VEGF-C expressing tumor-associated macrophages in lymph node positive breast cancer: impact on lymphangiogenesis and survival

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Background. *The ability of malignant tumors to metastasize presents a severe challenge in cancer treatment. Lymphatic vessels provide one of the main routes for tumor-metastasis on the way to regional lymph nodes. Increasing evidence suggests that inflammatory cells play an important role in tumor-associated angiogenesis and lymphangiogenesis. Recent data show that a specialized sub fraction of tumor-associated macrophages (TAMs) expressing the lymphoangiogenic growth factors vascular endothelial growth factor-C and -D (VEGF-C/D) at the tumor site, is related to lymphangiogenesis, lymphovascular invasion, and lymph node metastasis. Aim of this study was to clear the role of VEGF-C/D expressing TAMs in invasive breast cancer.*

Methods. *One hundred-seven cases of lymph node positive invasive breast cancer were included into the study. Lymphatic microvessel density (LMVD), lymphovascular invasion (LVI), peritumoral inflammatory reaction (PI), and VEGF-C expression in tumors (VEGF-C_T) and TAMs (VEGF-C_C) were evaluated by immunohistochemistry and in situ hybridization.*

Results. *Significant associations were seen between LMVD and LVI, LMVD and VEGF-C_T, and between VEGF-C_T and VEGF-C_C. Further significant correlations were evaluated between VEGF-C_C/VEGF-C_T and PI as well as between PI and LVI. LVI remained an independent prognostic factor for disease-free survival and overall survival.*

Conclusions. *Our data provide evidence that the peritumoral inflammatory reaction and VEGF-C expressing TAMs may play an important role in tumor lymphangiogenesis and lymphovascular invasion in invasive breast cancer, implying new potential anti-tumor targets.*

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The metastatic spread of neoplastic cells is responsible for the majority of cancer deaths, and, with a few exceptions, all cancers can metastasize. Metastasis of breast cancer is believed to occur, at least at the initial phase, primarily through the lymphatic system, and the extent of lymph node involvement is a key prognostic factor of the disease.

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Tumor-induced lymphangiogenesis was, until recently, a relatively unfamiliar term in the field of cancer research. However, due to the discovery of the two lymphatic endothelial growth factors, vascular endothelial growth factor-C (VEGF-C) and -D (VEGF-D), and the introduction of specific lymphatic endothelial cell markers, this formerly neglected area of tumor-biology has moved to the center of interest.[1] In many human neoplasms, VEGF-C or -D expression in neoplastic cells has been linked to lymphangiogenesis and correlates with lymph node metastasis.[2] Increasing evidence suggests that inflammatory cells play an important role in pathological angiogenesis and lymphangiogenesis.[3]

We recently showed that the release of VEGF-C and –D is linked to a circulating sub fraction of CD14+, VEGFR-3-expressing monocytes recruited

to and activated at the site of tumor growth. This VEGF-C expressing sub fraction of tumor-associated macrophages (TAMs) at the tumor site is related to lymphangiogenesis, lymphovascular invasion, and lymph node metastasis in cervical cancer.[4]

The aim of this study was to investigate the role of VEGF-C expressing TAMs in lymphangiogenesis, lymphovascular invasion, and clinical outcome in invasive breast cancer.

MATERIALS AND METHODS

Patients and tissues. The study population consisted of 107 randomly collected cases with invasive breast cancer UICC stages 1 to 4 that participated in one of five prospective, randomized, multicenter trials conducted between 1984 and 1990 by the Austrian Breast and Colorectal Cancer Study Group.[5,6] All neoplasms were re-graded according to Elston and Ellis.[7] Special care was taken to include only sections with sufficient amounts of normal breast directly adjacent to invasive tumor formations.

All patients underwent breast surgery and dissection of axillary lymph nodes, containing at least 10 nodes.

Immunohistochemistry. Rabbit anti-human podoplanin IgG was raised against the recombinant human homologue of the rat 43-kDa glycoprotein podoplanin as described previously.[8] Affinity purification of rabbit serum was carried out using nitrocellulose strips containing recombinant protein.[9] For immunohistochemical detection of VEGF-C protein expression, VEGF-C antibody (Zymed, South San Francisco, Calif) was used.

Immunohistochemistry was carried out on 4 μm-thick serial paraffin sections. After deparaffinization in xylol, sections were rehydrated and microwave pre-treated in citrate buffer at 600 W for 10 minutes. After cooling for 15 minutes and washing in PBS, endogenous peroxidase was blocked by using 3% hydrogen peroxide for 15 minutes, followed by incubation with PBS containing 10% normal goat serum for 30 minutes. For immunostaining of podoplanin, specimens were incubated at 20°C with the polyclonal rabbit antibody in a dilution of 1:200 for 1 hour. Detection of VEGF-C expression was carried out on a separate, subsequent section. For this task, the polyclonal anti-VEGF-C antibody was incubated at 20°C in a dilution of 1:800 for 1 hour.

Positive staining was detected using biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, Calif), respectively, for 30 minutes at 20°C followed by a streptavidin–peroxidase complex, according to the manufacturer's instructions. Peroxidase reaction product was visualized by diaminobenzidine (SERVA, Heidelberg, Germany). Slides were counterstained with hematoxylin.

Her2-neu-status was investigated using HERCEPtest (DAKO, Glostrup, Denmark) as described previously.[10]

In situ hybridization. Human VEGF-C antisense and sense RNA probes were generated from linearized (ApaI, KpnI) pCR2.1 Topo vector (Invitrogen, San Diego, Calif), corresponding to nucleotides 1033 to 1593 of human VEGF-C cDNA (sense 5′-TTCCCTGCCAGCA-ACACTACCA-3′, antisense 5′-CCAATATGAAGGG-ACACAACGACA-3′). Digoxigenin-labeled antisense mRNA was synthesized using T7 RNA polymerase and [DIG]UTP, and sense mRNA, using SP6 RNA polymerase and [DIG]UTP (Boehringer, Mannheim, Germany). In situ hybridization for VEGF-C mRNA expression was carried out on 5 μm thick formalin-fixed, paraffin-embedded tissue samples of 5 randomly selected cases, as described previously.[4]

Morphometry. Determination of lymphatic microvessel density (LMVD) assessed by immunostaining for podoplanin was carried out as suggested by Weidner and colleagues.[11,12] In brief, after scanning the immunostained section at low magnification (40×), the area of tissue with the greatest number of distinctly highlighted microvessels ("hot spot") was selected. LMVD was then determined by counting all immunostained vessels at a total magnification of 200× corresponding to an examination area of 0.74 mm^2. Determination of the staining reaction was confined strictly to the hot spots. LVI was considered evident if at least one tumor cell cluster was visible clearly inside the podoplanin-stained vascular space.[13] Microvessel counts were done by two independent observers (SFS, SU), naive to the patient's pathologic and clinical status. The mean values of microvessel densities observed by both investigators in each patient were entered into further calculations. In the case of inter-observer differences of greater than 30% in microvessel count, the respective slides were reinvestigated by both observers using a discussion microscope (evident in >10% of cases).

The number of VEGF-C positive stroma (VEGF-C_C) cells was determined in the area of their highest density ("hot spot"), at a magnification of 400× (field of view, 0.08 mm^2). Intensity of immunostaining of VEGF-C in cancer cells (VEGF-C_T) was graded as strong, medium, or weak expression. Peritumoral inflammation (PI) assessed in slides stained for hematoxylin-eosin, was graded as sparse, moderate/inhomogeneous reaction, or as

dense, homogenous inflammatory infiltrate, as described.[14] A block of cervical cancer used in a previous study served as positive control for VEGF-C and podoplanin.[14] For negative control, a slide was prepared from the same tissue block and a preimmune serum was used instead of the primary antibody.

Estrogen receptor density was determined using the dextran charcoal method from snap-frozen tumor samples as described previously.[15] For definition of estrogen receptor positivity, cut-off values of greater than 10 fmol/liter were used.[16]

Statistics. Association of LMVD, LVI, VEGF-C_T, VEGF-C_C and PI with clinical and pathohistologic parameters was investigated using Kruskal-Wallis test, subsequent pair-wise Mann-Whitney tests or Spearman's coefficient of correlation, as appropriate. Overall survival (OS) was assessed over the period from primary operation until death of the patient. Death from a cause other than breast cancer, or survival until the end of the observation period, was considered a censored observation. Disease-free survival (DFS) was defined from the end of primary therapy until first evidence of progression of the disease. Univariate analysis of OS and DFS was carried out as outlined by Kaplan and Meier.[17]

The Cox proportional-hazard model was used for multivariate analysis. LVI, VEGFC$_C$, VEGF-C_T, histologic grading according to Elston and Ellis,[7] patient's age, and tumor stadium according to UICC were entered into Cox regression. For all of the tests, a two-tailed P of less than or equal to .05 was considered significant. Continuous data are shown as mean ± standard deviation (SD).

RESULTS

Clinical data. The mean patient age at time of operation was 51 years (median, 48 years; range, 31 to 70 years). Sixty patients (56%) were pre-menopausal, 46 (43%) were post-menopausal, in 1 patient the status was not known. Mean estrogen receptor density was 70 ± 98 fmol/liter. As surgical treatment, breast conservation (usually wide excision) was carried out in 24 patients (22%), and mastectomy in 83 (78%). After breast conservation, the majority of patients were treated with adjuvant radiotherapy, except for a small subgroup of patients with minimal risk. After operation, 7 patients (7%) received no adjuvant therapy. In 47 patients, (44%) tamoxifen was administered for 5 years at a dose of 20 mg/day. Fifty-three patients (50%) received a combined adjuvant chemotherapy (6xCMF intravenously for 6 cycles, days 1 and 8, recycled on day 28, at the given doses: cyclophosphamide 600 mg/m^2, methotrexate 40 mg/m^2, and fluorouracil 600 mg/m^2 + tamoxifen).

Because all patients were treated within prospective clinical trials, documentation and follow-up is complete, comprehensive, and consistent. All 107 patients were staged lymph node-positive. Ninety-nine (92%) of the patients were staged N1, and 8 patients (8%) N2. Tumor grading was as followed: 8 tumors (8%) were graded G1, 57 (53%) G2 and 42 (39%) carcinomas were graded G3 according to Elston and Ellis.[7] Fifty-nine breast tumors (56%) were staged pT1 (≤2 cm or less in great dimension), 47 neoplasms (44%) pT2 (>2 cm but not >5 cm in dimension) and 1 tumor was staged (1%) pT3 (>5 cm in greatest dimension) according to UICC stages. Ninety-three patients (87%) had ductal NOS carcinomas and 14 patients (13%) had tumors histologically typed as lobular carcinomas. In 57 (53%) patients the tumor stadium was IIa, in 41 (38%) it was IIb, in 5 (5%) patients it was IIIa, and in 4 (4%) patients the stadium was IIIb, according to the UICC stages (Table I).

Immunohistochemistry and in situ hybridization. Intratumoral lymphatic vessels were observed in only one case, whereas most lymphatic vessels were located within the tumor stroma, at the border front to invasive tumor formations, as reported previously (Fig 1 *A*).[18] Lymphangiosis carcinomatosa was seen primarily in open lymphatic vessels but was absent in narrow or collapsed lymphatic spaces as described previously (Fig 1 *C*).[19]

Median LMVD was 9.2 microvessels/field (range, 1 to 19 vessels). LVI was observed in 40 cases (37%). In 57 cases (53%) peritumoral inflammation (PI) was sparse, in 35 (33%) it was moderate/inhomogeneous, and 15 tumors (14%) presented with dense, homogenous inflammatory infiltrate.

VEGF-C_T was graded as strong in 21 (20%), and as medium in 25 (23%) cases. Sixty-one neoplasms (57%) showed a weak VEGF-C protein-expression (Fig 1 *B*). VEGF-C expression was also observed regularly in peritumoral, TAMs by immunohistochemistry and in situ hybridization (Fig 1 *D*, *E*). The median number of VEGF-C_C was 8 cells/field (range, 0 to 65 cells/field).

An association was seen between LMVD and LVI ($P \leq .005$, Mann-Whitney test). Neoplasms with LVI had a greater amount of LMVD than neoplasms without lymphovascular invasion (11.8 ± 3.8 microvessels/field vs 7.8 ± 3.8 microvessels/field, $P < .05$). There was also an association between LVI and PI ($P = .028$, Mann-Whitney test). Cases with moderate/inhomogeneous inflammation presented more often with LVI than neo-

Table I. Patient characteristics (n = 107)

Age (y)	*Median, 48y Range, 31-70y (%)*
Stage	
T1	59 (55)
T2	47 (44)
T3	01 (1)
Grading	
G1	08 (8)
G2	57 (53)
G3	42 (39)
Histologic type	
Ductal NOS	93 (87)
Lobular	14 (13)
Hormone receptor status	
EgR+	94 (88)
EgR−	13 (12)
Her2neu status	
0/+	84 (79)
++	10 (9)
+++	13 (12)
Menopausal status	
Pre-	60 (56)
Post-	46 (43)
Unknown	01 (1)
Adjuvant therapy	
No further therapy	07 (7)
Hormonotherapy	47 (44)
Chemotherapy	53 (50)

plasms with sparse/absent inflammatory reaction (58% vs 42%, $P < .05$). We also observed an association between LVI and histologic grading ($P = .001$, Mann-Whitney test): whereas median grading was 2 in cases without LVI, it was 3 in cases with LVI.

Patients with LVI were younger (median, 46 years; range, 31 to 69 years) than those without LVI (median, 50.5 years; range, 31 to 70 years) ($P = .017$, Mann-Whitney test).

VEGF-C_T correlated positively with VEGF-C_C (r = 0.318, Spearman's coefficient of correlation), with LMVD (r = 0.242), and with staging (r = 0.222). VEGF-C_C correlated strongly with grade of PI (r = 0.766) (Table II). There was no statistical association between the number of involved lymph nodes or the lymph node staging and VEGF-C_T or VEGF-C_C Negative control, carried out as described above, showed no staining reaction.

A negative correlation between estrogen receptor density and LMVD was observed (r = −0.214, $P = .028$, Spearman's coefficient of correlation). There was also a correlation between HERCEP-test-score and VEGF-C_C expression (r = 0.265, $P = .006$).

Survival analysis. The mean observation time was 110 months (range, 1 to 170 months). During this observation time, 52 patients (49%) developed recurrent disease, and 39 (36%) died from their cancer. Of the recurrences, 11 (24%) were locoregional, 35 (69%) were systemic, and 6 (7%) were locoregional and systemic. In univariate survival analysis, differences in OS and DFS were found between patients with or without LVI ($P = .0153$ and $P = .0003$, respectively, log-rank test) (Fig 2 *A*, *B*), and histologic grading ($P = .012$ and $P = .054$, respectively, log-rank test). LMVD, VEGF-C_C, VEGF-C_T, and PI as well as the patient's age and other clinical and histopathologic parameters had no influence on OS and DFS in our collective ($P > .05$, log-rank test).

Histologic grading was of influence on OS ($P = .0119$) and on DFS ($P = .0012$) in univariate analysis (log-rank test).

In multivariate analysis of survival, for DFS only LVI ($P = .011$, all Cox regression) and grading ($P = .02$) remained independent prognostic factors. At analysis of OS only grading was an independent prognostic factor ($P = .027$).

DISCUSSION

The ability of malignant neoplasms to metastasize presents a severe challenge in cancer treatment. Clinical findings suggest that by providing a pathway for tumor cell dissemination, tumor-associated lymphatics play a key role in development of metastases. Although the (patho-) physiologic mechanism of lymphangiogenesis was unclear for a long time, vascular endothelial growth factors-C and -D (VEGF-C, D) have been identified, both promoting lymphangiogenesis both in vitro and in vivo through binding to their high affinity kinase-like receptor (VEGFR-3) expressed on lymphatics in their endothelium.[1]

In the past, cancer research focused on neoplastic cells, whereas during the last decade, the tumor stroma and the interaction between tumor, stroma and the immune system has moved to the center of interest. The lymphatic system, although relatively poorly studied for various reasons, forms the "first line of defense" once a given neoplasm has acquired the ability to move cells directly to its host.

In the field of tumor-lymphangiogenesis, a variety of cancer lymphatic vessels are nearly exclusively found in the adjacent stroma rather than within the neoplasm.[20] Recent studies suggest that inflammatory stroma cells play a crucial role in peritumoral lymphangiogenesis and lymphatic vessel invasion.[4,21]

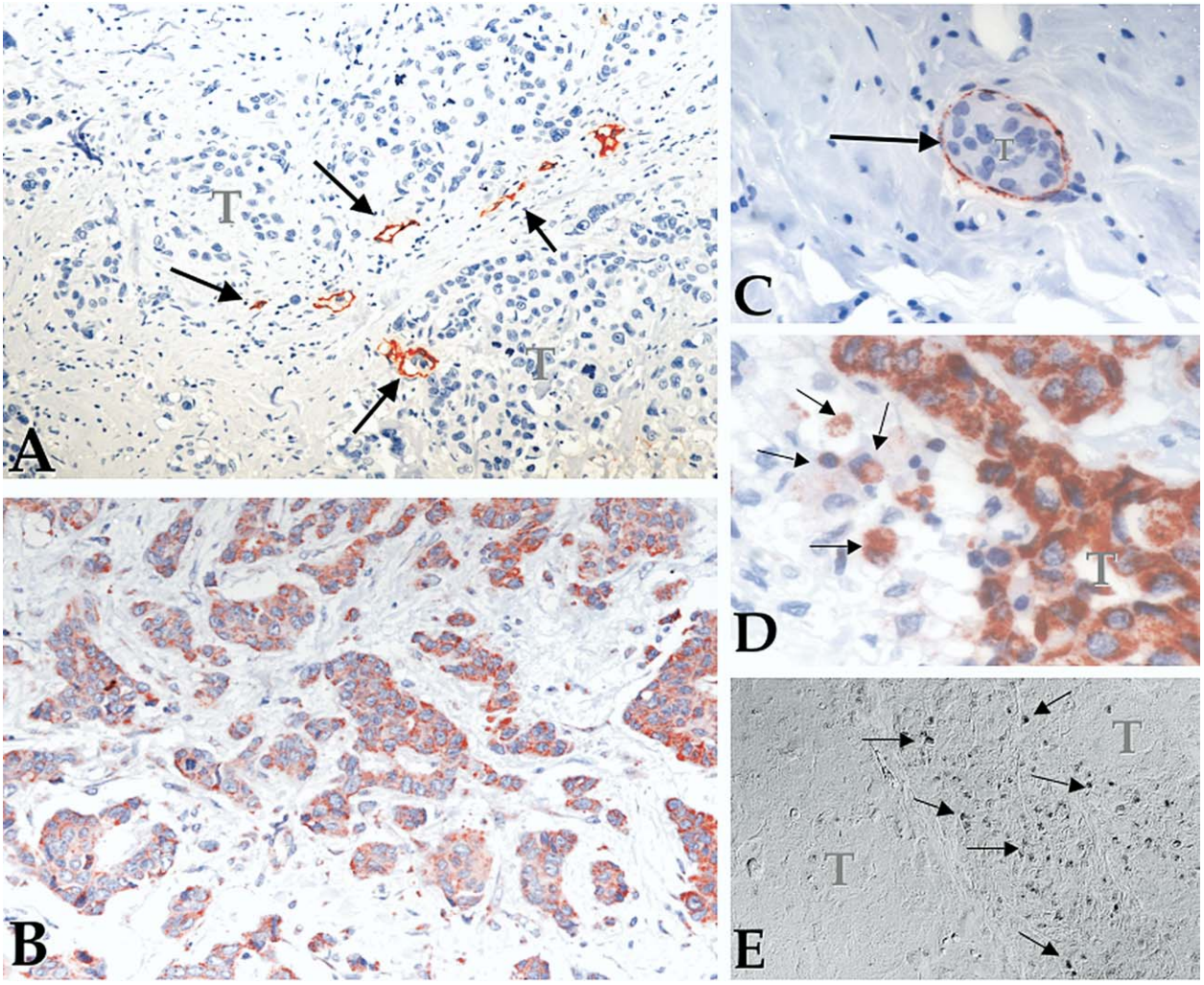

Fig 1. A, Immunohistochemistry of breast cancer specimen with anti-podoplanin antibody. Some peritumoral (T) lymphatic vessels are marked by arrows. Immunoperoxidase, original magnification ×200. **B,** Breast cancer specimen with high VEGF-C expression in tumor cells (T), Immunoperoxidase, original magnification ×400. **C,** Podoplanin-stained lymphatic vessel (*arrow*) with tumor cells inside (LVI). **D,** Arrows show peritumoral (T) VEGF-C expressing macrophages stained with anti-VEGF-C antibody, immunoperoxidase, original magnification ×600. **E,** Localisation of VEGF-C expressing peritumoral macrophages (T) by in situ-hybridization (some VEGF-C positive cells are marked by arrows).

In a previous work, we showed that a sub fraction of circulating VEGFR3+CD14+ monocytes express strongly VEGF-C and VEGF-D on recruitment to peritumoral sites, or in vitro stimulation causing peritumoral lymphangiogenesis.[4]

In the present study, we investigated the impact of VEGF-C expressing TAMs in a large collective of lymph node-positive breast cancers.

The fact that neoplasms with high LMVD have a much higher risk to develop LVI, correlates well with previously published data and can likely be explained by the concept of an extended 'lymphatic window,' during which tumor cells have the opportunity to enter lymphatic vessels.[19] Further a significant correlation between the amount of peritumoral inflammatory reaction (PI) and LVI was seen. Neoplasms with a high peritumoral inflammatory reaction present more often with LVI than neoplasms with sparse PI. Because our collective contains only lymph node-positive breast cancer patients at the point of tumor removal, the fact that LVI remains an independent prognostic factor and the PI-LVI association keeps statistically significant in this high-risk group underlines its prognostic value. PI was associated strongly with the quantity of VEGF-C expressing TAMs. Thus breast cancer combined with dense inflammation also pre-

Table II. Correlations between lymphatic parameters

	VEGF-CT	*VEGF-CC*	*PI*	*LMVD*	*LVI*
VEGF-CT	—	$P = .001$ (r = 0.318)	$P = .031$ (r = 0.209)	$P = .012$ (r = 0.242)	$P = .37$
VEGF-CC	P = .001 (r = 0.318)	—	$P = .001$ (r = 0.76)	$P = .413$	$P = .277$
PI	$P = .031$ (r = 0.209)	$P = .001$ (r = 0.76)	—	$P = .092$	$P = .028$
LMVD	$P = .012$ (r = 0.242)	$P = .413$	$P = .092$	—	$P = .005$
LVI	$P = .37$	$P = .277$	$P = .02$	$P = .005$	—
Staging	$P = .022$ (r = 0.22)	$P = .154$	$P = .319$	$P = .495$	$P = .612$
Grading	$P = .405$	$P = .269$	$P = .200$	$P = .455$	$P = .001$

LMVD, lymphatic microvessel density; LVI, lymphovascular invasion; PI, peritumoral inflammation; VEGF-CT, VEGF-C expression in tumor-cells; VEGF-CC, VEGF-C expression in tumor associated macrophages.

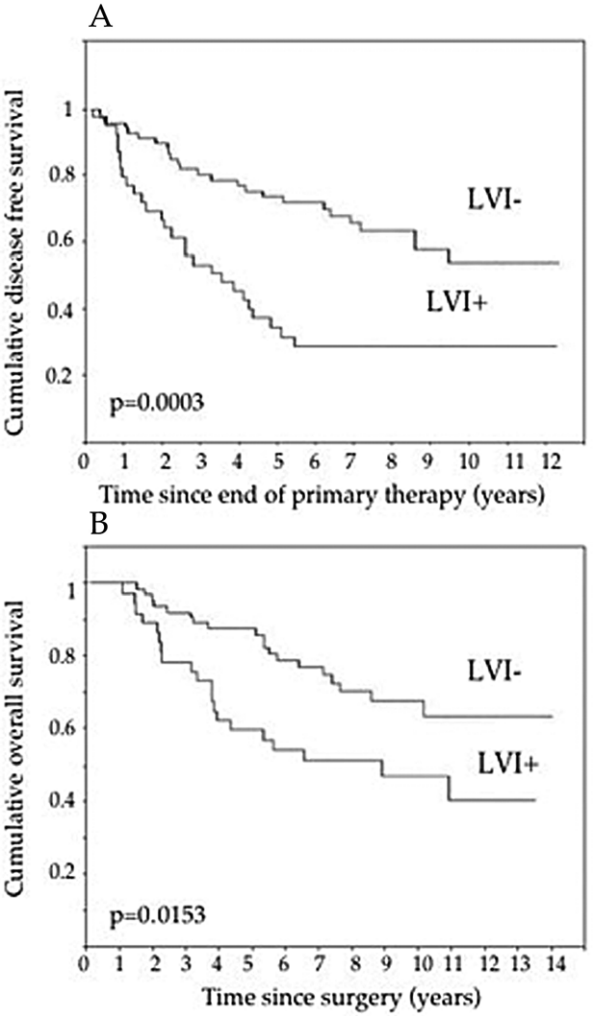

Fig 2. A, Disease-free survival of 107 breast cancer patients with (LVI+) or without (LVI) lymphatic vessel invasion. **B,** Overall survival of 107 breast cancer patients with (LVI+) or without (LVI) lymphatic vessel invasion.

sented with a high number of 'lymphogenic macrophages.'

Activated leucocytes are known to express and secrete a large number of cytokines and other regulatory peptides and proteins.[22,23] Most probably in addition to VEGF-C and VEGF-D, there are more unknown factors produced by inflammatory cells that also promote lymphangiogenesis. This possibility could explain why no direct association between TAMs and LMVD or LVI was found, although TAMs correlated strongly with the intensity of inflammation, whereas inflammation was associated with LVI, and LVI correlated with LMVD. As a consequence, the formation of lymphatic vessels might be understood as a reaction of the immune system against the malignant disease.

A primary requisite for successful immunologic response is that tumor antigens are brought into lymphatic organs through blood or lymph vessels. The stronger the local immunologic response (ie, peritumoral inflammation), the more pronounced lymphangiogenesis occurs. Tumor cells use these newly formed lymphatic routes that are formed originally to help the immune system to fight against generalization. Experimental anti-lymphoangiogenic strategies targeting VEGFR-3 mediated signalling have been reported to inhibit lymphangiogenesis and improve survival in animal models of metastatic cancer.[24] In a recent study, local depletion of VEGF-C and -D expressing macrophages by clodronate liposomes was shown to inhibit lymphangiogenesis in inflamed corneas.[25] This mechanism could inhibit TAM-driven lymphangiogenesis in cancer.

Additionally our patient cohort showed a strong association between the amount of VEGF-C_T and the peritumoral LMVD suggesting a complex tumor-stroma interaction where the immune system contributes to lymphangiogenesis. Data published recently on lymphangiogenesis in inflammatory breast cancer characterize this special entity of breast tumor as an extremely active tumor concerning angiogenesis and lymphangiogenesis. The authors explain the high metastatic potential of inflammatory breast cancer by the dense, active peritumoral inflammatory reaction.[21]

In our collective we found a statistically significant association between VEGF-C_T and c-erbB2 expression. This observation correlates well with recent in vitro and in vivo studies showing a regulatory link between VEGF-C and c-erbB2.[26,27] Further heregulin-β1, a ligand for c-erbB3 and c-erbB4, has been shown recently to up-regulate VEGF-C protein expression in breast cancer cell lines, an effect inhibited by the c-erbB2 blocking antibody trastuzumab (Herceptin®).[28] Data provides additional evidence for an important link between c-erbB2 and VEGF-C in invasive breast cancer.

In summary, our results demonstrate that in invasive, lymph node-positive breast cancer, the peritumoral inflammatory reaction and VEGF-C expressing TAMs play an important role in peritumoral lymphangiogenesis and lymphovascular invasion.

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