Inflammatory breast cancer (IBC) is a particularly aggressive form of locally advanced malignant breast disease that affects ~5% of women with breast cancer (1). Most patients with IBC have axillary lymph node involvement at the time of diagnosis and up to 30% have distant metastases (2). Prognosis therefore remains poor; despite the introduction of multimodality treatment, 3-year survival of IBC patients is only around 40% (3). The dissemination of tumor cells via the lymphatic system has occurred in nearly all patients with inflammatory breast cancer (IBC). The objective of this study was twofold: (a) to determine which is the most suitable marker of lymph vessels in primary breast tumors and (b) to compare histomorphometric lymph vessel variables in IBC and non-IBC.

**Experimental Design:** Serial sections of 10 IBCs and 10 non-IBC were immunostained for D2-40, LYVE-1, podoplanin, and PROX-1. Relative lymph vessel area, lymph vessel perimeters, and counts and lymphatic endothelial cell proliferation (LECP) were then measured in D2-40/Ki-67 double-immunostained sections of 10 normal breast tissues, 29 IBCs, and 56 non-IBCs.

**Results:** D2-40 was the most suitable antibody for staining peritumoral and intratumoral lymph vessels. D2-40-stained intratumoral lymph vessels were present in 80% of non-IBCs and 82.8% of IBCs (P = 0.76). In non-IBC, lymph vessels located in the tumor parenchyma were smaller and less numerous than those at the tumor periphery (P < 0.0001) whereas in IBC, intratumoral and peritumoral variables were not significantly different. The mean relative tumor area occupied by lymph vessels was larger in IBC than in non-IBC (P = 0.01). LECP at the tumor periphery was higher in IBC than in non-IBC: median LECP was 5.74% in ICB versus 1.83% in non-IBC (P = 0.005).

**Conclusions:** The high LECP in IBC suggests that lymphangiogenesis contributes to the extensive lymphatic spread of IBC.

**Abstract**

**Purpose:** At the time of diagnosis, metastatic dissemination of tumor cells via the lymphatic system has occurred in nearly all patients with inflammatory breast cancer (IBC). The objective of this study was twofold: (a) to determine which is the most suitable marker of lymph vessels in primary breast tumors and (b) to compare histomorphometric lymph vessel variables in IBC and non-IBC.

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**Conclusions:** The high LECP in IBC suggests that lymphangiogenesis contributes to the extensive lymphatic spread of IBC.
be associated with increased lymph vessel density in clinical samples of invasive breast carcinomas and with the presence of lymph node metastases (12). To resolve the current controversy and to determine the clinical significance of lymphangiogenesis in breast cancer, quantification methods of lymphangiogenesis need further optimization and standardization. During the last decade, several markers that show specificity for lymphatic endothelium have been discovered: PROX-1 (13, 14), a transcription factor, podoplanin (15), a glomerular podocyte membrane protein, LYVE-1 (16), a hyaluronan receptor, and more recently, the D2-40 antibody (17, 18); these have been used to detect lymph vessels in cancer. However, a debate still exists on which is the best marker to visualize lymph vessels in human cancer. The aim of the present study was therefore twofold: (a) to identify the most suitable lymph vessel marker to measure lymphangiogenesis in breast carcinoma and (b) to obtain quantitative morphologic data on lymphangiogenesis in IBC and non-IBC.

**Materials and Methods**

**Clinicopathologic data**

Breast carcinoma tissues were obtained from IBC (n = 29) and non-IBC (n = 56) patients treated at the General Hospital Sint-Augustinus, Wilrijk, Belgium, with written informed consent. IBC was diagnosed according to the criteria mentioned in the tumor-node-metastasis classification of the American Joint Committee on Cancer (19). All patients with IBC showed diffuse enlargement of the involved breast of sudden onset. There was erythema and edema of the skin involving more than one third of the breast. The presence of dermal lymphatic invasion as an isolated observation was not sufficient for the diagnosis of IBC. Tumor characteristics are listed in Table 1. Tumor size, lymph node status, estrogen receptor status, and progesterone receptor status were recorded by review of pathology files. Tumors were subtyped as ductal or lobular carcinoma and histologically graded from 1 to 3 according to the Nottingham modification of the Bloom and Richardson histologic grading scheme. In addition, the growth pattern of each tumor and the presence of a fibrotic focus, a scarlike area in a carcinoma, were evaluated using definitions described before (20). Expansively growing tumors form a well-circumscribed nodule consisting of carcinoma cells and desmoplastic connective tissue whereas breast carcinomas with an infiltrative pattern grow between preexisting structures without disturbing the tissue architecture. Mixed infiltrative-expansive breast tumors consist of a central expansive nodule surrounded by carcinoma cells showing an infiltrative growth pattern. Normal breast tissue (n = 10) was taken from reduction mammoplasty.

All protocols were reviewed and approved by the ethical committee of the General Hospital Sint-Augustinus.

**Immunohistochemical staining**

**Immunostaining for D2-40, LYVE-1, podoplanin, PROX-1, CD31, CD34, and factor VIII.** Serial 5-μm sections were cut from formalin-fixed paraffin-embedded tissue blocks, dewaxed in toluene, and rehydrated through sequential changes of alcohol and distilled water. All immunostainings were done on a DakoAutostainer (DakoCytomation, Glostrup, Denmark). For D2-40, CD31, and CD34 staining, antigen retrieval was done by heating slides for 3 minutes in citrate buffer (pH 6.0) at 98°C. For LYVE-1 and podoplanin staining, antigen retrieval was done by heating slides for 3 minutes in EDTA buffer (pH 9.0) at 98°C. No antigen retrieval was required for factor VIII immunohistochemistry. After antigen retrieval, a cooling off period of 20 minutes preceded preincubation for 15 minutes in wash buffer (DakoCytomation), after which endogenous peroxidase activity was quenched using Peroxidase-Blocking Solution (DakoCytomation). The slides were incubated with D2-40 antibody (Signet Laboratories, Inc., Dedham, MA; dilution 1:20), anti-LYVE-1 antibody (RELIAtech, Braunschweig, Germany; dilution 1:20), anti-PROX-1 antibody (RELIA-tech; dilution 1:200), anti-CD31 antibody (DakoCytomation; dilution 1:500), and anti-CD34 antibody (DakoCytomation; dilution 1:50) for 1 hour at room temperature, with anti-podoplanin antibody (RELIA-tech; dilution 1:200) for 2 hours at 4°C, and with anti-factor VIII antibody (DakoCytomation; dilution 1:10) for 15 minutes. Antibody binding was visualized using the EnVision+ Dual Link system and diaminobenzidine as chromogen (DakoCytomation), except for factor VIII, for which the LSAB kit (DakoCytomation) was used. The slides were counterstained with hematoxylin and mounted. Infratumoral lymph vessels were counted in a random 20× microscopic field and for each marker, the ratio (%) of marker-positive vessels to D2-40-positive vessels was calculated.

**Double immunostaining for D2-40 and Ki-67.** To detect dividing lymph vessel endothelial cells, a double immunostaining for D2-40 and the proliferation marker Ki-67 was done. First, a monoclonal antibody directed at Ki-67 (DakoCytomation; dilution 1:150) was applied to the rehydrated paraffin sections for 30 minutes after antigen retrieval in citrate buffer (pH 6.0) at 98°C. Sections were incubated with EnVision+ Dual Link solution before development with diaminobenzidine (DakoCytomation). Sections were then stained with the D2-40 antibody (Signet Laboratories; dilution 1:200) for 60 minutes. EnVision System alkaline phosphatase and Fast Red chromogen (DakoCytomation) were used to visualize binding of this second antibody.

**Table 1. Summary of clinicopathologic features**

<table>
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<tr>
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<th>Non-IBC</th>
<th>IBC</th>
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<tr>
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<td>56.0/25.6-83.2</td>
</tr>
<tr>
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<td>0/29</td>
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<tr>
<td>Tumor size</td>
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<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Large (&gt;2 cm)</td>
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<td></td>
</tr>
<tr>
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<tr>
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<tr>
<td>3</td>
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<td>Growth pattern</td>
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<tr>
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<td>0</td>
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<tr>
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</tr>
<tr>
<td>Positive (&gt;10%)</td>
<td>37</td>
<td>10</td>
</tr>
<tr>
<td>Negative (&lt;10%)</td>
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<tr>
<td>Progesterone receptor</td>
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<td></td>
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<td>Positive (&gt;10%)</td>
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</tr>
<tr>
<td>Negative (&lt;10%)</td>
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<td>20</td>
</tr>
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</table>

*In IBC, exact tumor size cannot be assessed clinically or pathologically due to the diffuse enlargement of the breast and the diffuse infiltrative growth pattern of the tumor.*
Assessment of relative lymph vessel area, lymph vessel perimeter, counts, and lymph vessel invasion

Immunostained tumor sections were scanned at low magnification (10× ocular and 10× objective) and the three most vascularized areas (hotspots) were chosen both in intratumoral and peritumoral areas. Intratumoral lymph vessels were defined as D2-40-positive vessels that were in close contact with tumor cells or located in desmoplastic stroma. Peritumoral lymph vessels were defined as D2-40-positive vessels located in preexisting mammary stroma at a maximal distance of 2 mm from the tumor periphery. Sections were examined using an Olympus BX41 microscope with Olympus DP50 digital camera and morphometric analyses of lymph vessels were done with Olympus DP soft version 3.2 to determine the lymph vessel number (LVN), average lymph vessel perimeter (LVP), and the relative area occupied by lymph vessels (LVA) in a field of examination of 1.2 mm². LVA was calculated as (area covered by lymph vessels) / (total area examined) × 100 (%). For each variable, the mean and maximal values are, respectively, the average and maximal values obtained in the three hotspots. Lymph vessel invasion (LVI) was considered evident when at least one tumor cell cluster was clearly visible inside a D2-40-positive lymph vessel.

Assessment of lymphatic endothelial cell proliferation

The fractions of proliferating lymphatic endothelial cells (LECP%) were calculated by two independent observers (I.V.d.A and G.V.d.E) in each hotspot as the number of lymphatic endothelial cells with Ki-67-stained nuclei per 100 lymphatic endothelial cells. Cases in which the average number of lymphatic endothelial cells in three hotspots was <10 were excluded for statistical analysis.

Statistical analysis

All statistical analyses were done using SPSS 12 (SPSS, Inc., Chicago, IL). The Mann-Whitney U test was used to determine statistically significant differences of the median of all lymphangiogenesis-related variables between different patient groups. Correlations between clinicopathologic data and lymphangiogenic variables were analyzed with the Spearman correlation coefficient. The relationships between LECPmean and other variables (estrogen receptor status, progesterone receptor status, tumor and node status, tumor grade, growth pattern, and presence or absence of a fibrotic focus) were examined with multivariate linear regression analysis. P ≤ 0.05 was considered significant.

Results

Immunostains with lymph vessel–specific markers

Immunostaining of serial breast tumor tissues from IBC (n = 10) and non-IBC (n = 10) patients was first used to assess the staining pattern of LYVE-1, podoplanin, PROX-1, and D2-40 for lymph vessels in breast cancer (Fig. 1A-D). The D2-40 antibody showed strongest immunoreactivity with lymph vessel endothelium, both in the tumor parenchyma and at the tumor periphery. Of D2-40-stained intratumoral lymph vessels, 50.5% and 35.1% were also positive for podoplanin and PROX-1, respectively. The number of D2-40-stained intratumoral vessels was significantly correlated with the number of podoplanin and PROX-1-stained vessels (r = 0.792, P < 0.001 and r = 0.585, P = 0.007, respectively). Although its expression on peritumoral lymph vessels was strong, LYVE-1 showed only weak or no immunoreactivity on intratumoral lymph vessels. Of D2-40-stained intratumoral lymph vessels, 37.9% showed (weak) immunoreactivity for LYVE-1. There was no correlation between the number of LYVE-1-positive vessels and those positively stained for D2-40, podoplanin, or PROX-1. One-way ANOVA analysis and the Bonferroni post hoc test revealed that more vessels were stained with the D2-40 antibody than with antibodies directed at podoplanin (P = 0.048), PROX-1 (P = 0.001), or LYVE-1 (P < 0.001). CD31 and CD34 stained both blood vessel and lymph vessel endothelium although immunoreactivity was weaker on the latter. D2-40 and factor VIII showed a mutually exclusive vascular expression pattern.

Relative lymph vessel area, lymph vessel perimeter, and counts in inflammatory and noninflammatory breast cancers

To characterize lymph vessels in normal breast tissue, IBC, and non-IBC, we did immunostainings with the D2-40 antibody, after which lymphangiogenesis-related variables were measured using a computer-aided system. Morphometric data on the lymphangiogenesis-related variables in IBC and non-IBC are summarized in Table 2.

We chose to measure all variables in three hotspots located in the tumor parenchyma and three hotspots at the tumor periphery. As a validation of this method, the identification of the hotspots was done by two different observers. On average, 4 of 6 (69%) hotspots could be reproduced (the minimum of hotspots that could be reproduced in all breast cancer cases was three).

All lymphangiogenesis-related variables (relative LVA, LVP, and LVN) were significantly higher in normal breast tissue than in non-IBC. Similarly, mean LVP and LVN were lower in IBC than in normal breast tissue. However, the mean and maximal relative LVA in IBC were not significantly different from the
LVA observed in normal breast tissue: median intratumoral and peritumoral relative LVA_{mean} was 1.25% in IBC and 3.42% in normal breast tissue ($P = 0.14$).

Intratumoral lymph vessels were present in 80% (44 of 55) of non-IBCs and 82.8% (24 of 29) of IBCs ($P = 0.76$, $\chi^2$ test). In non-IBC, these lymph vessels were smaller, less numerous, and occupied a smaller relative area than peritumoral lymph vessels ($P < 0.01$). Moreover, we found the presence of lymph vessels in the tumor parenchyma of non-IBC to be dependent on the growth pattern: the mean number of intratumoral lymph vessels was 1.6-fold higher in infiltratively growing tumors than in tumors with an expansive or mixed expansive-infiltrative growth pattern ($P = 0.05$). In IBC there were no differences in LVN, LVP, and relative LVA between the tumor parenchyma and the tumor periphery ($P > 0.05$).

When tumor specimens of patients with IBC and non-IBC were compared, peritumoral lymph vessels in non-IBC shared the morphology of those in IBC; none of the peritumoral lymphangiogenesis-related variables was significantly different in IBC and non-IBC. However, lymph vessels located in the tumor parenchyma of IBC covered a larger relative tumor area and were larger and more numerous than lymph vessels located in the tumor parenchyma of non-IBC (Table 2): median relative LVA_{mean} was 0.84% in IBC (range, 0.08-28.14%) and 0.33% in non-IBC (range, 0.03-16.02%; $P = 0.01$); median LVP_{max} was 432.64 μm in IBC (range, 99.14-4,286.26 μm) and 344.52 μm in non-IBC (range, 90.31-2,042.04 μm; $P = 0.04$); and median LVA_{max} was 9 in IBC (range, 0-42) and 6 in non-IBC (range, 0-29; $P = 0.05$).

### Correlations between lymphangiogenesis-related variables and clinicopathologic data

In non-IBC ($n = 56$), the presence of LVI was associated with maximal and mean LVA ($P = 0.03$ and $P = 0.02$, respectively), maximal LVP ($P = 0.02$), and maximal and mean LVA ($P = 0.01$ and $P = 0.01$, respectively).

The presence of lymph node metastases was associated with significantly higher maximal perimeter of intratumoral and peritumoral lymph vessels ($P = 0.001$) and with the presence of LVI ($P = 0.003$, $\chi^2$ test). However, there was no association between the presence of lymph node metastases and LVA or LVP.

The presence of a fibrotic focus ($P = 0.006$) and an expansive or mixed expansive/infiltrative growth pattern ($P = 0.027$) also resulted in larger LVP.

### Lymphatic endothelial cell proliferation in inflammatory and noninflammatory breast cancers

Proliferating lymphatic endothelial cells were detected in normal breast tissue, IBC, and non-IBC by a double immunostain for the lymph vessel marker D2-40 and the proliferation marker Ki-67 (Fig. 1E-F). The fractions of proliferating...
lymphatic endothelial cells determined by two different observers were strongly correlated ($r = 0.915, P < 0.001$).

Proliferating lymphatic endothelial cells were observed in 20% (2 of 10) of normal breast tissues; median LECP$_{mean}$ was 0% (range, 0-2.61%; Table 2).

The presence of dividing lymph vessel endothelial cells in the tumor parenchyma or at the tumor periphery in non-IBC was dependent on the growth pattern. Breast carcinomas with an expansive or mixed expansive-infiltrative growth pattern showed significantly higher LECP than breast carcinomas with an infiltrative growth pattern: intratumoral median LECP$_{mean}$ was 3.13% (range, 0-17.29%) versus 0.96% (range, 0-12.15%; $P = 0.02$); peritumoral median LECP$_{mean}$ was 2.36% (range, 0-14.47%) versus 0.61% (range, 0-6.75%; $P = 0.02$). The presence of a fibrotic focus in non-IBC was also associated with higher LECP: median LECP$_{mean}$ was 5.11% (range, 0-17.29%) versus 1.88% (range, 0-12.15%; $P = 0.08$).

Peritumoral lymph vessels in tumor specimens of patients with IBC had higher fractions of proliferating lymphatic endothelial cells than peritumoral lymph vessels in tumor specimens of patients with non-IBC ($P = 0.005$; Table 2). In intratumoral areas, the difference in LECP between IBC and non-IBC did not reach statistical significance.

Correlations between lymphatic endothelial cell proliferation and lymph vessel invasion or clinicopathologic data
An association between the presence of LVI and LECP$_{mean}$ and LECP$_{max}$ was found ($P = 0.06$ and $P = 0.04$, respectively). LECP was not correlated with relative LVA, LVP, or LVN in non-IBC or IBC. For non-IBC patients ($n = 56$), the following variables were entered for linear regression analysis: tumor grade, estrogen receptor status, progesterone receptor status, tumor status, node status, growth pattern, and fibrotic focus. Negative estrogen receptor status ($P < 0.001$), small tumor size ($P = 0.001$), and the presence of lymph node metastases ($P = 0.01$) were independent predictive factors of lymphatic endothelial cell proliferation.

Discussion
At the time of diagnosis, metastatic dissemination of tumor cells via the lymphatic system has occurred in nearly all patients with IBC. Recent evidence suggests that breast carcinoma cells might induce the formation of a new lymph vessel network, thereby providing a means of lymphatic dissemination. In the present study, we aimed to obtain quantitative morphologic data on lymphangiogenesis in IBC and non-IBC. We first determined the best marker of lymph vessels in breast cancer tissue and found the D2-40 antibody to be the most suitable antibody for that purpose. The monoclonal antibody D2-40 was reported to be a new selective marker of lymphatic endothelium and a suitable marker for the identification of tumor emboli in lymph vessels in paraffin sections of many primary tumors, including breast cancer (17, 18). Recently, Schacht et al. (21) showed that the monoclonal antibody D2-40 recognizes human podoplanin. Remarkably, although LYVE-1 expression on lymph vessels located at the tumor periphery was strong, no or weak immunoreactivity was found on intratumoral lymph vessels in nearly all cases. In a previous study, we also observed that the expression of LYVE-1 on hepatic blood sinusoids was lost towards the center of breast cancer liver metastases (22). Although LYVE-1 immunoreactive intratumoral lymph vessels were clearly shown in experimental animal models of breast cancer (7-9), studies in spontaneously developing human breast cancer have only been able to highlight intratumoral lymph vessels when using antibodies directed at podoplanin (12, 23, 24) whereas studies using LYVE-1 failed to show a substantial number of intratumoral lymph vessels (10, 11, 25). Maybe some factors in the tumor microenvironment of human tumors are different from experimental animal tumors and are responsible for a down-regulation of LYVE-1. Our findings suggest that when studying lymphangiogenesis in human cancer, several lymph vessel markers should be compared in their efficiencies of staining lymph vessels and that LYVE-1 is not useful to mark lymph vessels in human breast cancer.

In the second part of this study, the D2-40 antibody was used for the detection of lymph vessels in IBC and non-IBC. In contrast to previous studies (10, 11), the large majority of breast tumors contained intratumoral lymph vessels although the number of lymph vessels in breast cancer was reduced when compared with normal breast tissue, which could be explained by the partial destruction of the preexisting stroma by the invading tumor cells. Indeed, the presence of intratumoral lymph vessels in breast carcinomas was dependent on the growth pattern: more lymph vessels were found in tumors with an infiltrative pattern, which grow between preexisting structures without disturbing the tissue architecture, than in expansively growing tumors, which are characterized by replacement of the preexisting stroma by desmoplastic tissue.

We assessed the proliferation status of lymphatic endothelial cells and found higher fractions of dividing lymph endothelial cells in IBC versus non-IBC. This corresponds to the higher mRNA expression of lymphangiogenic molecules (VEGF-C, VEGF-D, VEGFR-3, and fibroblast growth factor-2) in IBC (5). Our findings in IBC show many similarities with those in experimental animal tumors overexpressing the lymphangiogenic factor VEGF-C (7, 8): numerous lymph vessels were present throughout the tumor, the lymph vessels were enlarged and frequently infiltrated by tumor cells, and high fractions of proliferating lymphatic endothelial cells were found. It has been suggested that not only the vicinity of lymphatic vessels to tumor cells but also the molecular interactions of activated lymphatic endothelial cells with tumor cells facilitate entry of the latter into the lymphatics thereby enhancing metastasis to regional lymph nodes (8).

Although we did not find significant differences in most lymphangiogenesis-related variables between lymph node–negative and lymph node–positive breast tumors in the non-IBC group, LECP and the maximal lymph vessel perimeter were higher in the lymph node–positive group, thereby providing a larger contact area between tumor cells and activated lymphatic endothelial cells. This may promote the dissemination of tumor cells into the lymphovascular lumina and to regional lymph nodes. Indeed, in non-IBC, the presence of LVI was associated with high LECP and a larger LVP. In the present study, the proliferation status of lymph vessels was also assessed in normal breast tissue and in non-IBC breast malignancies. In non-IBC, in contrast to normal breast tissue, proliferating lymphatic endothelial cells were often present both in the tumor parenchyma and at the tumor periphery.
This is in striking contrast with the study of Williams et al. wherein dividing lymph vessels were not detected in any of the invasive breast carcinomas. By analogy with hemangiogenesis (26), proliferating lymphatic endothelial cells are a reflection of ongoing lymphangiogenesis, which make our findings probably the strongest argument for the presence of lymphangiogenesis in breast cancer. Breast carcinomas with an expansive growth pattern showed higher LECP than breast carcinomas with an infiltrative growth pattern and also the presence of a fibrotic focus that was associated with higher LECP. These results are analogous with hemangiogenesis; in a previous study, we found that endothelial cell proliferation was highest in expansively growing breast tumors and was positively correlated with the presence of a fibrotic focus (20). Interestingly, VEGFR-2, the receptor that binds VEGF-A, VEGF-C, and VEGF-D, is not only present in blood vessel endothelium but also in lymph vessel endothelium (27), which implies that these angiogenic growth factors could be lymphangiogenic as well, possibly linking the process of hemangiogenesis to that of lymphangiogenesis. In this regard, it has been recently reported that VEGF-A induces active proliferation of VEGFR-2-expressing tumor-associated lymphatic vessels (28).

In conclusion, our data suggest that there is indeed lymphangiogenesis in breast cancer, the most compelling evidence being the presence of proliferating lymphatic endothelial cells. We propose D2-40/Ki-67 immunohistochemistry to quantify LECP as a standard histomorphometric technique for assessing lymphangiogenesis in breast cancer. Using this method, IBC was found to be more lymphangiogenic than non-IBC.

Acknowledgments

We thank the technical staff of the laboratories of pathology from the University Hospital Antwerp and the General Hospital Sint-Augustinus, and Ingrid Bernaert for the help with morphometric analyses.

References


Tumor Lymphangiogenesis in Inflammatory Breast Carcinoma: A Histomorphometric Study


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