Interplay between Mast Cells and Lymphatic Vessels in Different Molecular Types of Breast Cancer

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Abstract. *The particularities of lymphangiogenesis in different molecular types of breast cancer are virtually unknown and the contribution of microenvironment to this process has been ever less investigated. In the present study, we evaluated the relationships between lymphatic microvessel density (LMVD), mast cell density (MCD) and the different molecular subtypes of breast cancer. Molecular classification of breast tumors by immunohistochemistry was followed by the detection of mast cells and lymphatic vessels on the same slide by immunohistochemical double-stain method, using the lymphatic endothelial cell marker D2-40 and the mast cell tryptase. Mast cells and lymphatic vessels were simultaneously counted in the tumoral and peritumoral areas and results were compared with the molecular type, grade, lymphovascular invasion and lymph node status. Significant positive correlations were found between peritumoral MCD and LMVD for the luminal type-A breast cancers (p=0.025) and also for basal-like carcinomas (p=0.029). Moreover, a significant positive correlation was found between peritumoral and intratumoral MCD for basal-like carcinomas (p=0.009) and for overall MCD and LMVD. Low or inverse correlations between MCD and LMVD were also observed in other molecular subtypes of breast cancer. Our results strongly support that mast cells in the tumor microenvironment are keyplayers, involved in the development of tumor lymphatic vessels for some molecular subtypes of breast cancer.*

Breast cancer is the most frequent malignancy in females and despite efforts made in the field of early diagnosis and adjuvant therapy, the morbidity and specific mortality continue to increase (1). The large majority of breast malignant tumors are ductal invasive carcinomas, but conventional pathology gives us little data about prognosis for individual patients and is almost useless in selecting the adjuvant therapeutic strategy (2, 3). In the past 10 years, molecular profiling of breast cancer has fundamentally changed the understanding of breast cancer (1,4). Molecular classification of mammary tumors has revealed that breast cancer is a heterogeneous disease and five major distinct molecular subtypes have been characterized by gene analysis and immunohistochemistry. These molecular subtypes have different behavior, and a particular profile of response to therapy, reflected in the differential survival of patients (5, 6).

Few data are available concerning the specific profile of lymphangiogenesis in different molecular subtypes of breast cancer, despite being shown that their metastatic behavior is different. In breast tumor tissues, lymphatic vessels are major components of the tumor microenvironment. These vessels are newly-formed from pre-existing host vessels stimulated by lymphangiogenic factors secreted by tumor cells. More recently, it has been shown that triple-negative breast cancer correlates with higher lymphatic microvascular density and overexpression of vascular endothelial growth factor-C and -D. The HER2 subtype is one of the most aggressive molecular variants of breast cancer, frequently associated with lymph node metastasis and poor prognosis. A differential signature of lymphangiogenesis in different molecular subtypes of breast cancer was also reported by Raica *et al*, who found a positive correlation between VEGF-C, VEGFR-3 and LMVD in the HER2 type only, and a positive correlation in both HER2 and normal-like molecular subtype with VEGFR-3 expression in tumor cells (7).

The aggressive behavior of these tumors may be explained, in part, by VEGF-C expression in tumor cells. More recently, Schoppmann *et al*. (8) showed that HER2 overexpression is associated with high VEGF-C expression and high LMVD. These data support the clinical relevance of the association between HER2 and VEGF-C expression, and, thus blocking HER2 may reduce not only tumor progression, but also lymphangiogenic metastasis.

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Table I. *Antibodies and immunohistochemical procedures used for the assessement of the molecular classification, mast cells and lymphatic vessels in breast cancer.*

Antibody	Clone	Source	Dilution	HIER	WS
ER	1D5	DAKO, Glostrup, Denmark	Ready to use	Automated AR	Envision System
PR	Pgr636	DAKO, Glostrup, Denmark	Ready to use	system PT Link,	Envision System
HER2/neu	Polyclonal	DAKO, Glostrup, Denmark	Ready to use	30 min,	Visualisation System, Hercept Test Kit
CK5/6	D5/16B4	DAKO, Glostrup, Denmark	1:100	citrate pH=6	Labeled streptavidin-biotin complex, LSAB+
EGFR	Polyclonal	DAKO, Glostrup, Denmark	Ready to use		EGFR PharmDx Kit
p53	DO7	DAKO, Glostrup, Denmark	Ready to use		Labeled streptavidin-biotin complex, LSAB+
BCL2	124	DAKO, Glostrup, Denmark	Ready to use		Labeled streptavidin-biotin complex, LSAB+

HIER, Heat-induced epitope retrieval; WS, working system; ER, estrogen receptor; PR, Progesteron receptor; CK, cytokeratin; EGFR, epidermal growth factor receptor; HER2/neu, human epidermal growth factor receptor 2; BCL2, B cell lymphoma-2.

Epithelial stromal interactions play a crucial role in cancer initiation, progression, invasion, angiogenesis, and metastasis. However, the active role of stroma in human breast tumorigenesis is not well-characterized. Involvement of the tumor microenvironment in the behavior of breast cancer has been suggested by a few studies concerning T-cell receptor alpha (TCR-α) and zinc finger and (BRCA1)-interacting protein with a (KRAB) domain (ZBRK1), detected in leukocytes of (ER)-positive and endothelial cells of (ER)-negative tissues (9), by breast cancer cell-derived cytokines which stimulate macrophages to produce (TNF-α), improving the adherence of cancer cells to the endothelium, favoring breast cancer metastasis (10).

Mast cells (MCs) represent a controversial component of the breast cancer stromal compartment. Preliminary investigation suggests that during breast cancer progression, MCs may contribute to stromal remodeling and differentiation of myofibroblasts, through tryptase released in the stromal microenvironment (11). It has also been shown that MC tryptase increase MDA-MB- 231 cell migration and invasion in an *in vitro* experimental model and promotes breast cancer invasion *in vivo* (12).

Although MCs involvement in breast cancer angiogenesis has been studied in early breast cancer (13) and in human breast malignancies with or without lymph node metastases (14), the role of MCs in lymphangiogenesis of breast tumors is still not well-characterized. Moreover, association between MCs and lymphangiogenesis in molecular subtypes of breast cancer has not been yet reported.

Thus, the aim of the present study was to characterize the association between MCs and newly-formed LVs in the peri-tumoral and intra-tumoral areas from different molecular subtypes of breast cancer.

Materials and Methods

Patients' data. A total of 55 patients, aged between 26 and 81 years, admitted with breast cancer were investigated. Only ductal invasive carcinomas with tumor stage T2-T4 were selected, and of these, 26 (47.27%) showed lymph node metastasis on routine examination.

Specimens processing. Specimens were fixed in buffered-formalin for 48 h and embedded in paraffin using an automated system (Thermo-Shandon, Cheshire, UK). Five micrometers-thick sections were stained with hematoxylin-eosin and slides were reviewed for pathological diagnosis and tumors were graded according to the Nottingham modification of the Scarf Bloom Richardson method (3). A representative sample containing the invasive component was selected for the immunohistochemical evaluation. There was no attempt to select for areas characterized by any particular growth pattern.

Immunohistochemistry. To classify specimens of breast cancer according to the molecular profile, immunohistochemical staining was carried out to detect the expression of hormone receptors (ER and PR), HER2 protein, cytokeratin 5/6, epidermal growth factor receptor (EGFR), p53, and BCL-2. Details of the clone, source, dilution, antigen retrieval, and working system are included in Table 1. For all these methods, 3,3'-diaminobenzidine tetrahydrochloride was used as chromogen and nuclei were stained with Lillie's modified hematoxylin. Staining for ER and PR were scored according to Allred *et al*. (2), HER2 staining was scored according to the criteria used for HercepTest from 0 to +3, and cytokeratin 5/6, p53, EGFR and BCL2 were considered positive if a minimum 10% of the tumor cells were definitely stained with cytoplasmic (cytokeratin 5/6), membranar (EGFR and BCL2) and nuclear (p53) patterns.

To identify MCs and lymphatic vessels, two mouse monoclonal antibodies against the lymphatic endothelial cell (D2-40) and MC tryptase (clone AA1; DakoCytomation, Glostrup, Denmark) were used. Briefly, 3-μm-thick sections mounted on capillary gap slides (Dako REAL™) were de-waxed and rehydrated in graded ethanol and Tris-buffered saline (pH 7.6) and incubated with anti-D2-40 (30 minutes, ready-to-use) and anti-tryptase (30 minutes, ready-to-use), after antigen retrieval procedure with PT Link module (DakoCytomation, Carpinteria, USA) for 30 min at pH=6 and 99˚C. The working systems comprise of the Envision G2 Doublestain kit (Dako) and visualization was performed with 3,3' diaminobenzidine as chromogen for D2-40 (brown), and Fast Red as chromogen for MC tryptase (red). All immunohistochemical procedures were performed with DakoCytomation AutostainerPlus and nuclei were stained with Lillie's modified hematoxylin.

Table II. *Mean±standard deviation values of intratumoral and peritumoral mast cell density (MCD) and lymphatic microvessel density (LMVD) in the 5 subtypes of breast carcinoma.*

	MCD		LMVD	
Subtypes	Intratumoral	Peritumoral	Intratumoral	Peritumoral
Basal-like(N=7)	15.83±9.48	26.01±12.48	3.23±2.92	6.26±1.53
HER2(N=10)	43.21±13.37	24.07±9.14	2.92±2.91	6.52±3.05
Luminal A(N=26)	40.78±28.16	28.52±13.54	1.20±1.45	7.94±4.14
Luminal B(N=7)	51.56±59.51	24.38±19.06	2.46±2.49	5.40±2.05
Unclassified(N=4)	24.75±4.85	24.90±20.79	0.16±0.18	4.30±2.70

Values shown are the mean±SD.

MC and LV counts. MCs and LVs were simultaneously counted in the tumoral and peritumoral areas at ×200 magnification (covering an area of 0.74 mm^2) by two independent observers, using an Eclipse E600 light microscope (Nikon, Tokyo, Japan) and the hot-spot method. In each case, for both intra- and peritumoral areas, three fields with maximum density of MCs and LVs were chosen, and the arithmetic mean was calculated of the counts from each. All fields containing clusters of myoepithelial and/or myofibroblasts were excluded, in order to avoid an overestimation of the LMVD. Results were compared for molecular type of breast cancer with grade, lymphovascular invasion (detected on slides stained with hematoxylin-eosin and D2-40) and lymph node status.

Statistical analysis. All statistical analyses were performed with the SPSS statistical software package (SPSS Statistics 17.0; IBM, Chicago, IL, USA) for intratumoral and peritumoral MC and LMVD. Mean values±standard deviations (SD) were evaluated by two independent observers for each tumor type and in all series of sections. Correlations between intratumoral and peritumoral MC and LMVD, were assessed using the Pearson's correlation coefficient (r). A two-tailed, exact *p*-value less than 0.05 was considered statistically significant.

Results

Tryptase-positive MCs, (red) and D2-40-positive LVs (LMVD, brown) were identified in all specimens. In the normal mammary tissue adjacent to the tumor (available in 47 out of 55 cases), MCs were found in both the intra- and interlobular stroma, and LVs were found only in the interlobular connective tissue (Figure 1). In the intralobular stroma the median MC density was 7.66, and in the interlobular stroma, the median value was 24.66. Interlobular connective tissue had a median LMVD of 4.66 vessels.

When we evaluated the relationships between breast cancer types diagnosed by conventional histopathology, no significant correlation was obtained between MCD density and LMVD for overall (p=0.284), peritumoral (p=0.427) or intratumoral (p=0.945) assessement.

According to the molecular classification and based on the immunohistochemical profile previously mentioned, we found basal-like carcinoma in eight cases (14.54%), luminal A in 26 cases (47.27%), luminal B in seven cases (12.72%), HER2 in 10 cases (18.18%), and unclassified tumors in four cases (7.27%).

Assessement of MC and LMVD in molecular types of breast cancer showed results that were different from those found for conventional pathological classification of breast cancer (Figure 2a and b). Intratumoral MC density had heterogenous values among molecular subtypes of breast cancer (between 15.83±9.48 and 51.56±59.51), being higher in luminal A, luminal B and HER2 subtypes and lower in basal-like and unclassified types. Peritumoral MC density had relatively homogenous values, ranging between 24.07±9.14 and 28.52±13.54 for all molecular subtypes of breast cancer. The overall values for peritumoral LMVD were significantly higher compared with intratumoral LMVD for all types of breast cancer. The mean values for peritumoral and intratumoral MC density and LMVD in different molecular subtypes of breast cancer are shown in Table II.

As shown in Figure 3a, for luminal type A cases, LVs were found in all cases in the peritumoral area (n=1.66-16/×200) (Figure 2c) and in 19 out of 26 cases in the intratumoral area (n=0-6.66/×200) (Figure 2d). MCs were identified in all cases in both peri- (n=2.33-55.66/×200) and intratumoral area (n=3-123.33/×200) and have a compact or de-granulated appearance (Figure 2e and f).

In the luminal type-A carcinoma, no significant correlation was found for overall counting of MC and LMVD (p=0.389). When we performed differential counting for peritumoral *versus* intratumoral areas, a significant correlation was found between MC density and LMVD only for the peritumoral area (r=0.44, p=0.025). Low inverse-correlation was also observed between intratumoral MC density and peritumoral LMVD (r=–0.069, p=0.738)

For the HER2 type, MC density was significantly higher in the intratumoral area (n=25.33-62.33/×200) than in the peritumoral tissue (n=7-35.66/×200). LVs were found in 7 out of 10 cases in the intratumoral area (n=0-7/×200), and in all cases in the peritumoral tissue (n=2.66-13/×200) but only a low significant inverse correlation was found between intratumoral MCs and peritumoral LMVD (r=–0.075, p=0.837).

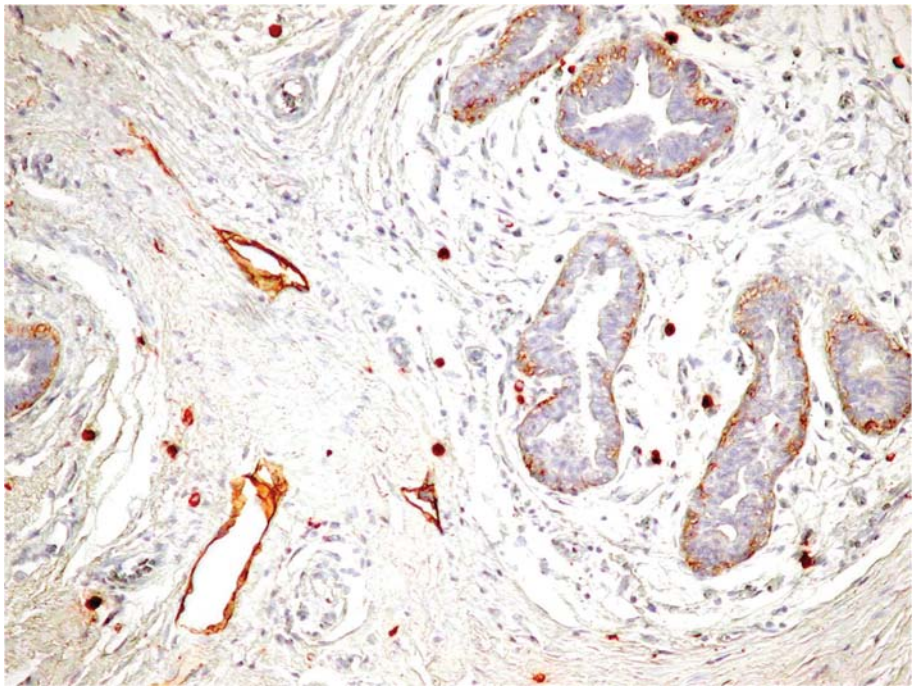

Figure 1. *Comparative distribution of mast cells (red) and lymphatic vessels (brown) in intralobular and interlobular stroma of the breast.*

In luminal type-B breast cancer, MCs density was not significantly different from values found in luminal A-type, but an increase in the number of intratumoral LVs was noticed (2.47 *vs*. 1.21); LMVD was closer that of HER2-subtype than to the luminal A-subtype. No significant differences were noticed between intratumoral and peritumoral MC density (24.75 *vs*. 24.91) whereas , LVs were significantly more numerous in the peritumoral tissue (4.33 *vs*. 0.16).

Basal-like carcinoma exhibited a particular behavior concerning MC and LVs. The overall count (without differentiating between peritumoral and intratumoral areas) of MC and LVs showed a significant correlation between these two parameters (p=0.003). Basal-like carcinoma was the only molecular subtype with a significant correlation found between intratumoral and peritumoral MC density (r=0.879, p=0.009). For this type, a strong significant correlation was also found between peritumoral MC density and LMVD (r=0.804, p=0.029) (Figure 3b)

No correlation was found for the unclassified type of breast cancer but this result should be re-evaluated due to the small number of cases analyzed in this study.

Discussion

Among stromal components, MCs remain one of the most controversial players (15) in the pathogenesis of mammary gland malignant transformation (16). In normal breast tissue, MCs can be found as cellular components of both intralobular and interlobular stroma but with different densities, having an increased number in the interlobular stromal compartment.

Breast cancer tumor stroma differs from its normal counterpart. MCs and cancer-associated fibroblasts (CAFs) crosstalk and influence each other in the process of tumor stroma development. CAFs turn to express podoplanin, a mucin-type glycoprotein which promotes cancer cell migration and invasiveness (17) and is involved in tumor lymphangiogenesis. Higher levels of (IL6) secreted by CAFs induce an increased number of MCs and stimulate tumor growth and invasion (18). Activated MCs become de-granulated and release (MMP1), (ADAM17), (ADAM19), tryptase, heparin and IL6, factors involved not only in the remodeling of the tumor microenvironment (11, 19, 20) but also in the malignant epithelial cell phenotype (11, 21).

Association between MCs and LVs was suggested a long time ago by many authors for various normal animal tissues such rat mesentery (22) or corneal limbus (23), but the role of MCs in tumor pathogenesis is still controversial.

Most of the observations about crosstalk between MCs and lymphatics were found and described in the conventional pathological types of breast cancer, especially for invasive ductal carcinoma and inflammatory breast cancer.

Previous data reported that MCs had a different behavior in the highly hormone-responsive (HHR, ER+/PR+) invasive

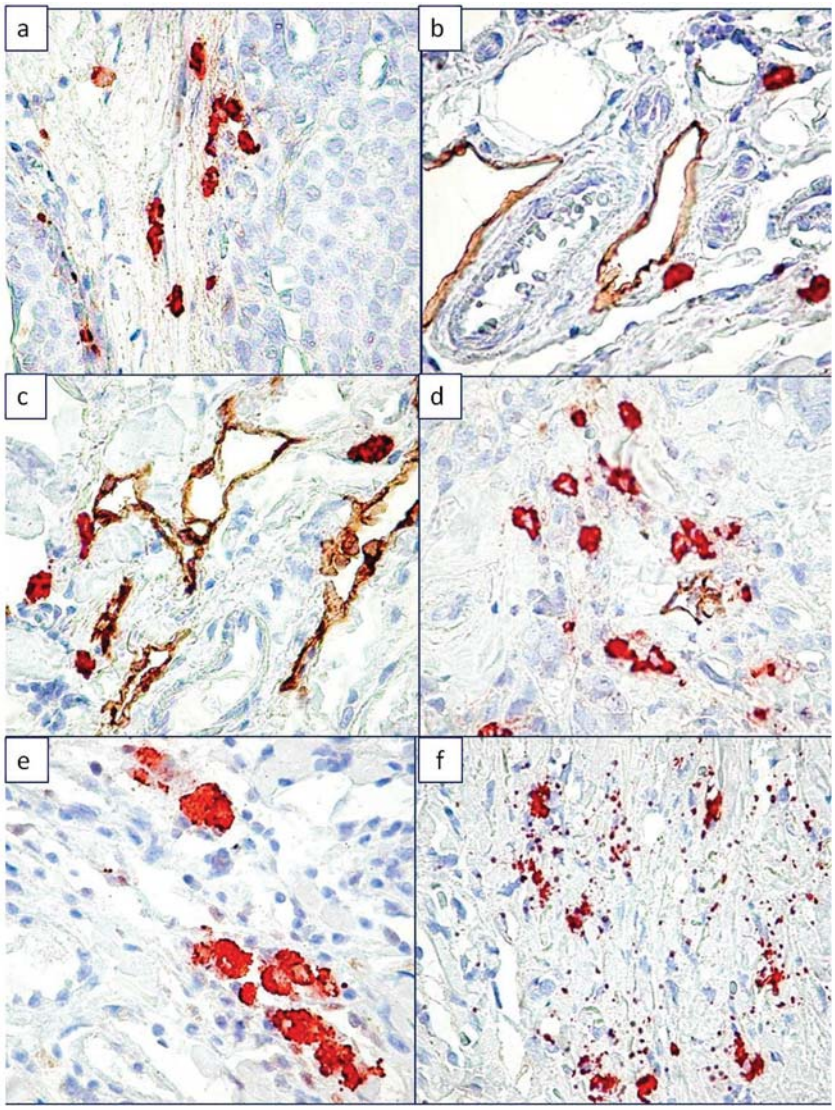

Figure 2. *Interrelations between mast cells (MCs), lymphatic vessels (LVs) and tumor mass in molecular subtypes of breast cancer. Although the unclassified type has fewer or no peritumoral lymphatic and a high number of peritumoral MCs with a high tendency to invade the tumor (a), luminal A-type has both a high number of peritumoral MCs and LVs (b), which explains the significant correlation found in the present study. Differences between the morphology and density of peritumoral and intratumoral LVs and MCs; Note the high number of LVs with sprout-like structures and split lumen together with numerous MCs in the peritumoral area (c) compared with the intratumoral area which had small LVs surrounded by normal and de-granulated MCs (d). Granulated (e) and de-granulated (f) MCs in molecular subtypes of breast cancer as a factor which might influence lymphovascular metastasis are shown.*

ductal carcinoma compared with minimally hormone-responsive (MHR, ER–/PR–) invasive ductal carcinoma. In a study performed by della Rovere *et al*. (24), the number of MCs was found to be higher in the peritumoral area of ER+/PR+ invasive ductal carcinoma cases and this finding was considered as a favorable prognostic factor in breast cancer. The authors also suggested a potential involvement of MCs in tumor angiogenesis. In the molecular classification of breast cancer, HHR cases partially correspond to luminal the type-A molecular profile. By immunohistochemical assessment of MCs in the molecular subtypes of breast cancer, we noticed a high number in the peritumoral area of luminal A breast cancer cases. Moreover, the significant correlation found in the present study between MCs and LVs in the peritumoral area from luminal type-A breast carcinomas strongly supports the involvement of MCs in the mechanism of tumor lymphangiogenesis and lymphovascular invasion. Recently, a few articles described

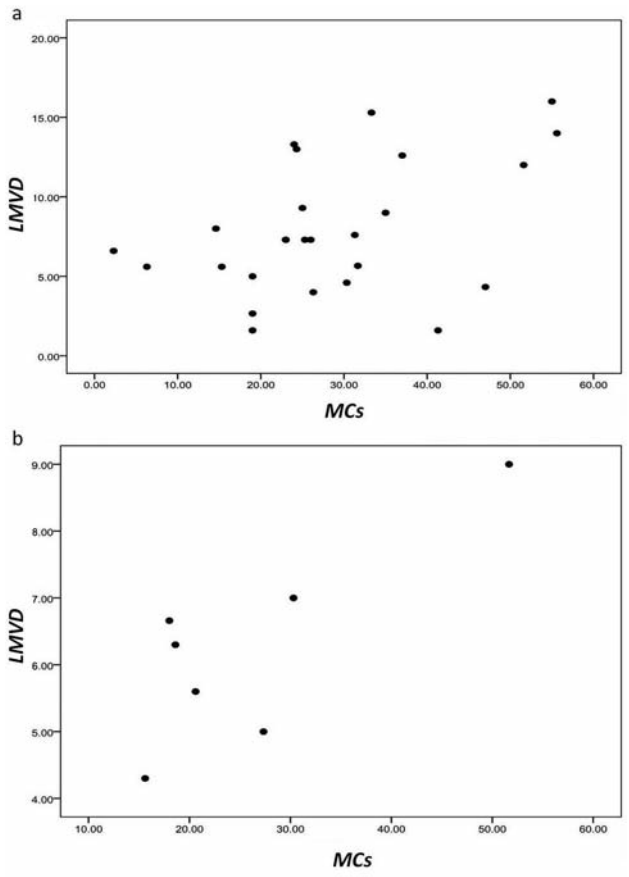

Figure 3. *Correlation between (MCs) and (LVs) in luminal A (a) and basal-like (b) types of breast cancer.*

the role of MCs in lymphangiogenesis from pre-malignant or malignant lesions (25), allergic and asthmathic conditions (26, 27) and aortic valvular stenosis (28). In breast cancer, indirect evidence suggests the role of MCs in the development of nodal metastasis (12, 14).

MC granules derived from tumor stromal MCs degranulation are able to penetrate the LV wall, trafficking to the sentinel lymph node and induce nodal lymphangiogenesis, most probably by a TNFα-mediated inflammatory mechanism (29) and/or by VEGF-C release.

Molecular subtypes of breast cancer have a different organ-specific preference for metastasis, but for all of them the most favored route for this process is lymphovascular invasion (30). Our data support these findings concerning the increased number of peritumoral and intratumoral LVs in all types of breast cancer. Among molecular subtypes of breast cancer, basal-like tumors have a higher rate of brain, lung, and distant nodal metastases. In the present study, we have shown three significant correlations concerning MC density and LMVD for basal-like breast cancer type. These findings support, in part, the nodal metastases preference for basal like tumors and also suggest that MCs are strongly involved in the development of the lymphovascular metastatic route.

Several studies reported the favorable prognostic role of MCs in breast cancer (31, 32). But Galinsky and Nechushtan (33) demonstrated that MCs have a positive or negative impact on tumor prognosis strictly dependent on tumor type. We reported here that MCs-tumor LVs crosstalk is specific for each molecular subtype of breast cancer and this can influence lymphovascular invasion dependent on each molecular tumor type.

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