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Microvascular Density of Breast Cancer in Bone Metastasis: Influence of Therapy

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Abstract. *Background: Bone is the most frequent site of systemic progression of breast cancer (BRC). Angiosuppressive therapy has now entered the management of progressing cancers, therefore it is clinically important to obtain information on vascular endothelial growth factor (VEGF) expression and microvessel density (MVD) of bone metastasis of BRC. Materials and Methods: VEGF expression and MVD were evaluated in bone metastases of BRC immunohistochemically in paraffin samples of 18 patients and compared to their primary tumors. MVD was determined by using the hot-spot method and the endothelial marker, CD34. Results: Chemo- and/or endocrine therapy-naïve BRC cases progressed to the bone with a concomitant increase in their angiogenic potential, suggesting that this is the "natural history" of BRC progression. On the other hand, this study revealed that vascularization of the bone metastases of BRC patients that had received adjuvant (chemo- and/or endocrine) therapy was significantly decreased compared to the corresponding primary tumors, also supported by a decreased VEGF expression in metastases, both suggesting that the treatment significantly affected the angiogenic phenotype of the progressing disease. Conclusion: Angiosuppressive therapy is a new approach to cancer management including BRC and is frequently applied in the advanced stage of disease. Tumors with a prominent angiogenic phenotype (high MVD and VEGF) are primary candidates for such regimes. The fact that chemo-endocrine adjuvant therapy of BRC resulted in a weaker angiogenic phenotype in bone metastases compared to non-treated cases may suggest that these latter patients are better candidates for angiosuppressive interventions.*

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Breast cancer (BRC) is now considered to be a systemic disease independent of our ability to detect disseminated cells (1), since hematogenous progression can start at a microscopic size of 10^6 tumor cells. This potential depends on a unique genetic feature of breast cancer cells, the metastatic signature (2), which can now discriminate non-metastatic primary tumors from metastatic ones. Survival of BRC patients strongly depends on the size, grade and estrogen receptor (ER) status of the primary tumor as well as the site of metastasis. Bone is the most frequent site of systemic progression of BRC and the survival of these patients is much better than any other form of metastatic disease, excluding regional lymph nodes (3).

Parathyroid hormone-related (PTHrP) protein expression in BRC cells is a significant mediator of bone metastatic potential, which is regulated by TGFb produced by osteoclasts (4, 5). However, recent molecular analysis revealed that bone metastatic BRC have a unique genetic signature characterized by an overexpresion of HIF1a (6), suggesting that regulation of hypoxia and, consequently, angiogenesis may play an important role in bone metastatization of BRC.

The microvessel density (MVD) of BRC was equivocally shown to be a promising new negative prognostic marker (7, 8). Neoangiogenesis in BRC depends on the expression of angiogenic cytokines aspecially vascular endothelial growth factor (VEGF), which is also a strong negative prognosticator (9). Meanwhile, it is not known how the angiogenic geno-phenotype and, consequently, the vascularization of the BRC metastases are altered at the metastatic site. Angiosuppressive therapy has now entered the management of progressing cancers (10, 11), therefore, information on the MVD of bone metastasis of BRC would have clinical impact.

Materials and Methods

Patients. Bone metastasis tumor samples were retrospectively selected from the pathology departments of three institutes (Departments of Orthopedics and Traumatology, Semmelweis University and National Institute of Traumatology, Budapest,

Table I. *Characteristiscs of patients and tumours.*

Case no.	Sex	Age (years)	Histology	Grade	pTNM	Therapy	Time to progression (months)	X-ray morphology of bone metastases	ER status in bone metastasis	ER status in primary tumours
1	female	44	ductal invasive cc.	3	pT3N0M0	E	211	lytic	-	-
2	female	45	ductal invasive cc.	2	pT1cN1Mx	C	138	lytic	-	-
3	female	54	ductal invasive cc.	3	pT2N1M1	CE	2	lytic	-	-
4	female	35	ductal invasive cc.	3	pT2N0M1	CE	10	lytic	-	-
5	female	47	ductal invasive cc.	2	pT2N1M0	E	19	lytic	+	-
6	female	50	lobular invasive cc.	2	pT2N1M0	CE	22	mixed	+	-
7	female	53	ductal invasive cc.	3	pT2N0M0	CE	14	lytic	+	-
8	female	66	ductal invasive cc.	3	pT2N1M0	E	20	lytic	-	-
9	female	51	ductal invasive cc.	2	pT2N1M0	no	80	mixed	+	+
10	female	72	lobular invasive cc.	2	pT2N0M0	no	35	lytic	+	+
11	female	56	ductal invasive cc.	1	pT2N0M0	no	195	lytic	+	+
12	female	57	ductal invasive cc.	3	pT3N1M1	no	33	mixed	+	-
13	female	64	ductal invasive cc.	3	pT2N1M0	no	21	lytic	+	+
14	female	47	ductal invasive cc.	2	pT3N1M0	no	76	lytic	+	-
15	female	48	ductal invasive cc.	2	pT2NxM1	no	2	lytic	+	-
16	female	53	ductal invasive cc.	3	pT1cN1M0	no	29	lytic	+	-
17	male	66	ductal invasive cc.	2	pT3NxM0	no	27	lytic	+	-
18	female	52	ductal invasive cc.	3	pT1bNxM0	no	10	lytic	+	-

ER: oestrogen receptor
E: endocrine therapy
C: chemotherapy
CE: chemo- and endocrine therapy

Hungary). The samples were open biopsies of bone metastases obtained during transfocal stabilization of impending or completed pathological fractures, or resected bone metastases.

Eighteen BRC patients were selected, from whom the primary tumour and its metachronous bone metastases were available. Informed consent was obtained from all patients. Necrotic areas were found in 8 out of 18 primary BRCs and in 5 out of 18 bone metastatic BRCs. The characteristics of the patients included in the study are shown in Table I.

Immunohistochemistry. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded 4-μm sections, using the avidin-biotin-peroxidase method. Staining for vascular endothelial cells was performed using a monoclonal mouse anti-human CD34, Class II antibody (DAKO, Glostrup, Denmark), at a 1:40 dilution in Tris-buffered saline (TBS). For the detection of oestrogen receptors (ER) in primary and metastastic BRC, a mouse monoclonal antibody against ER (Novocastra Laboratories, Newcastle-upon-Tyne, UK) was applied at a 1:40 dilution in TBS on adjacent sections. Staining for VEGF in primary and metastatic BRC cases was performed on adjacent sections using a polyclonal goat anti-human VEGF antibody (R&D Systems, Abingdon, UK), at a dilution of 1:80 in phosphate-buffered saline (PBS).

After deparaffination and rehydration, endogenous peroxidase was blocked by incubation with 1.5% hydrogen peroxide in methanol. Epitope retrieval was achieved in citrate buffer (0.1 M, pH 6), using a microwave oven for the detection of CD34-positive vessels and VEGF, as previously described (12), or by using a pressure cooker for the detection of ER.

After washing in TBS, the sections were blocked with 3% bovine serum albumin (BSA) for 30 min to inhibit non-specific immunoreactivity, followed by an overnight incubation at 4˚C with anti-CD34 or anti-ER antibody. The bound antibodies were detected using the avidin-biotin complex/horseradish peroxidase (HRP) (LSAB2 kit, DAKO) for the detection of CD34-positive vessels and ER, or by using the HRP-AEC System Goat Kit (R&D Systems) for the detection of VEGF, according to the manufacturers' instructions, and finally visualized using 3-amino-9-ethylcarbazole (AEC) (Vector Laboratories, Inc., Burlingame, CA, USA). Counterstaining was performed with haematoxylin. As negative controls, we used non-immune IgG1 immunoglobulin instead of primary antibodies or omitted primary antibody.

Assessment of microvessel density. Intratumoral MVD was determined according to previously described guidelines. (13) The areas containing the greatest numbers of microvessels (vascular hot-spots) were identified by scanning the stained sections at low magnification using a light microscope (Olympus B061, Olympus Optical Co. Ltd., Tokyo, Japan). Once these areas were recognized, individual stained microvessels were counted at x400 magnification using a square grid graticule. This corresponded to a field size of 0.0625 mm^2 (all figures in text are quoted per mm^2). Any CD34-positive endothelial cells or endothelial cell clusters clearly separated from adjacent microvessels, tumour cells and connective tissue elements were considered as single countable microvessels; branching structures were counted as one, unless there was a break in the continuity of the vessel, in which case it was counted as two distinct vessels. Three fields per tumour section were counted in the areas that appeared to contain the greatest number of microvessels on scanning at low magnification. MVD was defined as the mean score from all three fields/mm^2.

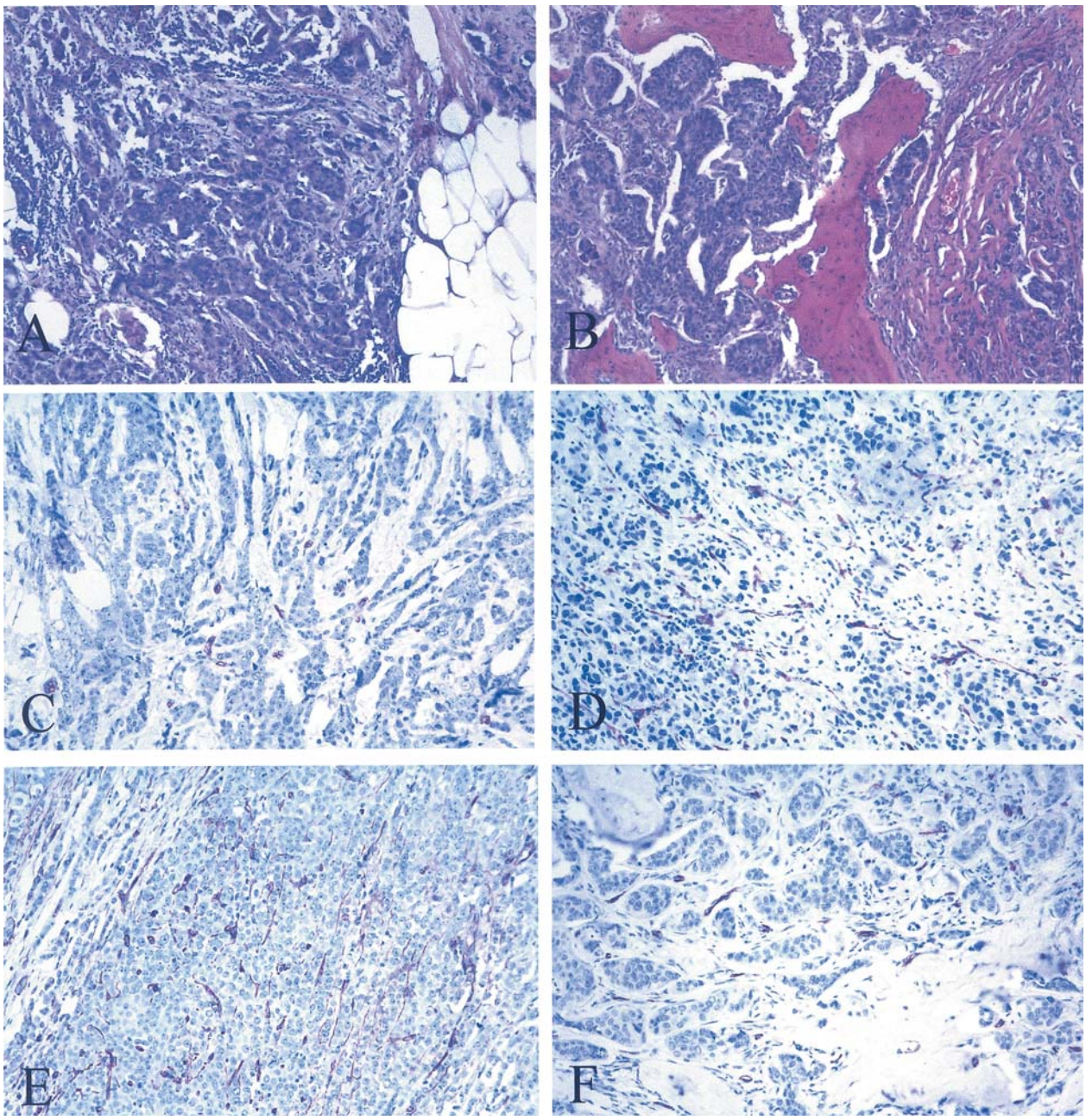

Figure 1. *Detection of microvessels in primary breast carcinoma as well as in bone metastasis using CD34 marker. (200x)*
A, B: H&E staining, A: primary tumour, B: bone metastasis. C, D: MVD in untreated patient. C: primary tumour, D: bone metastasis. E, F: MVD following chemo-endocrine therapy: E: primary tumour, F: bone metastasis.

Evaluation of VEGF expression. The intensity of VEGF staining of carcinoma cells was classified in two groups as previously described (12), with some modifications: absent-weak and moderate-strong.

Evaluation of ER expression. Determination of ER-labelling was performed qualitatively: tumours (primary as well as secondary) were defined as ER-positive if more than 10% of cells presented with a positive reaction (14).

Statistical analysis. Comparisons were made by Wilcoxon's rank sum test for paired variables and by Mann-Whitney *U*-test for unpaired variables. Spearman's rank-order correlation coefficient was used to assess the relationship between the vascularity of primary tumours and their matched bone metastases. Two-tailed p was considered significant when it was lower than 0.05. All analyses were undertaken using the SPSS 10. package for Windows.

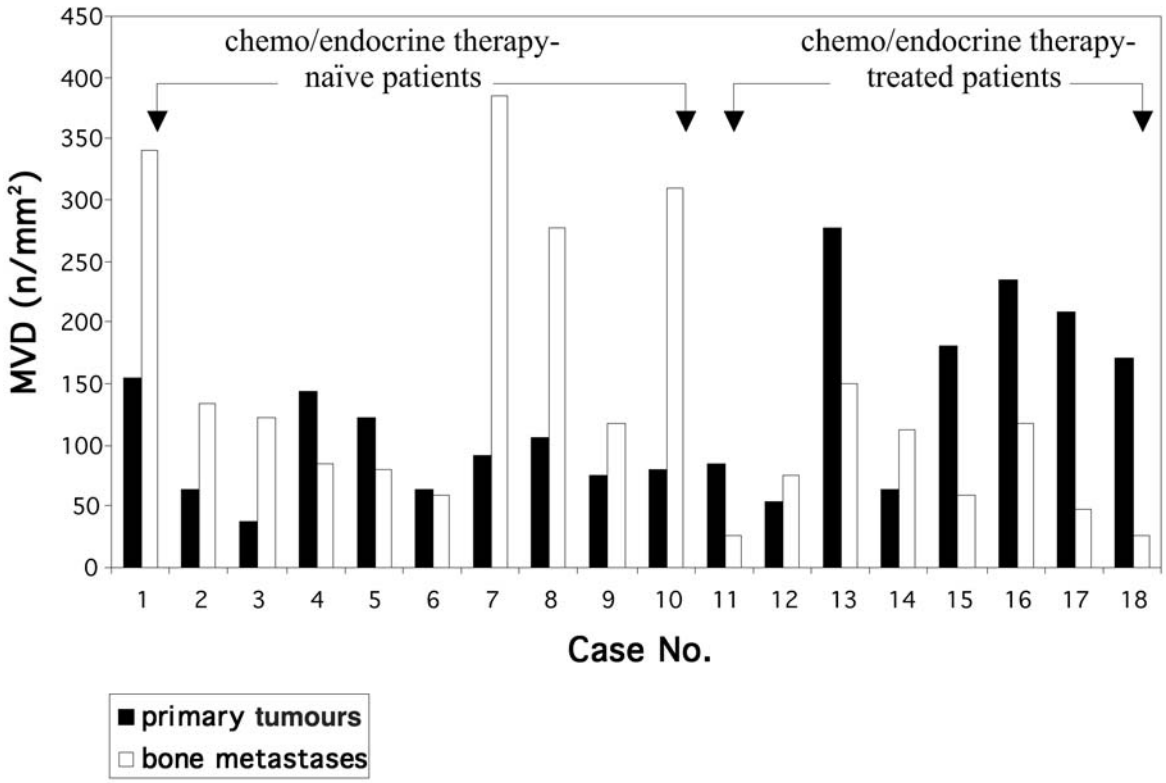

Figure 2. *Microvessel density (MVD) in primary breast cancers and their corresponding bone metastases. Data represent mean of MVD counted in three hot spots.*

Table II. *MVD and VEGF data of primary and bone metastatic BRC.*

Case No.	Therapy	MVD of the primary tumour (n/mm^2)	VEGF status of the primary tumour	MVD of the bone metastasis (n/mm^2)	VEGF of the bone metastasis
1	E	85.33	–	26.67	–
2	C	53.33	–	74.67	–
3	CE	277.33	+	149.33	–
4	CE	64.00	+	112.00	–
5	E	181.33	+	58.67	+
6	CE	234.67	+	117.33	–
7	CE	208.00	+	48.00	+
8	E	170.67	–	26.67	–
9	No	154.67	–	341.33	+
10	No	64.00	–	133.33	–
11	No	37.33	–	122.67	+
12	No	144.00	+	85.33	+
13	No	122.67	+	80.00	+
14	No	64.00	+	58.67	+
15	No	90.67	–	384.00	–
16	No	106.67	–	277.33	–
17	No	74.67	+	117.33	+
18	No	80.00	+	309.33	–

E: endocrine therapy;
C: chemotherapy;
CE: chemo- and endocrine therapy;
"–": absent/weak expression;
"+": moderate/strong expression

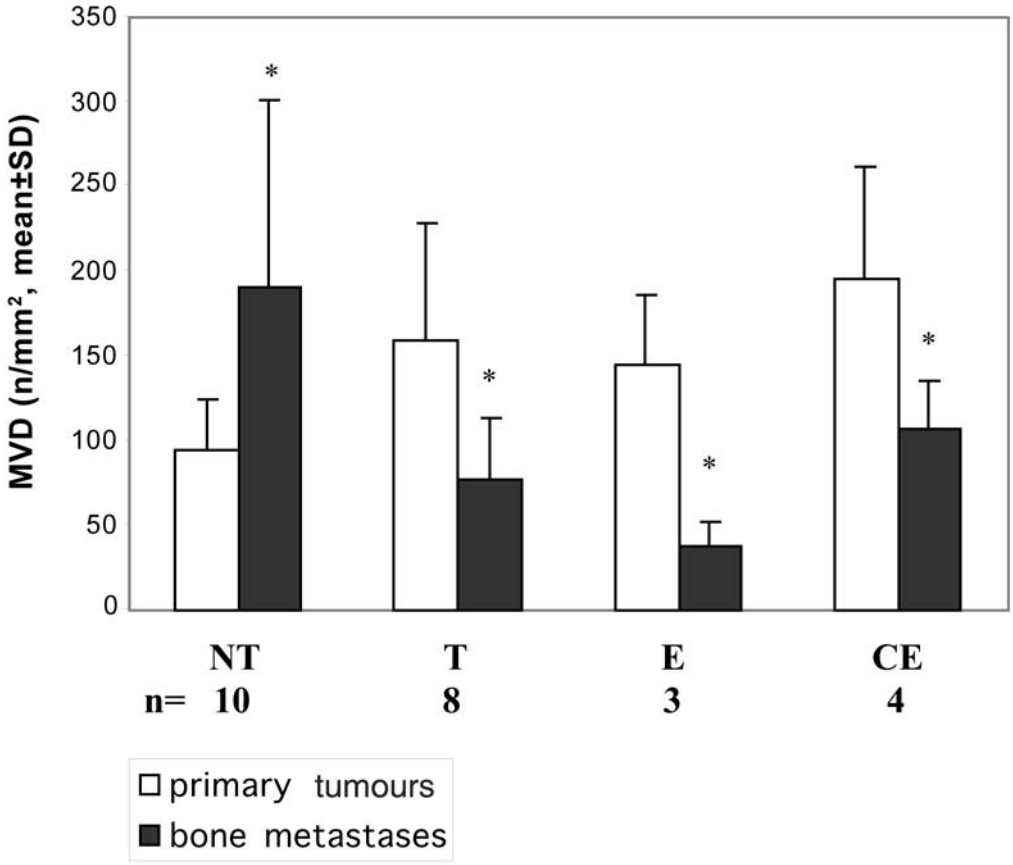

Figure 3. *Influence of therapy on microvessel density (MVD) of primary and bone metastatic breast cancers. Data are mean±SD of MVD in various groups of patients. NT: therapy-naïve, T: treated, E: endocrine therapy, CE: chemo-endocrine therapy. (*p<0.05)*

Table III. *Relation of VEGF status to MVD in primary and metastatic BRC.*

BRC	Primary	Metastatic
All cases (n=18)	10/18	8/18
Low density (<80/mm^2, n=7)	4/7	3/7
Medium density (80-160 mm^2, n=6)	2/6	3/6
High density (>160 mm^2, n=5)	4/5	2/5

Data represent incidence of VEGF+ (moderate/strong expression) cases.

Table IV. *VEGF status of primary and metastatic BRC in relation to therapy.*

BRC	Primary	Metastasis
All cases (n=18)	10/18	8/18
Non-treated (n=10)	5/10	6/10
Treated (n=8)	5/8	2/8

Data represent incidence of VEGF+ (moderate/strong expression) cases.

Results

Of the cohort of 18 BRC patients, 16 were ductal invasive carcinoma (DIC) samples, and 2 lobular invasive carcinoma (LIC) cases. The bone lesions were mostly lytic with a few mixed forms by X-ray analysis (Table I). The size of the metastatic lesions was significantly larger than the primary tumours. The majority of the primaries were ER+ (13/18), but the phenotype changed significantly in the bone metastases (4/18), in agreement with previous reports (Table I) (15).

CD34 immunostaining was performed on the primary tumours as well as the bone metastases (Figure 1) and the MVD was determined by morpho- metry using international standard (13). We considered the changes of MVD to be biologically relevant when a difference was greater than 30%.

The MVD of the primary BRCs was in the range of 37.3-277.7, while this feature was 26.7-384.0 n/mm^2 in bone metastases (Table II, Figure 2). The T stage or the ER status did not correlate with the MVD of the primary tumours (Tables I, II), while a higher MVD was detected in the node-positive primary BRCs, although this difference was not statistically

significant either. The incidence of modulation of MVD in bone metastases compared to the primary tumour was very frequent, however, both increase or decrease occurred with comparable frequencies. We established three BRC categories, based on the MVD values in the primary tumours as low (<80), medium (80-160) and high (>160) density and analysed the alterations in the metastases. In the case of the low primary MVD group, the MVD was increased frequently in the majority of cases in the metastases (6/7), similarly to the medium MVD group (3/6), while none of the metastases were found to have increased MVD in the high primary MVD group (0/5). The VEGF protein expression was similar in the primary BRCs compared to their bone metastases (10/18 *versus* 8/18). The MVD did not correlate to the VEGF expressions (Table III).

BRC cases were heterogenous in respect to their post-surgery history, since a significant proportion of cases were not treated by adjuvant chemo-endocrine therapy (10 patients), while others were treated by TMX, the CMF protocol or their combination (Table I). We have postulated that this might have affected the angiogenic phenotype of the bone metastases. MVD analysis was performed on therapy-naïve and treated patient samples. This study revealed that, in non-treated patients, the MVD of the metastases was significantly higher than those of the corresponding primaries and that observed in metastases of treated cases (Figure 3). On the contrary, the MVD of the metastases of adjuvant-treated patients was found to be significantly lower than those of the corresponding primaries (Figure 3), suggesting that the treatment significantly affected the angiogenic phenotype of the progressing disease. Since the treated patients were heterogenous with respect to the treatment (endocrine therapy alone or chemo-endocrine therapy), we analysed the MVD alterations separately in these two subgroups, but a similar trend was observed in the angiogenic phenotype (Figure 3).

The VEGF protein expression was similar in the primary and metastatic tumours of the therapy-naïve patient group, while it decreased significantly in the metastases following adjuvant therapy (Table IV). Loss of the ER expression in the bone metastases was similar in both groups of patients (Table I).

Discussion

This is the first study on the vascularization of BRC in bone metastases, which revealed great heterogeneity in the modulation of the angiogenic phenotype from the primary tumour to the metastatic lesion. Two patterns of angiogenic responses were identified. Decreased MVD in bone metastases of BRC occurred in cases with high MVD in the primary tumour, and *vice versa*: increased MVD developed exclusively from primary BRCs characterized by low MVD. This small cohort of BRC patients was heterogenous with respect to post-surgery treatments: besides the non-treated patients, the other part of this cohort was treated either by adjuvant endocrine (TMX) or chemo-endocrine (CMF+TMX) protocols. Analysis revealed that decreased MVD in bone metastasis characterized the adjuvant-treated group of patients independent of the form of treatment (TMX or CMF+TMX), while in therapy-naïve cases, the developing bone metastases became hyper-vascularized compared to the primary tumours. Interestingly, the VEGF protein expression of BRC cells decreased in the bone metastases of adjuvant-treated patients as well, unlike the therapy-naïve group, suggesting correlations between these events.

Previous studies (performed on primary tumours) did not show conclusively that chemotherapy of BRC affects vascularization (16-19). On the other hand, TMX was shown to decrease MVD of BRC in experimental and clinical settings (20, 21). In our study, decreased MVD and VEGF expressions characterized the BRC patient group, where adjuvant TMX was used following surgery with or without CMF chemotherapy. TMX may have multiple targets in BRC tissue including the ER+ cancer cells, the stroma and the vasculature. Endothelial cells express functional ER (22, 23) and oestrogen is a survival factor for them (24). TMX was shown to be antiangiogenic, inhibiting endothelial cell proliferation and migration (25). Recently, functional oestrogen-responsive elements have been identified in the promoter region of the VEGF gene (26) and TMX inhibited VEGF expression in vascular muscle cells (27) as well as BRC cells (28). These data collectively suggest that TMX (or other endocrine) treatment of primary BRC may significantly attenuate the angiogenic phenotype of cancer cells and/or directly inhibit tumour-induced neoangiogenesis. As a consequence, vascularization of the bone metastases of adjuvant-treated BRC patients was significantly decreased compared to the primary tumours. It is of note that, although the angiogenic phenotype of BRC was significantly inhibited by adjuvant treatments, this did not prevent the development of bone metastases, suggesting that the metastatic potential was not significantly affected in these cases. On the other hand, chemotherapy-naïve BRC cases progressed to the bone with a concomitant increase in their angiogenic potential, suggesting that it is the "natural history" of BRC progression.

Angiosuppressive therapy is a new approach to cancer management, including BRC, frequently being applied in the advanced stage of disease. Tumours with prominent angiogenic phenotype (high MVD and VEGF) are primary candidates for such regimes. The fact that chemo-endocrine adjuvant therapy of BRC resulted in a weak angiogenic phenotype in bone metastases compared to non-treated cases may suggest that these latter patients are suitable candidates for this new form of intervention.

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