

Angiogenesis in the Bone Marrow of Patients with Breast Cancer

Mariana Chavez-MacGregor,¹ Alejandro Aviles-Salas,² Dan Green,³ Adolfo Fuentes-Alburo,¹ Celedonio Gómez-Ruiz,² and Alvaro Aguayo³

Abstract Purpose: Pathologic angiogenesis has been correlated with tumor growth, dissemination, metastasis, and prognosis in solid tumors including breast cancer. Angiogenesis has also been implicated in the pathophysiology of, and shown to be a therapeutic target in tumors arising in the bone marrow. The status of angiogenesis in the bone marrow of breast cancer patients is unknown. The aim of this study was to estimate the extent of bone marrow angiogenesis in this subset of patients.

Experimental Design: We studied 42 women with breast cancer in whom a bone marrow biopsy was done. Bone marrow samples were sorted according to their infiltration status by breast cancer cells. In all bone marrow sections, blood vessels were highlighted by staining endothelial cells with an antibody directed against the CD34-related antigen. A hematopathologist blind to the status of infiltration of breast cancer did the bone marrow vessel count.

Results: Nineteen patients (45%) had bone marrow metastasis. The bone marrow microvessel density was significantly higher in patients with bone marrow metastases compared with patients without bone marrow metastases ($P < 0.0005$). Median bone marrow microvessel density was 2 for the negative bone marrow group, and 15 for the positive bone marrow group. An increased microvessel density was correlated with presence of disease at last follow-up.

Conclusions: This is the first study showing that bone marrow microvessel density is significantly higher in breast cancer patients with bone marrow metastases, when compared with breast cancer patients without evidence of bone marrow metastatic disease. Further research is needed to shed light into the prognostic and therapeutic relevance of this finding.

Angiogenesis refers to the formation of new blood vessels from an existing blood vessel network and occurs physiologically during embryonal growth, wound healing, and in the female genital tract during the menstrual cycle (1).

The role of abnormal, increased angiogenesis in the development and spread of cancer is well recognized (2). Growing evidence considers angiogenesis as an essential step in the growth of primary tumors and its metastatic potential (3), leading to the demonstration of a direct correlation between abnormal tumor vascularity and aggressiveness in solid tumors (4). Angiogenesis must occur to provide nutrients and oxygen to the growing tumor mass. It involves a multistep process that includes degradation of extracellular matrix proteins, activation, proliferation, and migration of endothelial cells and pericytes in which interaction of several positive and negative regulatory molecules are involved (5).

Recent studies have shown that angiogenesis is increased in bone marrow biopsies of patients with hematologic malignancies. This phenomenon suggests a dysregulation of the angiogenic growth factors produced by the tumor and/or accessory cells, leading to various models of autocrine and paracrine mechanisms of tumor aggressiveness (6–8).

Measurement of intratumoral microvessel density by immunohistochemistry seems to be a reliable method for measuring angiogenic activity. Anti-CD34 antibody is a marker of immature blood vessels, and is currently a widely used endothelial marker (9). In recent studies, increased microvessel density in areas of the primary tumor where vascularization was most intense (hotspots) showed to be a significant and independent prognostic indicator in early-stage breast cancer. There are also data supporting the hypothesis that evaluation of angiogenic activity in individual specimens might distinguish between indolent and aggressive tumors (10, 11), suggesting, at least in part, that breast cancer is an angiogenesis-dependent disease (12).

Identification of bone marrow metastases in breast cancer patients has been correlated with tumor size, lymph node involvement, and tumor grade (13). Gross metastatic and micrometastatic disease to the bone marrow is present in up to 25% to 36% of breast cancer patients and is associated with a significantly higher risk of distant metastases and death from cancer causes (14).

The status of angiogenesis within the bone marrow of patients with solid tumors is unknown. The aim of this study was to estimate the extent of bone marrow angiogenesis in patients with breast cancer. We hypothesized an increased microvessel density in the bone marrow of breast cancer patients with bone

Authors' Affiliations: Departments of ¹Breast Tumors and ²Pathology, Instituto Nacional de Cancerología and ³Department of Hematology and Oncology, Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán," Mexico, Mexico
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Requests for reprints: Mariana Chavez-Mac Gregor, Department of Breast Tumors, Instituto Nacional de Cancerología, Av. San Fernando 22 Col. Sección XVI, CP 14080, Mexico D.F., Mexico. Phone: 52-55-56456060; Fax: 52-55-56456060; E-mail: mariana.chavez@prodigy.net.mx.

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marrow metastases when compared with the microvessel density in the bone marrow of breast cancer patients without bone marrow metastases.

Patients and Methods

We evaluated breast cancer patients from the Breast Tumors Department at the Instituto Nacional de Cancerología in Mexico City, a tertiary-care, national referral-teaching center for cancer patients. Fifty-seven breast cancer patients were detected as having bone marrow biopsy done between 1998 and 2001. Fifteen samples were excluded (13 due to lack of adequate tissue, 2 because of hematologic malignancy). Charts of the remaining 42 evaluable patients were reviewed. Staging workup at diagnosis included physical examination, liver ultrasound, chest X-ray, bone scan, bone X-rays, blood chemistry, cell blood count, liver function tests, carcinoembryonic antigen, and Ca 15-3. The H&E-stained sections from each corresponding breast cancer biopsy were reviewed and the histologic diagnosis of bone marrow involvement by breast cancer cells was confirmed. Approval of the study by the Instituto Nacional de Cancerología Review Board was obtained.

Immunohistochemistry was done on formalin-fixed, paraffin-embedded bone marrow sections using the streptavidin-biotin peroxidase method. Briefly, the samples were stained with cytokeratin panel anti-AE1/AE3 (DAKO, Co., Carpinteria, CA) work dilution of 1:50 to evaluate the presence of micrometastases. Bone marrow samples were sorted as follows: In one group, we included bone marrow biopsies with evidence of breast cancer infiltration by H&E staining and/or cytokeratin-positive cells (positive bone marrow group), and in the other group we included bone marrow biopsies without breast cancer infiltration (negative bone marrow group).

In all bone marrow sections, blood vessels were highlighted by staining endothelial cells with an anti-CD34-related antigen antibody (anti-CD34rAg; DAKO) work dilution of 1:50. Microvessel density was evaluated in CD34-stained sections essentially as described by Perez-Atayde et al. (15); however, no computer assistance was used in this evaluation. A hematopathologist blind to the status of infiltration of breast cancer did the bone marrow vessel count. Microvessels were defined by their typical histologic appearance and reactivity with anti-CD34rAg, blood vessels were counted in 10 fields whenever possible, and the microvessel density was expressed as the mean number of microvessels per field.

Data analysis. Descriptive statistics methods were used to study population characteristics. Microvessel density was considered as having non-normal distribution; results of microvessel density by group are expressed together with 95% confidence intervals (95% CI). To analyze the microvessel density differences between groups, a nonparametric test was selected (Mann-Whitney test), and to calculate confidence intervals a *t* test was done. A multivariate model was developed, and adjustments were made to account for possible confounders. Odds ratios (OR) are presented, together with 95% CI values. Spearman's test was used to find correlations among microvessel density, and laboratory values at the time the bone marrow biopsy was done. Kruskal-Wallis test was used to compare the microvessel density in the bone marrow with stage at diagnosis and status at last follow-up. Differences were considered statistically significant at $P < 0.05$. The Statistical Package for Social Sciences version 11.5 was used to perform the analysis and all tests were two sided.

Results

Forty-two patients were studied and grouped according to their bone marrow status. Patient characteristics are listed in Table 1 and previous treatment strategies are summarized in Table 2.

The presence of bone marrow metastases was confirmed in 19 patients (45%); in 15 patients, the diagnosis was made in H&E sections and in four by cytokeratin staining. No differ-

ences in the characteristics of the patients were found between infiltrated biopsies diagnosed by H&E staining or by cytokeratin-positive cells.

The bone marrow microvessel density was significantly higher in patients with bone marrow metastases compared with patients without bone marrow metastases ($P < 0.0005$). Median bone marrow microvessel density was 2 (95% CI, 1.84-4.94) for the negative bone marrow group, and 15 (95% CI, 12.55-18.18) for the positive bone marrow group (Fig. 1). Figure 2 shows examples of expression of anti-CD34rAg in bone marrow sections. Considering the differences among groups, a multivariate model was developed to adjust for possible confounders, such as stage at diagnosis, surgery, and number of chemotherapy cycles received before the bone marrow biopsy was done. The crude and the adjusted OR for the bone marrow positive group was 1.68 (95% CI, 1.244-2.28) and 2.02 (95% CI, 1.17-3.48) respectively.

Bone marrow microvessel density was statistically different with respect to last follow-up status ($P = 0.050$) and stage at

Table 1. Patient characteristics according to bone marrow status

	Negative BM, n (%)	Positive BM, n (%)	All patients, n (%)
Age (mean, SD), <i>n</i> = 42	44.82 ± 9.93	39.41 ± 12.07	43.38 ± 11.15
Menopausal status, <i>n</i> = 42			
Premenopausal	14 (60.9)	13 (68.4)	27 (64.3)
Postmenopausal	9 (39.3)	6 (31.6)	15 (35.7)
Stage at diagnosis, <i>n</i> = 42			
II	11 (47.8)	2 (10.5)	13 (30.9)
III	10 (43.5)	6 (31.6)	16 (38.1)
IV	2 (8.7)	11 (57.9)	13 (31)
BC histology, <i>n</i> = 42			
Ductal	17 (73.9)	17 (89.5)	34 (81)
Lobular	4 (17.4)	2 (10.5)	6 (14.2)
Other	2 (8.7)		2 (4.8)
Tumor grade (SBR), <i>n</i> = 32			
Well differentiated (3-5)	2 (11.1)	1 (7.1)	3 (9.4)
Moderately differentiated (6-7)	8 (44.4)	4 (28.6)	12 (37.5)
Poorly differentiated (8-9)	8 (44.4)	9 (64.3)	17 (53.1)
Estrogen receptors, <i>n</i> = 31			
Negative, 0	9 (47.4)	5 (41.7)	14 (45.2)
Positive, +, ++	9 (47.4)	7 (58.3)	16 (51.6)
Positive, +++	1 (5.3)		1 (3.2)
Progesterone receptor, <i>n</i> = 31			
Negative, 0	9 (47.4)	6 (50)	15 (48.4)
Positive, +, ++	9 (47.4)	5 (41.7)	14 (45.2)
Positive, +++	1 (5.3)	1 (8.3)	2 (6.5)
HER2NEU, <i>n</i> = 22			
Negative	9 (69.2)	5 (55.6)	14 (63.6)
Positive	4 (30.74)	4 (44.4)	8 (36.7)

Abbreviations: BM, bone marrow; BC, breast cancer; SBR, Scarf Bloom Richardson.

Table 2. Treatment according to bone marrow status

	Negative BM, n (%)	Positive BM, n (%)	All patients, n (%)
Neoadjuvant chemotherapy	14 (60.9)	8 (42.1)	22 (52.4)
Surgical treatment (n = 42)			
None	3 (13)	7 (36.8)	10 (23.8)
Segmentectomy	2 (8.7)	2 (10.5)	4 (9.5)
Modified mastectomy	10 (43.5)	9 (47.4)	19 (45.2)
Radical mastectomy	8 (34.8)	1 (5.3)	9 (21.4)
Positive lymph nodes (n = 29)			
0	5 (26.3)	2 (20)	7 (24.1)
1-3	8 (42.1)	3 (30)	11 (37.9)
4-10	5 (26.3)	2 (20)	7 (24.1)
>10	1 (5.3)	3 (30)	4 (13.8)
Adjuvant chemotherapy (n = 42)	17 (73.9)	9 (47.4)	26 (61.9)
Palliative chemotherapy (n = 42)	14 (60.8)	16 (84.2)	30 (71.4)
No. chemotherapy cycles before BM biopsy (n = 42)			
0	1 (4.3)	1 (5.3)	2 (4.8)
4-6	11 (47.8)	6 (31.6)	17 (40.5)
7-10	5 (21.7)	6 (31.6)	11 (16.2)
>10	6 (26.1)	6 (31.6)	12 (28.6)
Radiation therapy (n = 42)	20 (87)	15 (78.9)	35 (83.3)
Hormone therapy (n = 42)	7 (30.4)	6 (31.6)	13 (31)

diagnosis, being higher in patients with advanced stages ($P = 0.007$). An inverse significant correlation was found between bone marrow microvessel density and hemoglobin levels ($r = -0.384$, $P = 0.006$), as well as with platelet count ($r = -0.267$, $P = 0.044$) at the time of bone marrow biopsy. A direct correlation between bone marrow microvessel density was found with serum Ca 15-3 levels ($r = 0.275$, $P = 0.048$). No correlation was found with WBC.

We compared the mean serum values of different laboratory data at the time of bone marrow biopsy. For the negative and the positive group, the mean value of hemoglobin (g/dL) was 10.86 versus 9.30 ($P = 0.175$), for platelets ($\times 10^9/L$) was 169.6 versus 139.5 ($P = 0.485$), and for WBC ($\times 10^3/mm^3$) was 3.06 versus 5.33 ($P = 0.011$), respectively. The carcinoembryonic antigen levels (ng/mL) in the bone marrow-negative group were significantly lower than in the bone marrow-positive group (39.46 versus 139.35, $P = 0.006$); no differences were found in Ca 15-3 (ng/mL) serum levels (247.9 versus 210.5, $P = 0.330$).

Median time from breast cancer diagnosis to bone marrow biopsy was 21.4 months (95% CI, 20.8-45.7); the indication to perform the bone marrow biopsy in this subset of patients was spontaneous cytopenia in 32 cases (56.2%), persistent post-chemotherapy cytopenia in 3 (7.1%), bone metastases in 2 (2.4%), and in 5 patients (11.9%) due to other reasons.

Median time of follow-up was 33.61 months (95% CI, 31.1-56.2). During that period, 9 patients in the negative bone marrow group had stable disease and 14 had progressive or

recurrent disease. In the positive bone marrow group, all patients had (other than bone marrow) progression or recurrence ($P = 0.006$). At the time of the analysis, 7 (31%) patients without bone marrow metastases were alive without evidence of disease; 10 (43%) were alive with disease and 6 (23%) died because of cancer-related causes. In the group with bone marrow metastases, 5 (26%) had evidence of disease at last follow-up, and 14 (74%) had died because of cancer-related causes.

Discussion

To the best of our knowledge, this is the first study looking into the status of angiogenesis in bone marrow of patients with breast cancer.

Our data showed that bone marrow microvessel density is significantly higher in patients with bone marrow metastases, compared with patients without evidence of bone marrow metastatic disease. These results are consistent with previous studies regarding hematologic malignancies (primary bone marrow malignancies) showing increased bone marrow angiogenesis. Perez-Atayde et al. (15) studied 61 bone marrow biopsies of 40 children with untreated acute lymphoid leukemia and 10 control biopsies, finding a significantly higher bone marrow microvessel density in acute lymphoid leukemia patients. Increased vascularity has been reported in bone marrow samples from patients with acute myeloid leukemia (16), myelodysplastic syndromes, chronic leukemias (17), multiple myeloma (18), and mastocytosis (19). These observations suggest that angiogenesis may have a role in the pathophysiology of hematologic malignancies, and its presence can be considered as a poor prognostic marker associated with both, progression-free and overall survival (20, 21). Angiogenesis may also be related to disease progression, Rajkumar et al. (22) showed that microvessel density progressively increases along the spectrum of plasma cell disorders.

Although angiogenesis is increased in bone marrow of hematologic malignancies, and according to our study, in

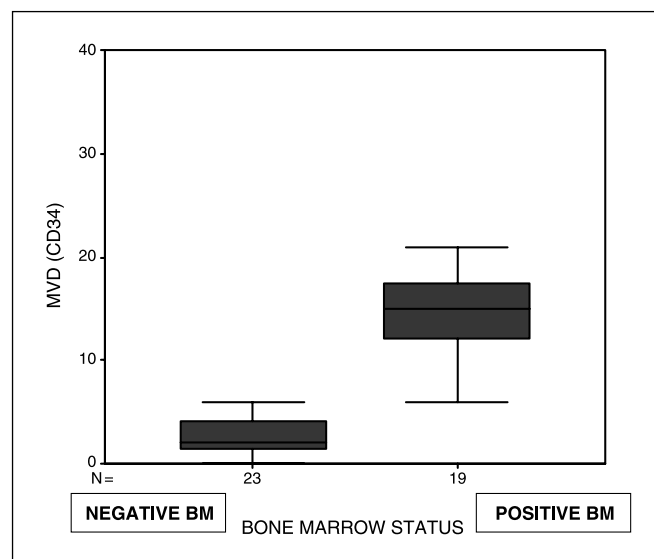
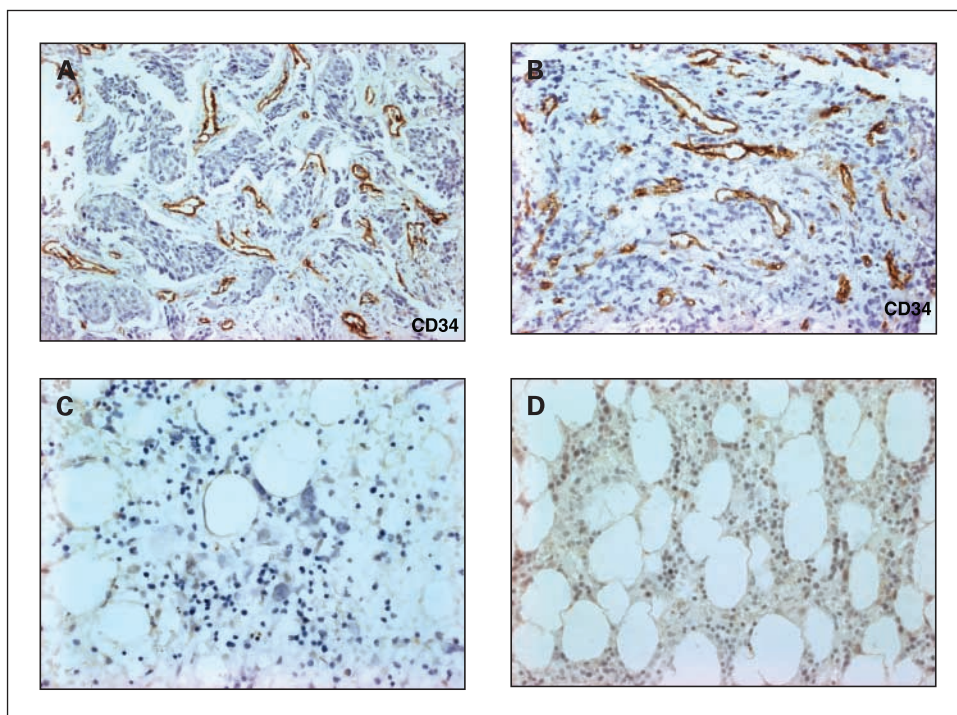


Fig. 1. Comparison of microvessel density according to bone marrow status. The microvessel density was statistically different among groups ($P < 0.0005$). In the negative bone marrow group, the median microvessel density was 2, compared with a microvessel density median of 15 in the positive bone marrow group.

Fig. 2. Representative areas of bone marrow sections infiltrated by ductal (A) and lobular breast carcinoma (B). All blood microvessels were highlighted with immunohistochemical CD34 staining. Microvessels are represented by brown staining, which stand out sharply from neoplastic tissue. Bone marrow sections of patients with ductal (C) and lobular (D) breast carcinoma without bone marrow infiltration.



breast cancer patients with bone marrow metastatic disease, the biological mechanism that explains this phenomenon is still unclear. A likely explanation to these observations would be the secretion of angiogenic factors by tumor cells, which, in turn, stimulate the development of new vessel beds as a mechanism of self-survival (6–8). The presence of angiogenic factors and their receptors has been shown in the bone marrow microenvironment (i.e., stromal cells, endothelial cells, and leukemic blasts), suggesting the involvement of both an autocrine and a paracrine loop, giving the cancer cells a survival advantage (23).

Within the bone marrow, angiogenic factors secreted by the malignant clone interact with other mechanisms, including differentiation, apoptosis, self-renewal, and inflammatory cytokine elaboration (24). We believe that similar mechanisms to those observed in primary bone marrow malignancies may also explain the behavior of breast cancer cells when they reach the bone marrow microenvironment.

Retrospective studies published in the 1990s showed that angiogenesis has an important role as a new prognostic indicator in early-stage breast cancer. It has also been shown that invasive breast cancer has a well-defined stromal vascular component and produces angiogenic factors (25). Braun et al. (26) found a similar bone marrow metastases rate in breast cancer patients with lymph node metastases and in those without it. This observation suggests that lymph node metastases are not necessarily associated with hematogenous spread of cancer, and that such dissemination of tumor cells in the bone marrow can be the origin of later recurrence.

In our series, most bone marrow metastases were found in patients that had received previous chemotherapy. Whether patients with high microvessel density in the bone marrow respond differently to chemotherapy remains to be studied. Angiogenesis may be a critical control point in tumor growth (27); our work raises the possibility of using angiogenesis inhibitors in addition to standard treatment as a novel

therapeutic strategy for breast cancer patients with bone marrow metastasis.

In our study, we found that bone marrow microvessel density was statistically different with respect to stage at diagnosis and with last follow-up status. We also observed that all patients in the positive bone marrow group had (other than bone marrow) progression or recurrence. Further research is needed to explore the relation between bone marrow angiogenesis in breast cancer patients and overall survival as well as disease-free survival; to determine whether bone marrow angiogenesis in breast cancer patients is an independent prognostic factor.

Our results should be interpreted with caution, taking into account the small sample size, which is understandable because bone marrow biopsies are not a standard procedure in breast cancer patients. Therefore, in this subset of patients, a possible selection according to the bone marrow biopsy indication cannot be ruled out. In the multivariate analysis, we could only include the most important possible confounders to build a stable model. Further research needs to be done to confirm these observations in larger samples and with molecular biology techniques, such as microarrays, reverse transcription-PCR, and protein studies. We are studying the expression of different endothelial and vascular markers in the bone marrow of breast cancer patients, as well as the relationship between angiogenesis in the bone marrow and the primary tumor.

In summary, our study shows for the first time that breast cancer bone marrow metastases are associated with an increased microvessel density. This observation may have implications for the understanding of breast cancer pathophysiology and the development of bone marrow metastatic disease. Our study also provides additional basis and rationale for initiating future studies on angiogenesis in patients with solid tumors and bone marrow metastases.

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