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Impact of tumor angiogenic profile on the outcome of patients with metastatic breast carcinoma treated with weekly docetaxel. A Hellenic Cooperative Oncology Group (HeCOG) study

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Summary. Background: Metronomic taxane administration has putative antiangiogenic properties. Herein, we examined the baseline tumor angiogenic profile of patients with metastatic breast carcinoma (MBC) in a prospective-retrospective translational research study. The interplay between the angiogenic factors expressed in the tumors and their prognostic value in MBC were investigated.

Patients and Methods: Tumor tissues from patients with MBC treated with weekly docetaxel (n=159) were examined by immunohistochemistry for VEGF-A, VEGF-C, VEGFR-1, VEGFR-2, VEGFR-3 and osteopontin (OPN) and by mRNA analysis for expression of VEGF-A, VEGFxxxa, VEGFxxxb, VEGF-C, thrombospondin-1 (THBS-1), hypoxia-inducible factor-1α (HIF-1α) and von Hippel-Lindau (VHL) genes. Associations between these parameters and outcome were statistically analyzed.

Results: Statistically significant correlations were identified between almost all biomarkers examined in continuous form, particularly at the mRNA level: VEGF-A with VEGFxxxa (rho=0.70); VEGF-C with VEGFxxxa, VEGFxxxb and VHL (rho=0.51, 0.60 and 0.44 respectively); HIF-1α with VEGF-C and THBS1 (rho=0.48 and 0.45). High VEGF-A mRNA was associated with worse survival (p=0.0279) and marginally with progression free survival (PFS). Intratumoral co-expression of VEGFR-1 and VEGFR-2 proteins was associated with more favorable survival (p=0.0337). In multivariate analysis, only high VEGF-A mRNA levels retained their prognostic role for worse PFS and survival (PFS: HR=2.34, 95% CI=1.25-4.40, p=0.0080; survival: HR=3.15, 95% CI=1.48-6.72, p=0.0029).

Conclusions: In MBC, this study confirms the adverse prognostic effect of high intratumoral VEGF-A mRNA and reveals the combined VEGFR-1/VEGFR-2

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protein expression as a potentially favorable prognosticator, which merits further evaluation in larger studies.

Key words: Metastatic breast cancer, Docetaxel, VEGF-A, VEGFR-1, VEGFR-2

Introduction

Angiogenesis is a major parameter in the development of tumor metastasis. The principal mediator of angiogenesis is vascular endothelial growth factor-A (VEGF-A), also known as VEGF (Fox et al., 2007), thought to induce endothelial cell proliferation, migration and motility (Kowanetz and Ferrara, 2006). VEGF-A is produced by most normal tissues as well as by tumors such as breast carcinoma, and acts primarily in a paracrine manner on adjacent endothelial cells (Koch et al., 2011). VEGF-A gene, through alternative proximal or distal splicing in the terminal eighth exon, produces two major families of isoforms, the pro-angiogenic VEGFxxxa isoforms, and the VEGFxxxb isoforms, which do not efficiently activate VEGF receptors, and thus are described as anti-angiogenic (Harper and Bates, 2008; Koch et al., 2011). In many normal tissues both isoforms are produced, while in angiogenic or non-angiogenic tissues the splicing switch favors, accordingly, the production of the corresponding isoforms (Nowak et al., 2010).

VEGF-A functions, primarily, through high affinity binding on two tyrosine kinase receptors. It promotes angiogenesis primarily after binding to VEGF receptor 2 (VEGFR-2, also known as KDR), expressed on endothelial cells. VEGFR-1, whose function remains more elusive, has a weaker signal transducing ability (Kerbel, 2008) and may act as a decoy receptor, limiting VEGF-A's availability to bind to VEGFR-2 (Park et al., 1994). VEGF-C is another angiogenic factor that mediates lymphangiogenesis through binding to VEGFR-3 and angiogenesis through binding to VEGFR-2. Breast carcinomas, among other tumors, express both VEGF-A, VEGF-C and their receptors, findings also observed by immunohistochemical examination, suggesting that a possible autonomous, autocrine mechanism may assist tumor growth and survival (Kerbel, 2008; Fountzilas et al., 2011). Immunohistochemically detected, VEGFR-1 has been associated with poor survival in patients with metastatic breast carcinoma (MBC), while VEGFR-3 has been associated with response to paclitaxel and bevacizumab in MBC (Fountzilas et al., 2011).

The hypoxia inducible factor-1 (HIF-1) is a key transcription factor, regulating the cellular oxygen and survival in hypoxic conditions, occurring in various circumstances such as tumor growth. It is comprised of the HIF-1 alpha (HIF-1α) and the HIF-1β subunits. HIF-1 activity is controlled through post-translational regulation of HIF-1α levels. At normal oxygen tension, HIF-1α is hydroxylated and subsequently recognized by von Hippel-Lindau (VHL) protein, with resultant ubiquitination and proteosomal degradation of HIF-1α. In hypoxia, HIF-1α stabilization results in dimerization with the HIF-1β subunit, and the HIF-1 complex activates transcription of several target genes, allowing the cell to survive through VEGF-A-induced angiogenesis, and switch to anaerobic glycolysis (Semenza, 1999). HIF-1α is associated with prognosis in breast carcinoma (Bos et al., 2003), tumor progression and metastasis in solid tumors (Vaupel, 2004), as well as with resistance to radiotherapy and to certain chemotherapeutic regimens (Unruh et al., 2003).

Osteopontin (OPN) is a secreted adhesive glycophosphoprotein expressed physiologically in several tissues, among which bone and breast, where it is involved in developmental processes, tissue differentiation, and vascular remodeling (Tuck et al., 2007). OPN is expressed by both tumor cells and cellular elements of the tumor microenvironment, and all these cellular constituents possess OPN receptors (Anborgh et al., 2010). OPN is involved in angiogenesis, as it increases the levels of VEGF in endothelial cells and recruits pro-angiogenic monocytes (Anborgh et al., 2010). Tumor-derived OPN has been associated with the establishment of the pre-metastatic niche (Anborgh et al., 2010), and with breast carcinoma metastasis in rodents. Circulating OPN levels are higher in MBC patients, and immunohistochemical expression of OPN is associated with worse prognosis in breast carcinoma (Rudland et al., 2002).

Thrombospondin-1 (THBS1) is expressed in platelets, endothelial cells, fibroblasts and macrophages, but also in several tumors, including breast carcinoma (Clezardin et al., 1993; Horiguchi et al., 2013). THBS1 has an anti-angiogenic function through inhibition of migration and induction of apoptosis of endothelial cells. Binding of THBS1 to endothelial cell membrane inhibits angiogenesis either through CD47 binding and nitric oxide (NO) signaling or through VEGF binding and inhibition of VEGFR-2 signaling (Chong et al., 2012). The data regarding THBS-1 and its role in metastasis are conflicting. THBS-1 has been proposed to have both pro-metastatic and anti-metastatic functions. Although THBS1 through its antiangiogenic role suppresses metastasis, in various tumor types it is thought to participate in various steps of the metastatic process, facilitating cell motility, invasiveness, intravasation, and at the metastatic site adhesion and extravasation (reviewed by Chong et al., 2012). In the breast carcinoma microenvironment of an animal model study, THBS-1 was found to inhibit angiogenesis and tumor growth but promote metastasis through tumor cell migration (Yee et al., 2009). Evidence from cell culture studies also suggests that THBS-1 accelerates metastasis in breast, thyroid, pancreatic and prostate carcinomas and is associated with invasion and lymph node metastases (Horiguchi et al., 2013).

Docetaxel is a major agent in the treatment of MBC.

Frequent and subtoxic doses of docetaxel, also referred to as metronomic, have been shown to inhibit angiogenesis (Hanahan et al., 2000; Kamat et al., 2007; Benelli et al., 2009). This antiangiogenic function is considered to occur through induction of THBS, (Benelli et al., 2009), decrease of VEGF (Benelli et al., 2009) and endothelial cell toxicity (Kamat et al., 2007).

In the present study we examined the expression of angiogenesis-related factors in the tumor at the protein and mRNA levels in a series of MBC treated with weekly docetaxel and analyzed the expression of these parameters to investigate possible angiogenic patterns in the tumors. We also correlated these parameters with outcome in the context of a phase II clinical trial.

Materials and methods

The patients were enrolled in the HE11/06 clinical trial of the Hellenic Cooperative Oncology Group (HeCOG), a prospective-retrospective translational research study examining angiogenesis-associated factors in patients with MBC, treated with weekly docetaxel. This study (HE11/06) was approved by the HeCOG Protocol Review Committee and the Institutional Review Board of the AHEPA University Hospital, as well as by the Bioethics Committee of the Aristotle University of Thessaloniki. Written informed consent was obtained from all patients. The results of this study have been recently published (Korantzis et al., 2012; Pectasides et al., 2012; Koutras et al., 2014).

Briefly, patients previously untreated for MBC received weekly docetaxel 35 mg/m^2 over 30 min for 12 weeks. Treatment could be continued beyond 12 cycles in responding cases, at the physician's discretion. Tumor tissue was obtained from the primary site in 79 patients and it was prospectively collected from a metastatic site in 12 patients. Estrogen and progesterone receptors (ER, PgR), Her-2 status, and Ki-67 were assessed centrally. Patients with HER2 overexpressing tumors (3+ by immunohistochemistry [IHC] or FISH positive, when 2+ by IHC) were treated with trastuzumab (8 mg/kg followed by 6 mg/kg every 3 weeks), concomitantly with docetaxel. Hormonal therapy, after the completion of chemotherapy, for cases with hormone receptor positive status included exemestane 25 mg daily for postmenopausal women and an LH-RH analogue combined with tamoxifen for premenopausal patients. Second-line chemotherapy was allowed only after progression of disease. Standard ECOG criteria for defining measurable disease and response were applied as previously stated (Pectasides et al., 2012) and the response was assessed by the investigators.

Biological material

The availability of biological material for IHC and mRNA analyses is depicted in Fig. 1 in a flow chart form according to the REMARK criteria. Formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks were retrieved from the HeCOG Tumor Tissue Repository for 91 patients as described above and tested for protein expression by IHC. In 71 cases, the tumor tissue in the paraffin block was adequate for the construction of tissue microarrays (TMAs) containing 2 cores per case of 1.5 mm in diameter. Each TMA block also included tissue cores of the same diameter in the first and the last column, which served as positive and negative controls for the tested antibodies. The remaining cases not included in the TMAs were evaluated as whole tissue sections.

Immunohistochemical analysis

Serial, 2.5μm thick sections from the original blocks or the TMA blocks were cut at the Laboratory of Molecular Oncology of the Hellenic Foundation of Cancer Research, School of Medicine, Aristotle University of Thessaloniki, mounted on adhesive microscope slides, and subjected to immunohistochemical labelling using the Bond Max™ (Leica Microsystems, Wetzlar, Germany) and i6000 (Biogenex, San Ramon, USA). The immunohistochemical protocols for VEGF-A, VEGF-C, VEGFR-1, VEGFR-2, VEGFR-3, and osteopontin were performed as described in Table 1.

Interpretation of IHC

The evaluation of all IHC sections was performed by one pathologist (HPK), experienced in breast cancer, blinded as to patients' clinical characteristics and survival data, according to previously proposed or established criteria. The assessment examined for cytoplasmic expression of proteins in the tumor cells. Briefly, for VEGF-A, VEGF-C, VEGFR-1, VEGFR-2, VEGFR-3, and osteopontin the H-score was calculated by the following method: H-score = (1 x percentage of weakly positive cells) + (2 x percentage of moderately strong positive cells) + (3 x percentage of strongly positive cells).

Table 1. Primary antibodies, source and staining conditions used in the present study.

Antibody	Clone/ Source	Dilution	Antigen Retrieval	Incubation Time
VEGF-A (m)	VG1 (1)	1:75	20′/EDTA	60′
VEGF-C (r, PL)	Z-CVC7 (2)	1:250	20′/CA	O/n
VEGFR-1 (r)	RB-1527 (3)	1:450	15′/CA	O/n
VEGFR-2 (r)	55B11 (4)	1:450	20′/EDTA	O/n
VEGFR-3 (m)	KLT9 (5)	1:50	15′/CA	O/n
Osteopontin(m)	OP3N (5)	1:80	20′/CA (3)	60′

CA, Citric acid; pH 6.0; EDTA, Ethylene diamine tetraacetate; pH 8.8; m, Mouse; O/n, Overnight; PL, Polyclonal; r, Rabbit. Source, (1), Dako, Glostrup DK; (2), Zymed™, Invitrogen, Carlsbad, CA, USA; (3) Thermo Fisher Scientific, Fremont, CA, USA; (4), Cell Signalling Technology, Beverly, MA, USA; (5), Novocastra™, Leica Biosystems, Newcastle Upon Tyne, UK

Evaluation of angiogenesis-related gene expression

Upon histological evaluation, 76 out of the above described 91 MBC FFPE tissue samples were deemed adequate for RNA extraction and evaluation. Based on a marked H&E section, macrodissection was performed where possible for tumor cell enrichment (≥50% in all cases). Deparaffinized tissue fragments were digested overnight at 56°C in a lysis buffer containing 10 mM NaCl, 50 mM Tris-HCl, pH 7.4, 20 mM EDTA, 1% SDS, and 0.8 mg/ml proteinase K. RNA was extracted from tissue lysates with TRIZOL-LS (Invitrogen / Life Technologies, Paisley, UK) and reverse transcribed with Superscript III and random hexamers (Invitrogen / Life Technologies), according to the manufacturers' instructions. cDNAs were normalized at 25 ng/ul and stored at -20°C until use. mRNA expression was evaluated with qPCR and TaqMan® Gene Expression Assays (hydrolysis probe assays, Applied Biosystems / Life Technologies) on cDNA templates for the following targets (assay ID; target reference sequence; location; amplicon size): HIF-1α (Hs00153153_m1; NM_181054.2, NM_001530.3; exons 4 – 5; 76bp), THBS1 (Hs_00170236_m1; NM_003246.2; exons 7 – 8; 109bp), total VEGF-A (VEGF-A Hs00173626_m1; NM_003376.5 and all splice variants; exons 1 – 2; 77bp), VEGF-C (Hs00153458_m1; NM_005429.2; exons 4 – 5; 126bp), and VHL (Hs00184451_m1; NM_000551.3; exons 2 – 3; 72bp). For the detection of VEGFxxxa and VEGFxxxb variant transcripts, custom assays were designed spanning exons 7b – 8a for VEGF-A165a, 189a, 206a, and exons 7b – 8b for VEGF165b, 189b, 206b (sense primer, antisense primer, and, Taqman® MGB probe, all sequences 5' – 3'): for xxxa, A A A C A C A G A C T C G C G T T G C A , AGAGATCTGGTTCCCGAAACC, and, CGAGGC-AGCTTGAG; for xxxb, AGGCGAGGCAGCTTG-AGTTA, ACGTTCTGTCGATGGTGATGGT, and, CGAACGTACTTGCAGATC. The size of xxxa transcripts was 129bp and of xxxb 122bp. Assay specificity was validated with dd-sequencing (forward – reverse). Relative quantification was assessed in comparison to GUSB (beta-glycuronidase, endogenous control Taqman® MGB expression assay, #4333767F, Applied Biosystems). Reactions at 10 μl (2 μl cDNA template) were run in duplicates in an ABI7500 real time PCR system under default conditions. GUSB was selected as the endogenous reference since it does not seem to be represented in pseudogenes, while it has also been independently identified as one of the best preserved mRNA targets in FFPE tissues (Sanchez-Navarro et al., 2010; Zhang et al., 2010). A commercially available reference RNA derived from multiple transformed cell lines (TaqMan® Control Total RNA, cat. no 4307281, Applied Biosystems) was applied in multiple positions in each run as positive control and for inter-run evaluation of PCR assay efficiency. No-template controls were also included. To obtain linear Relative Quantification (RQ) values, relative expression was assessed as (40-dCT), as previously described (Hennig et al., 2010), whereby dCT (or deltaCT) was calculated as (average target CT) – (average GUSB CT) from all eligible measurements. Samples were considered eligible for analysis when (a) both GUSB CTs in duplicates were <36 and when duplicate dCT's for the same sample within the same run were <0.75. The efficiency of all assays was considered as comparable, since the difference between inter-run RQ values for the reference RNA sample was <1 for all assays. Based on the criteria described above for sample eligibility, informative results for HIF-1α were obtained for 74 samples (86%); for THBS1 76 (88.4%); for VEGF-A 77 (89.5%); for VEGFxxxa 78 (90.7%); for VEGFxxxb 79 (91.9%); VEGF-C 74 (86%) and, for VHL 81 (94.2%). RQ values were assessed for single gene mRNA markers, as well as in combination by subtracting linear RQ values for VEGF-A vs.

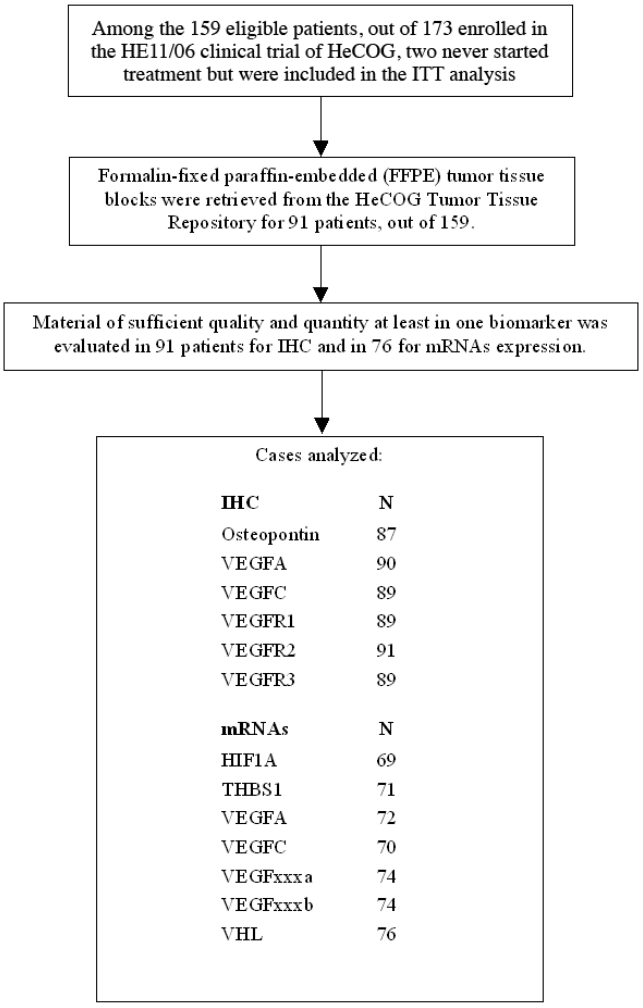

Fig. 1. Available biological material depicted in flow chart according to the REMARK criteria.

VEGFxxxa; VEGF-A vs. VEGFxxxb; and, VEGFxxxa vs. xxxb. The number of finally analyzed samples was lower, depending on the availability of clinico-pathological and follow-up data.

Statistical analysis

Categorical variables were presented as frequencies and percentages while various measures (mean, median, range etc.) were used for continuous variables. Associations among clinical characteristics, mRNA and IHC variables were examined. Chi-Square or Fisher's exact test, where appropriate, were used in order to examine possible associations among categorical variables. For testing categorical with continuous variables, Mann-Whitney or Kruskal-Wallis test was used. Correlations were calculated using the Spearman's rank correlation coefficient (Rho). For all the markers the quartiles (first or 25th percentile, median and third or 75th percentile) were examined as possible thresholds for prognostic significance in terms of survival or PFS. Given the limited literature regarding the pathways mediating the action of VEGFs and VEGFRs in neoplastic cells of breast carcinoma, we looked for co-expression patterns between the VEGF-A, VEGF-C, VEGFR-1, VEGFR-2 and VEGFR-3 IHC variables. Survival was measured from the date of treatment initiation to the date of patient's death or last contact,

Table 2. Patient and tumor characteristics.

	N=159 (whole sample)	N=91 (immunohisto-chemically analyzed cases)	N=76 (mRNA-analyzed cases)
Age			
Median /Range	60.7/ 27-87	60.7/ 32-87	60.8/ 32-83
	N (%)	N (%)	N (%)
Menopausal status			
Premenopausal	34 (21.4)	19 (20.8)	18 (23.6)
Postmenopausal	125 (78.6)	72 (79.2)	58 (76.4)
PS (ECOG)			
0	107 (67.2)	66 (72.6)	55 (72.4)
1	41 (25.8)	20 (22.0)	16 (21.0)
2	11 (7.0)	5 (5.4)	5 (6.6)
ER status			
Positive	106 (66.6)	65 (71.4)	56 (73.6)
Negative	48 (30.2)	26 (28.6)	20 (26.4)
Not reported	5 (3.2)		
PR status			
Positive	84 (52.8)	48 (52.8)	41 (54.0)
Negative	70 (44.0)	43 (47.2)	35 (46.0)
Not reported	5 (3.2)		
Her2 overexpression			
No	99 (62.2)	59 (64.8)	49 (64.4)
Yes	57 (35.8)	32 (35.2)	27 (35.6)
Not reported	3 (1.8)		
Subtypes			
Luminal A	16 (10.1)	16 (17.6)	14 (18.4)
Luminal B	30 (18.9)	30 (33.0)	25 (32.9)
Luminal HER2	38 (23.9)	22 (24.2)	18 (23.7)
HER2-Enriched	18 (11.3)	10 (11.0)	9 (11.8)
Triple-negative	25 (15.7)	13 (14.3)	10 (13.2)
Not reported	32 (20.1)		
Grade			
1	3 (1.8)	1 (1.0)	1 (1.4)
2	65 (40.8)	41 (45.0)	36 (47.4)
3	70 (44.0)	43 (47.2)	35 (46.0)
Not reported	21 (13.2)	6 (6.6)	4 (5.2)
Stage at diagnosis			
I/II/III	7 (4.4)/ 35 (22.0)/41 (25.8)	2 (2.2)/ 25 (27.4)/24 (26.4)	1 (1.4)/ 22 (29.0)/22 (29.0)
IV	60 (37.8)	33 (36.2)	28 (36.8)
Not reported	16 (10.0)	7 (7.6)	3 (4.0
Positive nodes			
0-3	63 (39.6)	36 (39.6)	31 (40.8)
4-9	20 (12.6)	15 (16.4)	14 (18.4)
>9	30 (18.8)	21 (23.0)	18 (23.6)
Not reported	46 (29.0)	19 (20.8)	13 (17.2)
Prior Adjuvant Treatment			
Adjuvant chemotherapy	80 (50.4)	49 (53.8)	40 (52.6)
Anthracycline-based	50 (31.4)	31 (34.0)	25 (32.8)
Taxane-based	29 (18.2)	18 (19.8)	16 (21.0)
CMF	48 (30.2)	29 (31.8)	25 (32.8)
Adjuvant hormonotherapy	58 (36.4)	35 (38.4)	29 (38.2)
Tamoxifen	51 (32.0)	32 (35.2)	26 (34.2)
Aromatase inhibitors	13 (8.2)	6 (6.6)	6 (7.8)
LH-RH analogues	4 (2.6)	2 (2.2)	2 (2.6)
Other	2 (1.2)	1 (1.0)	1 (1.4)
Adjuvant radiotherapy	61 (38.4)	40 (44.0)	32 (42.2)
Sites of metastases			
Locoregional	43 (27.0)	21 (23.0)	17 (22.4)
Nodes	27 (17.0)	15 (16.4)	12 (15.8)
Skin	11 (7.0)	6 (6.6)	5 (6.6)
Residual breast	16 (10.0)	8 (8.8)	6 (7.8)
Distant	145 (91.2)	83 (91.2)	71 (93.4)
Abdomen / ascites	5 (3.2)	1 (1.0)	1 (1.4)
Bones	79 (49.6)	49 (53.8)	45 (59.2)
Visceral	115 (72.4)	64 (70.4)	53 (69.8)
Soft tissue / nodes	39 (24.6)	22 (24.2)	19 (25.0)
Locoregional only	10 (6.2)	6 (6.6)	4 (5.2)
Distant only	112 (70.4)	68 (74.8)	58 (76.4)
Locoregional and distant	33 (20.8)	15 (16.4)	13 (17.2)
Number of metastatic sites			
1	49 (30.8)	29 (31.8)	23 (30.2)
2	64 (40.2)	37 (40.6)	30 (39.4)
≥3	42 (26.4)	23 (25.2)	22 (29.0)
Not reported	4 (2.6)	2 (2.2)	1 (1.4)
Treatment with trastuzumab	48 (30.2)	28 (30.8)	23 (30.2)
Outcome			
Follow-up (months)			
Median (range)	33.5 (2.8-45.0)	34.0 (6.2-45.0)	34.0 (6.2-45.0)
Survival (months)			
Median (95% CI)	27.7 (24.3-30.4)	30.4 (26.3-42.0)	31.3 (26.3- .)
PFS (months)			
Median (95% CI)	8.8 (6.6-9.8)	9.4 (8.3-12.7)	9.8 (8.3-14.2)
CR % (95% CI)	4.4 (1.8-8.9)	5.5 (1.8-12.4)	6.6 (2.2-14.7)
PR % (95% CI)	34.0 (26.7-41.9)	30.8 (21.5-41.3)	31.6 (21.4-43.3)
SD % (95% CI)	32.7 (25.5-40.6)	36.3 (26.4-47.0)	35.5 (24.9-47.3)
ORR % (95% CI)	38.4 (30.8-46.4)	36.3 (26.4-47.0)	38.2 (27.3-50.0)

while Progression-free survival (PFS) was measured from the date of treatment initiation to documented disease progression, death without prior documented progression or last contact, whichever occurs first. Survival status was updated in December 2009. Time-to-event distributions were estimated using Kaplan-Meier curves. The log-rank test was used to examine the prognostic significance of the markers for survival and PFS, while the Fisher's exact test or the Mann-Whitney test, where appropriate, were used to examine the association with the overall response rate (ORR). For all univariate tests significance level was set at α=0.05. Multivariate analysis was conducted among the following clinicopathological parameters: adjuvant chemotherapy, radiotherapy and hormonal therapy, histological grade, performance status, ER/PgR and triple negative classification. The parameters included in the model for survival were adjuvant chemotherapy, performance status and VEGF-A mRNA and for PFS adjuvant chemotherapy, adjuvant hormonal therapy, performance status and VEGF-A mRNA; significance threshold for keeping a variable in the final model was set at α=0.15. The design of the present study was prospective-retrospective, as described in Simon et al. (2009), while the analysis was fully compliant with the reporting recommendations for tumor marker prognostic studies (McShane et al., 2006). The SAS software was used for statistical analysis (SAS for Windows, version 9.3, SAS Institute Inc., Cary, NC, USA).

Results

The patient and tumor characteristics of the entire group, as well as of the subgroups studied by IHC and mRNA analyses are shown in Table 2. Among the 159 patients, two never started treatment but they were included in the intent-to-treat survival analysis. After a median follow-up period of 33.5 months (range=2.8-45.0), the median survival was 27.7 months (range=0.3-45, 95% CI= 24.3-30.4), whereas the median PFS was 8.8 months (range=0.3-45 95% CI=6.6-9.8). Overall, 38.4% of the patients achieved an objective response (95% CI 30.8-46.4%), 4.4% a complete response (CR) and 34.0% a partial response (PR), while 32.7% of the patients had stable disease (SD). The cohorts of the 91 and 76 patients with IHC and mRNA analyses had similar profiles.

The descriptive statistical values of the H-scores for each marker studied by IHC are depicted in Table 3 and Fig. 2, and the corresponding values for the mRNA analyses are shown in Table 4 and Fig. 3. Representative images of tumors with immunohistochemical scores above the median values for VEGF-A, VEGF-C,

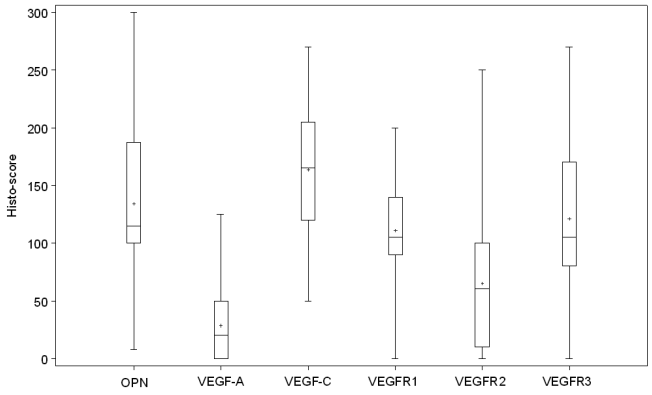

Fig. 2. Boxplots for immunohistochemically analyzed parameters.

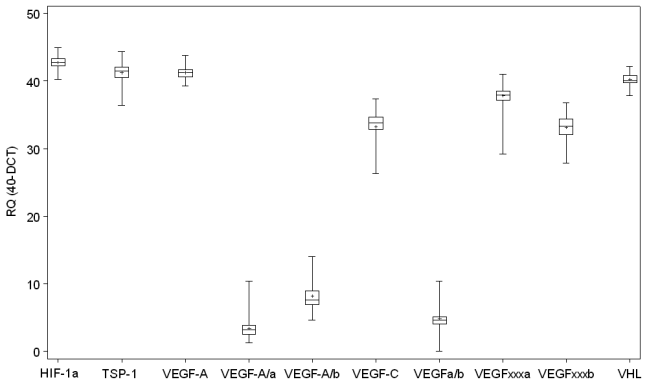

Fig. 3. Boxplots for mRNA analyses of angiogenesis-associated parameters. Abbreviations, VEGF-A/a: VEGF-A/VEGFxxxa; VEGF-A/b: VEGF-A/VEGFxxxb; VEGF-a/b: VEGFxxxa/VEGFxxxb.

Table 3. Statistical descriptives for immunohistochemical analyses.

	Descriptives							
	N	Mean	Std	Min	25th perc.	Median	75th perc.	Max
Osteopontin	87	134.0	61.6	7.5	100.0	115.0	187.5	300.0
VEGF-A	90	28.4	31.3	0.0	0.0	20.0	50.0	125.0
VEGF-C	89	163.5	49.9	50.0	120.0	165.0	205.0	270.0
VEGFR-1	89	110.6	47.1	0.0	90.0	105.0	140.0	200.0
VEGFR-2	91	65.1	55.9	0.0	10.0	60.0	100.0	250.0
VEGFR-3	89	121.1	62.3	0.0	80.0	105.0	170.0	270.0

VEGFR-1, VEGFR-2, VEGFR-3 and osteopontin are depicted in Fig. 4.

VEGF-A was usually weakly expressed in the cytoplasm of the neoplastic cells and was largely negative in the non-neoplastic breast elements. Only rarely were there scattered positive cells in the terminal ducts and lobules of the breast. Contrary to the low VEGF-A protein expression (H-score 0-125, median 20), VEGF-A transcript levels were generally high (39.3-43.7, median 41.2) and there was an inverse correlation between VEGF-A IHC and transcript values (rho=-0.25, p=0.0349). Although VEGF-A had higher H-score values in cases treated with hormonal treatment (p=0.036), it was not associated with ER/PR status or with the other clinicopathological parameters examined, but it was associated with the protein expression of VEGFR-2 (rho=0.30, p=0.0045) and VEGFR-3 (rho=0.29, p=0.0054).

VEGF-C showed granular cytoplasmic expression which was usually weak in benign lobules and ducts and stronger in the neoplastic cells. VEGF-C protein expression was not associated with the clinicopathological parameters examined but it showed a correlation with VEGFR-1 protein (rho=0.28, p=0.0081) and with the VEGFxxxa/VEGFxxxb mRNA ratio (rho=0.27, p=0.0227). VEGF-C mRNA levels were also associated with VEGFxxxa (rho=0.51, p<0.0001), VEGFxxxb (rho=0.60, p<0.0001), VHL (rho=0.44, p=0.0001) and inversely associated with the ratio VEGFxxxa/VEGFxxxb (rho=-0.32, p=0.0082). Higher VEGFC mRNA levels were also associated with older age (p=0.012).

VEGFR-1 was weakly or moderately expressed in the cytoplasm of the neoplastic cells but not in benign

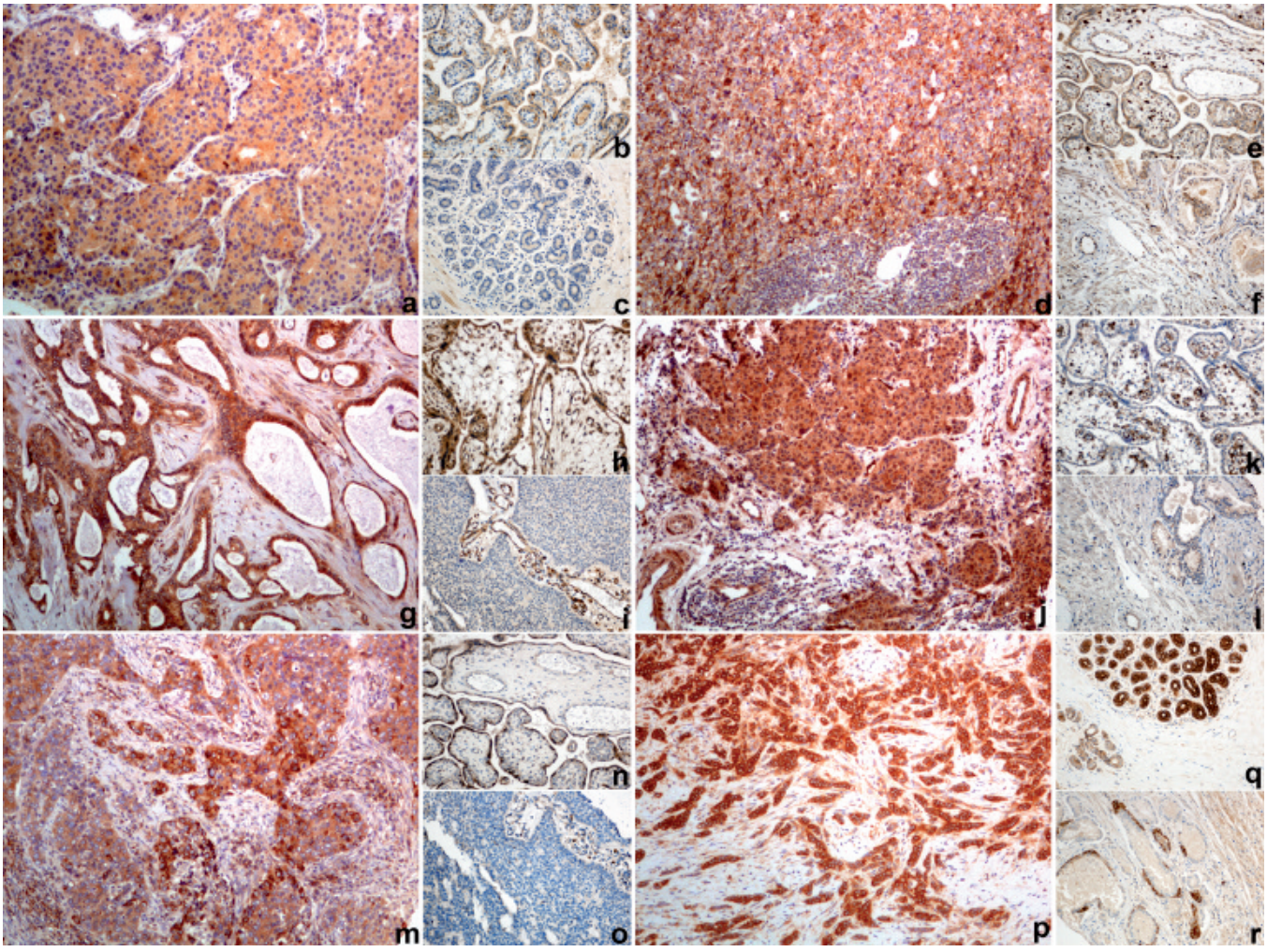

Fig. 4. Representative images of tumors (panels **a, d, g, j, m and p**), with corresponding positive controls (panels **b, d, h, k, n and q**) and negative control tissues (panels **c, f, i, l, o and r**) stained for VEGF-A (panels **a, b, c**), VEGF-C (panels **d, e, f**), VEGFR-1 (panels **g, h, i**), VEGFR-2 (panels **j, k, l**), VEGFR-3 (panels **m, n, o**) and Osteopontin (panels **p, q, r**). x 200.

breast tissue. High H-score values were, as previously stated, associated with VEGF-C and inversely associated with HIF-1α mRNA levels (rho=-0.25, p=0.0369) and with VEGFxxxa/VEGFxxxb (rho=-0.27, p=0.0217).

VEGFR-2 was weakly expressed in the cytoplasm of benign breast elements and more frequently weakly expressed in the cytoplasm of the tumor cells. High VEGFR-2 H-scores were associated with VEGFR-1 (rho=0.37, p=0.0003), OPN (rho=0.22, p=0.0430) and VEGF-A (as previously stated).

VEGFR-3 was weakly to moderately expressed in both benign and neoplastic breast tissue. VEGFR-3 H-score was associated with OPN (rho=0.29, p=0.0068) and VEGF-A.

OPN was expressed in a granular pattern in the cytoplasm of various cell types. It was intensely expressed in skeletal muscle, cutaneous squamous epithelium and benign lobules, moderately expressed in smooth muscle of the vasculature, Schwann cells of nerves and the luminal ductal cells, and moderately or weakly expressed in the cytoplasm of the neoplastic cells. Strong expression in the neoplastic cells was infrequent. Higher OPN H-scores were noted in triple negative tumors, a finding that did not reach statistical significance (p=0.079). OPN expression was associated with immunohistochemical data for VEGF-A (rho=0.23, p=0.0289), VEGF-C (rho=0.22, p=0.0407), VEGFR-2 and VEGFR-3. OPN expression was inversely associated with THBS-1 mRNA levels (rho=-0.27, p=0.028).

VEGFxxxa mRNA levels were associated with VEGF-A mRNA levels (rho=0.70, p<0.0001), HIF1A (rho=0.32, p=0.0068), THBS1 (rho=0.38, p=0.0010) and VEGF-C mRNA levels. VEGFxxxb mRNA levels were associated with VEGF-A mRNA levels (rho=0.41, p=0.0004), THBS1 mRNA levels (rho=0.40, p=0.0006)

Table 4. Statistical descriptives for mRNA analyses.

	Descriptives							
	N	Mean	Std	Min	25th perc.	Median	75th perc.	Max
HIF1A	69	42.7	1.0	40.2	42.2	42.7	43.3	44.9
THBS1	71	41.3	1.3	36.4	40.5	41.4	42.0	44.4
VEGF-A	72	41.2	1.0	39.3	40.5	41.2	41.7	43.7
VEGF-C	70	33.2	2.4	26.3	32.8	33.8	34.7	37.4
VEGFxxxa	74	37.9	1.6	29.2	37.2	37.9	38.6	41.0
VEGFxxxb	74	33.1	2.0	27.8	32.0	33.4	34.4	36.9
VHL	76	40.2	0.8	37.8	39.7	40.1	40.8	42.1
VEGFxxxa/b	73	4.8	1.5	0.0	4.1	4.6	5.2	10.4
VEGF-A/xxxa	71	3.3	1.2	1.3	2.5	3.2	3.8	10.4
VEGF-A/xxxb	70	8.2	2.0	4.6	6.8	7.6	8.9	14.0

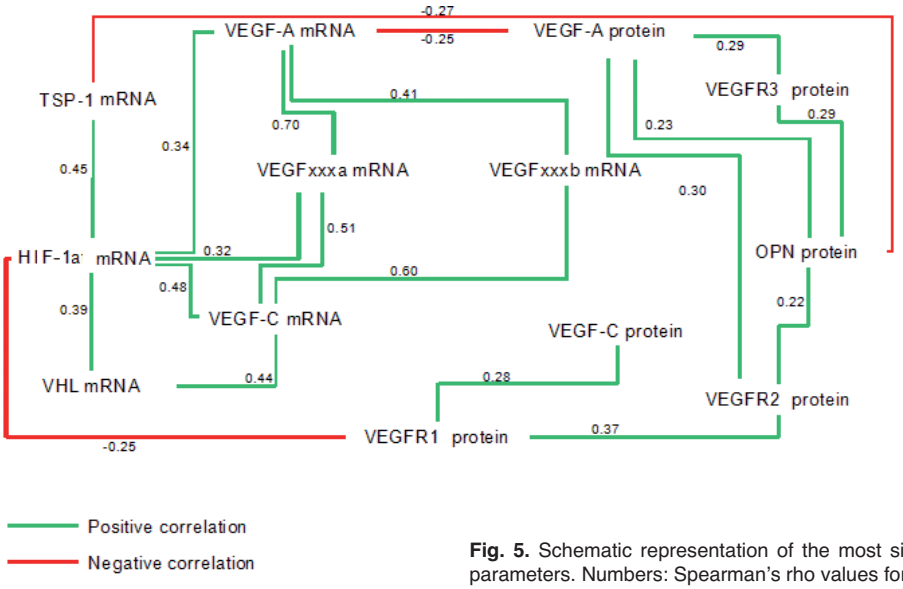

Fig. 5. Schematic representation of the most significant correlations between the angiogenesis parameters. Numbers: Spearman's rho values for positive and negative correlations.

and VEGF-C.

Higher VHL mRNA levels were associated with older age and postmenopausal status (p=0.033 and p=0.022, respectively), HIF-1α (rho=0.39, p=0.0008) and VEGF-C.

HIF-1α mRNA levels were associated with older age (p=0.001), VEGF-A (rho=0.34, p=0.0040), VEGF-C (rho=0.48, p<0.0001), THBS-1 (rho=0.45, p<0.0001), VEGFxxxa and VHL. All correlations among the angiogenesis parameters are schematically depicted in Fig. 5.

mRNA expression of all markers was compared to ER status (Table 5). mRNA expression in breast carcinoma IHC subtypes based on ER, PgR, HER2-status and Ki67 (Table 2) was not compared against outcome as it would result in groups too small for statistical inference. For the same reason, primary and metastatic tumors were not examined separately.

None of the factors examined by IHC or mRNA analysis was associated with response to treatment. Tumors with significantly high levels of VEGF-A mRNA (greater than the 75th percentile) were associated with worse survival (p=0.0279) (Fig. 6), while tumors with IHC co-expression of VEGFR-1 and VEGFR-2 using the median cut-off were associated with more favorable survival (p=0.0337) (Fig. 7). VEGF-A mRNA (at the cut-off at 75%) was also marginally associated with PFS (p=0.0581). VEGF-C IHC expression (greater than the 75th percentile) did not reach statistical significance for survival (p=0.0774). None of the IHC parameters was associated with PFS.

Only VEGF-A mRNA retained its significance in the multivariate setting. Adjusted for adjuvant treatment (chemotherapy/hormonal therapy) and performance

Table 5. mRNA-analyzed parameters by ER status.

		HIF1α				THBS1				VEGF-A			
		N	Median	Range	P-value	N	Median	Range	P-value	N	Median	Range	P-value
ER status	Negative	17	42.8	(41.2-44.9)	0.365	18	40.8	(36.4-44.4)	0.178	18	41.5	(39.3-43.7)	0.067
	Positive	52	42.7	(40.2-44.6)		53	41.6	(38.4-44.1)		54	41.0	(39.6-43.3)	
		VEGF-A/xxxa				VEGF-A/xxxb				VEGFC			
		N	Median	Range	P-value	N	Median	Range	P-value	N	Median	Range	P-value
ER status	Negative	18	3.0	(1.7-5.5)	0.606	18	8.1	(4.6-14.0)	0.136	17	33.8	(27.9-35.4)	0.498
	Positive	53	3.2	(1.3-10.4)		52	7.5	(5.1-13.3)		53	33.8	(26.3-37.4)	
		VEGFxxxa				VEGFxxxa/b				VEGFxxxb			
		N	Median	Range	P-value	N	Median	Range	P-value	N	Median	Range	P-value
ER status	Negative	20	38.2	(35.7-40.9)	0.324	20	4.6	(2.9-10.4)	0.282	20	33.5	(27.8-36.7)	0.855
	Positive	54	37.8	(29.2-41.0)		53	4.5	(0.0-8.0)		54	33.3	(29.2-36.9)	
		VHL											
		N	Median	Range	P-value								
ER status	Negative	20	39.8	(37.8-41.2)	0.128								
	Positive	56	40.2	(38.8-42.1)									

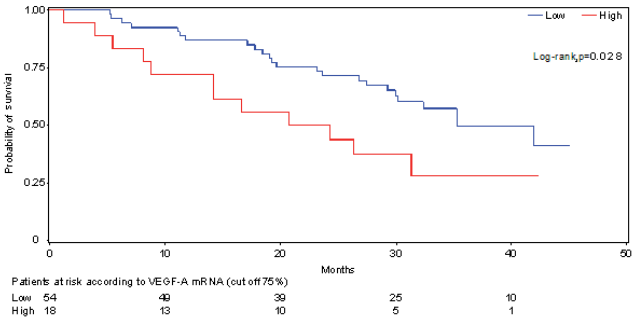

Fig. 6. Survival Kaplan-Meier curves for VEGF-A mRNA (cut-off 75%).

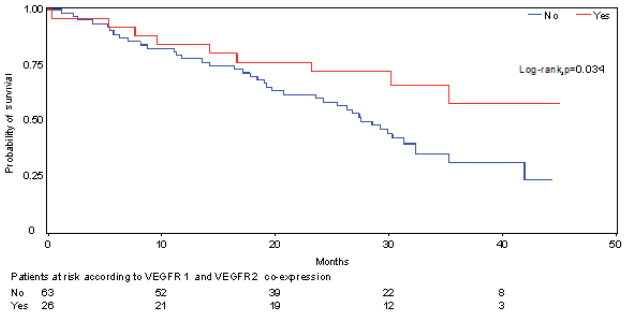

Fig. 7. Survival Kaplan-Meier curves for VEGFR-1 and VEGFR-2 co-expression, as determined by IHC.

status at study entry, high expression levels of VEGF-A mRNA are prognostic for worse PFS and survival (PFS: HR=2.34, 95% CI=1.25-4.40, p=0.0080; survival: HR=3.15, 95% CI=1.48-6.72, p=0.0029). Results are shown at Table 6.

Discussion

This study investigated a possible interplay between the angiogenic factors expressed in breast carcinomas and examined their potential prognostic value in patients with MBC treated with low-dose weekly docetaxel. In the majority of the cases primary tumors were examined, which only allows for indirect deductions on the impact of markers studied at baseline on the course of metastatic disease.

The herein observed association of THBS1 with VEGF-C mRNA links THBS1 to lymphangiogenesis. No clear association with angiogenesis could be established, since THBS1 positively correlated with both pro- (xxxa) and anti-angiogenic (xxxb) VEGF-A transcripts. In the same line, other investigators did not observe association of THBS1 with angiogenesis parameters (Ioachim et al., 2012), or with development of metastases (Wang-Rodriguez et al., 2003). Similarly conflicting are the results regarding THBS1 effect on disease outcome. In the present study, tumor THBS1 levels did not correlate with survival or response, unlike the findings from blood THBS1 mRNA indicating that high levels result in worse survival (Pectasides et al., 2012) or from tissue where low THBS1 levels were associated with increased risk of recurrence (Ioachim et al., 2012).

OPN is considered a pro-angiogenic factor. In endothelial cells, OPN induces VEGF expression thus promoting angiogenesis (Anborgh et al., 2010). In the present study, we also observed a parallel association between the tumor OPN and angiogenesis parameters, such as VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3, while an inverse association with the putatively anti-angiogenic THBS-1 was also noted. OPN in the tumor microenvironment is thought to down-regulate nitric oxide production, through down-regulation of the inducible nitric oxide synthase (iNOS), resulting in decreased apoptosis of the tumor cells (Anborgh et al., 2010). OPN has been associated with worse prognosis in stage 1 and 2 breast carcinoma (Rudland et al., 2002) and other tumors (Anborgh et al., 2010). In the present series assessing metastatic disease OPN was not associated with prognosis. However, a role for OPN on the outcome of MBC patients cannot be precluded from this finding which mainly reflects the expression of this molecule in primary tumors.

VEGF-A is likely the most important regulator of angiogenesis (Ferrara, 2002). VEGF-A transcription is induced by HIF-1 under hypoxic conditions and VEGF-A mRNA stabilization results in increased VEGF-A expression. Indeed, VEGF-A mRNA levels were associated with HIF-1α in this study. Unexpectedly, we observed an inverse correlation between VEGF-A protein and VEGF-A mRNA levels. Furthermore, although VEGF-A mRNA expression in tumor tissues was at the level of the endogenous control, i.e., relatively high, the detected protein corresponded to low H-scores. A possible explanation would implicate the antibody and immunohistochemical methodology implemented, which may produce a weaker immunohistochemical staining result. The antibody and conditions applied in the present study are similar to those reported in major previously published series, such as the retrospective analysis of E2100 trial for VEGF protein expression in the primary tumor (Schneider et al., 2008). Alternatively, it is known that cytokine mRNA expression can be altered by RNA-binding proteins and microRNAs. Nonetheless, the association of VEGF-A protein with VEGFR-2 and VEGFR-3 overexpression in the neoplastic cells would support not only angiogenesis and lymphangiogenesis, but also autonomous, autocrine growth of the neoplastic cells, as previously suggested (Kerbel, 2008; Fountzilas et al., 2011).

VEGFxxxa levels were higher than VEGFxxxb levels in the neoplastic tissue, suggesting an angiogenic phenotype. Of note, the corresponding assays used herein detected VEGF-165 transcripts and larger; smaller xxxa and xxxb molecules, which are more easily released into the circulation and more potent in promoting angiogenesis -at least the xxxa- (Harper and Bates, 2008) were not examined in this study. The present data on larger xxxa and xxxb are consistent with angiogenesis promoted by local hypoxia and HIF-1α.

VEGF-C overexpression was not associated with VEGFR-3, as in a previous study (Fountzilas et al., 2011), but with VEGFR-1. In all, the observed

Table 6. Multivariate analyses.

Survival (N=70)	HR	95% CI	Wald's p
Adjuvant Chemotherapy			
Yes vs. No	0.27	0. 12-0.60	0.0013
Performance Status (Study Entry)			
0 vs. 2	0.13	0.04-0.42	0.0007
1 vs. 2	0.22	0.06-0.79	0.0209
VEGF-A mRNA cut-off at 75%			
High vs. Low	3.15	1.48-6.72	0.0029
PFS (N= 67)	**HR**	**95% CI**	**Wald's p**
Adjuvant Chemotherapy			
Yes vs. No	0.44	0.21-0.92	0.0290
Adjuvant Hormonotherapy			
Yes vs. No	0.48	0.24-0.96	0.0384
Performance Status (Study Entry)			
0 vs. 2	0.22	0.07-0.67	0.0079
1 vs. 2	0.23	0.07-0.75	0.0147
VEGF-A mRNA cut-off at 75%			
High vs. Low	2.34	1.25-4.40	0.0080

correlations between transcript levels of VEGF-A (and variants), VEGF-C, VHL and THBS-1 suggest possible cross-talking between the angiogenic parameters.

With regards to outcome, no association was observed with VEGF-A protein expression, analyzed by IHC, a finding in agreement with previously published series (De Paola et al., 2002; MacConmara et al., 2002; Ludovini et al., 2003; Jubb et al., 2006; Kostopoulos et al., 2006; Mylona et al., 2007; Schneider et al., 2008; Fountzilas et al., 2011). In contrast to these reports VEGF-A was shown to have independent prognostic value for relapse free survival (Toi et al., 1994) or for survival in univariate analysis (Mohammed et al., 2007). Unlike IHC analysis, we observed association of high VEGF-A mRNA levels with worse survival and PFS in multivariate analysis. High VEGF-A mRNA levels have been previously associated with worse relapse free survival in breast cancer (Relf et al., 1997; Zhang et al., 2006).

VEGFR-2 is the receptor type mediating primarily VEGF-A responses in endothelial cells and its blockade inhibits tumor growth by interfering with tumor angiogenesis. In the present study VEGFR-2 IHC expression was studied in the neoplastic cells and it was not associated with outcome, similarly to previous studies (Schneider et al., 2008; Fountzilas et al., 2011). These findings indicate potentially differing functions of the receptors in the various cell types.

VEGFR-1 has a 10-fold higher affinity for VEGF-A compared to VEGFR-2 but a weak tyrosine kinase activity. Although VEGFR-1 is rather inhibitory in promoting angiogenesis, it is considered significant in tumor growth and metastasis through activation of monocytes and macrophages and production by these cells of VEGF-A and VEGF-C (Takahashi, 2011). In this study, VEGFR-1 was associated with VEGF-C, as mentioned above, but also with VEGFR-2, providing links to both angiogenesis and lymphangiogenesis. Its negative association with HIF-1α is in agreement with the concept of HIF-1α-induced angiogenesis, which would be hindered in the presence of high VEGFR-1 levels. In the present study where no direct anti-VEGF treatment was administered high VEGFR-1 levels were marginally associated with better survival, possibly because VEGFR-1 would sequester the available VEGF-A and prevent its angiogenic function. Given our previous findings (Fountzilas et al., 2011), we investigated the significance of co-expression of angiogenic parameters and we observed that co-expression of VEGFR-1 and VEGFR-2 was related to more favorable survival. These findings, if confirmed in larger studies, would point to simultaneous targeting of multiple VEGFRs in order to achieve more efficient antiangiogenic and possibly direct antitumor effect.

VEGF-C immunohistochemical overexpression was marginally associated with survival but not with PFS. VEGF-C protein overexpression was associated with worse survival and PFS in a meta-analysis examining lymphangiogenesis parameters in breast carcinoma (Wang et al., 2012). The relatively small sample size, which is a limitation of this study, may account for the only marginal association of VEGF-C with survival, noted herein.

In conclusion, multiple associations were identified among the angiogenesis parameters in MBC, highlighting potential intracellular pathways in the neoplastic cells. Further delineation of these pathways might be useful for testing novel treatment interventions for this currently incurable disease. Furthermore, this study confirms the prognostic role of VEGF-A mRNA in MBC and reveals VEGFR-1/VEGFR-2 protein co-expression as a new, putatively prognostic parameter, which needs further prospective validation in larger cohorts.

Conflict of interest. The authors declare that they have no conflict of interest.

Ethical standards. The authors declare that the research work presented herein is in compliance with the current Greek legislation.

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