



## Absence of lymphangiogenesis in ductal breast cancer at the primary tumor site

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### Abstract

Solid evidence for a relationship between lymphangiogenesis and prognosis in human breast cancer is still lacking. Evidence for ongoing lymphangiogenesis in breast cancer is only provided by animal studies. In the present study we investigated lymphatic vessel density as well as the expression level of the lymphangiogenic factors VEGF-C and -D in a series of 121 ductal breast cancer tissues using immunohistochemical stainings. We found that in the primary tumors the lymphatic vessel density, as well as the expression of both VEGF-C and -D, did not relate to grade, tumor stage, progression or patient survival. Furthermore, in tumors in which lymphatic vessels were present, a Ki-67/podoplanin double staining indicated the absence of proliferating lymphatic endothelial cells. In contrast, we did find a correlation between intratumoral lymphatic vessel density inside the lymph node metastases and patient survival. Another parameter that revealed prognostic value was the presence of tumor cells within the lymphatic vessels. This parameter did predict survival in patients with an age below 63 only. Interestingly, expression of VEGF-D was found to be related to the presence of intralymphatic tumor cells.

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### 1. Introduction

It is well established that angiogenesis plays a major role in tumor progression [1,2]. Furthermore, inhibition of angiogenesis has been shown to be an

effective anti-tumor therapy in vivo [3,4]. However, it is currently still uncertain whether human tumor growth and progression involve ongoing lymphangiogenesis. Despite the fact that lymphatic vessels in human tumors are rare and that there is very little evidence for an active role of lymphangiogenesis in tumor growth and metastasis formation, it might play a regulatory role in tumor biology. Evidence for ongoing lymphangiogenesis in breast cancer is mainly provided by animal studies [5–7], and it

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has been hypothesized that in human cancers lymphangiogenesis does not occur [8,9].

Lymphatic vessels can be identified using several recently developed antibodies [10–13]. In human tissues the detection of podoplanin appears the best option to quantify lymph vessels [14]. Most studies on lymphangiogenesis in breast cancer were performed by assessment of lymphatic vessel density (LVD), without relating it to clinical parameters or survival data [8,15–17]. Several studies concluded that ongoing lymphangiogenesis is not present in human breast cancer [8,15,18]. These studies only report on LVD and do not include studies on lymphangiogenic growth factors. The only studies that also included expression of the lymphangiogenic growth factors VEGF-C and/or VEGF-D, only present data on the RNA expression levels of these growth factors [19,20]. Other researchers used markers that were not exclusively specific for lymphatic vessels, e.g. VEGFR-3 [21–23].

To investigate whether lymphangiogenesis has prognostic value in human ductal breast cancer, we analysed LVD in primary tumor and lymph node metastasis samples of 121 breast cancer patients. Also the expression levels of the lymphangiogenic factors VEGF-C and -D were examined at the protein level. Furthermore, in primary tissue samples the number of proliferating lymphatic endothelial cells was assessed by a Ki-67/podoplanin double staining. We did not find a correlation between lymphatic vessel density (LVD) and tumor progression or patient survival. In addition, VEGF-C and -D expression was not related to lymphatic vessel density. Also, no proliferating lymphatic endothelial cells were observed, suggesting the absence of ongoing lymphangiogenesis in human breast cancer at the primary tumor site. Interestingly, the only prognostic marker found in the primary tumors was the presence of tumor cells inside the lymphatic vessels. These studies suggest that lymph node metastasis formation and breast cancer progression is independent of lymphangiogenesis.

## 2. Materials and methods

### 2.1. Patients and tumor samples

The study at final analysis included 121 women with invasive ductal breast cancer, diagnosed between 1990 and 1994 at the Maxima Medical Centre in Eindhoven.

Of these patients 51 presented with positive lymph nodes (because of tissue availability only 46 of these were included in the present study). Initially 187 patients diagnosed with invasive breast cancer were selected. Some patients were excluded because of 3 reasons: firstly many of patients were excluded from the study because the tissue from the primary tumor was not available, secondly the tumor tissue was not invasive ductal breast cancer or third because patients had presented with either breast cancer or a different kind of cancer previous to the diagnosis of breast cancer. Therefore 121 patients were used in the final analyses. The mean patient age was 61.4 years (standard deviation 12.2). The patients had a follow up of 12 years (144 months) and the mean disease free survival was 80.5 months and the mean overall survival was 92.2 months. Follow-up of all patients was completed until 1 January 2005. Information on the vital status of all patients was obtained from the municipal registries in the area of the Eindhoven Cancer Registry and the Central Bureau for Genealogy. The latter institution collects data on all diseased Dutch citizens via the civil municipal registries. Fifty-three of the cases had a positive progesterone receptor (PR) status and 65 patients had a positive estrogen receptor (ER) status, of which 43 patients were both positive for ER and PR. Furthermore, 51 patients presented with positive lymph nodes. The tissues were fixed in buffered formalin and paraffin embedded.

For comparing the expression of VEGF-D in tumor epithelium with normal epithelium a selection of 9 patient tissue samples containing either ductal breast cancer tissue or normal breast tissue from the Maastricht Pathology Tissue Collection (MPTC) were used.

### 2.2. Immunohistochemistry

Paraffin sections, 4  $\mu$ m thick, were deparaffinized in xylene. Then they were placed in methanol with 0.3% hydrogen peroxidase for 15 min. Subsequently, the sections were washed 3 times in PBS and then aspecific binding was blocked with 5% BSA in PBS. After this the sections were incubated with podoplanin antibody (AngioBio Co., Del Mar, CA, USA) for 60 min. The slides were then washed with PBS and subsequently incubated with a biotin labelled Rabbit anti-Mouse antibody (DAKO, Glostrup, Denmark) for 30 min. After this incubation the sections were washed again and the sections were then incubated for 30 min with avidin-biotin-peroxidase complex (DAKO, Glostrup, Denmark). After this the sections were washed again and the enzyme was visualized with 3,3'-diaminobenzidine (DAB, Sigma, Zwijndrecht, The Netherlands). The sections were counterstained with hematoxylin, dehydrated and mounted using entellan. For the staining of VEGF-C a rabbit polyclonal antibody (Zymed lab-

oratories, San Francisco, CA, USA) was used followed by a biotin labelled anti-rabbit antibody (DAKO, Glostrup, Denmark). For staining of VEGF-D a monoclonal antibody (R&D Systems, Abington, United Kingdom) was selected. Both the protocols for staining VEGF-C and VEGF-D included a microwave antigen retrieval step. For both stainings also positive controls and negative controls were included, among which heart tissue, placental tissue and colon carcinoma. These tissues were also used to optimize staining procedures. For the VEGF-C and -D staining all sections were stained on the same day and with exactly the same incubation times to avoid staining variations. A selection of the sections, positive for podoplanin staining, were double stained for podoplanin and Ki-67 (Neomarkers, Fremont, CA). The podoplanin in this case was visualized using Alkaline Phosphatase Blue (Vector Laboratories, Inc., Burlingame, CA) and the Ki-67 using DAB. Before embedding with entellan the sections were pre-treated with insolmount (Klinipath, Duiven, The Netherlands).

### 2.3. Microscopic examination

The intratumoral and stromal lymphatic vessel densities (LVD) were determined by counting the number of podoplanin positive vessels with lymphatic vessel characteristics in either the tumor tissue or the stromal tissue right next to or in between the tumor tissue at a 200 times magnification in a grid with an area of 0.25 cm<sup>2</sup> in four different randomly chosen microscopic fields. The lymphatic vessels were also examined for presence of tumor cells inside the lumen. The VEGF-C and -D staining were examined by three independent observers and the intensity of the staining was determined by evaluating the staining intensity of the tumor cells in the whole tissue area. The intensity was scored as 0, 1, 2, 3 or 4, representing negative, slightly positive, positive, bright staining and intense staining.

### 2.4. Statistics

For statistical analyses we used the SPSS 10.0 software (SPSS Inc., Chicago, IL, USA). Normal distribution was examined for the different parameters. The correlation between some of these parameters was examined using either Spearman correlation when the parameters were not normally distributed or Pearson when the values were normally distributed. Differences between groups were examined using Student's *t*-test or Mann-Whitney *U* test. Survival was analysed using Kaplan Meier Survival analysis with log rank test for univariate analysis and with Cox regression for multivariate analysis, which included possible confounders. Information about the clinical history of the patients was obtained from the files collected by the Comprehensive Eindhoven

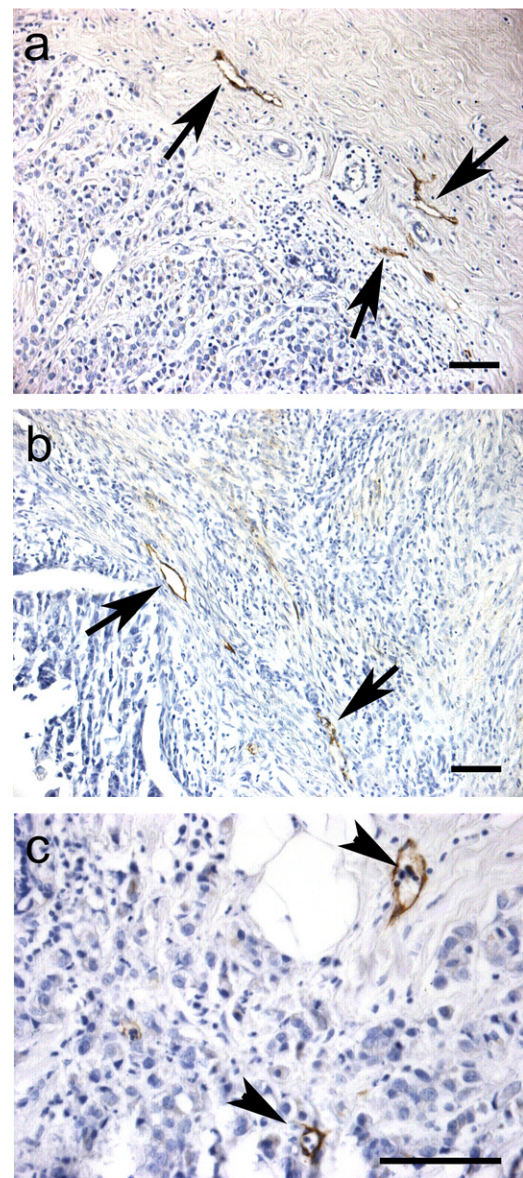


Fig. 1. Staining of lymphatic vessels in ductal breast cancer tissues. Ductal breast cancer tissues from primary tumors and lymph node metastases were stained for the lymphatic endothelial marker podoplanin. Examples of stromal and intratumoral lymphatic vessels are shown in (a,b), respectively. (c) The presence of tumor cells in podoplanin positively stained lymphatic vessels. Arrows indicate podoplanin stained vessels. Arrow heads indicate tumor cells inside lymphatic vessels. Bars represent 100  $\mu$ m.

Cancer Registry. In this file also information on the ER and PR status was present as well as the lymph node status, the tumor size, histological typing and grading. Also survival data were collected over a follow-up period until January 2005.

### 3. Results

#### 3.1. Lymphatic vessels are sparse in ductal breast cancer and presence does not correlate to clinical parameters

Lymphatic vessels were stained in primary tumor tissues and lymph node metastases of 121 patients with ductal breast cancer. The morphology of the lymphatic vessels was different from regular blood vessels, as indicated by a larger lumen, a more irregular shape, and the absence of a vast basal membrane and pericytes or smooth muscle cells. Lymphatic vessel density in the tumor was measured (i) inside the nests of tumor cells (Fig. 1b) and (ii) inside the stromal tissue of the tumor (Fig. 1a), as the number of podoplanin positive vessels per mm<sup>2</sup>. In general, the number of lymphatic vessels was very low, with most of the lymphatic vessels present in the stroma (on average  $4.68 \pm 3.98$  vessels/mm<sup>2</sup> in the stromal tissue and  $0.35 \pm 1.29$  inside the nests of tumor cells, Table 1). The lymphatic vessel density (LVD), in either tumor or tumor

stroma, did not correlate with age, hormone receptor (ER/PR) status, tumor size, tumor stage, differentiation grade, nodal status, or metastatic status.

For Kaplan Meier survival analysis the patient group was divided in higher and lower than median number of lymphatic vessels. We found that the number of stromal lymphatic vessels in the primary tumor did not predict overall patient survival (not shown) or disease free survival (Fig. 2a). Similar results were found in the lymph node metastases, although a slight trend ( $p < 0.3$ ) towards a better disease free survival in the group with a low stromal LVD was observed (Fig. 2b).

The number of intratumoral lymphatic vessels in the primary tumor did not correlate with patient overall (not shown) or disease free survival (Fig. 3a). However, we found that patients with a high intratumoral LVD inside the lymph node metastases had a shorter survival time (almost significant  $p = 0.0597$ , not shown) and a shorter disease free survival (Fig. 3b,  $p = 0.0244$ ).

Table 1

Comparison of intratumoral lymphatic vessel density (ITLVD) and stromal lymphatic vessel density (SLVD) between different patient groups

	Number of patients	Stromal LVD		Intratumoral LVD	
		Mean $\pm$ SD	Positive <sup>a</sup>	Mean $\pm$ SD	Positive <sup>a</sup>
<i>Primary tumor</i>					
Total	121	4.68 $\pm$ 3.98	55/121(45%)	0.35 $\pm$ 1.29	12/121(10%)
Lymph node +	51	4.59 $\pm$ 4.29	22/51(43%)	0.29 $\pm$ 1.06	5/51(10%)
Lymph node –	70	4.74 $\pm$ 3.80	33/70(47%)	0.04 $\pm$ 1.44	7/70(10%)
Age $\leq$ 63	58	4.29 $\pm$ 3.65	25/58(43%)	0.16 $\pm$ 0.75	3/58(5%)
Median >63	63	5.03 $\pm$ 4.25	30/63(48%)	0.35 $\pm$ 1.29	9/63(14%)
<i>Tumor size</i>					
T1	38	5.40 $\pm$ 4.48	18/38(47%)	0.24 $\pm$ 1.30	2/38(5%)
T2	61	3.77 $\pm$ 3.34	24/61(39%)	0.54 $\pm$ 1.48	10/61(16%)
T3	4	5.00 $\pm$ 4.73	1/4(25%)	0.00	0/4(0%)
T4	13	6.23 $\pm$ 3.47	8/13(62%)	0.00	0/13(0%)
Missing	5				
<i>ER</i>					
Positive	65	5.14 $\pm$ 4.12	32/65(49%)	0.37 $\pm$ 1.41	6/65(9%)
Negative	20	2.50 $\pm$ 2.24 <sup>*</sup>	4/20(20%)	0.70 $\pm$ 1.78	4/20(20%)
Missing	36				
<i>PR</i>					
Positive	53	4.81 $\pm$ 4.26	23/53(43%)	0.25 $\pm$ 1.05	5/53(9%)
Negative	32	3.84 $\pm$ 3.28	11/32(34%)	0.63 $\pm$ 1.86	4/32(13%)
Missing	36				
<i>Grade</i>					
I	14	5.86 $\pm$ 6.87	7/14(50%)	0.07 $\pm$ 0.27	1/14(7%)
II	24	4.63 $\pm$ 3.75	9/24(38%)	0.00 $\pm$ 0.00	0/24(0%)
III	20	5.00 $\pm$ 2.71	11/20(55%)	0.50 $\pm$ 1.24	4/20(20%)
Missing	63				
Inside lymph node metastasis	45	0.93 $\pm$ 0.90	21/45(47%)	0.067 $\pm$ 0.25	5/45(11%)

Statistical significance,  $p < 0.05$ .

<sup>a</sup> Positive indicates presence of lymphatic vessels inside the stroma or the tumor tissue.

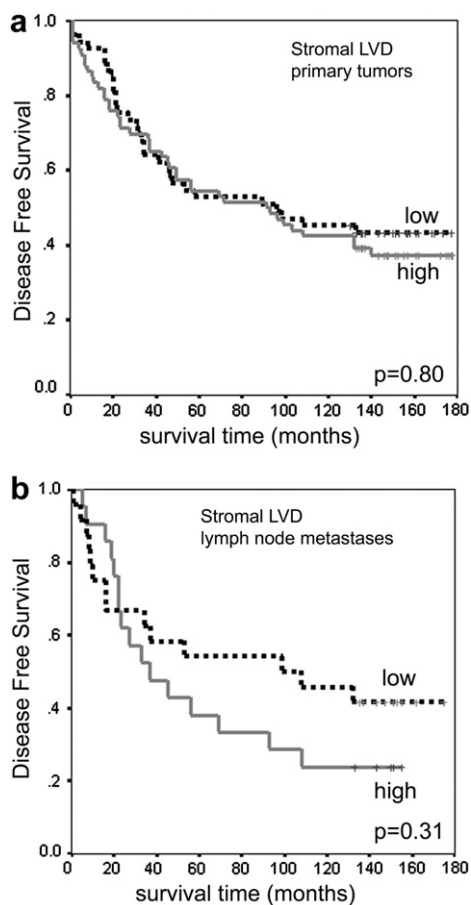


Fig. 2. Stromal lymphatic vessel density does not predict patient survival. Kaplan Meier survival analysis for the relationship between the amount of stromal lymphatic vessels and disease free survival (a) in primary ductal breast cancer tissues and disease free survival in metastases (b). The group is separated in higher and lower than median (log rank test).

### 3.2. Prognostic value of lymphangiogenic factors in primary ductal breast cancer

Since lymphangiogenesis is regulated by the lymphangiogenic factors VEGF-C and VEGF-D, we examined the expression levels of these growth factors. Within the primary tumors the VEGF-C expression was found in the majority of tumors, only three samples scored negative. All primary tumor specimens were positive for VEGF-D expression. VEGF-D was also expressed in the epithelial cells in the normal breast tissue and at a similar expression level as in the cancerous tissue (scores were  $2.773 \pm 0.958$  and  $2.708 \pm 0.674$ , respectively). The expression of VEGF-C and VEGF-D in the lymph node metastases was not higher than in the primary tumors.

Both the VEGF-C and VEGF-D expression levels were not correlated to the stromal LVD or the intratumoral LVD. Neither VEGF-D nor VEGF-C was significantly

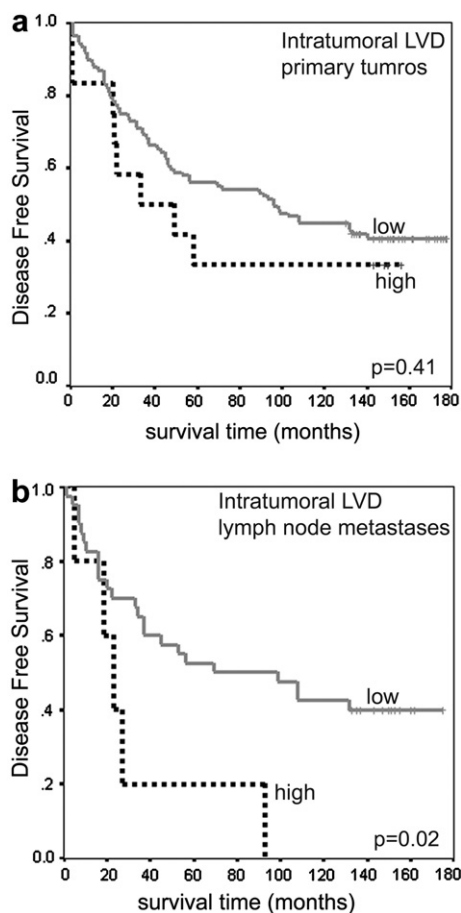


Fig. 3. Patients with intratumoral lymphatic vessels in the lymph node metastases have a shorter survival. Kaplan Meier survival analysis on disease free survival (a) for intratumoral lymphatic vessels density in the primary tumors and in the metastases (b). The group is separated in higher and lower than median (log rank test).

related to nodal status or the presence of metastases or to tumor grade as determined by Nottingham score (data not shown). VEGF-D was also analysed for its role in survival. There was no significant relationship between VEGF-D expression and survival time, although there was a trend (log rank  $p = 0.063$ ) in the patient group with a higher VEGF-D for longer survival (Fig. 5f). When the relationship between VEGF-C or -D and survival was analysed within subpopulations no significant relationship was found between VEGF expression and survival within groups divided according to e.g. age, PR status or nodal status (data not shown). Furthermore, VEGF-D expression was not related to the occurrence of lymph node metastases, or recurrences or other metastases (data not shown). The same was the case for VEGF-C (data not shown).

Next to using growth factors as lymphangiogenic markers and the quantification of lymphatic vessels in a selection of 10 tissues in which both intratumoral and

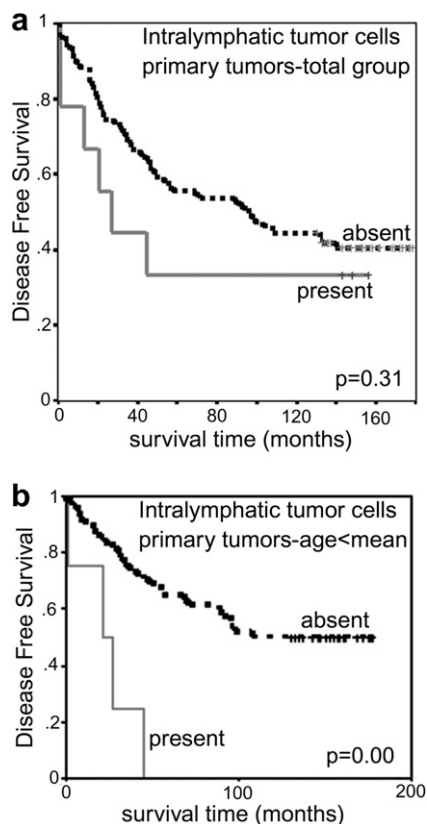


Fig. 4. Presence of intralymphatic tumor cells is a marker of poor prognosis in ductal breast cancer. (a) A Kaplan Meier survival curve (disease free survival) for patients with or without intralymphatic tumor cells in the primary tumor and (b) shows this for the patient group below median age 63.

stromal lymphatic vessels were observed a double staining for podoplanin and Ki-67 was performed. The number of nuclei that were positive for Ki-67 in the podoplanin stained vessels were evaluated. In all 10 tissues investigated double positive nuclei were not observed (data not shown). As a control for the method we also included Ki-67 double staining with CD31/CD34. In the regular blood vessels 30% of the tissues (36 of 121) showed proliferating endothelial cells (data not shown).

### 3.3. The presence of intralymphatic tumor cells is a marker of poor prognosis

Next to LVD and the lymphangiogenic growth factors VEGF-C and -D also the presence of tumor cells inside the lymphatic vessels within the primary tumors (Fig. 1c) was determined. We found a trend of shorter survival in cases with intralymphatic tumor cells present (Fig. 4a). When this analysis was restricted to the group of patients younger than 63 (mean age) a very significant difference in survival was found (Fig. 4b, log rank

$p < 0.0004$ ). Also, the relationship between presence of intralymphatic tumor cells and VEGF-C and VEGF-D expression was examined. Surprisingly, although VEGF-D expression was not related to the LVD, we found that VEGF-D expression was significantly higher in the group that had tumor cells present in the lymphatic vessels compared to the group that did not have tumor cells inside the lymphatic vessels (mean 2.53 versus 3.50 in, respectively, the ILTC negative and positive groups,  $p < 0.01$ ,  $n = 47$  and 6, respectively).

## 4. Discussion

Although many studies on lymphangiogenesis have been reported in animal models [5,6,24], the presence of ongoing lymphangiogenesis in human tumors is still a subject of debate. For breast cancer several papers report on the absence of lymphangiogenesis during breast cancer progression [8,9,20,25]. Other reports clearly demonstrate a relationship between lymphangiogenesis or lymphatic vessel density in breast cancer and progression or metastasis formation [17,19].

We show that intratumoral lymphatic vessels in ductal breast carcinoma are only sparsely present in a minority of cases (14 out of 121, 9%). The presence of these vessels, as well as the expression of VEGF-C and VEGF-D in the primary tumor, did not correlate to clinical parameters. The expression level of both growth factors also did not correlate to the lymphatic vessel densities in primary tumors and lymph node metastases. Neither did we see a difference in the expression of VEGF-C and VEGF-D between normal and cancerous tissues. Furthermore, we did not observe any proliferating lymphatic endothelial cells in the primary tumor tissue. These results lead us to conclude that ongoing lymphangiogenesis is absent in ductal breast cancer. A very recent study by Van den Eynden et al. described the presence of proliferating lymphatic endothelial cells in lymph node metastases [26]. This is an interesting observation because this is in line with our hypothesis that lymphangiogenesis is an ongoing process in certain metastases of aggressive tumors. Furthermore, we found that a higher LVD in the metastases was related to a shorter survival. This is also in correspondence with the findings by Van den Eynden et al. [26].

The only parameter that we found to be of prognostic value was the presence of tumor cells within the lymphatic vessels. This correlated directly with disease free survival. Interestingly, this parameter

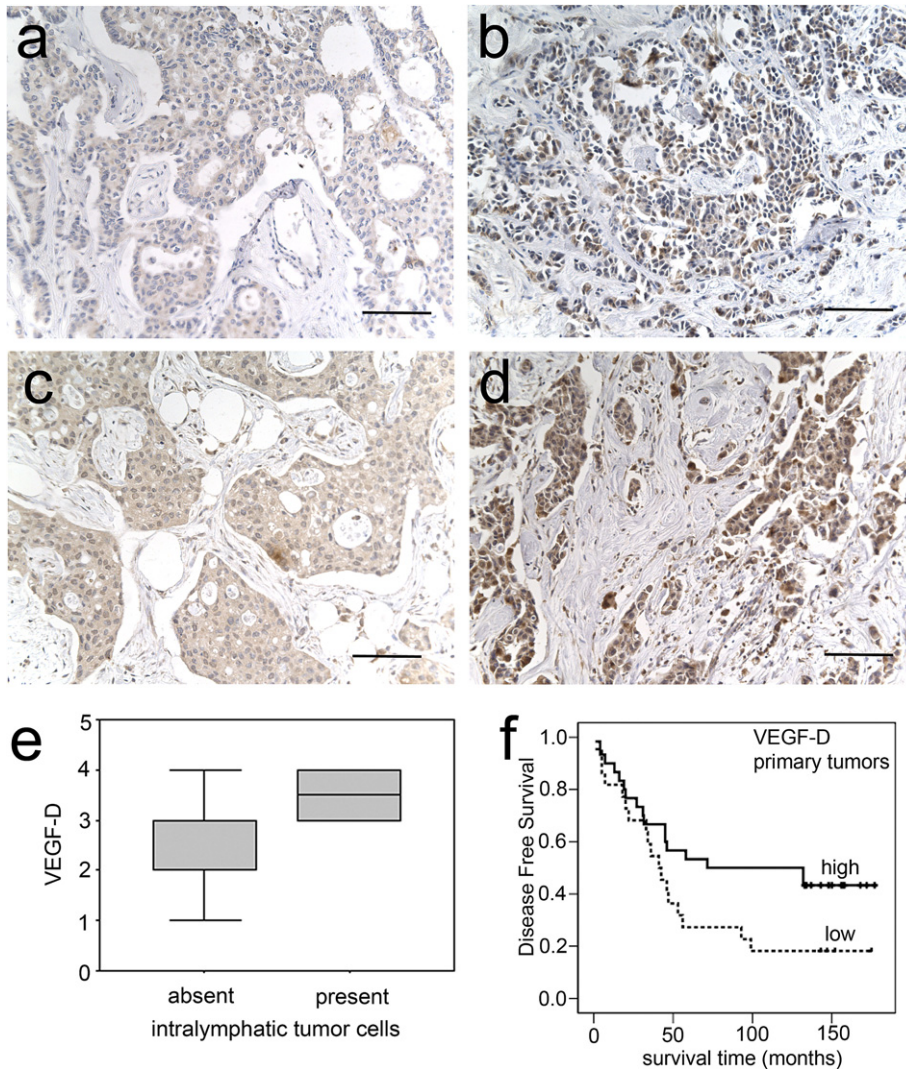


Fig. 5. Expression of VEGF-C and -D does not relate to lymphatic vessel density or survival. (a) An example of a ductal breast cancer tissue with low VEGF-C expression (score 1) and (b) with high VEGF-C expression (score 4). (c) An example of a ductal breast cancer tissue with low VEGF-D expression (score 1) and (d) with high VEGF-D expression (score 4). Magnification 200 times, bar represents 50  $\mu$ m. (e) The difference in VEGF-D expression in tissues without (mean value 3) and tissues with intralymphatic tumor cells (mean value 3.5). The boxes represent quartiles, the error bars indicate 100% of the values. (f) Kaplan Meier survival curve (disease free survival) for patients with high versus low VEGF-D expression in the primary tumor.

was dependent on the expression of VEGF-D. This result suggests that VEGF-D is involved in the permeabilisation of lymphatic vessels and/or that VEGF-D facilitates the transport of tumor cells via lymphatic vessels, both being able to support metastasis formation. Alternatively, VEGF-D expression could have an autocrine function on tumor cells themselves. The finding that VEGF-D, next to being an angiogenic or lymphangiogenic factor, has been found to be a growth or survival factor for the tumor cells [27,28], could be relevant in this

regard. Although these are attractive hypotheses, we did not find a significant relationship between VEGF-D expression and nodal status or the occurrence of distant metastases. In a previous study, however, we did find that VEGF-D and VEGF-C are able to reduce the expression of the leukocyte adhesion molecule ICAM-1 on endothelial cells, which indicates that these growth factors in this way suppress leukocyte infiltration in the tumor. This could give the tumor a growth advantage [29]. The relationship between presence of tumor

cells in lymphatic vessels and patient survival could also relate to a metastasis formation advantage of the tumor cells instead of a more permeabilized phenotype of the lymphatic vessels, e.g. it might be that the tumor cells express more adhesion molecules or matrix metallo proteinases which enable them to migrate. This hypothesis would need further attention in future studies.

Also, there were lymphatic vessels present in the stroma surrounding the tumor cells. This suggests that it is more likely that the tumor grows around existing lymphatic vessels instead of stimulating the generation of new lymphatics. Although based on animal studies, most studies have described the expression of VEGF-C and -D in relation to lymphangiogenesis [6,7]. A recent publication suggests that VEGFR3, which is the receptor for VEGF-D, is not just expressed by lymphatic endothelial cells, but also by endothelial cells in blood vessels in breast cancer tissues [22]. Furthermore, Currie et al. suggest that VEGF-D expression in breast cancer was not related to lymph node metastasis status [30]. These results together with results from previous research papers suggest an alternative role for VEGF-C and -D in breast cancer, possibly angiogenesis stimulation. In our study we also did not find a relationship between VEGF-C or VEGF-D expression and lymphatic vessel density, neither in the stromal tissue nor in the tumor tissue. Furthermore, we also did not find a correlation between the expression of one of these growth factors and differentiation status.

In conclusion, we suggest that lymphatic vessel density assessment and the measurement of VEGF-C or VEGF-D level, are of limited use to measure ongoing lymphangiogenesis in human breast cancer. The presence of intralymphatic tumor cells, however, did predict a poor patient outcome. Furthermore, in the lymph node metastases a higher LVD was observed in patients with shorter survival. Nevertheless, we favour the view that lymphangiogenesis is not an active process in human breast cancer at the primary tumor site.

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