Prognostic Significance of *VEGFC* and *VEGFR1* mRNA Expression According to HER2 Status in Breast Cancer: A Study of Primary Tumors from Patients with High-risk Early Breast Cancer Participating in a Randomized Hellenic Cooperative Oncology Group Trial

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Abstract. *Background: Vascular endothelial growth factor C (VEGFC) and vascular endothelial growth factor receptor 1 (VEGFR1) mRNA overexpression has recently been shown to have strong predictive and prognostic value in patients with high-risk early breast cancer undergoing adjuvant chemotherapy. The present study evaluated associations of VEGFC and VEGFR1 with human epidermal growth factor receptor 2 (HER2) and their prognostic value dependent on HER2 status. Patients and Methods: RNA was isolated from 298 formalin-fixed paraffin-embedded tumor tissue samples from the HeCOG 10/97 (HE10/97) trial, evaluating adjuvant dose-dense sequential chemotherapy with epirubicin followed by cyclophosphamide, methotrexate and 5-fluorouracil therapy with or without paclitaxel (E-T-CMF vs. E-CMF). A fully-automated method based on magnetic beads was applied for RNA extraction, followed by one-step quantitative reverse*

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transcription-polymerase chain reaction. Results: At 13.3 years of median follow-up, 116 patients (38.9%) had experienced relapse and 115 (38.6%) had died. There were strong associations between VEGFC/VEGFR1 mRNA expression and HER2 and estrogen receptor/progesterone receptor status. In multivariate analysis, both VEGFC and VEGFR1 were found to be associated with risk for death or relapse, but such associations depended on HER2 status and treatment group. High VEGFC was a negative prognostic factor for disease-free survival [hazard ratio (HR)=1.79, 95% confidence interval (CI)=1.05-3.05, Wald's p=0.032], with a trend for overall survival (HR=1.80, 95% CI=0.94-3.47, p=0.078) in patients treated with E-CMF adjusted for clinicopathological characteristics, while high VEGFR1 was associated with increased risk for death, yet non significantly in patients with HER2-negative disease (HR=1.51, 95% CI=0.82-2.77, p=0.18), regardless of treatment. Conclusion: VEGFC and VEGFR1 mRNA overexpression is of prognostic value, dependent on HER2 status, in patients with high-risk early breast cancer undergoing adjuvant treatment. Among HER2-negative cases, these angiogenic markers could identify more aggressive tumors with worse prognosis. Further studies are warranted to validate VEGFC and VEGFR1 as potential biomarkers in adjuvant therapy and their use in identifying sub-groups that could benefit from anti-VEGF strategies.

The recognition of the role of the human epidermal growth factor receptor 2 (*HER2*) gene and the advent of targeted-therapies against its protein product remains one of the major advances in the management of breast cancer (1). *HER2/neu* gene amplification occurs in 15-30% of breast cancers and is associated with an aggressive tumor phenotype and poor prognosis. HER2 status has become an important indispensable component of the evaluation of patients with breast cancer and the determination of biological behavior and therapy response (2).

Tumoral angiogenesis is important for tumor cell growth and progression (3). Vascular endothelial growth factor (VEGF) is important in breast carcinogenesis (4). However, it remains unclear whether the effect of VEGF expression on survival varies in breast cancer according to HER2 status, while overall, the prognostic significance of VEGF expression in breast cancer remains controversial. The VEGFs and their receptors (VEGFRs) have a central function in angiogenesis and the formation of vascular networks. Today we recognize five VEGFs (VEGFA to -E), with the first three being better-characterized. VEGFA and -B are considered mainly angiogenic, while VEGFC is thought to be more lymphangiogenic. Their binding partners are three different tyrosine kinase receptors, VEGFR1 (or FLT1), VEGFR2 (or KDR/FLK1) and VEGFR3 (or FLT4) (5, 6). VEGFC was initially identified as a ligand for the tyrosine kinase receptor VEGFR3, which is associated with lymphatic vasculature (7). VEGFC is also a ligand for VEGFR2, shared with VEGFA and -D.

A number of recent studies have investigated the role of VEGFC in human tumors (8); however, few have explored its role in breast cancer. In those that have, VEGFC is proposed to be an inducer of tumor lymphangiogenesis and therefore an important promoter of breast cancer metastasis (9-12). VEGFC overexpression, a subsequent increase in lymphangiogenesis, and a higher rate of lymphovascular invasion have been shown to worsen breast cancer prognosis (9, 12, 13). In the human breast cancer cell line MCF-7HER2, kinase stimulation by heregulin-b1 was shown to up-regulate VEGFC expression (14). In addition, the HER2 tyrosine kinase inhibitor PD153035 was shown to inhibit such VEGFC overexpression, thus indicating the importance of HER2 in regulating VEGFC-dependent tumor lymphangiogenesis (14). There is very limited information regarding the predictive role of any of the VEGF family members in patients with breast cancer who are undergoing systemic treatment, hormonal therapy or chemotherapy, and even less regarding their role for specific sub-groups, *i.e.* based on HER2 status.

We recently showed that *VEGFC* and *VEGFR1* mRNA overexpression holds a strong predictive and prognostic value in patients with high-risk early breast cancer undergoing adjuvant chemotherapy, utilizing a one-step quantitative reverse transcription-polymerase chain reaction (qRT-PCR) technique (15). Quantitative RT-PCR is a powerful tool that offers accurate relative quantification of mRNA levels of specific biomarkers in formalin-fixed paraffin-embedded (FFPE) tumor tissue samples (16, 17).

The aim of the present study was to explore and evaluate the significance and clinical relevance of the mRNA expression of *VEGFC* and *VEGFR1* according to HER2 status in patients with high-risk early breast cancer that participated in a randomized adjuvant chemo-hormonotherapy trial.

Patients and Methods

Patient population. FFPE tumor tissue samples were retrospectively collected from patients with high-risk operable breast cancer who participated in a prospective randomized phase III study (HE10/97) by the Hellenic Cooperative Oncology Group of dose-dense sequential chemotherapy with epirubicin, followed by intensified cyclophosphamide, methotrexate and 5-fluorouracil therapy with (E-T-CMF) or without (E-CMF) paclitaxel (Taxol®; Bristol Myers-Squibb, Princeton, NJ, USA). The clinical study randomized a total of 595 patients with high-risk ($T_{1-3}N_1M_0$ or $T_3N_0M_0$) breast cancer from 1997 to 2000 in order to explore the effect of dose-dense sequential chemotherapy with or without paclitaxel (E-T-CMF *vs.* E-CMF), primarily on disease-free survival (DFS) and secondarily on overall survival (OS). Due to the retrospective nature of the present translational research study, collection of FFPE tumor tissue samples was possible for 298 patients only (50% of the 595 randomized patients) due to logistical/organizational barriers. Comparisons of basic

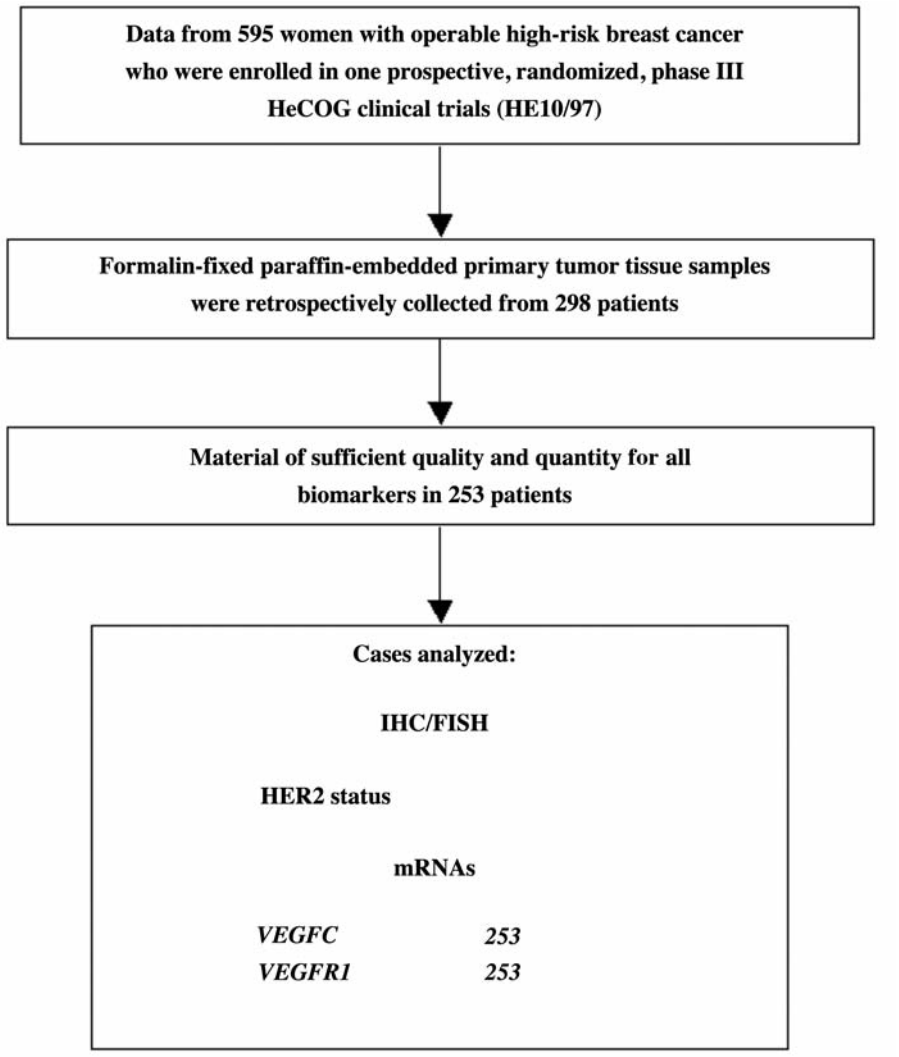


Figure 1. *REMARK diagram*.

patient and tumor characteristics between those patients included in the analysis (FFPE tumor tissue samples available) and the rest of the HE10/97 trial are shown in Table I. The results of the HE10/97 trial have previously been reported (18). The trial was included in the Australian New Zealand Clinical Trials Registry and allocated the following Registration Number: ACTRN12611000506998.

Chemotherapy cycles were administered every 2 weeks and patients received granulocyte-colony stimulating factor support. The present study was approved by the Bioethics Committee of the Aristotle University of Thessaloniki (485/05-07-13) and patients provided written informed consent prior to enrolment. All participating patients also gave written informed consent for research use of their biological material. The REMARK diagram (19) for the present study is shown in Figure 1.

Data collected for this retrospective study included treatment arm, age, menopausal status, interval from operation, number of positive nodes, tumor size, histological grade and adjuvant radiotherapy/hormonotherapy. Primary tumor diameter and axillary nodal status were obtained from the pathology report. Histological grade was evaluated according to the Bloom and Richardson system (20).

Tissue microarray (TMA) construction. Representative hematoxylin-eosin-stained sections from the tissue blocks were reviewed by a pathologist and the most representative tumor areas were marked for the construction of the TMA blocks, as previously described (21). Each case was represented by two tissue cores, 1.5 mm in diameter, with each TMA block also containing cores from various neoplastic, non-neoplastic and reactive tissues serving as assay controls. Cases not represented, damaged or inadequate on the TMA sections were re-cut from the original blocks and were used for protein and gene analysis.

Immunohistochemistry (IHC). IHC for estrogen receptor (ER) (clone 6F11; Novocastra™, Leica Biosystems, Newcastle, UK), progesterone receptor (PgR) (clone 1A6; Novocastra™, Leica

Biosystems) and HER2 (A0485 polyclonal antibody; Dako, Glostrup, Denmark) was performed on serial 2.5 μm-thick TMA sections using a Bond Max™ autostainer (Leica Microsystems, Wetzlar, Germany), as previously described (22). All cases were also stained for vimentin (clone V9; Dako) and cytokeratin 8/18 (clone 5D3; Novocastra™ , Leica Biosystems), which were used as control stains for tissue immunoreactivity and fixation, as well as identification of tumor cells. Tissue samples negative for the above antibodies were excluded from the study. The evaluation of all IHC sections was performed by experienced breast cancer pathologists, blinded to the patient clinical characteristics and survival data.

Interpretation of the IHC results. ER, PgR and HER2 protein expression was evaluated according to established or proposed criteria (23, 24). The ER and PgR immunostaining was scored using the Histoscore method (maximum score=400). Tissue sections stained for ER/PgR were considered to be positive when 1% or more of neoplastic cells displayed nuclear immunoreactivity (23). HER2 protein expression was scored according to the recent guideline recommendations (scores 0 to 3+) (24). HER2 was considered to be positive in cases with an IHC score of 3+ (uniform, intense membrane staining in >30% of invasive tumor cells).

Fluorescence in situ hybridization (FISH). TMA sections or whole-tissue sections (5 μm-thick) were used for FISH analysis, using ZytoLight® SPEC *HER2/TOP2A*/CEN17 triple color probe (ZytoVision, Bremerhaven, Germany), as previously described (25). Four carcinoma cell lines (MDA-MB-231, MDA-MB-175, MDA-MB-453, and SK-BR-3) from the Oracle HER2 Control Slide (Leica Biosystems) with known *HER2* gene status were also used as a control for the FISH assays and analyzed for genomic *HER2* status.

For the evaluation of the *HER2* gene status, non-overlapping nuclei from the invasive part of the tumor were randomly selected and scored. The virtual slides of HER2, ER or PgR stains were used for selecting the invasive part of the tumor in each TMA. The virtual slides were created as previously described (22). Twenty tumor nuclei were counted according to Press *et al*. (26). The *HER2* gene was considered to be amplified when the ratio of the gene probe to centromere probe was 2.2 or more (24) or the *HER2* copy number was greater than 6 (27). In cases with values at or near the cut-off (*i.e*.1.8-2.2), an additional 20 or 40 nuclei were counted and the ratio was recalculated. In cases with a borderline ratio at 60 nuclei, additional FISH assays were performed in whole sections. *HER2* was considered to be positive when amplified (ratio ≥2.2 or copy number >6) by FISH or a HER2 score of 3+ was obtained by IHC.

RNA isolation from FFPE tissue and qRT-PCR assessment. Hematoxylin eosin-stained sections from all available FFPE tissue specimens were evaluated histologically by a certified pathologist who recorded the percentage of tumor cell content. Prior to RNA isolation, macrodissection of tumor areas was performed in most of the FFPE sections with <50% tumor cell content. The tumor cell content was >30% in practically all (97%) of the samples and >50% in the majority (76%) of the samples. More than one FFPE section was used for RNA extraction when the tumor surface of a given sample was less than 0.25 cm2 in an effort to minimize the rate of technical failures in RNA extraction.

Sufficient RNA was isolated from 257 FFPE specimens followed by qRT-PCR, as previously described (28). From each FFPE section or macrodissected tissue fragments (10 μm-thick), RNA was isolated using a standardized fully automated isolation method for total RNA from FFPE tissue, based on silica-coated magnetic beads (VERSANT Tissue Preparation Reagents; Siemens Healthcare Diagnostics, Tarrytown, NY, USA) in combination with a liquid handling robot, as previously described in detail (17). The method involves extraction-integrated deparaffinization and DNase I digestion steps. DNA-free total RNA was eluted with 100 μl elution buffer and stored at −80˚C.

One-step qRT-PCR was applied for the relative quantification of *VEGFC* and *VEGFR1* mRNA expression using gene-specific TaqMan® based assays. Forty cycles of nucleic acid amplification were applied and the cycle threshold (CT) values of the target genes were identified. CT values were normalized by subtracting the CT value of the reference gene ribosomal protein L37a (*RPL37A*) from the CT value of the target genes (ΔCT). RNA results were then reported as 40−ΔCT values, which correlate proportionally to the

Table I. Basic patient and tumor characteristics in cases included (formalin-fixed paraffin-embedded tumor tissue samples available) and not included in the analysis.

Entire HE10/97 cohort: 595 patients	Included	Not included	*p*-Value
Patients			
N	298	297	
Age (years)			
Median	51	49	**0.041**
Range	22-78	24-75	
Number of nodes removed			
Median	19	17	**0.014**
Range	4-59	3-53	
Number of positive nodes			
Median	7	5	**0.035**
Range	0-54	0-49	
	N (%)	N (%)	
Treatment group			
E-CMF	159 (53.5%)	135 (45.3%)	**0.045**
E-T-CMF	138 (46.5%)	163 (54.7%)	
Number of positive nodes			
0-3 nodes	67 (22.6%)	96 (32.2%)	**0.008**
≥4	230 (77.4%)	202 (67.8%)	
Menopausal status			
Pre-menopausal	151 (50.8%)	170 (57.0%)	0.13
Post-menopausal	146 (49.2%)	128 (43.0%)	
Type of operation			
Modified radical mastectomy	228 (76.8%)	223 (74.8%)	0.58
Breast-conserving surgery	69 (23.2%)	75 (25.2%)	
Tumor size			
≤2 cm	98 (33.0%)	87 (29.2%)	0.23
2-5 cm	149 (50.2%)	170 (57.0%)	
>5 cm	50 (16.8%)	41 (13.8%)	
Histological grade			
I-II	150 (50.5%)	139 (47.0%)	0.39
III-IV	147 (49.5%)	157 (53.0%)	

N, Number; E-CMF, epirubicin plus cyclophosphamide, methotrexate and 5-fluorouracil; E-T-CMF, E-CMF plus paclitaxel (taxol). Significant *p*-values are shown in bold.

Table II. Basic patient and tumor characteristics according to HER2 status.

	HER2 status			
	All patients	Negative	Positive	*p*-Value
Patients				
N	298	222	76	
Age (years)				
Median	51	52	49	0.28
Range	22-78	22-78	24-74	
Number of nodes removed				
Median	19	18	21	0.082
Range	4-59	4-59	5-56	
Number of positive nodes				
Median	7	6	8	**0.003**
Range	0-54	0-54	0-35	
N (%)	N (%)	N (%)		
Treatment group				
E-CMF	160 (53.6%)	125 (56.3%)	35 (46.1%)	0.12
E-T-CMF	138 (46.4%)	97 (43.7%)	41 (53.9%)	
Number of positive nodes				
0-3 nodes	68 (22.8%)	55 (24.8%)	13 (17.1%)	0.17
≥4	230 (77.2%)	167 (75.2%)	63 (82.9%)	
Menopausal status				
Pre-menopausal	150 (50.4%)	108 (48.6%)	42 (55.3%)	0.32
Post-menopausal	148 (49.6%)	114 (51.4%)	34 (44.7%)	
Type of operation				
Modified radical mastectomy	228 (76.6%)	167 (75.2%)	61 (80.3%)	0.37
Breast-conserving surgery	70 (23.4%)	55 (24.8%)	15 (19.7%)	
Interval from operation				
<2 Weeks	40 (13.4%)	32 (14.4%)	8 (10.5%)	0.089
2-4 Weeks	144 (48.4%)	99 (44.6%)	45 (59.2%)	
>4 Weeks	114 (38.2%)	91 (41.0%)	23 (30.3%)	
Tumor size				
≤2 cm	98 (32.8%)	81 (36.5%)	17 (22.4%)	0.078
2-5 cm	149 (50.0%)	105 (47.3%)	44 (57.9%)	
>5 cm	51 (17.2%)	36 (16.2%)	15 (19.7%)	
Histological grade				
I-II	150 (50.5%)	125 (56.6%)	25 (32.9%)	**0.001**
III-IV	147 (49.5%)	96 (43.4%)	51 (67.1%)	
ER/PgR status				
Negative	64 (21.5%)	36 (16.2%)	28 (37.3%)	**<0.001**
Positive	223 (78.5%)	186 (83.8%)	47 (62.6%)	
Missing data	1		1	
Adjuvant RT				
No	56 (18.9%)	48 (21.7%)	8 (10.7%)	**0.035**
Yes	240 (81.1%)	173 (78.3%)	67 (89.3%)	
Missing data	2	1	1	
Adjuvant HT				
No	30 (10.1%)	22 (10.0%)	8 (10.5%)	0.89
Yes	267 (89.9%)	199 (90.0%)	68 (89.5%)	
Tamoxifen	230 (77.4%)	173 (78.3%)	57 (75.0%)	0.55
LH-RH agonist	118 (39.9%)	87 (39.5%)	31 (40.8%)	0.85
Aromatase inhibitor	10 (3.4%)	9 (4.1%)	1 (1.3%)	0.25
Other	4 (1.3%)	4 (1.8%)	0 (0.0%)	0.24

N, Number; E-CMF, epirubicin plus cyclophosphamide, methotrexate and 5-fluorouracil; E-T-CMF, E-CMF plus paclitaxel (taxol); ER, estrogen receptor; PgR, progesterone receptor; RT, radiation therapy; HT, hormonal therapy; LH-RH, luteinizing hormone-releasing hormone. Significant *p*-values are shown in bold.

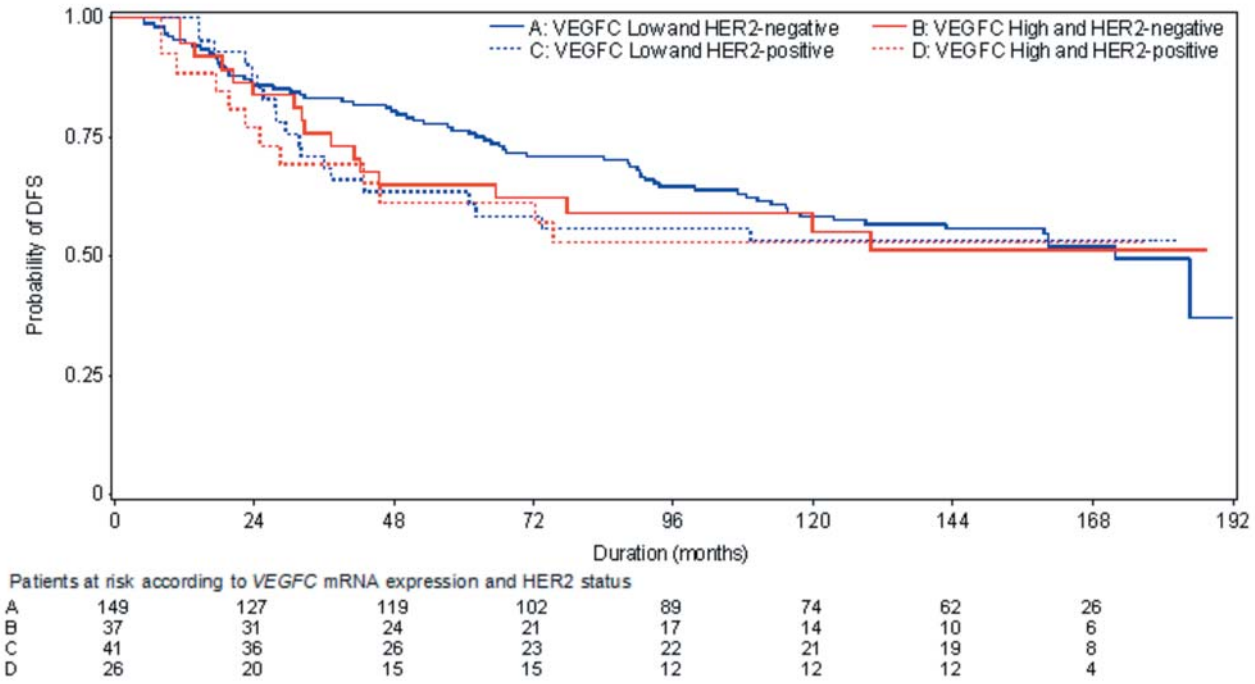
mRNA expression level of the target genes. For assessment of DNA contamination, a qPCR analysis specific for the progestagen-associated endometrial protein (*PAEP*) gene was performed, without the preceding reverse-transcription step. Samples were considered to be substantially free of DNA when CT values above 38 were recorded. In cases of DNA contamination, samples were manually re-digested with DNase I. The quantity of RNA following isolation (yield) was checked by measuring *RPL37A* expression as a surrogate marker for amplifiable mRNA. Samples with average *RPL37A* CT values <32 were considered to have sufficient RNA and were eligible for analysis. Only four of the 257 extracted samples (1%) had an average *RPL37A* CT value of ≥32 and were therefore excluded from further analysis, resulting in successful RNA extraction from 98% of the samples.

Expression of the target genes, as well as the reference gene *RPL37A*, was assessed in triplicate by qRT-PCR using the SuperScript III PLATINUM One-Step Quantitative RT-PCR System with ROX (Invitrogen, Karlsruhe, Germany) in an ABI PRISM 7900HT (Applied Biosystems, Darmstadt, Germany) (16). The lengths of the amplicons detected by the *VEGFC*, *VEGFR1*, and *RPL37A* assays were 77 bp, 85 bp, and 65 bp, respectively, with PCR efficiencies [$E=1^{(10\text{-slope})}$] of 88.2%, 95.7%, and 86.0%, respectively. A commercially available human reference RNA (Stratagene qPCR Human Reference Total RNA; Agilent Technologies, Waldbronn, Germany) was used as positive control. No-template controls were assessed in parallel to exclude contamination.

The Primer/Probe sets used for amplification of the target and reference genes were the following (5' -> 3'): VEGFC: probe TTGAGTCATCTCCAGCATCCGAGGAAA, forward primer: CCA CAGATGTCATGGAATCCAT, reverse primer: TGCCTGGCTCA GGAAGATTT; VEGFR1: probe TGCTGTCGCCCTGGTAGTCA TCAAACA, forward primer: CATGGGAGAGGCCAACAGA, reverse primer: AACCTTTGAAGAACTTTTACCGAATG; RPL37A: probe: TGGCTGGCGGTGCCTGGA, forward primer: TGTGGTTCCTGCAT GAAGACA, reverse primer: GTGACAGCGGAAGTGGTATTGTAC.

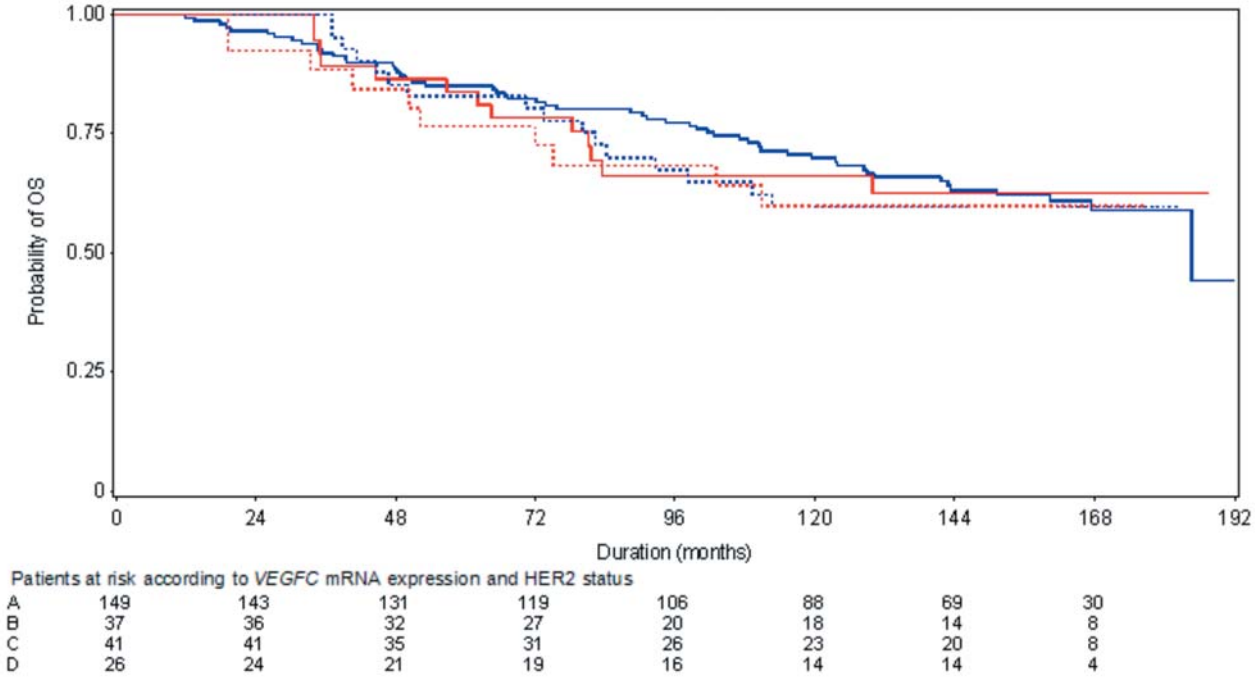
Statistical analysis. OS was measured from the date of randomization until death from any cause. Surviving patients were censored at the date of last contact. DFS was measured from the date of randomization until recurrence of tumor, secondary neoplasm or death from any cause (29). Time-to-event distributions were estimated using Kaplan–Meier curves. Continuous variables are presented as medians with the corresponding range and categorical variables as frequencies with the respective percentages. Associations of ligands and receptors with basic patient and tumor characteristics were examined using the chi-square or Fisher's exact test for categorical variables and the Mann–Whitney or the Kruskal–Wallis tests, where appropriate, for continuous variables.

Correlations between *VEGFC*, *VEGFR1* and HER2 and ER/PgR status were calculated using the Spearman's rank correlation coefficient (Rho). Cox regression analyses were performed to assess the relationship between markers and OS or DFS. Interactions between markers and treatment group, as well as HER2 and ER/PgR status were also explored in the Cox models. In the multivariate Cox regression analysis, a backward selection procedure with a removal criterion of $p>0.15$ based on the likelihood ratio test was performed to identify significant variables among the following: treatment group (E-CMF *vs*. E-T-CMF), menopausal status (post *vs*. pre), time interval from breast surgery (>4 weeks *vs*. 2-4 weeks *vs*. <2 weeks), histological grade (III-IV *vs*. I-II), tumor size (>5 cm *vs*. 2-5 cm *vs*. ≤2 cm), number of



Patients at risk according to *VEGFC* mRNA expression and HER2 status

A	149	127	119	102	89	74	62	26
B	37	31	24	21	17	14	10	6
C	41	36	26	23	22	21	19	8
D	26	20	15	15	12	12	12	4



Patients at risk according to *VEGFC* mRNA expression and HER2 status

A	149	143	131	119	106	88	69	30
B	37	36	32	27	20	18	14	8
C	41	41	35	31	26	23	20	8
D	26	24	21	19	16	14	14	4

Figure 2. *Disease-free survival (DFS) and overall survival (OS) according to vascular endothelial growth factor C (VEGFC) mRNA expression and human epidermal growth factor receptor 2 (HER2) status.*

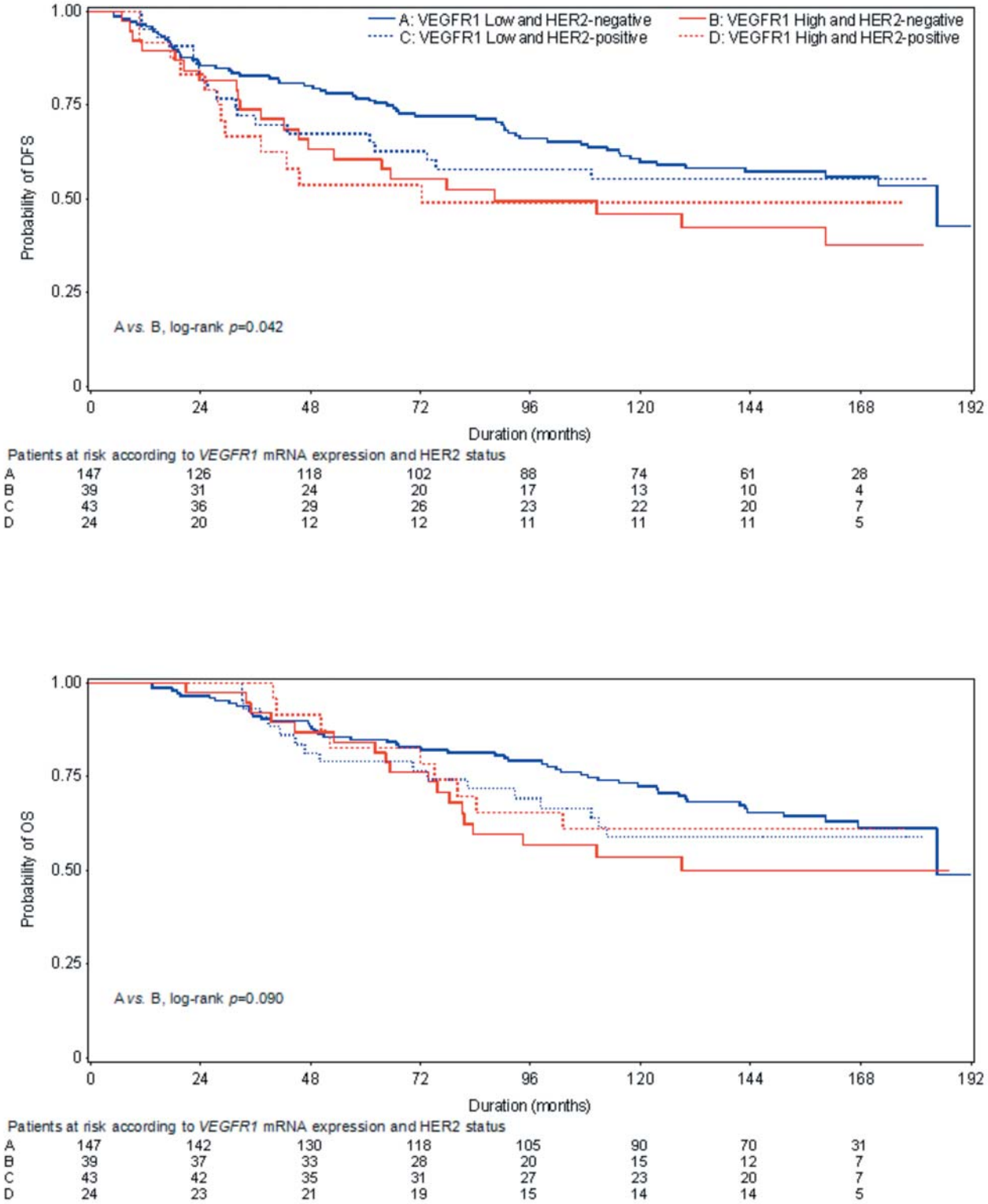


Figure 3. *Disease-free survival (DFS) and overall survival (OS) according to vascular endothelial growth factor receptor 1 (VEGFR1) mRNA expression and human epidermal growth factor receptor 2 (HER2) status.*

Table III. *Association of vascular endothelial growth factor C and vascular endothelial growth factor receptor 1 mRNA expression with basic patient and tumor characteristics. Cut-off values were set at the 75th percentile of the marker's distribution.*

	VEGFC mRNA expression (N=253)			VEGFR1 mRNA expression (N=253)		
	High (n=63) N (%)	Low (n=190) N (%)	*p*-Value	High (n=63) N (%)	Low (n=190) N (%)	*p*-Value
HER2 status						
Negative	37 (58.7%)	149 (78.4%)	**0.002**	39 (61.9%)	147 (77.4%)	**0.016**
Positive	26 (41.3%)	41 (21.6%)		24 (38.1%)	43 (22.6%)	
Age (years)						
<50	24 (38.1%)	98 (51.9%)	0.058	25 (39.7%)	97 (51.3%)	0.11
≥50	39 (61.9%)	91 (48.1%)		38 (60.3%)	92 (48.7%)	
Treatment group						
E-T-CMF	24 (38.1%)	95 (50.0%)	0.10	26 (41.3%)	92 (48.4%)	0.32
E-CMF	39 (61.9%)	95 (50.0%)		37 (58.7%)	98 (51.6%)	
Menopausal status						
Pre-menopausal	28 (44.4%)	103 (54.2%)	0.18	26 (41.3%)	105 (55.3%)	0.054
Post-menopausal	35 (55.6%)	87 (45.8%)		37 (58.7%)	85 (44.7%)	
ER/PgR status						
Negative	20 (32.3%)	32 (16.8%)	**0.009**	19 (30.6%)	33 (17.4%)	**0.025**
Positive	42 (67.7%)	158 (83.2%)		43 (69.4%)	157 (82.6%)	
Missing data	1 (1.6%)			1 (1.6%)		
Number of positive nodes						
0-3 nodes	10 (15.9%)	45 (23.7%)	0.19	8 (12.7%)	47 (24.7%)	**0.045**
≥4	53 (84.1%)	145 (76.3%)		55 (87.3%)	143 (75.3%)	
Type of operation						
Modified radical mastectomy	52 (82.5%)	145 (76.3%)	0.30	52 (82.5%)	145 (76.3%)	0.30
Breast-conserving surgery	11 (17.5%)	45 (23.7%)		11 (17.5%)	45 (23.7%)	
Tumor size						
≤2 cm	26 (41.3%)	54 (28.4%)	0.10	25 (39.7%)	55 (28.9%)	0.28
2-5 cm	30 (47.6%)	99 (52.1%)		28 (44.4%)	101 (53.2%)	
>5 cm	7 (11.1%)	37 (19.5%)		10 (15.9%)	34 (17.9%)	
Histological grade						
I-II	36 (57.1%)	96 (50.5%)	0.36	34 (54.0%)	98 (51.6%)	0.74
III-IV	27 (42.9%)	94 (49.5%)		29 (46.0%)	92 (48.4%)	
Adjuvant RT						
No	7 (11.1%)	36 (19.1%)	0.14	4 (6.5%)	39 (20.6%)	**0.010**
Yes	56 (88.9%)	152 (80.9%)		58 (93.5%)	150 (79.4%)	
Interval from operation						
<2 Weeks	7 (11.1%)	28 (14.7%)	0.77	8 (12.7%)	27 (14.2%)	0.57
2-4 Weeks	31 (49.2%)	91 (47.9%)		34 (54.0%)	88 (46.3%)	
>4 Weeks	25 (39.7%)	71 (37.4%)		21 (33.3%)	75 (39.5%)	
Adjuvant HT						
No	8 (12.7%)	16 (8.4%)	0.32	8 (12.7%)	16 (8.4%)	0.32
Yes	55 (87.3%)	174 (91.6%)		55 (87.3%)	174 (91.6%)	
Tamoxifen						
No	14 (22.2%)	38 (20.0%)	0.71	13 (20.6%)	39 (20.5%)	0.99
Yes	49 (77.8%)	152 (80.0%)		50 (79.4%)	151 (79.5%)	
LH-RH agonist						
No	46 (73.0%)	107 (56.6%)	**0.021**	45 (71.4%)	108 (57.1%)	**0.044**
Yes	17 (27.0%)	82 (43.4%)		18 (28.6%)	81 (42.9%)	
Aromatase inhibitor						
No	62 (98.4%)	182 (95.8%)	0.33	62 (98.4%)	182 (95.8%)	0.33
Yes	1 (1.6%)	8 (4.2%)		1 (1.6%)	8 (4.2%)	
Other						
No	62 (98.4%)	188 (98.9%)	0.73	63 (100.0%)	187 (98.4%)	0.32
Yes	1 (1.6%)	2 (1.1%)			3 (1.6%)	

N, Number; E-CMF, epirubicin plus cyclophosphamide, methotrexate and 5-fluorouracil; E-T-CMF, E-CMF plus paclitaxel (taxol); ER, estrogen receptor; PgR, progesterone receptor; RT, radiation therapy; HT, hormonal therapy; LH-RH, luteinizing hormone-releasing hormone. Significant *p*-values are shown in bold.

positive axillary nodes (≥4 *vs*. 0-3), ER/PgR status (positive *vs*. negative *vs*. missing data), HER2 status (negative *vs*. positive), hormonal therapy (yes *vs*. no), radiotherapy (yes *vs*. no), *VEGFC* (high *vs*. low, at the 75th percentile), *VEGFR1* (high *vs*. low, at the 75th percentile).

The design of the study is prospective-retrospective as described in Simon *et al*. (30). Results of this study are presented according to reporting recommendations for tumor marker prognostic studies (19). SAS software was used for statistical analysis (SAS for Windows, version 9.2; SAS Institute Inc., Cary, NC, USA). No adjustment for multiple comparisons is reported.

Results

Patients' and tumor characteristics. A total of 298 primary tumor tissue samples were analyzed, as stated in the Patients and Methods section (Table I). Basic patient and tumor characteristics according to HER2 status were well-balanced (Table II) except for the number of positive nodes (Fisher's exact test, p=0.003), histological grade (p=0.001), ER/PgR status (p=0.001) and adjuvant radiotherapy (p=0.035). The median follow-up period was 13.3 years (range=7-192 months). A total of 116 patients developed a relapse (38.9%) and 115 patients died (38.6%). The median OS was 185 months [95% confidence interval (CI)=185 months-not reached yet], while median DFS was 185 months (95% CI=129 months-not reached yet). The 5-year OS rate was 83% (95% CI=79-87%) and the 7-year OS rate 74% (95% CI=69-89%). The 5- and 7-year DFS rates were 69% (95% CI=64-75%) and 64% (95% CI=58-69), respectively.

Associations of VEGFC and VEGFR1 mRNA expression with patient and tumor characteristics. The mRNA expression of *VEGFC* and *VEGFR1* was evaluated for associations with the following patient and tumor characteristics: HER2 status, age, treatment group, menopausal status, ER/PgR status, number of positive nodes, type of and interval from operation, tumor size, histological grade and adjuvant treatment (hormonal and radiation therapy). Cut-off values were set at the 75th percentile of the marker's distribution (Table III). High mRNA expression of *VEGFC* was associated with a trend for higher age (≥50 years, Fisher's exact test, p=0.058), while ER/PgR-negative tumors were more frequent in patients with high *VEGFC*-expressing tumors (32.3% in high *vs*. 16.8% in low, p=0.009). Concerning *VEGFR1*, ER/PgR-negative tumors were more frequent in patients with high *VEGFR1*-expressing tumors (30.6% in high *vs*. 17.4% in low, p=0.025), while high expression of *VEGFR1* was associated with an increased number of positive nodes (p=0.045) and adjuvant radiotherapy (p=0.010).

With regards to HER2 status, there was a strong association between HER2 status and *VEGFC* and *VEGFR1* mRNA expression: HER2-positive tumors were more frequent in patients with high *VEGFC*-expressing tumors (41.3% in high *vs*. 21.6% in low, Fisher's exact test, p=0.002) and similarly in patients with high *VEGFR1*-expressing tumors (38.1% in high *vs*. 22.6% in low, p=0.016). Nonetheless, continuous values of *VEGFC* and *VEGFR1* did not differ according to HER2 status. Overall, high *VEGFC* and *VEGFR1* expression was more frequent in patients with ER/PgR-negative and HER2-positive tumors. The number of positive lymph nodes did not seem to be associated with the expression of VEGFC (p=0.19).

Univariate analysis for prognostic significance. Regarding co-expressions, not taking into consideration the treatment effect, HER2 status did not differentiate the *VEGFC* effect regarding OS and DFS in the univariate setting (Figure 2). On the contrary, patients with HER2-negative disease with high *VEGFR1* mRNA expression had a trend for worse DFS and OS in comparison to those with low *VEGFR1* expression (HR=1.61, 95% CI=0.99-2.62, Wald's p=0.055, log-rank p=0.042; and HR=1.57, 95% CI=0.92-2.69, Wald's p=0.10, log-rank p=0.090, respectively) (Figure 3).

Multivariate analysis for predictive and prognostic significance. In multivariate analysis, this pattern was examined for prognostic and predictive significance when accounting for treatment. Basic patient and tumor characteristics according to treatment arm were well-balanced (Table IV), except for histological grade (Chi-square test, p=0.001), in agreement with the corresponding results in the full cohort presented in the clinical article (18).

The Cox multivariate regression analysis for OS (Figure 4) revealed that the risk for death at any time was significantly higher for patients with high histological grade (HR=1.43, 95% CI=0.92-2.21; p=0.11) and more than three positive nodes (HR=4.01, 95% CI=1.92-8.39; p<0.001) and lower for patients that received hormonal therapy (HR=0.48, 95% CI=0.25-0.90; p=0.021). The same clinicopathological factors, except for histological grade, had significant prognostic value for DFS (Figure 4): four or more positive nodes (HR=2.66, 95% CI=1.51-4.69, p<0.001) and hormonal therapy (HR=0.50, 95% CI=0.28-0.90, p=0.020).

High *VEGFC* and *VEGFR1* expression was associated with increased risk for death and relapse but their effect depended on HER2 status or treatment group. For both DFS and OS, the interaction of *VEGFC* with treatment group had a p-value of 0.004, while for OS, the interaction of *VEGFR1* and HER2 had a p-value of 0.13.

In terms of DFS (Figure 4), regardless of HER2 status, high *VEGFC*-expressing tumors were found to be associated with increased risk for relapse in patients treated with E-CMF (HR=1.79, 95% CI=1.05-3.05, Wald's p=0.032), while in terms of OS, a trend for increased risk for death was observed (HR=1.80, 95% CI=0.94-3.47, Wald's p=0.078). Finally, in terms of OS (Figure 4), regardless of treatment,

high *VEGFR1*-expressing tumors were found to be associated with increased risk for death, yet non significantly, in patients with HER2-negative disease (HR=1.51, 95% CI=0.82-2.77, Wald's p=0.18).

Discussion

The quest for predictive markers of response to breast cancer treatment, initially based primarily on ER levels, is now been extensively explored using HER2 and is rapidly expanding to other molecular classes (31, 32). The significant role of angiogenesis in breast cancer progression and metastasis is being further recognized, as evidence both from *in vitro* and clinical studies is rapidly accumulating (33, 34). We recently showed that among the VEGF family members, *VEGFC* and *VEGFR1* mRNA overexpression holds a strong predictive and prognostic value in patients with high-risk early breast cancer undergoing adjuvant chemotherapy (15). It has been demonstrated that HER2 overexpression is associated with high VEGF levels in breast cancer (35) and, more recently, the associations of HER2 with lymphangiogenesis and VEGFC have also been explored (36). The present study was designed to evaluate the associations of *VEGFC* and *VEGFR1* mRNA expression with HER2 and the prognostic value of *VEGFC* and *VEGFR1* according to HER2 status. Indeed, a statistically significant association was shown between *VEGFC* and *VEGFR1* mRNA overexpression and HER2 overexpression (p<0.001), adding to the recent evidence on the interactions between HER2 and angiogenic factors.

High VEGF expression levels have been associated with HER2 overexpression (37), in accordance to preclinical data, as HER2 is known to up-regulate *VEGF* in human tumor cell lines (38, 39). With regards to VEGFC, several pre-clinical studies indicate its significant role in breast cancer progression via stimulation of lymphangiogenesis, as well as direct effects on cancer cell migration and proliferation (40, 41). Similarly, in clinical studies with breast carcinomas, VEGFC expression was associated with a high risk of lymph node metastasis and worse outcome (9, 10, 42). Interesting data in non-small cell lung cancer showed that VEGFC expression is mediated by transactivation of HER2 *via* the SRC kinase pathway, thus indicating a direct association between HER2 and lymphangiogenesis (43). Further pre-clinical studies in human breast cancer cells provided evidence on the migration-promoting role of VEGFC and its receptors and their dependence on HER2 (44). The monoclonal antibody to HER2, trastuzumab, inhibited angiogenesis in an animal model of HER2-overexpressing breast cancer (38), as well as inhibiting lymphangiogenesis by significantly reducing *VEGFC* mRNA and protein expression in breast cancer cells (14).

In a recently published study, our group analyzed the mRNA expression of well-recognized VEGF family members, including receptors (VEGFR1, 2 and 3) and their

Table IV. *Basic patient and tumor characteristics according to treatment arm.*

	Treatment group		
	E-CMF	E-T-CMF	p-Value
Patients			
N	160	138	
Age (years)			
Median	51.0	51.8	0.62
Range	22.5-78.0	23.8-75.9	
Number of nodes removed			
Median	19.0	19.0	0.73
Range	4-50	5-59	
Number of positive nodes			
Median	6.0	7.0	0.39
Range	0-35	0-54	
	N (%)	N (%)	
HER2 status			
Negative	125 (78.1%)	97 (70.3%)	0.12
Positive	35 (21.9%)	41 (29.7%)	
Number of positive nodes			
0-3 nodes	42 (26.3%)	26 (18.8%)	0.13
≥4	118 (73.8%)	112 (81.2%)	
Menopausal status			
Pre-menopausal	83 (51.9%)	67 (48.6%)	0.57
Post-menopausal	77 (48.1%)	71 (51.4%)	
Type of operation			
Modified radical mastectomy	123 (76.9%)	105 (76.1%)	0.87
Breast-conserving surgery	37 (23.1%)	33 (23.9%)	
Interval from operation			
<2 Weeks	23 (14.4%)	17 (12.3%)	0.72
2-4 Weeks	74 (46.3%)	70 (50.7%)	
>4 Weeks	63 (39.4%)	51 (37.0%)	
Tumor size			
≤2 cm	58 (36.3%)	40 (29.0%)	0.33
2-5 cm	74 (46.3%)	75 (54.3%)	
>5 cm	28 (17.5%)	23 (16.7%)	
Histological grade			
I-II	95 (59.4%)	55 (40.1%)	**0.001**
III-IV	65 (40.6%)	82 (59.9%)	
ER/PgR status			
Negative	28 (17.6%)	36 (26.1%)	0.076
Positive	131 (82.4%)	102 (73.9%)	
Missing data	1 (0.6%)		
Adjuvant RT			
No	32 (20.1%)	24 (17.5%)	0.57
Yes	127 (79.9%)	113 (82.5%)	
Adjuvant HT			
No	19 (11.9%)	11 (8.0%)	0.27
Yes	141 (88.1%)	126 (92.0%)	
Tamoxifen	118 (73.8%)	112 (81.8%)	0.10
LH-RH agonist	62 (39.0%)	56 (40.9%)	0.74
Aromatase inhibitor	6 (3.8%)	4 (2.9%)	0.69
Other	2 (1.3%)	2 (1.5%)	0.88

N, Number; E-CMF, epirubicin plus cyclophosphamide, methotrexate and 5-fluorouracil; E-T-CMF, E-CMF plus paclitaxel (taxol); ER, estrogen receptor; PgR, progesterone receptor; RT, radiation therapy; HT, hormonal therapy; LH-RH, luteinizing hormone-releasing hormone. Significant p-values are shown in bold.

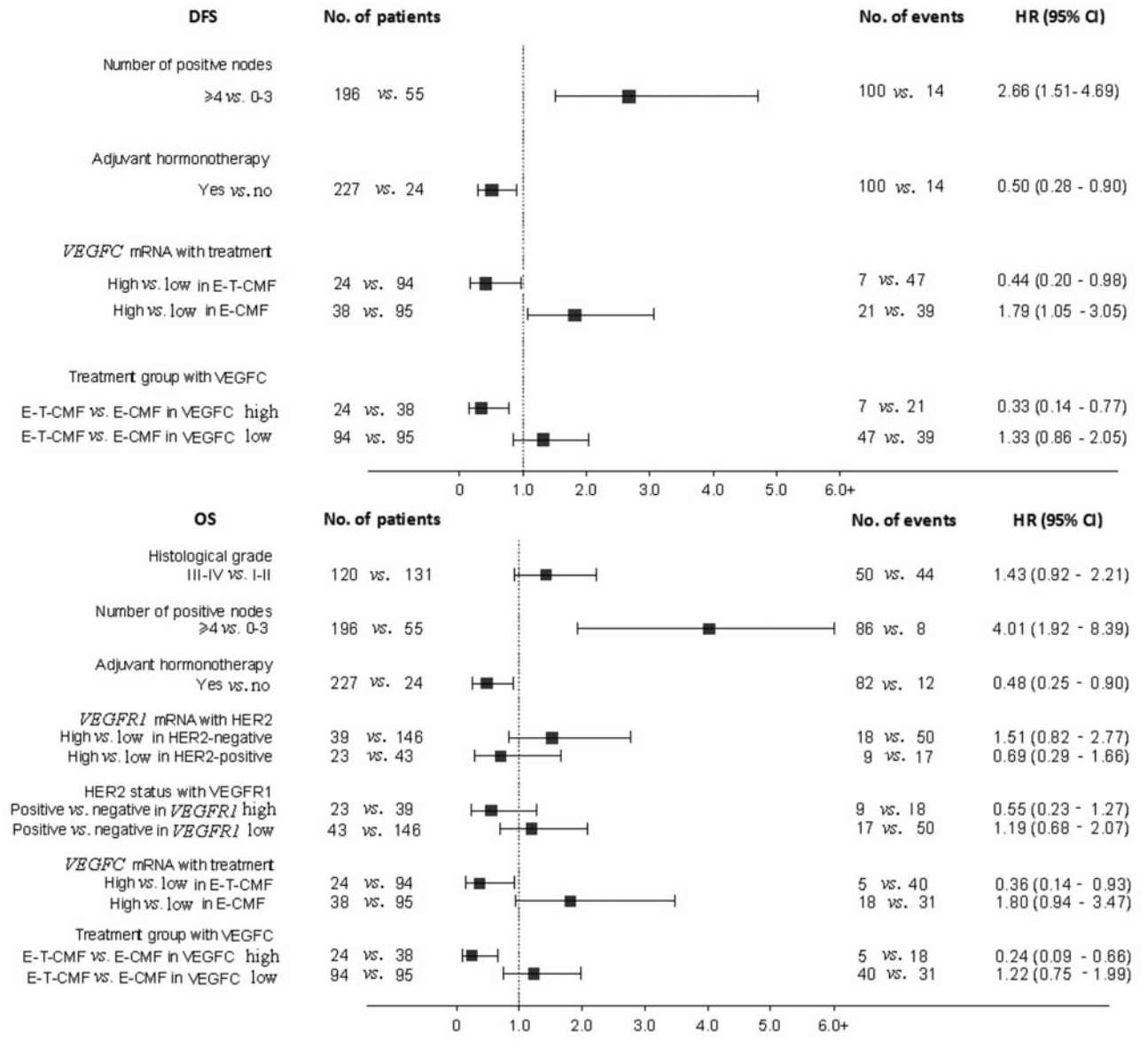


Figure 4. *Multivariate Cox regression analysis for disease-free survival (DFS) and overall survival (OS) presented by forest plots.*

ligands (VEGFA, -B and -C) in an attempt to identify individual members with prognostic or predictive significance (15). The same patient cohort, with extended follow-up, was utilized for the present study and included patients with early breast cancer with high-risk characteristics: half were pre-menopausal, the majority had four or more positive axillary lymph nodes, large tumor size in most cases, and almost half had high-grade tumors, while 21.5% had ER/PgR-negative and 25.5% HER2-positive tumors. These patients were randomized to receive adjuvant anthracycline-based chemotherapy with or without a taxane (E-T-CMF *vs*. E-CMF). In this high-risk population, the most important emerging factor was expression of *VEGFC*, a significant member of the VEGF family; in agreement with recent evidence from a number of studies (9-11, 36, 45, 46) associations were found with *VEGFC* and aggressive phenotype characteristics. Furthermore, high *VEGFC* and *VEGFR1* mRNA expression was more frequently seen in patients with HER2-negative tumors, indicating that certain VEGF family members could prove to be even more useful when analyzed in combination with other markers, with potential, for instance, to recognize patients with poor prognosis among the HER2-positive or, more importantly, the HER2-negative populations. The above indications

formed the basis of the present evaluation of *VEGFC* and *VEGFR1* mRNA expression according to HER2 status in an attempt to identify clinically relevant associations.

Indeed, in the present analysis, strong interactions were observed between HER2 status and *VEGFC* and *VEGFR1* mRNA expression. Regarding both DFS and OS, it seems that the high-risk category for disease progression and death involves patients with HER2-positive and high *VEGFC*- or *VEGFR1*-expressing tumors. But more interestingly, *VEGFC* or *VEGFR1* mRNA expression allowed for the identification of a sub-group of patients with worse prognosis among patients with HER2 negativity. Patients with highly *VEGFR1*-expressing tumors had worse DFS than those with low *VEGFR1* expression among the HER2-negative group, while high *VEGFR1* mRNA expression was associated with a trend for worse OS among patients with HER2-negative disease. This could have significant clinical consequences, not only because it provides further tools to sub-categorize patients with breast cancer with worse prognosis among groups with favorable characteristics, but also because these could specifically be those patients that might benefit from treatment with targeted compounds directed towards VEGF, in the near future. Recent evidence of the strong predictive value of VEGF in pre-menopausal patients with early breast cancer (47), as well as the predictive significance of tumor angiogenesis in those with high-risk early breast cancer (48), underlines the need for additional studies that could possibly support or clarify these findings.

Large research groups are including extensive biomarker programs in clinical trials, such as the AVADO trial with a combination of bevacizumab with first-line chemotherapy, which was shown to significantly improve PFS in HER2-negative metastatic breast cancer. However, identification of patients benefiting most from bevacizumab remains elusive, with plasma VEGFA emerging as a potential predictive biomarker, currently under prospective evaluation in the MERiDiAN trial in patients with metastatic breast cancer (49).

In conclusion, the present study reports for the first time that *VEGFC* and *VEGFR1* mRNA overexpression, as assessed by qRT-PCR, has prognostic value dependent on HER2 status, in patients with high-risk early breast cancer undergoing adjuvant anthracycline-containing treatment, providing evidence for a clinically relevant association between HER2 status and *VEGFC* and *VEGFR1* mRNA expression in breast cancer. Among patients with HER2-negative disease, the mRNA expression of these angiogenic markers could identify patients with worse prognosis and more aggressive tumors. Further studies are warranted to validate *VEGFC* and *VEGFR1* as potential biomarkers in adjuvant therapy and to identify patients that could benefit from anti-VEGF strategies among biologically distinct groups with better prognosis, such as those with HER2-negative disease.

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