphovascular invasion, but not LVD, were independent poor prognostic factors. No association was found between LVD and MVD. Proliferating lymphatics were detected in 29% of specimens and were significantly associated with dense inflammatory infiltrate. In conclusion, lymphatics are located primarily in the peritumoural and peripheral areas in breast cancer and seem to play an important role in disease progression by being routes for tumour dissemination. The lack of correlation between lymphangiogenic and angiogenic characteristics suggests two distinct processes and the presence of active lymphangiogenesis, albeit in a small portion of specimens, may have important therapeutic implications.

Keywords Angiogenesis · Breast cancer · D2-40 · Lymphangiogenesis and prognosis

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Introduction and aims

Although the lymphatic system was first described by the Italian anatomist Asellius in 1627, as vein-like vessels carrying whitish fluid, research in this area has progressed slowly compared with that in blood vessels [1]. One of the major limitations has been the lack of histological, ultrastructural, and immunohistochemical markers that can accurately differentiate blood endothelial cells (BECs) from lymphatic endothelial cells (LECs). Recently molecular lymphatic markers have been characterized and used successfully to differentiate lymphatics from blood vessels. These include podoplanin/D2-40, LYVE-1, Prox-1, mannose receptor and VEGFR-3. Several articles and reviews have been published describing their structures, functions and roles in lymphangiogenesis [2–7].

Characterization of these molecules has lead to a surge in research investigating the role of lymphatics in normal physiology and in diseases, particularly in cancer progression. Clinicopathological data suggest that lymphatics are most likely to be the initial route for the spread of certain solid tumours, such as breast cancer [8]. Although numerous

studies have investigated lymphatics in breast cancers, many questions remain unanswered. There is still debate whether lymphatics are present within tumours or located only at the periphery. Equally, debate still occurs on whether active lymphangiogenesis is a feature in breast cancer.

Initial studies on xenografted tumours reported that lymphatics were only present in peritumoural areas and absent from the intratumoural compartment [9, 10]. Such studies also suggested that these vessels were the main route for metastasis and that lymphangiogenesis did not seem to play a role in this process. Opposing results have been reported by later studies where functional intratumoural lymphatics were detected in xenografted tumours [11]. With human breast cancer certain studies have reported an absence of both intratumoural lymphatics and of lymphangiogenesis [12–14] whereas others have detected lymphatics both inside and around the tumour [15–17]. The importance of the lymph vessel density (LVD) in promoting metastatic spread of breast cancer is questionable [18, 19] and which vessels, newly formed lymphatics or pre-existing ones, play the more important role in the initial dissemination of tumour cells is uncertain.

Detection of actively dividing LECs along lymph vessels has been considered a sign of active lymphangiogenesis. Double immunohistochemical staining of tumour sections with a lymphatic marker such as D2-40 or LYVE-1 and a proliferative marker such as Ki-67/MIB-1, or proliferating cell nuclear antigen (PCNA), have been used to address this question however, as with the aforementioned, results are controversial [20, 21].

We, like others, have shown in previous work that lymphangiogenesis and angiogenesis may share some common regulatory features [22]. It is now appreciated that angiogenesis has an important role in the progression of breast cancer, through providing essential nutrients for tumour cell growth and proliferation [23–25]. Microvessel density (MVD); as a measurement for angiogenesis, has been found to be strongly associated with features of tumour aggression as larger size and poor differentiation, however its role as a prognostic factor has not been firmly established [26].

The aims of the current study were (a) to study, in detail, the topography and characteristics of lymphatic vessels in breast cancer and examine their association(s) with clinicopathological criteria and influence on patient prognosis in a well characterized series of breast cancer with long-term follow-up, (b) to investigate the angiogenic characteristics in the same tumour specimens to discover if tumours share the common, and related, angiogenic and lymphangiogenic characteristics, and (c) to assess the proliferative status of tumoural lymphatics (as an indicator of lymphangiogenesis) and of tumour cells within lymphatics.

Material and methods

Patients and specimens

This study was conducted on pre-treatment primary invasive breast cancer specimens that have previously been described, in detail [8, 22]. Briefly, 177 consecutive paraffin-embedded archival specimens were obtained from the Department of Histopathology, Nottingham University Hospitals, City Hospital Campus. The median age of patients at time of diagnosis was 57 years (range, 32–70 years), 52 (29%) patients had positive lymph nodes with most tumours presenting as either stage I ($n = 121$ (68.4%)) or stage II ($n = 43$ (24.3%)) disease.

The clinical management of patients was based on tumour characteristics; by the Nottingham Prognostic Index (NPI) and by hormone receptor status. After surgical treatment, patients with an NPI score $\leq$3.4 received no adjuvant therapy, those with a NPI score >3.4 received Tamoxifen if oestrogen receptor (ER) positive ($\pm$ Zoladex if pre-menopausal) or classical cyclophosphamide, methotrexate and 5-fluorouracil (CMF) if ER negative and fit enough to tolerate chemotherapy [27]. Patients that received breast conserving surgery or mastectomy may receive a course of radiotherapy according to the risk of regional recurrence that is determined by the multidisciplinary team. Tumours were graded according to a modified Bloom–Richardson scoring system [28] and size was categorized according to the TNM staging criteria [29]. NPI was calculated as previously described [30]. Survival data, including survival time and disease free interval, was maintained on a prospective basis. The disease-free interval was defined as the interval (in months) from the date of the primary surgical treatment to the first loco-regional or distant recurrence. Overall survival was taken as the time (in months) from the date of the primary surgical treatment to the time of death. Complete clinical follow-up information was available for all 177 patients. The median follow-up period was 96 months (range, 2–184 months). Forty-one patients developed regional recurrence by the time of the last follow-up and sixteen patients died from the disease. Ethical approval for this study was obtained for analysis from Nottingham Local Research Ethics Committee (REC C2020313).

One hundred and thirty two of the patients (72.4%) were treated by surgery and subsequent adjuvant therapy. Of the 49 patients (27.6%) that did not receive any adjuvant therapy one died from the disease and eight developed a recurrence. Survival analysis was conducted only on those patients that received identical treatment i.e. those who were treated by surgery and adjuvant therapy. REMARK criteria were followed throughout, as recommended by McShane et al. [31].

Immunohistochemistry

For visualization of lymphatics and of blood vessels, 3 consecutive sections from each specimen were stained with the lymphatic marker D2-40 (SIGNET, 730-16, Cambridge, UK) and with blood vascular markers CD34 (SEROTEC, MCAP547, Oxford, UK) and CD31 (Dako-Cytomation, 0823, Denmark) as descried previously [22].

For examination of the proliferative status of lymphatics, and of tumour cells within lymphatics, a double IHC technique with MIB-1 (Monoclonal Mouse Ki-67 Antigen, Clone MIB-1, Code-No. M 7240, DAKO, Denmark) and D2-40 was used. Briefly, 4 um-thickness sections were deparaffinized in xylene for 20 min and then were rehydrated by incubation in a sequence of descending concentration of Ethanol ($\sim$100, 70 and 50%). Sections were immersed in distilled water for 2 min. For antigen retrieval, sections were put in 0.01 mol/l sodium citrate buffer (pH 6.0) in microwave for 20 min before incubation in endogenous enzyme block solution, containing 0.5% hydrogen peroxide for 5 min to block any endogenous peroxidise activity. MIB-1 was then applied to sections for 1 h at room temperature (RT). Excess unbound antibody was washed with Tris-buffered saline (TBS). Sections were incubated in Polymer/HRP (a Dextran polymer conjugated with horseradish peroxidise and immunoglobulins) for 30 min at RT and then washed for 5 min with TBS. For visualization of MIB-1, sections were incubated in 5% 3,3′-diaminobenzidine tetrahydrochloride chromogen solution and substrate for 10 min and then washed with TBS for 5 min. Sections were then incubated for 3 min in blocking solution and then washed with TBS. Following blocking, D2-40 (monoclonal mouse AB) diluted in 1:100 was applied for 1 h at RT. In the next step Rabbit/Mouse (LINK) was added for 30 min and then sections were washed in TBS, followed by incubation with the Polymer/AP reagent. To visualize the second staining with D2-40, Permanent Red Chromogen was applied for 5 min and then washed in running water for 3 min before counterstaining with haematoxylin and mounting with glycerol.

Assessment of lymphangiogenic and angiogenic characteristics

Two characteristics were noted for lymph vessels (1) distribution/location within and around the tumour and (2) total LVD. The majority of studies have assessed LVD by counting lymphatics in three or five hot spots (areas with highest density of lymphatics) [32–34], in a fashion akin to that for the determination of MVD of vascular endothelium. As we were interested to examine density and distribution of lymphatics in the whole tumour, we used a modified method by counting all vessels in the whole section. First, each section was examined at low magnification ($\times$40) and divided into three areas, the central 2/3 core, the intratumoural (IT) area, the outer 1/3 of the tumour tissue, the peripheral tumoural area (PP) and the normal area surrounding the tumour, the peritumoural area (PT) as shown in Fig. 1. Vessels were counted in each area using $\times$100 magnification with a surface area of 3.46 mm^2. The sum of LVD in all fields in each zone was divided by the total surface areas of all fields to give the LVD for that zone, presented as vessels/mm^2. This method gave the LVD in the peritumoural area (LVD-PT), in the peripheral tumour area (LVD-PP), and in the intratumoural area (LVD-IT) with the sum of three giving the total LVD for the specimen. Although this method of assessment is difficult and time consuming it allows all lymphatics across the whole tumour section to be assessed thereby enabling distribution information to be obtained. Assessment of MVD was conducted using the Chalkely method as described previously [22]. Lymphvascular invasion (LVI) was identified when tumour clusters were detected within D2-40-positive vessels.

Assessment of the proliferation fraction of lymphatics, of tumour emboli and of the invasive component of the tumour

Lymphatic nuclei were only scored as proliferating (MIB-1 positive) if they fulfilled features of an endothelial cell nucleus i.e. plump oval nuclei lying within the contour of the red LEC, Fig. 2f. Cells that did not fulfil such criteria, as shown in Fig. 2i, were excluded as they could represent

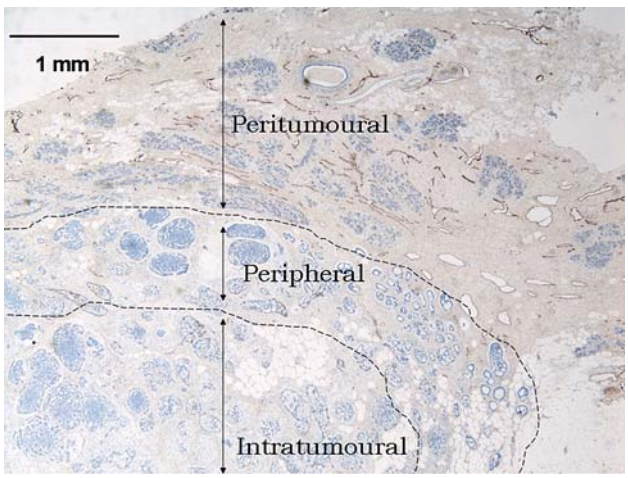

Fig. 1 Tumour section with dashed lines showing how the section was divided into three areas; intratumoural (the inner 2/3 core of the tumour), peripheral (the outer 1/3 of the tumour) and peritumoural (all the normal tissue surrounding the tumour), magnification $\times$20

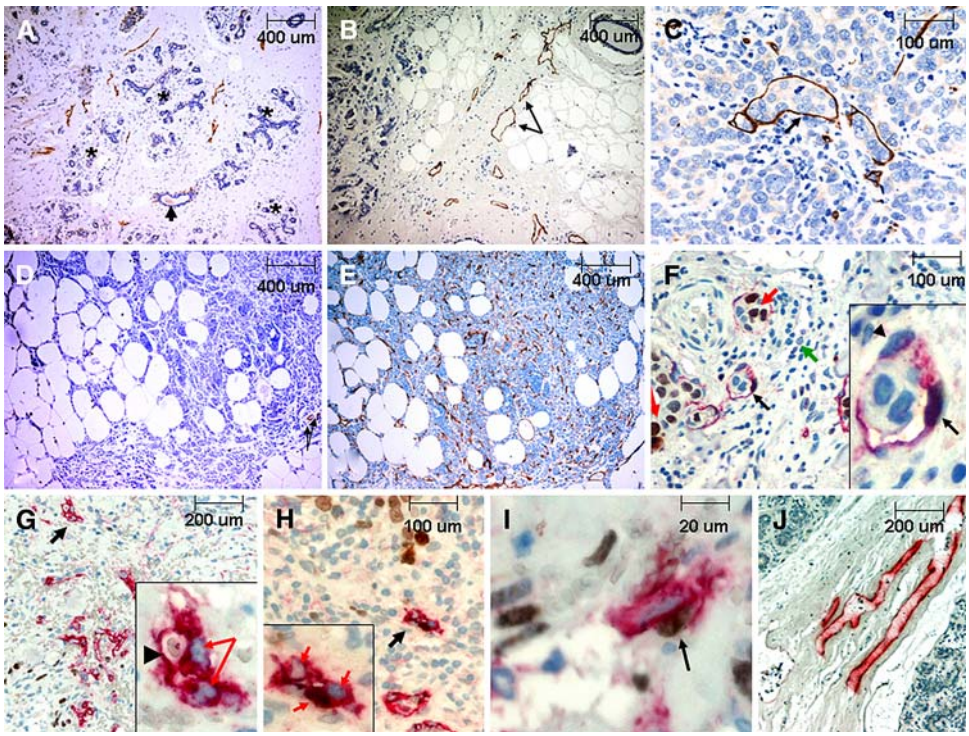

Fig. 2 (**a**) Normal breast tissue showing the structural unit of the breast; a central duct (arrow) surrounded with multiple lobules (asterisk). Lymphatics are distributed within the interlobular stroma and completely absent from the intralobular stroma, ×40 magnification. (**b**, **c**) Two infiltrating ductal carcinomas stained with D2-40 showing multiple lymphatics at the peritumoural area (**b**, ×100) and intratumoural lymphatics loaded with multiple tumour cells (**c**, ×200). (**d**, **e**) infiltrating ductal carcinoma stained with D2-40 and CD34 respectively showing few lymphatics (arrow) in (**d**) yet high number of blood capillaries in (**e**), an example of a tumour with low LVD yet high MVD, ×40. (**f**–**j**) are tumours stained by double IHC with D2-40 and MIB-1. (**f**) Tumour section with a lymphatic capillary showing a proliferating LEC with a MIB-1-positive nucleus (black arrow) and a MIB-1-negative LECs nucleus (arrow head). Proliferating tumour cells with MIB-1-positive nuclei (red arrows), and inflammatory infiltrate (green arrow) are also seen. (**g**) Morphology of some intratumoural lymphatics (black arrow) showing irregular outline, sprouting, intracytoplasmic lumina (arrow head inset), and multiple nuclei (red arrows inset) (**h**) A cross section of an intratumoural lymphatic with multiple nuclei (red arrows inset) one of which is MIB-1 positive suggesting newly formed lymphatics, in comparison with the regular lymphatics at the normal peritumoural areas in (**j**) where they have regular thin outline with flat endothelial cells. (**i**) MIB-1 positive nuclei that do not meet the criteria of endothelial nuclei, as shown here (arrow) and described in the text, were not counted as proliferating LEC

a proliferating tumour or inflammatory cell infiltrating through the lymphatic vessel wall.

No standard method is currently used for the assessment of the proliferation fraction of lymphatics (PF-L) in human tumours. Different approaches have been used with certain studies simply reporting the presence or absence of MIB-1 positive LEC nuclei [35], with others reporting the number of positive nuclei/100 LECs nuclei [20], and yet others counting the number of positive nuclei in three high power fields [21]. As both the proliferation fraction of the tumoural lymphatics (PF-L) and of the tumour emboli (PF-E) were to be assessed, specimens which had LVI were selected for the double IHC procedure; they were 54 specimens of which 41 specimens were characterized with high LVD. In seven of the 54 specimens, tumour emboli were small and lost by deeper sectioning of the paraffin blocks so that PF-E was assessed in 47 specimens. To allow statistical comparison between PF-L in specimens with high LVD and specimens with low LVD, an additional 31 specimens with low LVD were randomly selected from the study population giving a total of 85 specimens. PF-L was assessed by counting the total number of MIB-1 positive LEC nuclei in the whole tumour section and dividing it by the total LVD. A similar approach has been used previously [36].

To assess the proliferation fraction of tumour emboli (PF-E) within the lymph vessels, the number of MIB-1-positive tumour cell nuclei was divided by the number of total cells within the emboli. The proliferation fraction of the invasive component (i.e. not including ductal carcinoma in situ) of the tumour cells outside lymph vessels (PF-T), was scored using a method similar to that described by Potemski et al. and by Rudolph et al. [37, 38]. First the tumour fields with highest reactivity for MIB-1 were selected and then the number of MIB-1 positive tumour nuclei was counted in 500 tumour cells and this gave the

percent of the proliferating tumour cells (500 tumour cells were present in 2–3 high magnification fields, ×400 according to amount of tumoural stroma). About 10% was chosen as a cut-off point to categorize the specimens into tumours with low PF-T and tumours with high PF-T.

A second observer re-examined 20% of specimens with good concordance between both readings; κ value >85%.

Statistical analysis

For assessment of associations between LVD, MVD and clinicopathological criteria, specimens were categorized into two groups according to the median and then Chi squared and 2 × 2 tables were used to assess statistical significance. To assess prognostic significance of LVD and MVD, univariate analysis was first conducted using the Kaplan–Meier method where statistical significance of differences in the cumulative survival curves between groups was evaluated by the long-rank test. Multivariate analysis was performed using the Cox's proportional hazard method. Concordance between results from both observers was evaluated using Kappa (κ) agreement test. Statistical analysis was conducted using the software SPSS for windows, version 14. Statistical analysis was two sided and significance was defined if $P < 0.05$.

Results

Distribution of lymphatics in normal breast tissue and in tumours

The distribution of lymphatics in areas of normal breast tissue surrounding the tumour and in the three tumoural areas was examined. In the normal areas, lymphatics were found to be more or less evenly distributed in the interlobular and interlobar areas but completely absent from the intralobular compartment (Fig. 2a). In tumours, the majority of lymphatics were located mainly in the peripheral and peritumoural areas. Peritumoural lymphatics (PT-L) were detected in all 177 tumours while peripheral lymphatics (PP-L) were detected in 152 tumour (85.9%) compared with 73 tumour (41.2%) having intratumoural lymphatics (IT-L), Fig. 2b, c.

IT-LVD ranged from 0.0 to 2.7/mm^2 (median 0.00/mm^2), PP-LVD ranged from 0.00 to 3.50/mm^2 (median 0.30/mm^2) and PT-LVD ranged from 0.015 to 3.94/mm^2 (median 0.83/mm^2) Total-LVD ranged from 0.015 to 8.59/mm^2 (median 1.37/mm^2). The means of the IT-LVD, PP-LVD, PT-LVD and total-LVD were 0.3/mm^2 (±0.03), 0.5/mm^2 (±0.04), 1.02/mm^2 (±0.06) and 1.7/mm^2 (±0.1), respectively. Figure 3 illustrates distribution of lymph vessels in the three tumoural areas. By using the median as a cut-off point, 55 (31.1%) tumours were characterized with high LVD.

Relationship between LVD, clinicopathological characteristics and patient prognosis

No association was found between LVD and tumour size or type. Significant association was found between high-grade tumours and higher IT-LVD ($P = 0.048$). Higher LVD at the intratumoural, peripheral, peritumoural or total LVD were associated with the presence of LN metastasis; P-values were 0.009, <0.001, <0.001, <0.001, <0.001 respectively. Also a strong association between high LVD, poor NPI and advanced tumour stage were detected; the mean LVD was 1.4 ± 0.12, 1.8 ± 0.17 and 2.9 ± 0.38, in good, intermediate and poor prognostic groups respectively (P-value = 0.009). The relationships between high LVD in different tumour areas and other clinicopathological criteria of tumours are summarized in Table 1. Associations indicate that tumours with aggressive features have higher LVDs. Vascular invasion (VI); encompassing both lymphovascular invasion (LVI) and blood vascular invasion (BVI) was assessed in the same specimens and results reported previously [8]. Briefly, by using differential expression of blood and lymphatic markers, VI was detected in 56 specimens (31.6%) of which 54 (96.4%) were purely LVI with no BVI. A highly significant association was found between high total LVD and the presence of LVI ($P < 0.001$) (Table 2).

As illustrated by the Kaplan–Meier curve (Fig. 4a, b), in univariate analysis of the 132 patients treated with adjuvant therapy, total LVD was significantly associated with shorter overall survival $P = 0.015$, and DFI $P = 0.01$. However in multivariate analysis, LVD lost significance when adjusted to tumour grade, size, LN status, and the presence of LVI indicating it is not an independent prognostic factor. Only tumour grade ($P = 0.009$), LN status ($P = 0.015$) and LVI ($P = 0.022$) retained significance in multivariate analysis (Table 3).

Evidence of lymphangiogenesis in breast cancer

MIB-1-positive LECs nuclei were detected in 25 (29%) of specimens (Fig. 2f) with the proliferation fraction (PF-L) ranging from 0 to 2.2%. Figure 4c shows the distribution frequencies of MIB-1-positive LECs in the 85 specimens. When the presence of proliferating LECs was compared between specimens, according to the LVD, no significant association was found; 37.8% of specimens with high LVD had positive MIB-1 LEC nuclei compared with 20% of specimens with low LVD ($P = 0.073$) (Table 4).

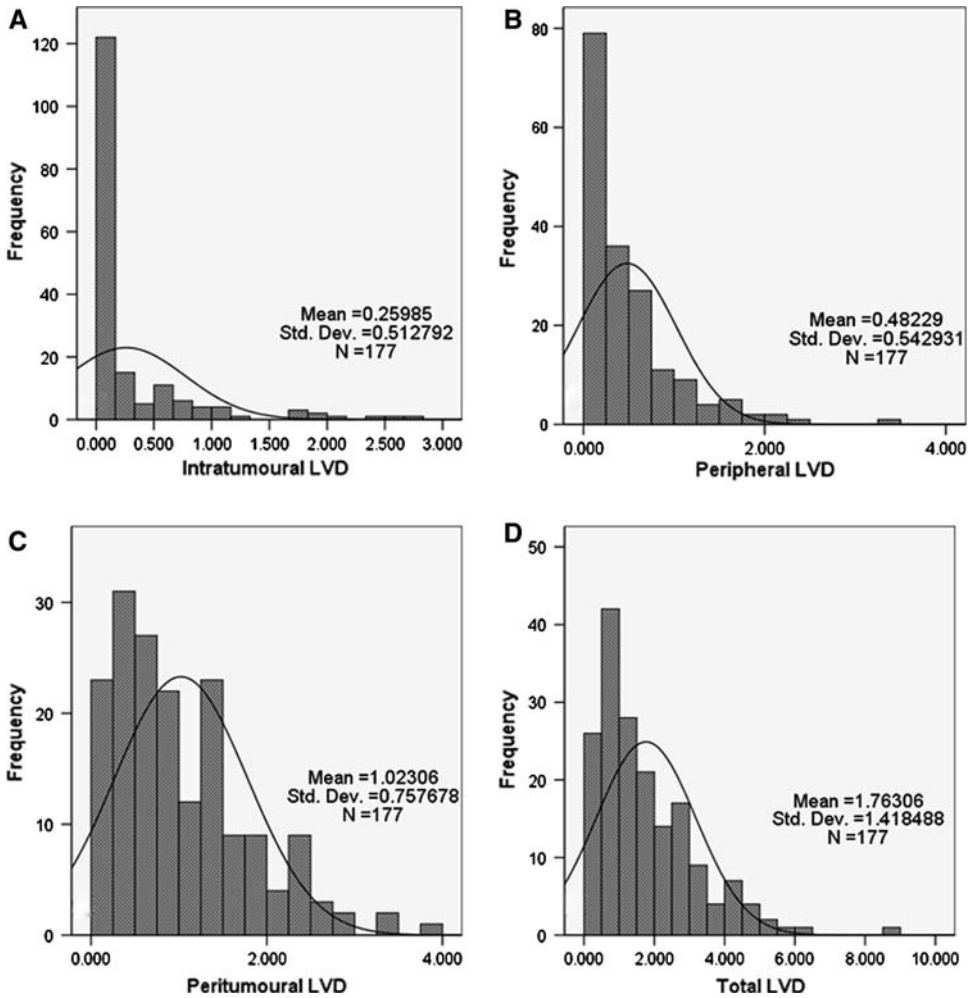

Fig. 3 Histograms showing distribution of intratumoural LVD (**a**), peripheral LVD (**b**), peritumoural LVD (**c**) and total LVD (**d**) in the 177 studied breast cancer specimens

A different morphology of the intratumoural and peripheral lymphatics in comparison with lymphatics in the peritumoural areas was observed. Lymphatics with MIB-1 positive nuclei were characterized with three features; (a) irregular outline with multiple sprouting, (b) more than one endothelial nucleus in the cross section of small lymphatic capillary, which is unusual (Fig. 2h), and (c) the presence of intracytoplasmic lumina within the cytoplasm of the LEC (Fig. 2g). Lymphatics in the peritumoural areas were, in comparison, more regular, had thinner LEC nuclei with the larger vessels invariably negative for MIB-1 (Fig. 2j). Certain lymphatic vessels in the peritumoural area did, however, show MIB-1 positivity i.e. the smaller vessels immediately adjacent to the peripheral tumour area.

Proliferative status of tumour cells within lymphatics

As reported previously [8], it appears that, with the current cohort of specimens, breast tumour cells preferentially invade lymphatics rather than blood vessels. It was therefore of interest to assess the proliferative fraction of tumour cells (emboli) within such vessels (PF-E). PF-E was assessed in 47 specimens in whom LVI was still detected after deeper sectioning. From those 47 specimens MIB-1 positive nuclei of invading tumour cells were detected in 40 (93.8%) specimens. In seven (8.2%) specimens, tumour emboli were MIB-1-negative. The PF-E ranged from 0 to 90% with a median of 26% and mean of 32.5 ± 3.9%. Figure 4d shows the distribution frequencies of PF-E in the 47 specimens. To examine whether there was an association between the PF-T and PF-E, a Kruskal–Wallis test was conducted. Tumours which were characterized by high PF-T also had a high PF-E. Twenty-five of the 47 specimens were characterized with high PF-T. Sixteen out of them had high PF-E, $P = 0.006$ (Table 5).

Relationship between density of tumoural inflammatory infiltrate, LVD and proliferation fraction of lymphatics

Examination of the density of inflammatory cells in tumour sections (stained by the double staining technique) showed that 66 specimens (77%) had mild infiltrate compared with

Table 1 Association between LVD at the three tumour areas and total LVD and clinicopathological criteria

	IT-LVD				PP-LVD				PT-LVD				Total-LVD			
	Low	High	Total	*P*-value	Low	High	Total	*P*-value	Low	High	Total	*P*-value	Low	High	Total	*P*-value
Stage																
I	81(67)	40(33)	121	**0.005**	75(62)	46(38)	121	**0.001**	73(61)	47(39)	121	**<0.001**	102(85)	19(15)	121	**<0.001**
II	17(39)	26(61)	43		8(18)	35(81)	43		14(33)	29(67)	43		14(33)	29(67)	43	
III	64(6)	75(4)	13		5(39)	8(61)	13		2(15)	11(85)	13		6(46)	7(54)	13	
Total			177				177				177				177	
ER																
Negative	23(41)	33(59)	56	**0.003**	21(38)	35(62)	56	**0.049**	24(43)	32(57)	56	0.191	33(59)	23(41)	56	**0.050**
Positive	74(66)	38(34)	112		61(55)	51(45)	112		61(55)	51(45)	112		83(74)	29(26)	112	
Total			168				168				168				168	
PR																
Negative	34(47)	38(52)	72	**0.028**	33(46)	39(54)	72	0.638	34(47)	38(53)	72	0.435	45(63)	27(38)	72	0.089
Positive	60(65)	33(36)	93		47(51)	46(49)	93		50(54)	43(46)	93		70(75)	23(25)	93	
Total			165				165				165				165	
RR																
No	84(62)	52(38)	136	0.151	71(52)	65(48)	136	0.304	69(51)	67(49)	136	1.000	98(72)	38(28)	136	0.124
Definite	20(49)	21(51)	41		17(41)	24(59)	41		21(51)	20(49)	41		24(39)	17(41)	41	
Total			177				177				177				177	
DM																
Yes	92(59)	64(41)	156	0.464	80(51)	76(48)	156	0.081	81(52)	75(48)	156	0.320	112(72)	44(28)	156	0.029
No	9(50)	9(50)	18		5(28)	13(72)	18		7(39)	11(61)	18		8(44)	10(56)	18	
Total			174				174				174				174	
Age																
≤50 years	26(51)	25(49)	51	0.238	27(53)	24(47)	51	0.621	25(49)	26(51)	51	0.868	40(78)	11(22)	51	0.106
>50 years	78(62)	48(38)	126		61(48)	65(52)	126		65(52)	61(48)	126		82(65)	44(35)	126	
Total			177				177				177				177	
Size																
≤1.5 cm	45(69)	20(31)	65	0.321	38(58)	27(42)	65	0.087	31(48)	34(52)	65	0.539	48(74)	17(26)	65	0.315
>1.5 cm	59(53)	53(47)	112		50(45)	62(55)	112		59(53)	53(47)	112		74(66)	38(34)	112	
Total			177		88	89	177		90	87	177		122	55	177	
Grade																
I	28(62)	17(38)	45	**0.048**	23(51)	22(49)	45	0.077	23(51)	22(49)	45	0.634	33(73)	12(27)	45	0.849
II	43(69)	19(31)	62		37(60)	25(40)	62		29(47)	33(53)	62		40(65)	22(35)	62	
III	33(47)	37(53)	70		28(40)	42(60)	70		38(54)	32(46)	70		49(70)	21(30)	70	
Total			177				177				177				177	

Table 1 continued

	IT-LVD				PP-LVD				PT-LVD				Total-LVD			
	Low	High	Total	*P*-value	Low	High	Total	*P*-value	Low	High	Total	*P*-value	Low	High	Total	*P*-value
LN status																
Negative	81(65)	44(35)	125	**0.009**	77(62)	48(38)	125	**<0.001**	77(62)	48(38)	125	**<0.001**	104(83)	21(17)		**<0.001**
Positive	23(44)	29(56)	52		11(21)	41(79)	52		13(25)	39(75)	52		18(35)	34(65)		
Total			177				177				177				177	
NPI																
Good	51(69)	23(31)	74	**0.007**	4460	3040	74	**0.007**	38(51)	36(49)	74	**0.038**	58(78)	16(22)	74	**0.009**
Intermediate	48(54)	40(46)	88		4147	4753	88		49(56)	39(44)	88		37(65)	31(35)	88	
Poor	53(3)	10(67)	15		320	1280	15		3(20)	12(80)	15		7(47)	8(53)	15	
Total			177				177				177				177	
Tumour type																
NOS	47(54)	40(46)	87	0.225	41(47)	46(52)	87	0.549	45(52)	42(48)	87	0.881	59(68)	28(32)	87	0.880
Others	57(63)	33(45)	90		47(52)	43(48)	90		45(50)	45(50)	90		63(70)	27(30)	90	
Total			177				177				177				177	

ER—estrogen receptor, PR—progesterone receptor, RR—regional recurrence, DM—distant metastasis

IT-LVD, intratumoural lymph vessel density. PP-LVD, Peripheral lymph vessel density, PT-LVD, peritumoural lymph vessel density, NPI, Nottingham prognostic index. NOS not otherwise specified

Significant *P*-values are bold

Table 2 Association between LVD and LVI

LVD	LVI (%)			*P*-value
	Absent	Present	Total	
Low	109(89)	13(11)	122	<0.001
High	14(24)	41(75)	55	
	123	54	177	

19 (22%) with dense infiltrate. The presence of dense inflammatory infiltrate was significantly associated with the presence of proliferating LEC where 58% of tumour with dense inflammatory infiltrate had positive MIB-1 LEC nuclei (Fig. 2f) compared with 21% of tumours with mild inflammatory infiltrate, $P = 0.002$ using Chi squared test (Table 6).

Angiogenic characteristics and relationship with lymphangiogenesis and survival

The relationship between MVD and LVD was of interest for aforementioned reasons. No statistically significant relationship was detected between high MVD and high LVD; certain tumours with high MVDs had low LVDs and vice versa (Fig. 2d, e). Tumours with high MVD were highly significantly associated with larger tumour size ($P < 0.001$), higher grade ($P < 0.001$), presence of LN status ($P < 0.001$) and negativity of hormonal receptors; $P = 0.001$ for estrogen receptors and $P = 0.006$ for progesterone receptors (Table 7). There was a trend for patients whose tumours had high MVD to have poorer survival. The 10-years OS was 93% in patients with low MVD compared with 88.2% of patients with high MVD, however this trend did not reach statistical significance ($P = 0.316$).

Discussion

The topography of lymphatics in malignant tumours, particularly in breast cancer, has, for some time, been a hotly debated issue in the literature. Initial studies investigated lymphatics in xenografted sarcomas and reported that vessels were only found in peritumoural areas and absent from the intratumoural compartment [9, 10]. With advances in imaging techniques, intratumoural lymphatics were detected and found to be functional, with tumour cells detected flowing within the vessels [11]. Controversy exists regarding lymphatics in human breast cancer, with some studies reported absence of intratumoural lymphatics

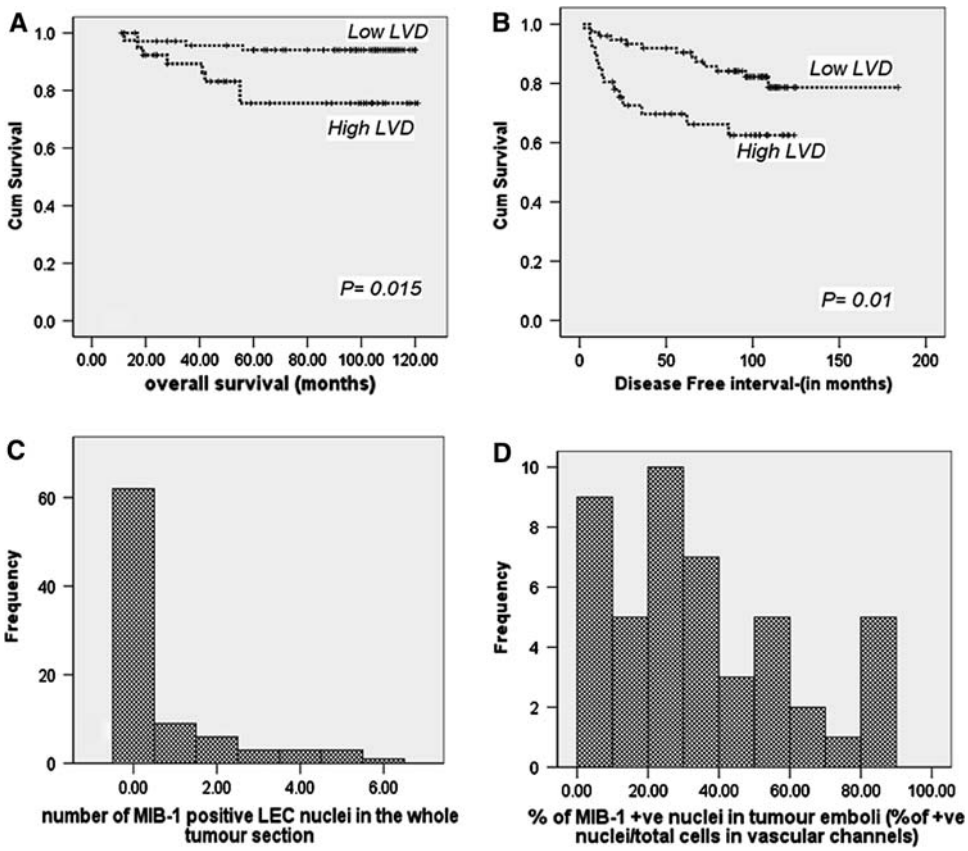

Fig. 4 (**a**) and (**b**) are Kaplan–Meier curves showing positive relationship between higher LVD and shorter overall survival (**a**) ($P = 0.015$) and disease free interval ($P = 0.01$) (**b**). (**c**) Histogram shows distribution of MIB-1-positive LECs in whole tumour sections. (**d**) Histogram showing the distribution of proliferation fraction of tumour cells (emboli) within lymphatics (PF-E) with the majority tumour emboli having MIB-1-positive reactivity and the PF-E ranging from 20 to 90%

Table 3 Multivariate analysis of LVD and MVD adjusted to other prognostic factors

	Sig.	Exp(B)	95.0% CI for Exp(B)	
			Lower	Upper
Size (≤ vs. >1.5 cm)	0.891	0.888	0.162	4.865
Grade (I vs. II & III)	**0.009**	4.423	1.771	19.137
LVD status (high versus low)	0.604	0.603	0.089	4.070
MVD status (high versus low)	0.346	0.550	0.159	1.906
LVI (negative versus positive)	**0.022**	4.157	1.352	18.218
LN status (negative versus positive)	**0.015**	4.829	1.222	19.057

Significant *P*-values are bold

Table 4 Association between LVD and proliferation of tumoural lymphatics

LVD status	MIB-1-Negative LEC nuclei	MIB-1-positive LEC nuclei	Total	*P*-value
Low	32 (80%)	8 (20%)	40 (47.1%)	0.07
High	28 (62.2%)	17 (37.8%)	45 (52.9%)	
	60	25	85	

Table 5 Association between proliferation fraction of the tumour emboli within lymphatics (PF-E) and the PF of tumour cells outside the vessels (PF-T)

PF of tumour emboli	PF of the tumour ells outside the vessels		Total	*P*-value
	High	Low		
High	16	6	22	0.006
Low	7	18	25	
Total	23	24	47	

Table 6 Association between density of inflammatory infiltrate within the tumour and proliferation of tumoural lymphatics

Density of inflammatory infiltrate	MIB-1-Negative LEC nuclei	MIB-1-positive LEC nuclei	Total	*P*-value
Low	52 (79%)	14 (21%)	66	0.002
High	8 (42%)	11 (58%)	19	
Total	60	25	85	

[12–14] and others reporting their presence [17, 19]. In the present study, lymphatics were detected in the peritumoural area in all specimens while 86 and 41% of specimens have additional lymphatics in the peripheral and in the inner 2/3 core of the tumour, respectively.

The controversy regarding the presence or absence of intratumoural lymphatics may exist for many reasons. Breast cancer is a heterogeneous disease; therefore the patient population will vary considerably from one study to another. A contributory factor to conflicting results is that different terms have been used to describe the location of tumoural lymphatics. In the current study, intratumoural lymphatics referred to vessels within the inner 2/3 core of the tumour including vessels both among the tumour cells and/or in the tumour stroma. Other studies refer to intratumoural lymphatics as any vessels within the tumour area, either in the peripheral part or in the inner core [15]. Intratumoural lymphatics have also been referred to as vessels present only among tumour cells, while those present within the tumour stroma were separately counted and analyzed [39]. In a recent study, lymphatics at the peripheral area or the tumour were referred to as lymphatics at the advanced tumour edge while peritumoural lymphatics were referred to as lymphatics in the non-tumour area [40]. Results from the current study are in concordance with previous studies showing that, in breast cancer, tumoural lymphatics are mainly found in the peritumoural and advancing edge of the tumour. Intratumoural lymphatics are, however, found in a number of patients, seem to be functional, as discussed below, and may have important clinical consequences.

The relationship between LVD and clinicopathological criteria has not been firmly established as yet. The present study, as with others, found a significant positive association between high LVD and the presence of LN metastasis and tumours with negative hormonal receptor status [16, 19]. Such associations were not found in other studies [18, 39]. This may be due to different approaches used to assess LVD where it was assessed by counting the immunostained vessels in selected tumoural hot spots [16–19] or in randomly selected fields [39].

Although high LVD was significantly associated with shorter OS, the prognostic power of LVD was not strong enough to retain significance when adjusted to other prognostic factors such as tumour grade, LN status, tumour size and LVI. A similar conclusion has been reported both in studies on breast cancer [19] and in other types of cancers [41]. Current results suggest that rather than it being the number of the lymph vessels that is important in determining whether tumour cells will metastasise, it is the ability of the tumour cells to invade through the vessel that is of greatest importance. As such it was of interest to examine if these lymphatics were newly formed or were

Table 7 Association between MVD and clinicopathological criteria

MVD (%)									
	Low	High	Total	*P*-value		Low	High	Total	*P*-value
Age					*ER*				
<50 years	26(51)	25(49)	51	0.383	Negative	20(36)	36(64)	56	**0.001**
>50 years	75(49)	51(41)	126		Positive	72(64)	40(36)	112	
			177					168	
Size					*PR*				
<1.5 cm	81(79)	14(21)	65	**<0.0001**	Negative	30(42)	42(58)	72	**0.006**
>1.5 cm	50(45)	62(55)	112		Positive	60(65)	33(35)	93	
			177					165	
Grade					*NPI*				
I, II	75(70)	32(30)	107	**<0.0001**	Good	62(83)	12(16)	74	**<0.0001**
III	26(37)	44(63)	70		Intermediate	32(36)	56(63)	88	
			177		Poor	7(46)	8(53)	15	
LN status					*RR*				
Negative	82(66)	43(34)	125	**<0.0001**	No	79(58)	57(42)	136	0.880
Positive	19(37)	33(63)	52		Definite	22(54)	19(46)	41	
			177					177	
Stage					*LVD*				
I	79(65)	42(35)	121	**0.001**	Low	74(61)	48(39)	122	0.189
II	17(40)	26(60)	43		High	27(49)	28(51)	55	
III	5(38)	8(62)	13					177	
			177						

ER—estrogen receptor, PR—progesterone receptor, NPI—Nottingham prognostic index, RR—regional recurrence

Significant *P*-values are bold

pre-existing. In the study of Van der Auwera et al. [20]. Ki-67-positive LECs were detected in 16 (~50%) non-inflammatory and 12 (80%) inflammatory specimens whereas the study by van der Schaft et al. [39] reported no proliferative LECs (10 specimen were studied). The present study examined 85 specimens and proliferative lymphatics were detected in 25 (29%) with PF-L ranging from 0 to 2.2%. PF-L ranged from 0 to 3% in studies of other tumour types, [36, 42, 43]. The low PF-L is undoubtedly due to the inability of the methodology to accurately detect all dividing LECs within tumours, and is most probably underestimated as sprouting of new vessels begins at different levels along the lymphatic vessel [44]. Furthermore, new vessels may originate from circulating progenitors or from non-endothelial cells via trans-differentiation [45].

Intriguingly the presence of large number of lymphatics in tumour areas which were densely infiltrated by inflammatory cells was noted. This finding is supported by studies investigating the role of macrophages and inflammatory cells in inducing lymphangiogenesis in tumours and other tissues [46, 47], and could be due to macrophages being a source of angio- and lymphangiogenic growth factors [48].

The present study also found that most of the tumour cells within lymphatics were actively proliferating and that the PF of these tumour emboli positively correlates with the PF of the surrounding tumour cells. Such findings indicate that, in terms of proliferation, the cell population within lymphatics have a similar phenotype to the rest of the tumour cells outside the vessels supporting the notion that the ability of the tumour cells to invade and to metastasis is not a clonal trait [49]. However there was no association between the PF of the tumour and the presence of LVI i.e. tumours with high proliferation do not necessarily invade vessels suggesting that proliferation is not the only determinant of invasiveness.

In concordance with other studies [17, 34], no association was found between high LVD and high MVD suggesting that although both processes share some common mechanisms of molecular/cellular regulation [22, 44], the tissue regulation can vary significantly from each other. A significant association was found between high MVD and lager tumour size, higher grade, negativity of hormonal receptors, the presence of LN metastasis and advanced tumour stage. Similar results were reported in the study of Hansen et al. [50], which is the largest study conducted investigating angiogenesis in breast cancer (836 patients).

These associations reflect the importance of angiogenesis in tumour progression. However, unlike their results, we could not find a significant association between high MVD and DFI or OS. In an interesting review, and meta-analysis, by Uzzan et al., examining 87 breast cancer angiogenesis studies; 25 reported significant association between MVD and DFI and 11 with OS [26]. The authors concluded that inter-study variation could result from patient selection criteria, staining techniques and methodology used in counting blood vessels and in selection of the cut-off level for MVD assessment. They also stated that MVD is a significant, although weak, prognostic factor in breast cancer and that standardisation of MVD assessment is needed. Recommendations for such are mentioned in the review by Fox and Harris with regards the different methodologies used to assess angiogenesis [51].

In conclusion, the current study shows that, in breast cancer, lymphatics are located primarily in the peritumoural and peripheral areas and seem to play an important role in disease progression by being likely routes for tumour cell dissemination. It should be noted, however, that proliferating intratumoural lymphatics also occur and appear to be functional in that tumour emboli are observed within them. LVI, but not LVD, is an independent prognostic factor. Tumours differ in their lymphangiogenic and angiogenic characteristics indicating two distinctive biological processes. Lymphangiogenesis does occur in breast cancer, albeit in a small proportion of specimens, with inflammatory cell infiltrate seeming to play an important role in its regulation.

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