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Lymphatic microvessels density, VEGF-C, and VEGFR-3 expression in 25 cases of breast invasive lobular carcinoma

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Abstract

Invasive lobular carcinoma (ILC) is the second most common type of invasive breast cancer, having distinct morphologically but also prognostic and therapeutic features. This type of breast cancer shows a higher rate of multiple metastases with a more frequent axillary-lymph-node involvement. Related to these dissemination and metastatic features, we aimed to study the immunohistochemical expression of D2-40, VEGF-C and VEGFR-3 in 25 cases of ILCs stratified according to the histopathological and molecular classification. Regardless of histopathological or molecular subtype, the statistical tests proved that for ILC, the highest D2-40 lymphatic microvessels density (LMVD) was in the peritumoral areas. In classical subtype, the LMVD values were positively correlated with the degree of tumor differentiation and pTNM clinical stages and when these cases were classified based on the molecular criteria the highest recorded values were found in the luminal B subtype. In addition, regardless of the histopathological and molecular subtypes, the D2-40 LMVD varied in the same direction for both VEGF-C and VEGFR-3 categories, with the highest LMVD values recorded in those cases with the highest VEGF-C and VEGFR-3 reactivity, especially in the peritumoral areas. Considering only the molecular luminal A and B subtypes, we have noted that VEGF-C and VEGFR-3 expression was significantly higher in luminal A subtype compared to luminal B. This immunoprofile suggests the existence of a tumor type-specific lymphangiogenesis that may have certain prognostic and therapeutic implications.

Keywords: invasive lobular carcinoma, lymphatic microvessels density, VEGF-C, VEGFR-3.

Introduction

Invasive lobular carcinoma (ILC) is the second most common overall type of breast cancer, accounting for 5–15% of the cases in the most Western reports [1–4]. Data from the literature reported an increasing incidence of ILC [3, 5], especially among postmenopausal women [3] and seems to be related to the use of hormonal therapy [2, 6, 7]. The morphological diagnosis is difficult due to several distinct variants of ILC that have been reported [8–13]. Even more, according to the new molecular classification five distinct subclasses of breast cancer have been identified: ER-positive, which include luminal A and B tumors; and ER-negative that include HER2 type, basal-like tumors and normal-like breast tumors [14, 15].

In addition, ILC seems to have a distinctive clinico-pathologic profile, showing a higher rate of multiple metastases [16], with a distinct pattern of metastasis [16–18], and a lower rate of lymphatic-vascular invasion [19]. In breast cancer, lymph node metastasis occurs in more than one third of the cases and according to the most recent data in breast ILC is a higher rate of axillary-lymph-node involvement [20–22].

Since tumor lymphangiogenesis promotes lymphatic metastasis, this process was extensively investigated in breast cancer in the last decade, the lymphatic micro-vascular density (LMVD) has been shown to correlate with lymph node metastasis [23–25].

Few data are available concerning the specific profile of lymphangiogenesis in ILC and its molecular

subtypes. Thus, in the present study we have investigated the correlations between D2-40 LMVD, VEGF-C/ VEGFR-3 expression and different histopathological and molecular types of ILC by immunohistochemistry.

Materials and Methods

Patients, samples and histopathological processing were described in detail in a previous article [26]. Based on ER, PR, and HER-2/neu receptors status, and according to the molecular criteria of breast classification [27–30], the 25 breast invasive lobular carcinoma studied were grouped in the following subclasses: luminal A, luminal B, HER2 type and basal-like tumors.

Immunohistochemical processing

The methodology of ER, PR, and HER-2/neu immunohistochemical expression was previously described [26]. To establish the lymphangiogenic status, namely the immunoreactivity for D2-40, VEGF-C, and VEGFR-3 we proceeded as follows.

The sections were first subjected to antigen unmasking by heat induced epitope retrieval in DakoCytomation Target Retrieval solution, code S1700, and as visualization system it was used the LSAB2 (Dako, Redox, Romania, code K0675) and the following primary antibodies: Podoplanin (D2-40, mouse anti-human, monoclonal, Dako, Redox, Romania, code M3619) diluted as 1:100, VEGF-C (polyclonal rabbit anti-human, Invitrogen, Antisel, Romania, code 18-2255) diluted as 1:100, and Flt-4 (BB49, mouse anti-human, monoclonal, Santa Cruz, Redox, Romania, code sc-74011) diluted as 1:100, incubated overnight at 4^0C.

As chromogen, we used 3,3'-diaminobenzidine tetrahydrochloride (Dako, Redox, Romania, code K3468) and for nuclei counterstaining Mayer's Hematoxylin.

Negative controls were obtained by omitting the primary antibodies, and as external positive control were used normal breast tissues specimens.

Lymphatic microvessels density (LMVD) assessment

Slides immunostained with anti-D2-40 antibody were scanned at low-power magnification (×40) by two independent observers to identify three areas with the greatest number of lymphatic vessels (hotspots), for both intra- and peritumoral areas in each investigated cases.

Microvessels were counted under ×200 magnification (covering an area of 0.74 mm^2) considered the mean number of vessels in these areas in each sample.

Vessels with muscular walls and clusters of myoepithelial and/or myofibroblasts were disregarded in microvessels counts.

VEGF-C and VEGFR-3 immunostaining assessment

Their expression was quantified in the various samples examined using a semi-quantitative scoring method. A mean percentage of positive tumor cells was determined in at least five areas at a magnification of ×400, and assigned to one of the three following scores: 0 (no reaction), 1 (positive in less than 10% of the total tumor cells), 2 (positive in less than 50% of the total tumor cells) and 3 (positive in more than 50% of the total tumor cells).

The images were acquired utilizing a Nikon Eclipse 55i microscope (Nikon, Apidrag, Bucharest, Romania) equipped with a 5-megapixel cooled CCD camera and the Image ProPlus AMS7 software (Media Cybernetics Inc., Buckinghamshire, UK).

Statistical analysis

Statistical analysis was done in SPSS version 16.0 for Windows, using the χ^2-test for dependence assessment; Student's *t*-test and ANOVA testing being used for paired or multiple inter-group comparisons, all results were considered statistically significant for a *p*-value <0.05.

As we did not have enough cases in different histopathological and molecular groups, we grouped the data for further analysis as classic/non-classic for the histopathological typing and excluding basal-like and Her2 from the molecular classes.

In the same line, the reactivity for VEGF-C and VEGFR-3 was deemed as positive (including here scoring 2 and 3), or negative (including scoring 0 and 1).

Results

As we shown in the previous article, the general median onset age was of 58 years (range 45–69 years) with a slight tendency for the non-classic cases to develop in older people.

In most cases, the tumor degree was 2 (48%), but in the classic subtype grade 1 (63.63%) prevailed [26].

Regarding the stage of the disease and lymph node status, we observed that while most of the cases were in the stage II (48%) and with no lymphatic metastases (60%), the non-classical variants have been diagnosed more frequently in more advanced stages (50% of these in stage III) and with lymph node metastasis (in 42.85% of these cases).

Histopathologically, our casuistry was dominated by the classical type with 11 (44%) cases, followed by the solid subtype with four (16%) cases and histiocytoid variant with four cases.

The tubulolobular and plemorphic subtypes were diagnosed in two cases each one, while the alveolar and trabecular subtypes have been found only in a single case each.

According to the molecular classification, as shown in Table 1, the most encountered type was luminal B (11 cases), followed by luminal A (10 cases) and HER2 (three cases).

Statistical analysis revealed that the molecular subtype luminal A was more frequently associated with the classical histopathological type of ILC [$\chi^2(1, N=22) = 24.489$, $p<0.001$].

Table 1 – ***The D2-40 LMVD and VEGF-C/VEGFR-3 immunohistochemical assessment according to the histopathological and molecular stratification of the investigated breast adenocarcinoma cases***

ILC subtypes (No.)	ER, PR, HER2 status				LMVD		VEGF-C	VEGFR-3
	Basal like	*Her2*	*Luminal A*	*Luminal B*	*Intratumoral*	*Peritumoral*	*Intratumoral*	*Peritumoral*
Classic (1)				+	6	11	0	0
Classic (2)				+	5	12	2	2
Classic (3)			+		0	0	0	0
Classic (4)			+		3	14	3	3
Classic (5)			+		4	15	3	2
Classic (6)			+		0	0	0	0
Classic (7)			+		5	13	2	1
Classic (8)				+	7	11	1	0
Classic (9)	+				0	0	0	0
Classic (10)			+		3	10	1	1
Classic (11)				+	6	11	2	2
Alveolar (1)			+		0	4	2	2
Solid (1)			+		5	14	3	1
Solid (2)				+	5	13	3	2
Solid (3)			+		4	15	2	3
Solid (4)			+		0	0	0	0
Tubulolobular (1)				+	6	10	1	0
Tubulolobular (2)				+	4	12	2	1
Trabecular (1)				+	3	11	0	0
Pleomorphic (1)		+			8	15	3	3
Pleomorphic (2)				+	6	13		2
Histiocytoid (1)				+	0	0	0	0
Histiocytoid (2)		+			0	0	2	1
Histiocytoid (3)		+			3	7	2	1
Histiocytoid (4)				+	2	6	1	0

D2-40 expression and LMVD assessment

In the resection margins, at the level of residual glandular parenchyma we noticed a D2-40 positive reaction in lymphatic vessels from the interlobular stroma, which were elongated and with a linear prevalent morphology (Figure 1A).

With variable intensity, we observed that myoepithelial cells of normal ducts and lobules were also positive to D2-40, with a granular, branching membranous staining pattern (Figure 1B).

In addition, a weak positive reaction was detected as a thin or discontinuous membranous staining pattern around foci of lobular carcinoma *in situ* (Figure 1C). In addition, some stromal myofibroblasts were weakly positive for D2-40.

Overall, in the invasive tumor specimens we noticed a D2-40 positive reaction in 17/25 (68%) cases. The mean±SD peritumoral LMVD was about 8.68±5.64 (range 0–15), whereas for intratumoral lymphatic vessels we found only 3.4±2.55 (range 0–8). The difference was highly statistically significant (p<0.001).

Regarding their morphology, the intratumoral lymphatic vessels were small, linear and flattened (Figure 1D), while the peritumoral lymphatics were widely opened and tortuous (Figure 1, E and F).

Occasional invasion of the carcinoma cells into the lymph vessels was observed, mainly in peritumoral tissue.

Regarding the histological type, we noticed overall significant differences of LMVD between all classes (p<0.001). When grouping the data depending of the luminal A and B molecular subtypes, we found that LMVD was higher in the peritumoral areas (for both types), and the values were significantly higher in luminal A subtype but only for the intratumoral areas (p<0.01).

In the classical subtype, we found a significant higher LMVD in G2 histological grading type (p<0.05). For pTNM stages, we observed that LMVD increased from stage I to stages II and III, with higher values for peritumoral areas compared to intratumoral areas (p<0.05) (Figure 2).

Grouping the data from the classical subtype of ILC depending on molecular subtypes, we found that luminal A values were constantly lower than luminal B values for both intratumoral and peritumoral areas (p<0.05).

Statistically, regardless of the histopathological and molecular subtypes, the LMVD varied in the same direction for both VEGF-C and VEGFR-3 categories, with the highest values being recorded for the peritumoral positive areas (with the 2 and 3 scoring for both markers) [$F(1,92)=17.86$, p<0.001 for VEGF-C data and $F(1,92)=21.36$, p<0.001 for VEGFR data].

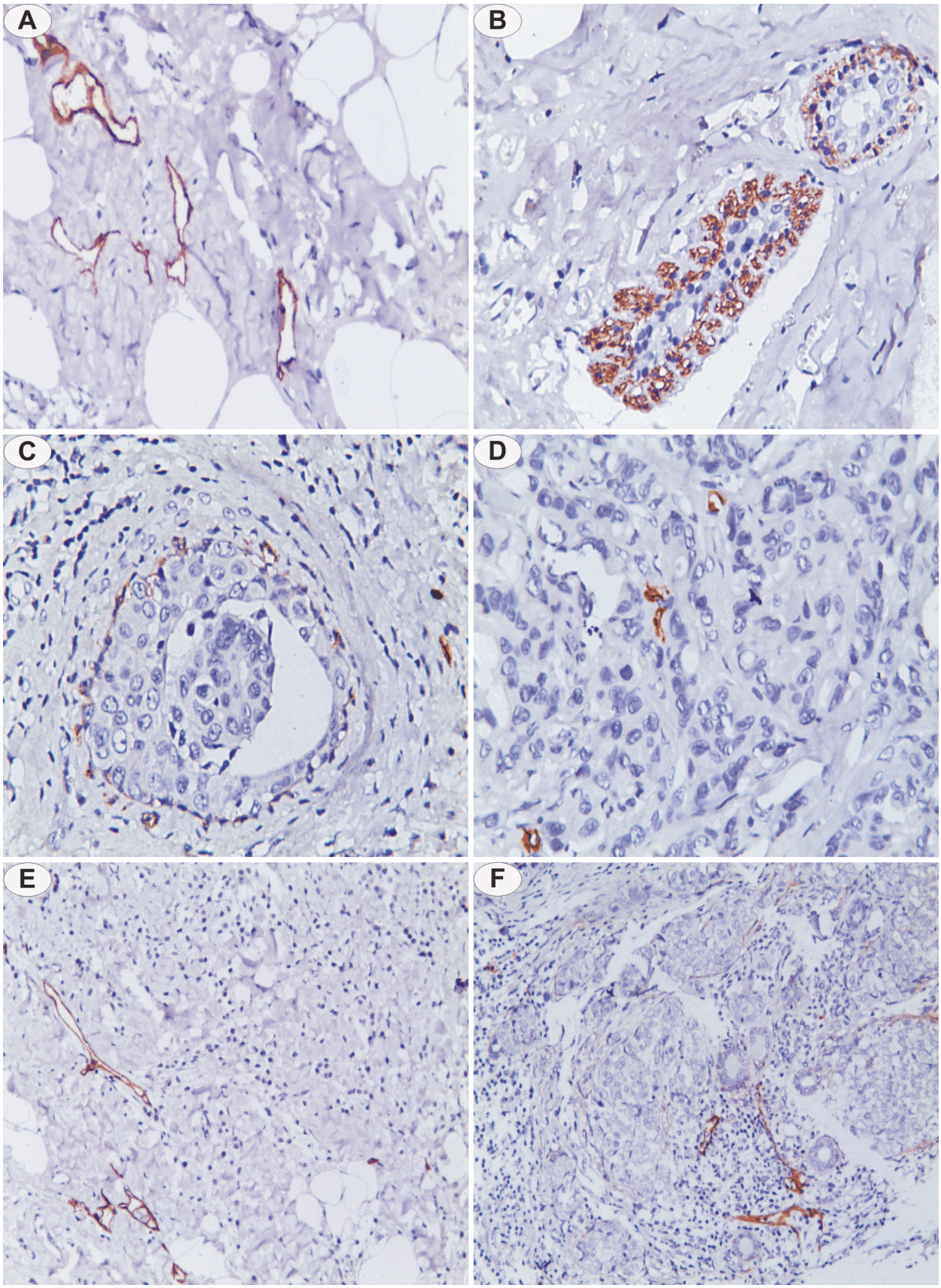

Figure 1 – ***Breast invasive lobular carcinoma: (A) D2-40 positive reaction in lymphatic vessels from the normal interlobular stroma, DAB, ×100; (B) D2-40 positive reaction in myoepithelial cells of normal ducts and lobules, DAB, ×100; (C) D2-40 thin or discontinuous membranous staining pattern around foci of lobular carcinoma in situ, ×200; (D) D2-40 positive intratumoral lymphatic vessels form ILC solid type, DAB, ×100; (E and F) D2-40 positive peritumoral lymphatic vessels from ILC classical and solid types, DAB, ×100.***

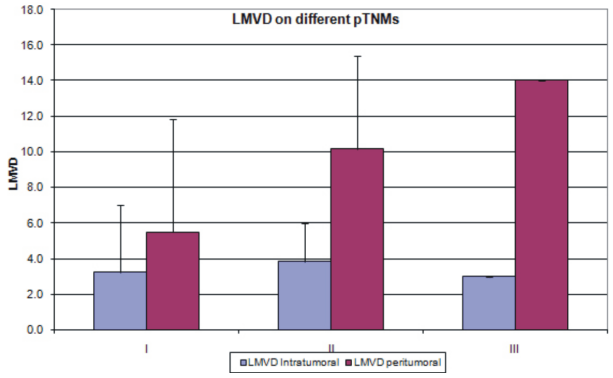

Figure 2 – ***The average intratumoral and peritumoral LMVD for stage II pTNM differ significantly (p<0.05, Student's t-test). Error bars represent standard deviation.***

VEGF-C expression and its assessment

The VEGF-C protein was expressed as diffuse cytoplasmic staining in tumor cells and with only a weak intensity in the normal mammary tissue adjacent to the tumor (Figure 3A). In addition, a positive reaction was present in lobular carcinoma *in situ* (Figure 3B) and lymphatic endothelial cells.

Positive reaction of tumor cells to VEGF-C was detectable in 18 (72%) cases, with a heterogeneous distribution within the tumor mass. The negative cases were represented by four cases of classic ILC, and by one case from the solid, trabecular and tubulolobular histopathological ILC subtypes. According to the molecular classification, these negative VEGF-C cases belonged to the luminal A (three cases), luminal B (three cases) and basal like (one case). The semiquantitative VEGF-C immunoreactivity assessment proved that most reactive cases, with score three, belong to the classic ILC (two cases), solid type (two cases) and pleomorphic (one case) (Figure 3, C–E). The most reactive to VEGF-C were the luminal A molecular subtype (three cases with score 3), followed by luminal B and HER 2 subtypes (each with one cases with score 3) (Table 1).

In addition, a positive VEGF-C reaction was noticed in seven (28%) cases in stromal cells with polygonal (possible macrophages) or spindle morphology (Figure 3F). No positive reaction was detected in the blood vessels. Statistically, the VEGF-C expression was significantly higher in luminal A subtype compared to luminal B [$\chi^2(1, N=22)=22.916, p<0.001$].

VEGFR-3 expression and its assessment

The VEGFR-3 staining had a granular cytoplasmic pattern, being expressed both in tumor cells and in lymphatic endothelium. In normal breast tissue adjacent to tumors, we noticed a weak reaction in epithelial ductal cells (Figure 4A) and vessels from interductal stroma (Figure 4B). In addition, a positive reaction was observed in myoepithelial cells surrounding normal ducts and ducts with lobular carcinoma *in situ* (Figure 4C).

VEGFR-3 expression in tumor cells was found in 16 (64%) cases. The highest average score (3) was noticed in classic (one case), solid (one case) and pleomorphic (one case) histopathological ILC subtype (Figure 4, D and E). At the opposite pole were cases of histiocytoid, trabecular and tubulolobular subtypes. As regarding the molecular subtype, the luminal A and HER2 seem to be the most reactive, while the lowest reactivity was recorded in luminal B and basal-like subtypes (Table 1). In addition, we observed a positive VEGF-3 reaction in the stromal cells, sometimes with more intense reaction than tumor cells.

VEGFR-3 expression in the lymphatic endothelium was found in nine cases (36%). Lymphatic vessels were stained mainly in the periphery of the tumor, including adipose tissue (Figure 4F). Also at the tumor periphery, we noticed blood vessels that were positive to VEGF-C, recognized by the presence of erythrocytes in their lumen. The intratumoral lymphatic vessels positive to VEGFR-3 were much less numerous and usually of small size. Statistically, the VEGFR-3 expression was significantly higher in luminal A subtype compared to luminal B [$\chi^2(1, N=22)=22.916, p<0.001$].

Discussion

ILC seems to have a distinctive clinicopathologic profile, showing a higher rate of multiple metastases [16], with a distinct pattern of metastasis involving with predilection peritoneal and leptomeningeal surfaces, gastro-intestinal tract and ovaries [16–18], a trend towards later locoregional recurrence [31, 32], and a lower rate of lymphatic-vascular invasion [19].

Overall, in breast cancer, lymph node metastasis occurs in more than one third of the cases and is one of the most important prognostic factors for this human malignancy [33]. Especially for early-stage breast cancer, it was showed that axillary-lymph-node status is the most important prognostic factor [34, 35]. Data regarding the differences of axillary-lymph-node status between ILC and invasive ductal carcinoma of the breast are contradictory. Although most studies did not find any significant differences [1, 6, 36–39], the most recent data reported a higher rate of axillary-lymph-node involvement in breast ILC [21, 22, 30]. Moreover, axillary nodal metastasis is more commonly seen in pleomorphic subtype of ILC [40].

It is well known that tumor lymphangiogenesis promotes lymphatic metastasis [42–43] and particularly in breast cancer was proved that LMVD correlates with lymph node metastasis [23–25]. It was suggested that lymphatic metastasis in breast cancer may be achieved through the formation and invasion of newly induced lymphatics both within the tumor and in the tumor periphery [24].

Few data are available concerning assessment of LMVD in breast ILC and its correlation with different histopathological and molecular subtypes of this breast cancer variety. Thus, van Iterson V *et al.* found a significant correlation between LYVE-1+ peritumoral lymph-vessel density and presence of lymph node metastases and the number of metastatic lymph nodes [44].

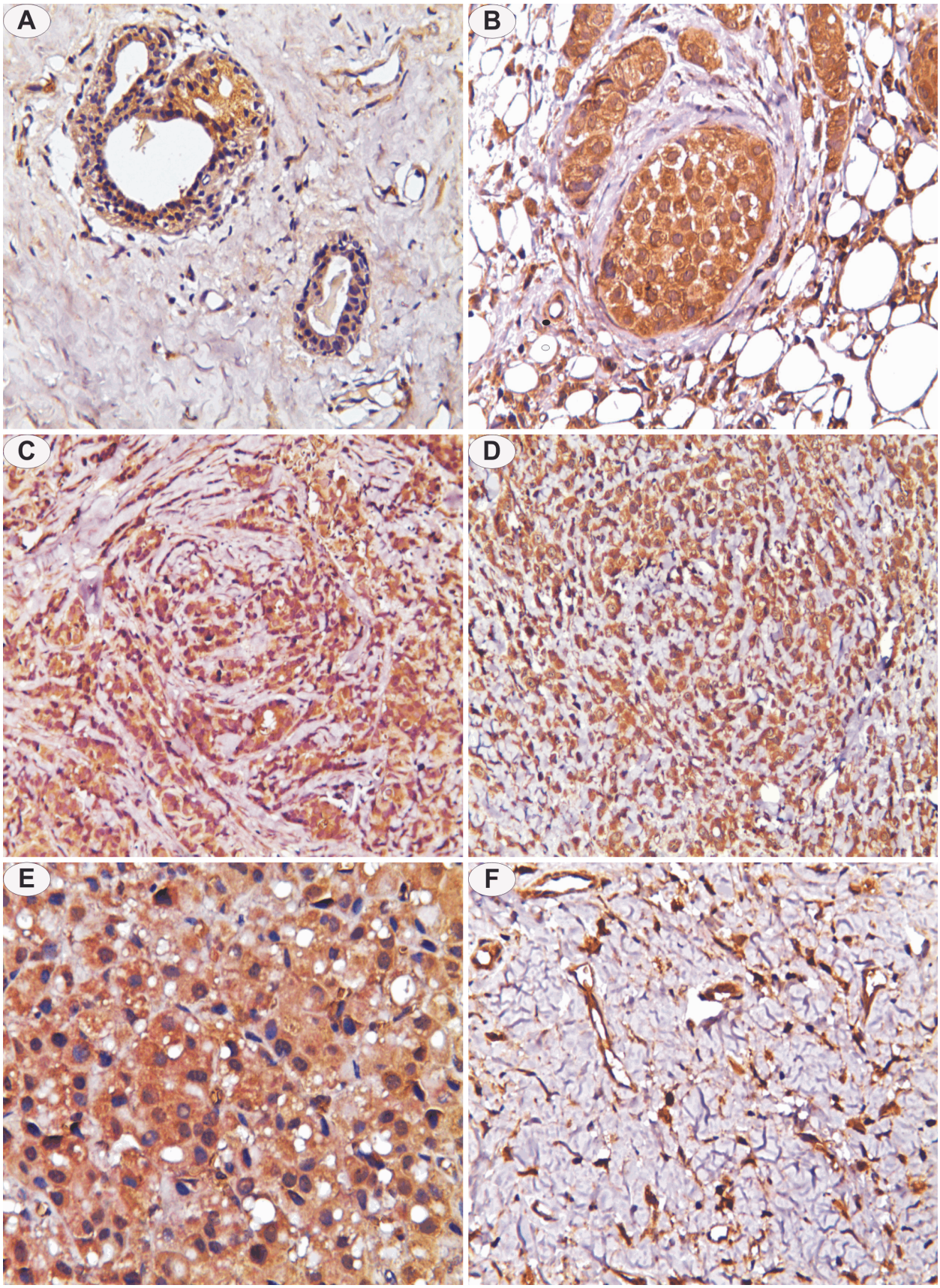

Figure 3 – ***Breast invasive lobular carcinoma: (A) VEGF-C expression in the normal mammary tissue adjacent to the tumor, DAB, ×200; (B) VEGF-C expression in tumor cells from lobular carcinoma in situ, DAB, ×200; (C) VEGF-C expression in tumor cells from ILC classical type, score 3, DAB, ×100; (D) VEGF-C expression in tumor cells from ILC solid type, score 3, DAB, ×100; (E) VEGF-C expression in tumor cells from ILC pleomorphic type, score 3, DAB, ×200; (F) VEGF-C expression in stromal cells and lymphatic endothelial cells , DAB, ×200.***

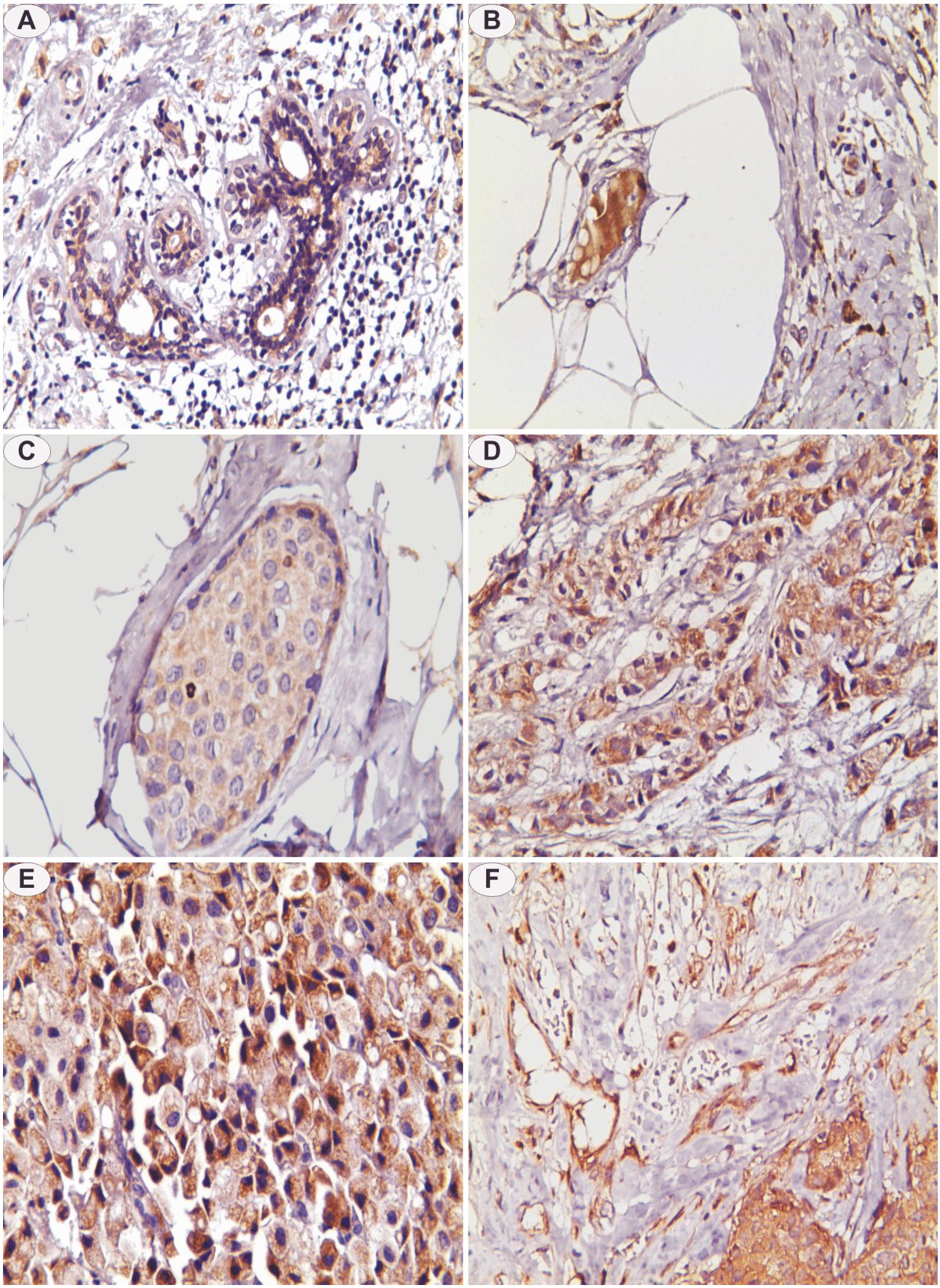

Figure 4 – ***Breast invasive lobular carcinoma: (A) VEGFR-3 weak reaction in the normal epithelial ductal cells, DAB, ×200; (B) VEGFR-3 expression in vessels from the normal interductal stroma, DAB, ×200; (C) VEGFR-3 expression in tumor cells from lobular carcinoma in situ, DAB, ×100; (D) VEGFR-3 expression in tumor cells from ILC classic type, score 3, DAB, ×100; (E) VEGFR-3 expression in tumor cells from ILC pleomorphic type, score 3, DAB, ×200; (F) VEGFR-3 expression in the lymphatic endothelial cells from the periphery of the tumor, DAB, ×100.***

To our knowledge, this is the first study that has assessed D2-40 LMVD in breast ILC evaluating cases stratified in histopathological and molecular subtypes. Overall, we found significant differences of LMVD between all classes, with highest values at the periphery of tumors. For the classical subtype, the LMVD values increased with the degree of differentiation and in more advance pTNM stages. When we stratified the classical ILC cases on molecular criteria, we noticed that the luminal B cases had the highest LMVD values. In addition, regardless of the histopathological and molecular subtypes, the LMVD varied in the same direction for both VEGF-C and VEGFR-3 categories, with the highest values being recorded for the peritumoral positive areas. It seems that the highest values of LMVD correlated with the highest expression of both VEGF-C and VEGFR-3 markers, especially in the peritumoral areas.

Raica M *et al.* assessing D2-40 LMVD in different molecular types of breast cancer but without histopathological stratification of the investigative cases found the highest scores in the HER2 type, both for intratumoral and peritumoral lymphatic vessels density [45]. These results confirm that HER2 subtype is one of the most aggressive molecular variants of breast cancer, frequently associated with lymph node metastasis and poor prognosis [46–48]. Moreover, as we have shown more important is the peritumoral D2-40 LMVD, Zhao YC *et al.* proving that it was significantly associated with lymph node metastasis, lymphatic vessel invasion and TNM clinical stage, serving as an independent predictor of lymph node metastasis and prognostic factor in breast carcinoma [49].

VEGF-C plays an important role in tumor progression by both stimulating lymphangiogenesis and tumoral proliferation via direct and/or autocrine action on cancer cells [50, 51]. In breast cancer, there have been reported high levels of VEGF-C expression in more than 30–40% of the investigated tumors and a strong correlation with lymphatic vessel invasion, lymph node metastasis and poorer disease-free survival times [49, 52, 53]. In the present study, we found VEGF-C expression in 72% of the cases and a higher reactivity especially in classical and solid histopathological subtypes, respective in the luminal and HER2 molecular subtypes, even if we did not reveal significant statistically differences. However, when the data were further grouped in classical and non-classical type and respective only in luminal A and luminal B, statistically we noticed that the highest values of LMVD correlated with the highest expression of VEGF-C, especially in the peritumoral areas. In addition, the VEGF-C expression was significantly higher in luminal A subtype compared to luminal B. These results are consistent with those obtained by van Iterson V *et al.*, which did not prove any correlation between VEGF-C expression and peritumoral and intratumoral lymph vessel densities [44]. The authors concluded that VEGF-C has limited role in the dissemination of breast ILCs. However, Zhao YC *et al.* investigating 73 cases of breast cancers without any histopathological stratification proved that only the peritumoral LVD was closely related to the expression of VEGF-C and VEGF-D, suggesting that tumor-derived VEGF-C/-D induce lymphangiogenesis around tumors, but not within breast tumors [49].

Liu HT *et al.* showed that basal-like, HER2 and normal-like subtypes of breast cancers correlate with both intratumoral and peritumoral LMVD, and with the expression of VEGF-C [47]. Moreover, the aggressive behavior of HER2 subtype was explained in part by VEGF-C expression in tumor cells [45, 46]. At the same time, the correlation between HER2 and VEGF-C expression from these tumors justify the usefulness of an HER2 blocking therapy that could reduce not only tumor progression, but also lymphangiogenic metastasis [48].

VEGFR-3 acting as a functional trigger and signaling molecule for angiogenesis, lymphangiogenesis and regional metastasis, then blocking of VEGFR-3 signaling could suppress tumor-induced lymphangiogenesis and regional lymph node metastasis in animal lung and breast cancer models [54, 55].

VEGFR-3 is expressed in both lymph and blood vessel endothelium of breast cancers [55, 57] but its correlation with lymph node status is controversial. Thus, while Jacquemier J *et al.* and Gunningham SP *et al.* have not obtained a significant relationship between VEGFR-3 expression profile and lymph node metastasis [58, 59], Nakamura Y *et al.* proved that VEGFR-3 positive vessel density in breast cancer was correlated with lymph node status [24]. van Iterson V *et al.* indicated that invasive lobular cancers producing VEGF-D, surrounded by VEGFR-3+ vessels, showed a significant correlation with peritumoral lymph vessel density and lymph node status [44]. In our study, the lymphatic endothelium VEGFR-3 expression was obviously seen in the periphery of the tumor, and some small blood vessels positive to this marker were also noticed.

In the present study, tumor cells with VEGFR-3 expression was found in 64% of cases with the highest reactivity in the classic and solid histopathological subtype and respective in the luminal A and HER2 molecular breast ILC types, but without any significant statistic correlations (most likely due to the small number of cases). However, when the data were further grouped in classical and non-classical type and respective only in luminal A and luminal B, statistically we noticed that the highest values of LMVD correlated with the highest expression of VEGFR-3, especially in the peritumoral areas. In addition, the VEGFR-3 expression was significantly higher in luminal A subtype compared to luminal B. Raica M *et al.*, without considering the histopathological classification but considering molecular classification found a correlation between HER2 subtype and VEGF-C and VEGFR-3 expression in tumor cells and lymphatic endothelium, respectively, and LMVD [45]. In addition, Wülfing P *et al.* found VEGF-C and VEGFR-3 expression even in ductal carcinoma *in situ* suggesting that lymph-angiogenesis could be an early event during breast carcinogenesis [60].

Conclusions

Regardless of histopathological or molecular subtype, the statistical tests proved that for ILC the highest D2-40 LMVD was in the peritumoral areas. In the classic subtype, LMVD values were positively correlated with the degree of tumor differentiation and pTNM clinical stage and when

these cases were classified based on the molecular criteria the higher LMVD values were recorded in the luminal B subtype. In addition, regardless of the histopathological and molecular subtypes, the D2-40 LMVD varied in the same direction for both VEGF-C and VEGFR-3 categories, with the highest LMVD values recorded in those cases with the highest VEGF-C and VEGFR-3 reactivity, especially in the peritumoral areas. Considering only the molecular luminal A and B subtypes, we have noted that VEGF-C and VEGFR-3 expression was significantly higher in luminal A subtype compared to luminal B. Such immuno-profile suggests the existence of a tumor type-specific lymphangiogenesis, having certain future therapeutic implications.

Contribution Note

All authors contributed equally to the manuscript.

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