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Lymphatic and blood vessel morphometry in invasive breast carcinomas: Relation with proliferation and VEGF-C and -D proteins expression

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Summary. Introduction: The aim of the present study was to investigate the distribution of both lymphatics and blood microvessels in invasive breast carcinomas and the clinicopathological and prognostic significance of their density and size related parameters as well as their correlation with the proliferative potential of the tumor and VEGF-C and -D expression. Methods: Both single and double immunohistochemistry were applied on a series of 146 paraffin-embedded breast tissue specimens to detect VEGF-C and -D as well as lymphatics and blood microvessels, respectively. Computer-assisted morphometry was performed to evaluate the blood and lymphatic vessel density (BVD and LVD respectively) as well as various vascular size related parameters. Results: Lymphatics were detected within the stroma at the tumor border, while blood vessels were located in both the interior of the tumor mass and peritumor stroma. BV major axis, minor axis and perimeter inversely correlated with ER (p=0.011, p=0.023 and p=0.008 respectively), while LV major axis, minor axis and the perimeter inversely correlated with tumor nuclear grade (p=0.045, p=0.037 and p=0.032 respectively) and topoisomerase IIα (p=0.015, p=0.024 and p=0.045 respectively). The same LV parameters were found to positively correlate with cancerous VEGF-C (p<0.0001, p=0.092 and p=0.012 respectively) and VEGF-D in the stromal fibroblasts surrounding neoplastic cells (p=0.011, p=0.041 and p=0.026 respectively). High BVD exerted an unfavorable impact on both disease-free (p=0.021) and overall survival (p=0.031) of the patients. High LVD correlated with poor disease-free and overall survival only in the subgroup of patients with ER-negative tumors (p=0.056 and p=0.0312 respectively). Conclusion: These findings, for the first time, correlate lymphatic size with tumors of limited proliferative potential and higher nuclear differentiation. Moreover, they suggest that VEGF-C and -D expression influence lymphatic size rather than being involved in the increase of lymphatic vessel number.

Key words: Angiogenesis, Breast cancer, D2-40, Immunohistochemistry, Lymphangiogenesis, Morphometry, Proliferation.

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Introduction

Angiogenesis and lymphangiogenesis are of pivotal importance in tumor growth, invasion and metastasis (Folkman, 1997; Saharinen et al., 1997). The estimation of angiogenesis, usually by microscopic estimation of microvessel density on tissues stained with a variety of endothelial markers, has proceeded that of lymphagniogenesis and blood vessel density (BVD) has been reported either to correlate or not with clinicopathological parameters and patient outcome in breast cancer (Ludovini et al., 2000; Medri et al., 2000). The results are debateable however due to the variety in the endothelial markers used, with some of them not distinguishing blood from lymphatic endothelium, as well as the different cut off values and microvessel counting techniques used (Uzzan et al., 2004).

In comparison to blood vessels, lymphatics have been poorly studied until recently, partly due to lack of specific lymphatic entothelial markers (Stacker et al., 2002). Today, there are several antibodies [(VEGFR-3, podoplanin, lymphatic vessel endothelial HA receptor-1 (LYVE-1) (Stacker et al, 2002), D2-40 (Kahn and Marks, 2002)] which more or less specifically recognize lymphatic endothelium. The use of these antibodies in breast cancer has shown the almost absolute lack of lymphatics from the interior of the tumor (Williams et al., 2003; Bono et al., 2004; Schoppmann et al., 2004;

Vleugel et al., 2004; Agarwal et al., 2005), thus supporting the suggestion that intratumoral lymphatics are absent rather than collapsed or not functional (Pepper, 2001). However, the findings remain controversial with regards to the clinicopathological and prognostic significance of lymphatic vessel density (LVD).

Moreover, molecular regulation of lymphangiogenesis is another issue of interest. While vascular endothelial growth factors (VEGF)-C and -D have been shown to promote endothelial cell proliferation in vitro (Joukov et al., 1997; Achen et al., 1998), and lymphangiogenesis and lymph node metastasis in vivo (Stacker et al., 2001; Veikkola et al., 2001), it is unclear whether lymphatic spread is activated through the expansion and invasion of pre-existing lymphatics at the tumor periphery, or by active de novo intratumoral lymphangiogenesis (Pepper, 2001). Thus, the question remains whether VEGF-C and -D contribute to lymph node metastasis by increasing the number of lymphatic and blood vessels or by promoting dilation of peritumoral lymph vessels (Alitalo and Carmeliet, 2002).

The aim of the present study was to investigate the distribution of both lymphatics and blood microvessels by double immunohistochemical method in the same sections of invasive breast carcinoma, using CD34 and D2-40. Moreover, we studied the clinicopathological and prognostic significance of both vessel type density and size related parameters as well as their correlation with the proliferative potential of the tumor and VEGF-C and -D expression.

Materials and methods

Patients and samples studied

One hundred and forty six paraffin blocks with tumor samples were available from patients with resectable breast cancer who had undergone surgery. We only selected women with histologically proven, clearly invasive breast carcinomas, regardless of their initial stage, in whom axillary lymph node dissection had been performed and who had all their resected materials studied histologically. The patients were aged from 25 to 86 years (mean age 56.89 years). None of them had received radiation or chemotherapy preoperatively. Lastly, an informed consent was obtained from patients in order for the material derived from them to be used in research.

Routine histological examination was performed with heamatoxylin-eosin staining. All carcinomas were classified according to the criteria of the World Health Organization (Tavassaoeli and Davilee, 2003) and were recorded as invasive ductal or invasive lobular. The combined histological grade (1, 2 and 3) of infiltrating ductal carcinoma was obtained according to a modified Scarff-Bloom-Richardson histologic grading system with guidelines as suggested by Nottingham City Hospital pathologists (Robins et al., 1995). Nuclear grading was based on nuclear polymorphism. Staging at the time of diagnosis was based on the TNM system (Union International Contre Cancer, 1992). Tumor size (<2cm, 2-5 cm, >5 cm) and lymph node status were evaluated separately. The clinicopathological characteristics of the series are shown in Table 1. During the immunohistochemical procedure some specimens were destroyed and were considered to be too small to be evaluated for both immunomarkers. Therefore, the cases finally included in the statistic analysis were 146 for CD34 and 111 for D2-40.

Follow up was available for 110 patients. Mean survival time was 96.7 months (range 5 to 135 months). Patient outcome was defined as disease-free and overall survival. All patients received conventional post-operative treatment depending on the extent of the disease, including radiation therapy and medical antiestrogen therapy, when indicated. Premenopausal patients with axillary involvement were treated with six courses of adjuvant chemotherapy.

Immunohistochemistry

Single immunohistochemical staining

Immunohistochemical staining for VEGF-C and VEGF-D was performed on 4 μm thick formalin-fixed paraffin sections, using an avidin-biotin peroxidase technique, after overnight heating at 37°C and subsequent deparaffinization in xylene and rehydration through graded alcohols. After quenching of endogenous peroxidase activity using a hydrogen peroxide solution (0.3% in TBS for 30 min), we proceeded to microwave mediated antigen retrieval in 10 mM, pH 6.0, citrate buffer at 750 W for 10 min. In the case of VEGF-D, the deparaffinization, rehydration and antigen retrieval were performed in one step, using the commercially available reagent, Trilogy (Cell Marque), in the microwave oven for 15 min. After rinsing with TBS, normal horse serum was applied for 30 min to block non-specific antibody binding. Subsequently, sections were incubated overnight at 4°C with the primary antibodies. A standard avidin biotin-peroxidase complex technique (Vectastain Elite, Vector Laboratories, Burlingame, CA) was used for visualization, with diaminobenzidine as a chromogen. Finally, sections were then counterstained with hematoxylin and mounted.

Polyclonal antibodies against VEGF-C (goat/C-2, sc:1881) and VEGF-D (rabbit/H-144, sc:13085) (Santa Cruz Biotechnology Inc, CA, USA) were used at a dilution of 1:80 and 1:120 respectively.

The evaluation of the immunohistochemical staining was performed by two pathologists, independently, through light microscopic observation, without knowledge of the clinical data of each patient. Cases of disagreement were reviewed jointly to arrive at a consensus score. The score resulted as the average of 10 distinct high-power fields observed under x400 magnification. As positive controls, formalin-fixed,

paraffin-embedded sections from normal human placenta were stained for VEGF-C and VEGF-D. Negative controls had the primary antibody omitted and replaced by nonimmune, normal serum from the same species as the primary antibody or TBS. Staining intensity and the number of stained cells were taken into consideration all through the evaluation process and the staining was scored on a scale of 0 to 3 in half steps. A score of 0 was given when no staining or weak staining in <10% of positive cells was detected, score 1 if weak to moderate staining in 11-20%, score 2 if moderate to strong staining in 21-50% and score 3 if strong staining in more than 50 % of cells was detected.

The immunostaining results of ER, PR and topoisomerase IIα (topoIIα) were obtained from our data files (Nakopoulou et al., 2000).

Double immunohistochemical staining

For detection of blood and lymphatic vessels a double immunohistochemical staining was applied, on the same sections, using the monoclonal primary antibodies: anti-CD34 [QBEnd/10] (Biogenex, San Ramon, USA) and D2-40 (Signet Laboratories Inc, Dedham, MA, USA), at a dilution of 1:50 and 1:20, respectively. Specifically, single immunohistochemistry was first applied, as previously described, for the detection of CD34 antigen on blood vessel endothelium, with NovaRED (#Cat SK-4800, Vector) as a chromogen substrate (red color) and then, after applying normal horse serum for 30 min to block non-specific binding, sections were incubated overnight at 4°C with D2-40. Subsequently, a standard avidin biotin-peroxidase

Table 1. Correlation of BVD and LVD with clinicopathological parameters, ER/PR, topoIIα and VEGF-C and –D proteins expression.

	BVD			LVD		
	Total	Median (range)	p value	Total	Median (range)	p value
Menopausal Status						
Before	45	96 (36-217)	NS	38	11 (4-30)	NS
After	101	86 (10-274)		73	9 (3-21)	
Stage						
1	29	75 (17-274)	NS	23	10 (4-23)	NS
2	95	88 (10-223)		72	10 (3-30)	
3	21	81 (26-211)		14	8 (4-21)	
Histologic Type						
Ductal	118	86 (10-238)	NS	88	10 (3-30)	NS
Lobular	27	88 (26-274)		22	8,5 (4-21)	
Nuclear Grade						
1	49	81 (10-274)	NS	41	8 (4-30)	NS
2	49	81 (12-223)		39	10 (3-21)	
3	47	100 (24-211)		36	12,5 (4-23)	
Histologic Grade						
1	18	65 (10-238)	NS	15	8 (4-16)	NS
2	88	91 (12-274)		66	10 (3-30)	
3	35	88 (31-211)		26	13 (4-21)	
Tumor Size						
< 2 cm	36	78,5 (17-274)	NS	31	10 (4-30)	NS
2-5 cm	89	88 (10-223)		64	10 (3-21)	
> 5 cm	20	90,5 (26-210)		15	8 (4-21)	
Lymph Nodes						
Non-Infiltrated	60	88 (17-274)	NS	44	9,5 (3-23)	NS
Infiltrated	85	86 (10-223)		65	10 (4-30)	
ER						
Negative	70	99,5 (24-223)	NS	49	13 (4-21)	NS
Positive	75	75 (10-274)		61	8 (3-30)	
PR						
Negative	76	87 (12-238)	NS	57	10 (3-30)	NS
Positive	69	87 (10-274)		53	8 (4-23)	
topoIIα						
Negative	83	87 (10-217)	NS	62	9 (3-30)	NS
Positive	38	99,5 (24-274)		28	12,5 (4-23)	
VEGF-C cytopl						
Negative	53	80 (24-274)	NS	43	10 (4-19)	NS
Positive	80	92 (10-238)		61	10 (3-30)	
VEGF-D stromal						
Negative	79	80 (17-274)	NS	55	9 (3-21)	NS
Positive	55	100 (10-238)		46	12 (4-30)	

complex technique was used for visualization with diaminobenzidine bound with NiCl (#Cat SK-400, Vector) as a chromogen substrate (black color). Finally, sections were counterstained with hematoxylin and mounted. Paraffin sections of normal small intestine were used as a positive control for CD34 and D2-40 antibodies staining.

Microvessel counting and image analysis

Immunohistochemically stained slides for CD34 and D2-40 were examined field per field by two experienced investigators, without knowledge of each patient's clinicopathological data or outcome, to identify the areas of highest vascularization ("hot spot" areas) (Weidner et al., 1991). The selection of hot spot areas was based on scanning of the tumor sections at low power (25x and 100x) and identifying the areas of invasive carcinoma with the highest number of discrete vessels staining for CD34 (red) and D2-40 (black). Nine blood and five lymphatic vessel hotspots were chosen in each section. Each hotspot was examined in turn at 200x magnification. The average score was then determined for each section. The hot spot areas were photographed and stored as JPEG files (1600x1200 pixels, 16.7 million colors). Each microvessel was defined as a lumen surrounded by a rim of endothelial cells, clearly separated from adjacent microvessels and highlighted by immunostaining with anti-CD34 or anti-D2-40 antibody. Quantification of digital images was performed by one investigator by using the Image Proplus Media Software (Cybernetics, Canada) on a Pentium III PC. In each selected field the outline of each individually stained vessel was identified and traced to determine a) blood and lymphatic vessel density (BVD and LVD respectively) defined as the number of vessels per mm^2, b) The relative blood and lymphatic vascular area (BVA and LVA), defined as the percentage of the area of the lesion covered by microvessels, c) the major axis length (the distance between the two points along the vessel periphery that are further apart), d) the minor axis length (the longest axis perpendicular to major axis formed by two points along the vessel periphery) and e) perimeter (Fig. 1). The variables entered into the statistical analysis were the mean values of the measurements in the nine and five fields.

Statistics

Correlations were assessed using the Spearman rank correlation test for two continuous variables and the Mann-Whitney U-test and Kruskall-Wallis test when one variable was continuous and the other categorical. In order to investigate the effect of blood and lymphatic vessel parameters on clinical outcome we used the median value and the upper quartile as the cut-off value, as previously described (Franchi et al., 2004). Overall and disease-free survival curves were obtained using the Kaplan Meier test and log-rank statistics, followed by Cox's proportional hazards regression model. For all tests, a two-tailed p of < 0.05 was considered significant. All statistical tests were performed using SPSS software (release 10.0, SPSS Inc., Chicago, IL).

Results

CD34, D2-40 and VEGF-C and –D expression in breast cancer

CD34 positive blood vessels and D2-40 positive lymphatic vessels were detected in all cases. Blood vessels were negative for D2-40, as can be seen from the surrounding D2-40 negative vessels with intraluminal erythrocytes (Fig. 2a,b). Lymphatic vessels were almost exclusively detected within an area of 500 μm from the tumor border (peritumor stroma) (Fig. 3a). Intratumoral lymphatic vessels were extremely rare (observed in 3 cases). This was in marked contrast to blood vessels, which were located in both the interior of the tumor mass (Fig. 3b) and the surrounding peritumoral tissue (Fig. 3a). Median blood vessel density (BVD) and lymphatic vessel density (LVD) were 87 microvessels/mm^2 (range, 10-274) and 10 microvessels/mm^2 (range 3-30) respectively. The median values of the rest of the computer-assisted morphometric vessel parameters are shown in table 2. The majority of tumors expressed the proteins VEGF-C and VEGF-D (50.9 and 65.8% respectively). VEGF-C and -D proteins were detected in the cytoplasm of the cancer cells as well as in the stromal fibroblasts surrounding the neoplastic cells (Fig. 4a,b).

Correlations among the various morphometric variables

A substantial correlation was found between BVD and LVD ($r=0.811$; $p<0.0001$), as well as between the BVA and LVA ($r=0.707$; $p<0.0001$). Moreover, significant correlations were found among the size related parameters of the blood and lymphatic vessels. In

Table 2. Median values and range of the various computer-assisted morphometric vessel parameters.

	Median	range
BVD	87	10-274
BVA	2.34	0.21-7.3
BV major axis	28.11	20.6-44.18
BV minor axis	12.29	9.97-20.22
BV perimeter	72.22	55.00-116.32
LVD	10	3-30
LVA	1.99	0.12-7.01
LV major axis	37.02	20-73.91
LV minor axis	15.69	5.55-34.38
LV perimeter	91.39	58.01-208.626

BVD: blood vessel density; LVD: lymphatic vessel density.

particular, BVA correlated with BV major axis (r=0.206; p=0.012), minor axis length (r=0.401; p<0.0001) and perimeter (r=0.243; p=0.003) and BV perimeter correlated with BV in both major and minor axis length (r=0.977 and r=0.655 respectively, with p<0.0001 for both). In proportion to the aforementioned for blood vessels, LVA correlated with LV major (r=0.392; p<0.0001), LV minor axis length (r=0.353; p<.0001) and perimeter (r=0.394; p<0.0001) and lymphatic perimeter correlated with LV in both major and minor axis length (r=0.931 and r=0.844 respectively, with p<0.0001 for both).

Correlations between CD34 and D2-40 expressing vessel parameters, clinicopathological parameters, tumor growth potential and VEGF-C and -D proteins expression

Statistical analysis recognized no significant association between BVD and LVD with the classical clinicopathological parameters (Table 1). Moreover, none of the remaining morphometric parameters of the vessels was related to any of the clinicopathological parameter (data not shown) except for BV major axis, minor axis and perimeter that inversely correlated with ER expression (p=0.011, p=0.023 and p=0.008 respectively, Mann-Whitney test) (Fig. 5) and LV major axis, minor axis and perimeter which inversely correlated with tumor nuclear grade (p=0.045, p=0.037 and p=0.032 respectively, Kruskal Wallis test) (Fig. 6a). With regards to tumor proliferative potential, LV major axis, minor axis and perimeter were inversely associated with topoisomerase IIα (p=0.015, p=0.024 and p=0.045 respectively, Mann Whitney test) (Fig. 6b), while the same parameters were found to positively correlate with VEGF-C within neoplastic cells (p<0.0001, p=0.092 and p=0.012 respectively, Mann Whitney test) (Fig. 6c) and fibroblastic localization of VEGF-D (p=0.011, p=0.041 and p=0.026 respectively, Mann Whitney test) (Fig. 6d).

Survival

As far as the clinical impact is concerned, BVD>119 microvessels/mm^2 (upper quartile) was found to exert unfavorable impact on both disease-free and overall survival of the patients (p=0.040 and p=0.013 respectively) (Fig. 7a,b). Moreover, increased BVD correlated with poor overall and disease-free survival in the subgroups of patients with tumor size 2-5cm (p=0.0002 and p=0.0049 respectively) (Fig. 8a,b) and with ER-negative tumors (p=0.0002 and p=0.002 respectively) (Fig. 8c, d). LVD>15 vessels/mm^2 (upper quartile) correlated with poor overall and disease-free survival only in the subgroup of patients with ER-negative tumors (p=0.0312 and p=0.056 respectively) (Fig. 9a,b). None of the remaining morphometric parameters was found to have any clinical significance in our series of patients. Multivariate analysis in the entire cohort, adjusted for age, menopausal status, histological type, histological and nuclear grade, tumor size, lymph node metastasis, ER, PR, received therapy and blood vessel morphometric parameters, selected increased BVD and the stage of the disease as the

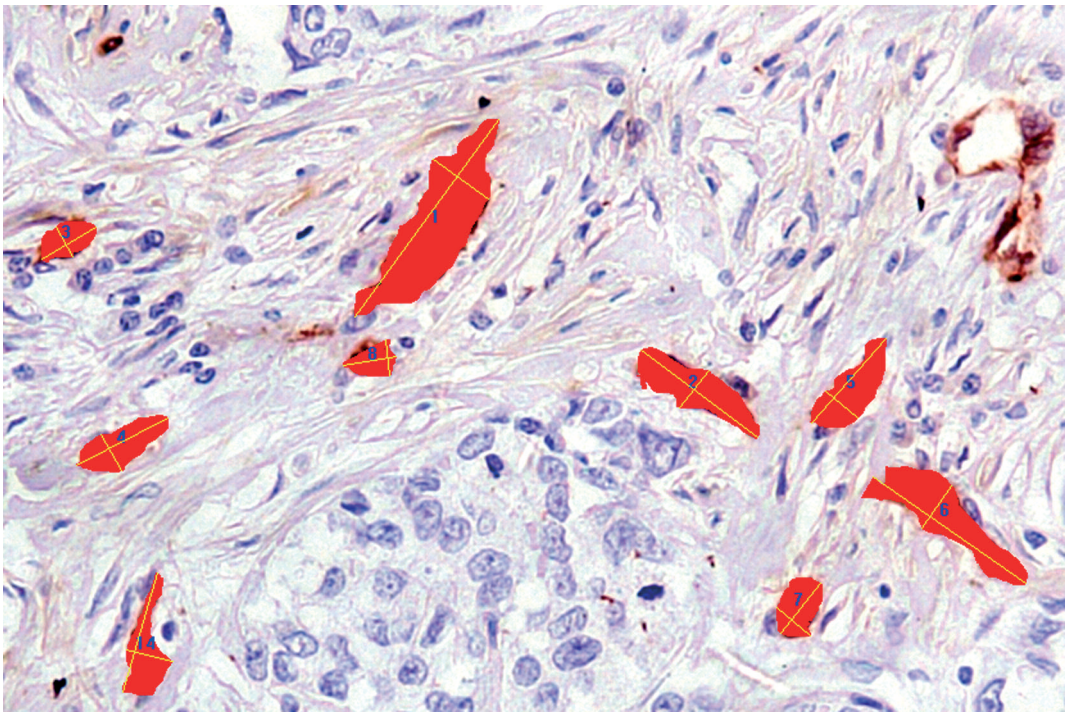

Fig. 1. Digital image. processed with SigmaScan Pro5, showing the major and minor axis of the positively stained blood vessels (ABC/HRP, x 400).

independent prognostic indicators of both disease-free and overall survival (Table 3).

Discussion

The clinicopathological and potential prognostic significance of angiogenesis and lymphangiogenesis as well as their molecular regulation are issues that have intensively preoccupied the scientific community and that, nevertheless, have not been elucidated yet. Although in previous studies, series of breast cancer were separately stained with blood vessel and lymphatic endothelium markers, simultaneous staining with CD34 and D2-40, as well as evaluation of blood and lymphatic vessel size related parameters in relation to the tumor growth potential and VEGF-C and -D proteins expression, was not carried out.

Table 3. Contribution of parameters of statistical significance to patients' overall and disease-free survival through stepwise forward Cox's proportional hazard regression model.

	Overall Survival			
	Coefficient	SE	p value	95% CI
Stage	1.578	0.359	<0.0001	2.396-9.805
High BVD	0.877	0.406	0.031	1.084-5.333
	Disease-free Survival			
	Coefficient	SE	p value	95% CI
Stage	1.578	0.302	<0.0001	2.681-8.754
High BVD	0.797	0.344	0.021	1.130-4358

SE: standard error. 95% CI: 95% coefficient interval

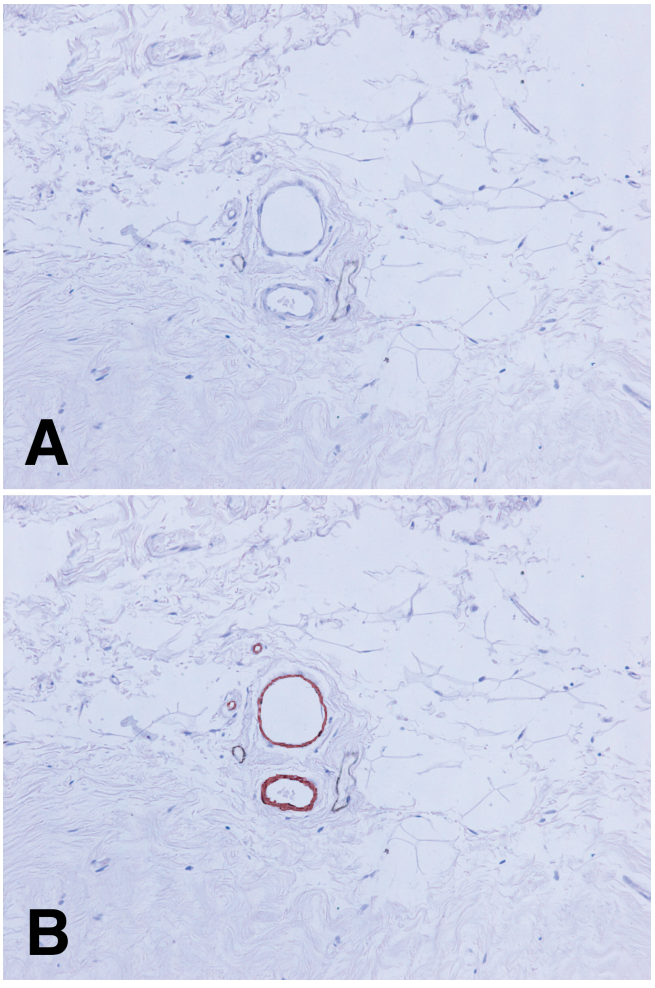

Fig. 2. Single immunohistochemical staining for D2-40. Lymphatics were stained black while blood vessels remained unstained **(a)**. The same section after double immunostaining with D2-40 and CD34. The previously unstained with D2-40 blood vessels, are positive for CD34 **(b)** (ABC/HRP, x 200).

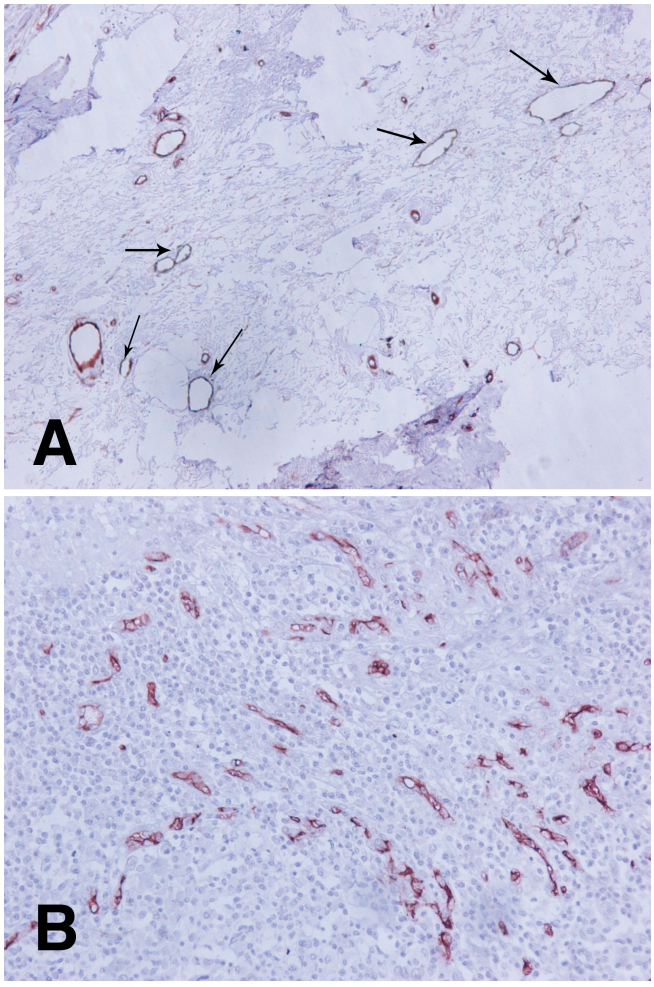

Fig. 3. D2-40 detected lymphatic vessels (black, arrows) and CD34 detected blood vessels (red) within the tumor stroma **(a).** CD34 stained blood vessels were also located in the interior of the tumor mass, where no lymphatics were observed **(b)** (ABC/HRP, x 200).

Our findings showed that D2-40 stains lymphatics, not stained with CD34 which further validates D2-40 specificity as a lymphatic marker. In the present study, lymphatic vessels were almost exclusively detected within peritumor stroma and not within the tumor mass, a finding that is in accordance with recently published data (Williams et al., 2003; Bono et al., 2004; Schoppmann et al., 2004; Vleugel et al., 2004; Agarwal et al., 2005). However, there are studies reporting lymphatic detection both in the malignant tissue and the peritumor stroma (Choi et al., 2005, Nakamura et al., 2005). Moreover, the strong positive correlation between BVD and LVD indicated that angiogenesis and lymphangiogenesis are two closely associated processes which may be subjected to similar regulatory mechanisms.

The clinicopathological and prognostic significance of angiogenesis by microscopic estimation of microvessel density has been extensively assessed with various results. For instance, a recent study reported microvessel density, detected visually by anti-Factor VIII antibody, to be an independent predictor of disease-free and overall survival in a series of Japanese patients (Tsutsui et al., 2003). Furthermore, Uzzan (2004) in a meta-analysis of 43 independent studies linking BVD to patient survival ascertained a large variability in the results regarding the prognostic value of BVD, although the conclusion was that BVD seems to be a significant but weak prognostic factor in women with breast cancer (Uzzan et al., 2004). Similar variability exists regarding the clinicopathological value of BVD as well. Most studies reported no relation between BVD and any of the well established pathological parameters (Axelsson et al., 1995; Medri et al., 2000), while others found a relation with tumor size, LN metastasis and stage (Choi et al., 2005), or biological markers such as HER2 oncoprotein (Ludovini et al., 2000). In the present study, blood vessel size related parameters inversely correlated with ER, indicating that the size of blood vessels is associated with better differentiated breast carcinomas. This finding is further supported by the statistic selection of BVD as an independent prognostic indicator of both disease-free and overall survival. The above mentioned variability in the reported clinicopathological and prognostic significance of BVD may lie in the differences in microvessel counting techniques, antibodies and cut off values used.

As far as the lymphatic vessels are concerned, the present study recognized no relation between LVD and size related parameters with any of the well known clinicopathological parameters, but for the inverse correlation between LV major and minor axis length and

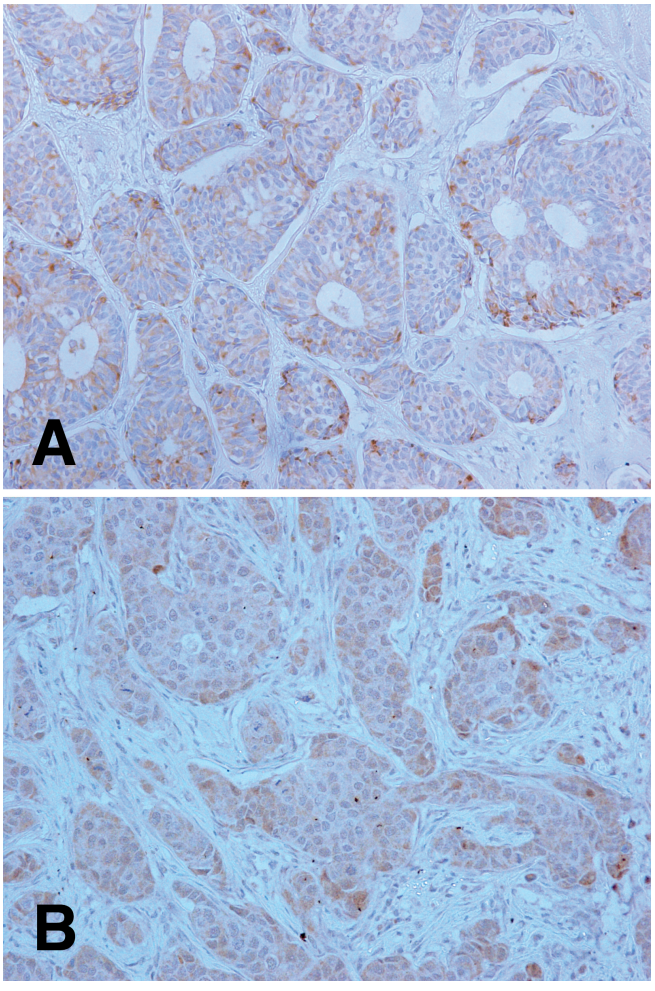

Fig. 4. Immunohistochemical staining of VEGF-C in the cytoplasm of the malignant cells **(a)** and VEGF-D in both the cytoplasm of the malignant cells and the peritumoral stroma **(b)** of an invasive breast carcinoma (ABC/HRP, x 200).

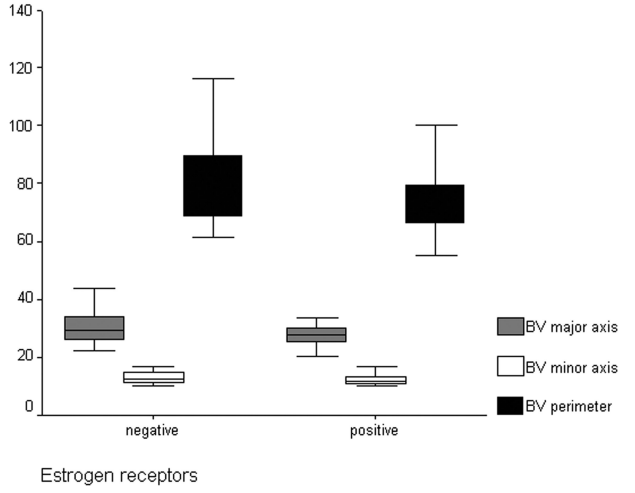

Fig. 5. Schematic representation of the relation of BV major axis, minor axis and perimeter with ER expression.

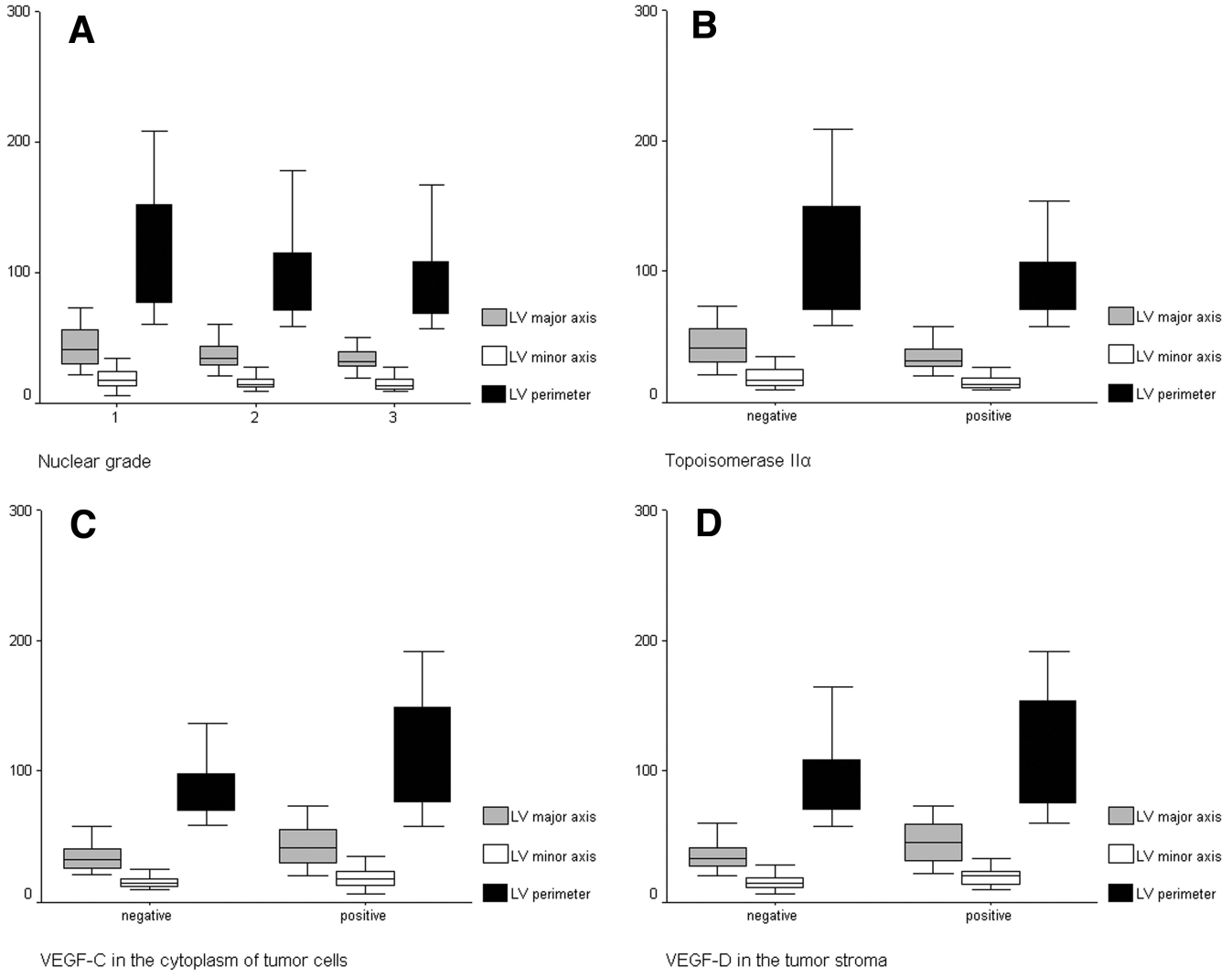

Fig. 6. Schematic representation of the relation of LV major axis, minor axis and perimeter with tumor nuclear grade **(a)**, topoisomerase IIα **(b)**, cancerous VEGF-C **(c)** and stromal VEGF-D **(d)** expression.

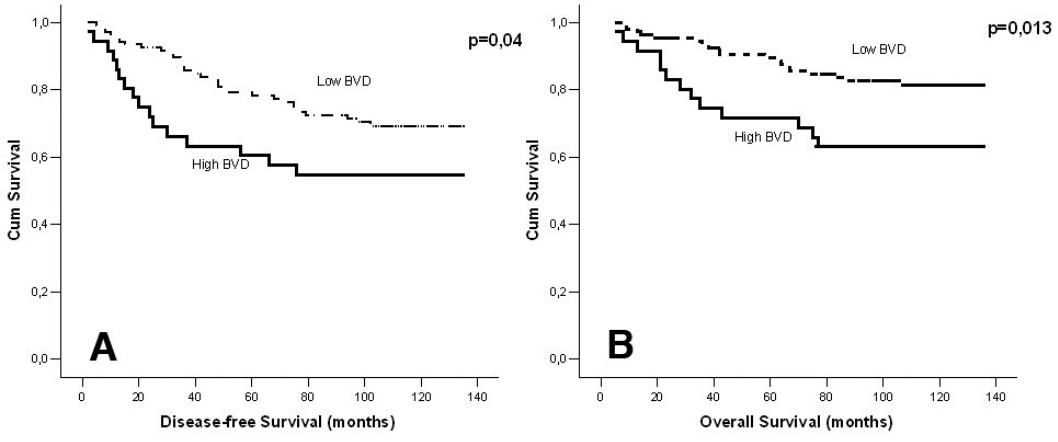

Fig. 7. Schematic representation of the impact of high BVD on the disease-free **(a)** and overall **(b)** survival of the patients.

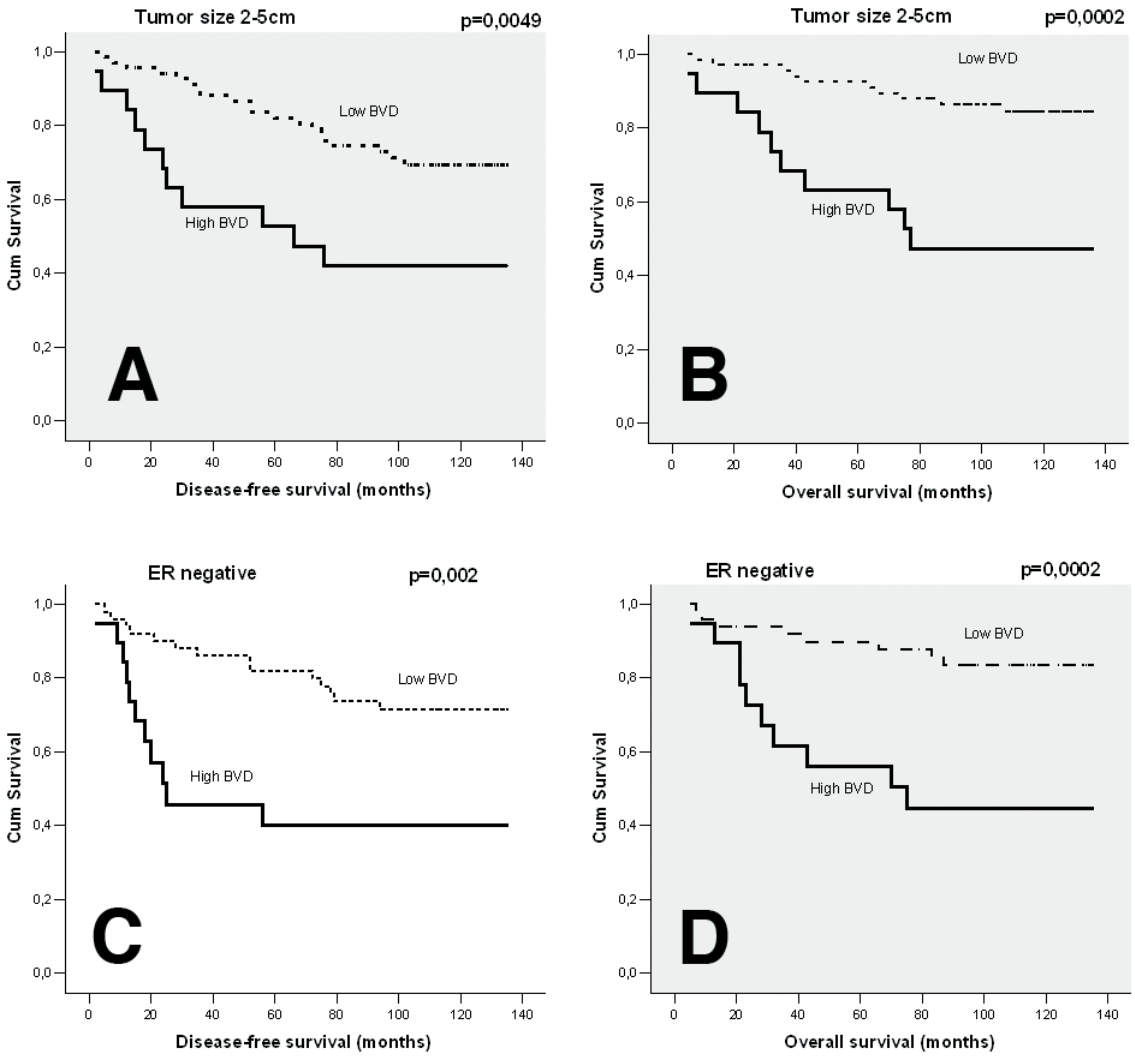

Fig. 8 Schematic representation of the impact of high BVD on the disease-free and overall survival of the patients with tumor size 2-5 cm **(a,b)** and ER-negative tumors **(c,d)**.

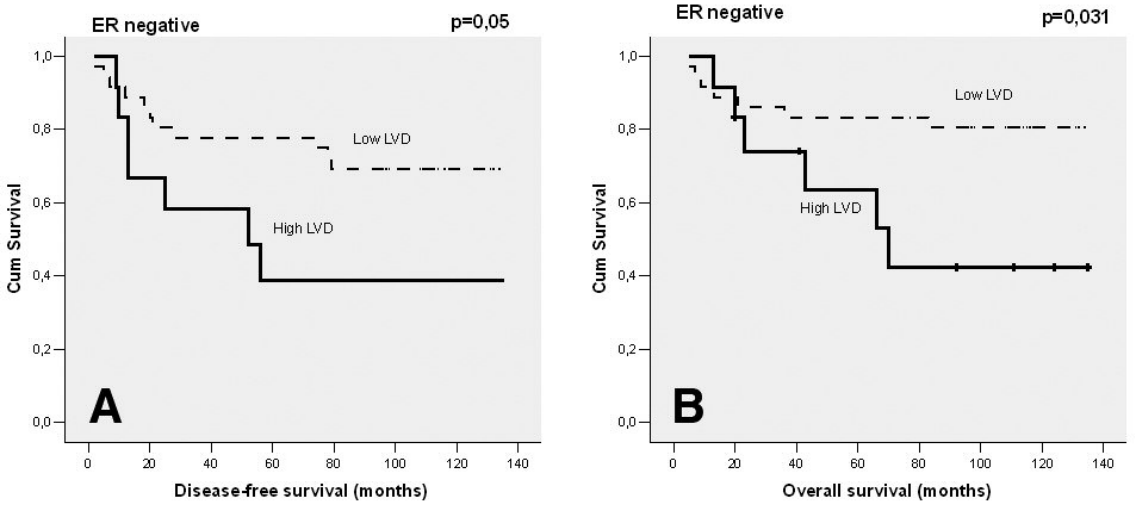

Fig. 9. Schematic representation of the impact of high LVD on the disease-free **(a)** and overall survival **(b)** of the patients with ER-negative tumors.

perimeter with tumor nuclear grade, which indicates an association of the larger lymphatics with tumors of better differentiation. This finding is further supported by the inverse correlation of lymphatic size related parameters with topoisomerase IIα expression, suggesting an association between greater lymphatic size and lower proliferative potential of the tumor. Similarly to blood microvessels, there is no unanimity with regards to lymphatic vessel density clinicopathologic and prognostic value. Nakamura et al. (2005) and Bono et al. (2004) having used podoplanin and LYVE-1 as lymphatic endothelial markers respectively, found LVD to correlate with lymph node metastasis (Nakamura et al., 2005) and the number of the infiltrated lymph nodes (Bono et al., 2004), as well as to be a prognosticator of poor disease-free and overall survival in univariate analysis (Bono et al., 2004; Nakamura et al., 2005). Choi et al. (2005) reported a positive association between the computer-assisted estimation of the percentage of total field area (x 400) positively stained for D2-40 and the stage of the disease in a series of 29 invasive breast carcinomas (Choi et al., 2005). Agarwal et al. (2005), having applied three lymphatic endothelial markers (D2-40, podoplanin and Prox-1) reported no clinicopathological significance for LVD (Agarwal et al., 2005). The aforementioned discrepancies among the analyzed studies, including the present one, could be assigned to the different methodologies and targeted endothelial antigens used, as well as the different cut off values selected for the determination of the LVD impact on clinical outcome. The present study found high LVD to unfavorably influence the clinical outcome of only the patients with ER-negative tumors and is the first to evaluate the lymphatic size related parameters and to find them to inversely correlate with tumor proliferative potential and nuclear differentiation. This finding may be associated with the possibility that larger lymphatics drain away factors promoting growth of the tumor more effectively.

Another controversial issue is the molecular regulation of lymphangiogenesis, in humans. Although VEGF-C and -D have been reported to promote lymphangiogenesis in vitro (Joukov et al., 1997; Achen et al., 1998) and in animal models (Stacker et al., 2001; Veikkola et al., 2001), only few, if any, studies of human cancers have revealed any correlation between VEGF-C and -D levels and LVD, despite the correlation with nodal metastasis (Ohta et al., 2000; Bono et al., 2004). It therefore remains a possibility that, in vivo, VEGF-C and -D may influence nodal metastasis independently of lymphangiogenesis, being involved in the dilation of preexisting lymphatics (Leu et al., 2000). In the present study, no correlation was found between LVD and VEGF-C and -D expressions. However, size related lymphatic parameters were positively related to VEGF-C and -D indicating that growth factors might be involved in the increase of lymphatic vessel size rather than their number.

In conclusion, this is the first study to perform double immunohistochemical staining for CD34 and D2-40 on breast cancer specimens and to correlate D2-40 detected lymphatic size with tumors of limited proliferative potential and higher nuclear differentiation. Moreover, VEGF-C and -D expressions seem to influence lymphatic size rather than being involved in boosting lymphatic vessels' number.

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