Expression of angiogenic markers in the peripheral blood of docetaxel-treated advanced breast cancer patients: A Hellenic Cooperative Oncology Group (HeCOG) study

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Abstract. It is well known that low-dose metronomic chemotherapy has antiangiogenic activity. The aim of the present trial was to investigate the antiangiogenic properties of weekly docetaxel in patients with metastatic breast cancer. In total, 157 metastatic breast cancer patients received 35 mg/m^2 docetaxel weekly as a recommended treatment. Blood samples were collected before the start of chemotherapy (baseline) and during treatment. Nitric oxide (NO) and vascular endothelial growth factor A (VEGF-A) plasma levels were measured at baseline and during treatment, while *VEGF-A*, endothelial nitric oxide synthase (*eNOS*) and thrombospondin-1 (*THBS-1*) peripheral blood mRNA levels were measured at baseline, in 127 patients and 39 female healthy controls. In general, the treatment was well-tolerated. Sixty-one patients (38%) achieved an objective response (4% complete and 34% partial response), while 52 (33%) had stable disease and 27 (17%) progressed. At a median follow-up of 33.5 months (range 2.8-45.0), 118 patients (74%) demonstrated disease progression and 94 (59%) had died. Median progression-free survival (PFS) was 8.8 months and median overall survival (OS) was 27.7 months. Median baseline level of plasma NO was significantly lower in patients than in healthy controls (p=0.010), while none of the plasma markers significantly changed upon docetaxel treatment. In addition, the median relative quantification value for *THBS-1* mRNA was significantly higher (p<0.001) in patients as compared to healthy controls. NO plasma levels were positively associated with the number of organs involved (p=0.008). In multivariate analysis, low *eNOS* mRNA levels showed adverse prognostic significance for OS and high *THBS-1* mRNA levels were found to be associated with shorter OS and PFS, independently from established clinical prognostic factors. Although an antiangiogenic activity of weekly docetaxel was not demonstrated in the present study, some interesting observations regarding the prognostic role of a number of blood angiogenic markers could be made.

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Introduction

During the past two decades, new chemotherapeutic options have been developed in the treatment of metastatic breast cancer (MBC). Docetaxel (D) has demonstrated significant activity, as a single agent or combined with other drugs, in several phase II and III clinical trials in the first-line setting, as well as in anthracycline-resistant MBC. There is no conclusive evidence that D mono-therapy given 3-weekly is more effective than the corresponding weekly regimen in MBC patients (1-3), although the superiority of the former was demonstrated in the adjuvant setting (4). Weekly D at a dose of 35-40 mg/m^2 induces a

response rate (RR) of 30-40% and leads to a time-to-progression (TTP) of 5-7 months (5), while 3-weekly D treatment is associated with an RR of 30-48% and a TTP of 4.2-6.5 months in phase II trials (6). However, the weekly regimen seems to have a better toxicity profile causing less neutropenia, mucositis and neurotoxicity (1-3).

On the other hand, enough data from *in vitro* and animal model experiments suggest that D at low doses has antiangiogenic activity (7,8). D was shown to induce the expression of antiangiogenic thrombospondin-1 (*THBS-1*) (8), decrease vascular endothelial growth factor (VEGF) (8) and to cause endothelial cell toxicity *in vitro* (7), while reducing microvessel density in animal models (7). In addition, frequent and subtoxic doses of chemotherapeutics, namely metronomic treatment, were found to inhibit angiogenesis. Notably, it was reported after our study had been initiated, that bevacizumab improved progression-free survival (PFS) when combined with paclitaxel (9) or D (10). The latter results strengthen the need to explore the antiangiogenic properties of taxanes in MBC, as not enough data from clinical studies exist in the literature.

The aim of the present study was to examine the antiangiogenic properties of weekly D, considered at the time to be a standard treatment in patients with advanced breast cancer, by measuring the levels of angiogenic markers in the blood.

Patients and methods

Treatment plan and response evaluation. In this prospective translational research study, patients with MBC were treated with weekly D 35 mg/m^2 over 30 min for 12 weeks, as a recommended first-line chemotherapy schedule by the Hellenic Cooperative Oncology Group (HeCOG). Patients with partial response (PR) could continue treatment beyond 12 cycles, at their physician's discretion. Pre-medication for prophylaxis from possible adverse reactions (hypersensitivity, edema) was given to all patients 30 min before each D dose and consisted of dexamethasone 8 mg, dimethidene maleate 4 mg and cimetidine 150 mg intravenously, while the same dose of dexamethasone was also administered 12 h after each chemotherapy infusion. Patients with HER2-overexpressing tumors [3+ by immunohistochemistry (IHC) or FISH-positive, when 2+ by IHC] were treated with trastuzumab (initially 8 and then 6 mg/kg every 3 weeks) as well, concomitantly with D. Of note, the policy of our group was to continue treatment with trastuzumab beyond disease progression in all patients with an expected survival of >3 months and no serious cardiac problems. Patients with osseous metastases were allowed to receive bisphosphonates during the entire chemotherapy period and thereafter, at the discretion of the treating physician. Tailoring hormonal therapy (HT) after the completion of chemotherapy for patients with hormone receptor-positive status included, exemestane 25 mg daily until progression for postmenopausal women and an LH-RH analogue combined with tamoxifen for pre-menopausal patients. Second-line chemotherapy was allowed only after progression of disease. Standard ECOG criteria were used to define measurable disease, evaluable disease, response and toxicity (11).

Translational research study. This prospective translational research study (HE11/06) was approved by the HeCOG Protocol Review Committee and the Institutional Review Board of the AHEPA University Hospital, as well as the Bioethics Committees of the Aristotle University of Thessaloniki School of Medicine. Written informed consent was obtained from all patients.

Determination of plasma protein levels. Nine milliliters of blood were collected in EDTA tubes, in the morning (between 08.30 and 11.00 h), from 127 patients with MBC before the initiation of the treatment, and from 70 patients during the treatment (at least 4 weeks after treatment initiation). Morning blood samples were also collected from 39 healthy female controls. The plasma was separated by centrifugation (at 2,000 rpm), aliquoted and frozen at -80˚C within 20 min from blood collection.

Plasma concentrations of nitric oxide (NO) and VEGF-A were measured, in duplicates, by solid-phase enzyme-linked immunosorbent (ELIZA) assays using commercially available kits (NO from Assay Designs, Ann Arbor, MI, and VEGF-A from R&D Systems, Minneapolis, MN, USA). Due to the transient and volatile nature of NO, the assay used estimated NO blood levels by measuring its two stable breakdown products, nitrate (NO_3) and nitrite (NO_2), that can be easily detected by photometric means. The lower limit of detection was 3.13 μmol/l for the NO assay and 31.25 pg/ml for the VEGF-A assay. Both assays were specific without significant cross-reactivity with other related molecules. The intra- and inter-assay coefficients of variation, established by the manufacturers for concentrations close to the center of the standard curves, were 3.1 and 4.2% for the NO assay, and 4.5 and 7.0% for the VEGF-A assay, respectively.

Peripheral blood mRNA measurements. Baseline peripheral blood was drawn in PAXgene tubes and frozen at -80˚C. Total RNA, corresponding to transcripts produced mainly in blood monocytes, was extracted with the PAXgene blood RNA kit, according to manufacturer's instructions (PreAnalytiX; Qiagen), within 6 months from collection. The additive contained in the PAXgene tubes reduces *in vitro* RNA degradation and minimizes *in vitro* gene induction. RNA was reverse transcribed into cDNA with random hexamers and Superscript III Reverse Transcriptase, followed by an incubation step with RNase H (all reagents from Invitrogen/Life Technologies). The expression of the following transcripts was investigated: *VEGF-A* exons 3-5, amplicon size 194 bp and Tm 83.3˚C, with forward 5'-GTACATCTTCAAGCCATCCT-3' and reverse 5'-CTTTCTTTGGTCTGCATTCAC-3' primers; *THBS-1* exons 3-4, amplicon size 187 bp and Tm 81.8˚C, with forward 5'-TTGTCTTTGGAACCACACCA-3' and reverse 5'-CTGGACAGCTCATCACAGGA-3' primers; and *eNOS* exons 9-10, amplicon size 180 bp and Tm 85.5˚C, with forward 5'-GTTACCAGCTAGCCAAAGTC-3' and reverse 5'-GGAAATAGTTGACCATCTCCTG-3' primers. A GAPDH transcript was used as endogenous control for the assessment of template adequacy (size 183 bp; Tm 82.2˚C). Relative expression of the above genes was assessed with quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and SYBR Green labeling of the reaction products by using the Power SYBR Green PCR Master Mix (Applied Biosystems/Life Technologies) and an ABI7500 DNA sequence detection

system equipped with SDS v1.4 software. Briefly, samples were run in duplicates in 20-μl reactions, including 50 ng cDNA and 1 μM each primer; reactions were incubated at 50˚C for 2 min and at 95˚C for 10 min, and then amplified for 45 cycles at 95˚C/15 sec and 60˚C/60 sec. Melting curves were obtained by incubating reaction products once at 95˚C for 15 sec, 60˚C for 60 sec and 95˚C for 15 sec, and assessed for PCR product Tm's for the evaluation of PCR product specificity. Values ± 0.5 of the predicted Tm for each target were accepted as indicative of the presence of the given target in the sample. The size of PCR products for all assays from the two initial runs was validated upon agarose electrophoresis. Cycle threshold (CT) values were obtained by default readings. Criteria for excluding samples from analysis were i) GAPDH Tm values <81.7 or >82.7; ii) GAPDH CT values >36; iii) differences in GAPDH CT values >1 for duplicates in the same run. Overall, out of a total of 349 samples that were evaluated with qRT-PCR, 12 (3.4%) were excluded from analysis due to aberrant GAPDH Tm values. The remaining of the samples had CT_{GAPDH} mean 25.71; median 27.18 and SD ±12.45. Relative quantification (RQ) of target mRNA expression was performed with the 40-ΔCT method ($\Delta CT = CT_{target} - CT_{GAPDH}$). Patient samples were analyzed in comparison to 33 samples obtained from an equal number of female healthy controls.

In order to evaluate reproducibility of results (inter-assay variability), 19 randomly selected samples from patients and healthy controls were evaluated in a separate run that was performed with the same assays under the same conditions several months after the first one (technical repeat). RQvariability = mean of ($RQ_{replicate\ run} - RQ_{first\ run}$) was calculated for each marker as follows: *eNOS* RQvariability 1.13, SD ±0.72; THBS-1 RQvariability -0.28, SD ±1.23; VEGF-A RQvariability 1.60, SD ±0.76. Variability of ±1 CT between runs corresponds to <2-fold differences in the evaluated relative mRNA expression, that is, an acceptable variation when examining complex templates; alarming variability results of more than ±3 CTs, which would correspond to >10-fold differences in gene expression in a given sample, were not observed. Since for some samples RQvariability was extremely small (<0.1), the difference observed in relative gene expression may be due at least to both sample and assay performance. Nevertheless, given that i) there is no universal cut-off for accepting reproducibility of results (12); and ii) that these were blood samples (templates derived from heterogeneous cells) and not cell line samples (templates from homogeneous cells), where methods are usually evaluated, these results were considered acceptable for method reproducibility.

Statistical analysis. Descriptives for all markers are presented as median and corresponding range. Comparisons for paired plasma samples (baseline vs. during treatment) were made using the Wilcoxon signed ranked test, while Mann-Whitney tests were used to compare values from independent groups. Fisher's exact tests for the association of markers with selected patient characteristics and Spearman's coefficient for the correlation among markers were also used.

Overall survival (OS) was measured from the date of initiation of treatment with docetaxel to the date of patient's death or last contact. PFS was measured from the date of treatment initiation to documented disease progression, death without prior documented progression or last contact. Survival status was updated in December 2009.

Univariate Cox regression analysis, Kaplan-Meier analysis and the log-rank test were used to examine the prognostic significance of the baseline samples using the median cut-off, to dichotomize biomarker's expression in low and high expression. To examine predictive significance, the difference between the two samples (during treatment sample-baseline sample) was associated with overall response rate (ORR). For the multivariate Cox regression model, a backward selection procedure with removal criterion p>0.10 was used to select the predictors among the following: age, ER/PgR status (negative vs. positive), menopausal status (pre vs. post), adjuvant chemotherapy/hormonotherapy (no vs. yes), visceral metastasis (no vs. yes), number of organs involved (1 vs. 2 vs. ≥3), locoregional metastasis only (no vs. yes), distant metastasis only (no vs. yes), locoregional and distant metastasis (no vs. yes), VEGF-A protein/mRNA expression (low vs. high), plasma NO levels (low vs. high), *eNOS* mRNA expression (low vs. high) and *THBS-1* mRNA expression (low vs. high). For all tests significance level was set at α=0.05. The results are presented in accordance to the REMARK criteria (13). SPSS 15.0 software was used for statistical analysis.

Results

Patient population. From February 2006 to January 2008, 173 patients were enrolled in the study. Fourteen patients were ineligible, because of concomitant chemo- and hormonotherapy (10 patients), or because of prior history of chemotherapy for metastatic disease (4 patients). Among the 159 eligible patients, 2 never started treatment, but they were included in the intent-to-treat survival analysis. Basic patient and tumor characteristics are shown in Table I. Half of the patients had a history of adjuvant chemotherapy, as described in Table I.

Treatment and toxicity. In total, 126 patients (79%) received at least 12 cycles of D (110 received 12 cycles). Thirty-one discontinued their treatment because of death (3 patients), toxicity (5 patients), progression (12 patients), doctor's decision (3 patients), patient refusal (4 patients) and other or unknown reasons (4 patients). The total number of chemotherapy cycles was 1760 (median 12 cycles, range 1-24). Median dose intensity was 32.3 mg/m^2/week (range 10.7-51.7) and relative dose intensity was 0.92 (range 0.31-1.48). Trastuzumab was administered concomitantly with D in 48 patients (30%) and bisphosphonates in 58 (36%).

The regimen was well-tolerated. Among 148 patients with available data, most frequent hematological grade 3 toxicities were the following: neutropenia in 10 patients (7%), febrile neutropenia in 3 (2%), anemia in 1 (1%) and thrombocytopenia in 1. Antibiotics, G-CSF and recombinant erythropoietin were administered in 28 (18%), 23 (15%) and 27 patients (17%), respectively. Seven patients (4%) required packed red blood cells and 1 (1%) platelet transfusion. Grade 3 non-hematological toxicities were infrequent: fatigue in 8 patients (5%), nausea in 1 (1%), diarrhea in 4 (3%), mucositis in 2 (1%), nail changes in 3 (2%), rash/photosensitivity in 3, dacryrrhea in 2 (1%), edema in 1 (1%), hypothyroidism in 1 and infusion reactions in 3 (2%). Finally, grade 3 electrolyte/liver function

Table I. Basic patient and tumor characteristics.

	No. (%)
Age (years; n=159)	
Median (range)	60.7 (27.3-86.7)
Menopausal status (at study entry)	
Pre-menopausal	34 (21)
Postmenopausal	125 (79)
Performance status	
0	107 (67)
1	41 (26)
2	11 (7)
ER/PgR status	
Negative	43 (27)
Positive	111 (70)
Not available	5 (3)
HER2 overexpression	
No	99 (62)
Yes	57 (36)
Not available	3 (2)
Triple negative disease	25 (16)
Grade	
I	3 (2)
II	65 (41)
III	70 (44)
Not available	21 (13)
Histological subtype	
Ductal	105 (66)
Lobular	20 (13)
Inflammatory	7 (4)
Mucinous/mixed/other	17 (11)
Not available	10 (6)
Prior breast surgery	114 (72)
Prior adjuvant treatment	
Adjuvant chemotherapy	80 (50)
Anthracycline-based	50 (31)
Taxane-based	29 (18)
CMF	48 (30)
Adjuvant HT	58 (36)
Tamoxifen	51 (32)
Aromatase inhibitors	13 (8)
LH-RH	6 (3)
Adjuvant RT	61 (38)
Site of metastases	
Locoregional	
Nodes	27 (17)
Skin	11 (7)
Residual breast	16 (10)
Distant	
Bones	79 (50)
Visceral	115 (72)
Soft tissue/nodes	39 (24)
Locoregional only	10 (6)
Locoregional and distant	33 (21)
Distant only	112 (70)
Not available	4 (2)
No. of organs involved	
1	49 (31)
2	64 (40)
≥3	42 (26)
Not available	4 (2)
Treatment with trastuzumab	48 (30)

test disorders were recorded in 12 patients (8%) and grade 4 in 2 (1%). One early toxic death occurred, because of infection in a patient with a history of diabetes mellitus.

Response and survival. Response was evaluated in 140 patients (88%). Of the remaining 19 non-evaluable patients, 9 (6%) discontinued treatment, 1 (1%) died because of toxicity prior to evaluation, 2 (1%) did not have measurable lesion at the time of the initiation of treatment, 2 never started treatment, while for 3 patients (2%) no data were available for response.

Overall, 61 patients (38%, 95% CI 30.8-46.4) achieved an objective response, 7 (4%) a complete response (CR) and 54 (34%) a PR, while 52 (33%) had stable disease (SD) and 27 (17%) progressed (PD). At a median follow-up of 33.5 months (range 2.8-45.0), 118 patients (74%) demonstrated disease progression and 94 (59%) had died. Median PFS was 8.8 months (95% CI 7.4-10.1) and median OS was 27.7 months (95% CI 24.7-30.6). Three-year PFS was 15% and 3-year OS 39.4%. Among the 48 HER2-positive patients treated with D/trastuzumab combination, 22 (46%) responded [CR=1 (2%), PR=21 (44%)], 12 (25%) had SD and 8 (17%) progressed, while 3 (6%) had non-measurable disease and 3 discontinued treatment prior to evaluation. Median PFS was 10.4 months (95% CI 5.8-15.0) and median OS was 30.5 months (95% CI 24.3-36.7).

Translational research results. Blood samples were collected from 127 patients. A baseline (before the initiation of treatment) sample was available from 127 patients (122 plasma samples and 120 RNA samples), while for 70 patients a plasma sample was collected during the treatment (at least 4 weeks after treatment initiation). In addition, blood samples were collected from 39 female healthy controls. Thirty-eight were analyzed for VEGF-A and NO plasma levels, and 33 for *VEGF-A*, *eNOS* and *THBS-1* mRNA levels.

When angiogenic marker levels were compared between patients and healthy controls, a significant difference was noted only for plasma NO levels, *eNOS* mRNA and *THBS-1* mRNA. Breast cancer patients had significantly lower ($p=0.010$) median baseline levels of plasma NO compared to healthy controls. Also, median RQ value of *eNOS* mRNA was significantly lower ($p=0.014$) and median RQ value of blood *THBS-1* mRNA was significantly higher ($p<0.001$) in patients as compared to healthy controls (Table II). The above markers did not significantly change during D treatment (Table II).

Associations of blood markers with clinical and tumor characteristics are illustrated in Table III. Of note, plasma NO

Table II. Levels of plasma markers and blood mRNA expression in healthy controls and patients.

	Controls		Patients		
			Treatment initiation (1st sample)	During treatment (≥4 weeks; 2nd sample)	
	Median (n, range)	p-value[a]	Median (n, range)	Median (n, range)	p-value[b]
Plasma markers					
NO (μmol/l)	282.50 (38, 107.0-479.0)	0.010	201.50 (122, 29.0-825.0)	198.00 (70, 72.0-732.0)	0.404
VEGF-A (pg/ml)	103.60 (38, 31.2-854.8)	0.413	120.10 (122, 31.2-874.8)	109.10 (70, 31.2-877.9)	0.421
Blood mRNA (40-ΔCT)					
eNOS mRNA	32.18 (33, 29.80-34.12)	0.014	31.37 (120, 20.07-39.06)		
THBS-1 mRNA	33.68 (33, 31.57-37.07)	<0.001	36.87 (120, 20.07-43.39)		
VEGF-A mRNA	33.33 (33, 31.95-34.01)	0.743	33.42 (120, 20.07-39.82)		

[a]Mann-Whitney test for the comparison of markers in controls and in the 1st sample of patients. [b]Wilcoxon signed ranked test for the comparison of paired samples (1st vs. 2nd sample).

levels were positively associated with the number of organs involved (p=0.008), low *eNOS* mRNA levels were associated with triple negative disease (p=0.019) and high *VEGF-A* mRNA levels with slightly older age (p=0.038).

The correlation between baseline marker levels was also studied (Spearman's test). Although plasma VEGF-A levels did not correlate with plasma NO (Rho=0.11, p=0.21), there was a significant correlation between *VEGF-A* mRNA and *eNOS* mRNA levels (Rho=0.60, p<0.001), and a similarly strong correlation between *VEGF-A* mRNA and *THBS-1* mRNA levels (Rho=0.43, p<0.001), while *eNOS* mRNA levels were only weakly correlated with *THBS-1* mRNA (Rho=0.25, p=0.006). There was no correlation between plasma NO and *eNOS* mRNA levels, nor between VEGF-A protein and mRNA levels (p>0.050).

Baseline marker levels, as well as their change during treatment (the difference between the sample taken during treatment and the baseline sample) did not show a predictive significance for response (p>0.050 for all markers).

Concerning prognostic factor analysis, the median value was used as a cutoff. High levels (above median) of plasma NO were found to be associated with shorter OS (HR=1.64, 95% CI 1.05-2.58, p=0.030), while high *eNOS* mRNA levels with longer OS (HR=0.47, 95% CI 0.29-0.75, p=0.001) and PFS (HR=0.61, 95% CI 0.41-0.90, p=0.012). By contrast, in multivariate analysis (Table IV), plasma NO levels lost their prognostic significance, *eNOS* mRNA kept it for OS and notably high *THBS-1* mRNA levels were found to be associated with shorter OS and PFS.

Discussion

Although treatment efficacy was not the primary endpoint of the present prospective translational research study, it should be mentioned that weekly D treatment was accompanied by significant response and survival rates in the metastatic setting. Nevertheless, the aim was to investigate the possible antiangiogenic activity of this regimen. For this purpose, established proangiogenic and antiangiogenic markers were measured in blood before the initiation of the treatment and during treatment. As it is already known, blood cells, namely myeloid cells, and platelets seem to play a significant role in angiogenesis and the metastatic process (14). We aimed to measure the plasma levels of angiogenic factors (VEGF-A and NO) and, for the first time, the transcriptional activity of angiogenic (*eNOS* and *VEGF-A*) and antiangiogenic (*THBS-1*) genes in the peripheral blood, and to examine their prognostic and predictive significance.

VEGF-A plays a central role in the angiogenic process. Multiple studies have demonstrated the prognostic significance of plasma VEGF-A concentrations as well as their suppression by antiangiogenic treatment (15-18). The advantage of our study was that VEGF-A protein levels measured in plasma do not require correction, as is the case when they are measured in serum, because of VEGF release from activated platelets (19). In contrast to what has been reported from other investigators (18,19), VEGF-A protein and mRNA levels in the blood of breast cancer patients were not significantly different compared to those in healthy controls. Furthermore, VEGF-A protein and mRNA levels were not correlated, which may be due to a number of reasons, such as the fact that all mRNA transcripts are not translated, or that the VEGF-A protein is also produced in significant amounts from other sources other than blood cells. The most important finding however, was that VEGF-A plasma levels were not significantly changed by the treatment, nor was their change associated with response. It should be mentioned that, although experimental data suggest that weekly D treatment has antiangiogenic activity (7), clinical data (20) do not support such a suppressive effect on serum VEGF levels.

VEGF has been shown to activate eNOS in endothelial cells, which in turn produces NO, a multifunctional highly reactive free radical displaying a significant angiogenic role (21). It is well known that peripheral monocytes express eNOS and, upon stimulation, iNOS (22). iNOS action on inflammatory cells results in high concentrations of NO with a potent cytotoxic

Table III. Association of markers with selected clinicopathological characteristics.

	Plasma NO no. (%)			Plasma VEGF-A no. (%)			*eNOS* mRNA no. (%)			*THBS-1* mRNA no. (%)			*VEGF-A* mRNA no. (%)		
	Low	High	p-value	Low	High	p-value	Low	High	p-value	Low	High	p-value	Low	High	p-value
Age (years)			0.260			0.210			0.430			0.370			0.038
Median	58.6	61.8		58.5	61.8		61.8	59.9		61.1	60.6		58.2	62.6	
Menopausal status			0.650			0.180			0.990			0.990			0.071
Pre-menopausal	11 (18)	14 (23)		16 (26)	9 (15)		13 (22)	12 (20)		12 (20)	13 (22)		17 (28)	8 (13)	
Postmenopausal	50 (82)	47 (77)		45 (74)	52 (85)		47 (78)	48 (80)		48 (80)	47 (78)		43 (72)	52 (87)	
Performance status			0.380			0.620			0.200			0.690			0.890
0	43 (70)	36 (59)		40 (65)	39 (64)		35 (58)	44 (74)		41 (68)	38 (64)		40 (67)	39 (65)	
1	14 (23)	18 (29)		17 (28)	15 (25)		19 (32)	11 (18)		13 (22)	17 (28)		14 (23)	16 (27)	
2	4 (7)	7 (12)		4 (7)	7 (11)		6 (10)	5 (8)		6 (10)	5 (8)		6 (10)	5 (8)	
ER/PgR status			0.999			0.990			0.092			0.680			0.300
Negative	16 (27)	17 (29)		17 (29)	16 (27)		20 (35)	11 (19)		14 (25)	17 (29)		18 (32)	13 (22)	
Positive	43 (73)	42 (71)		42 (71)	43 (73)		38 (65)	47 (81)		43 (75)	42 (71)		39 (68)	46 (78)	
HER2 overexpression			0.850			0.253			0.999			0.572			0.481
No	39 (65)	37 (63)		35 (58)	41 (70)		36 (62)	38 (63)		38 (65)	36 (60)		37 (64)	37 (62)	
Yes	21 (35)	22 (37)		25 (42)	18 (30)		22 (38)	22 (37)		20 (34)	24 (40)		21 (36)	23 (38)	
Triple negative disease			0.601			0.114			0.019			0.999			0.443
No	52 (88)	49 (83)		54 (92)	47 (80)		45 (78)	55 (93)		50 (86)	50 (85)		48 (83)	52 (88)	
Yes	7 (12)	10 (17)		5 (8)	12 (20)		13 (22)	4 (7)		8 (14)	9 (15)		10 (17)	7 (12)	
Grade			0.052			0.990			0.700			0.078			0.70
I-II	21 (40)	32 (60)		28 (50)	25 (50)		26 (50)	29 (55)		30 (63)	25 (44)		27 (55)	28 (50)	
III	32 (60)	21 (40)		28 (50)	25 (50)		26 (50)	24 (45)		18 (37)	32 (56)		22 (45)	28 (50)	
Histological subtype			0.410			0.870			0.770			0.690			0.710
Ductal	41 (70)	45 (80)		46 (77)	40 (73)		41 (76)	43 (74)		43 (77)	41 (72)		39 (71)	45 (78)	
Lobular	8 (13)	5 (9)		6 (10)	7 (13)		5 (9)	8 (13)		5 (9)	8 (14)		7 (13)	6 (10)	
Other	10 (17)	6 (11)		8 (13)	8 (14)		8 (15)	8 (13)		8 (14)	8 (14)		9 (16)	7 (12)	
No. of metastatic sites			0.008			0.230			0.390			0.890			0.990
1	21 (36)	13 (22)		14 (24)	20 (34)		16 (28)	20 (34)		19 (33)	17 (29)		18 (31)	18 (31)	
2	29 (49)	22 (37)		30 (51)	21 (36)		22 (39)	26 (44)		24 (41)	24 (42)		24 (41)	24 (41)	
≥3	9 (15)	24 (41)		15 (25)	18 (30)		19 (33)	13 (22)		15 (26)	17 (29)		16 (28)	16 (28)	
Visceral metastasis			0.843			0.999			0.693			0.999			0.693
No	17 (28)	19 (31)		18 (30)	18 (30)		17 (28)	20 (33)		18 (30)	19 (32)		20 (33)	17 (28)	
Yes	44 (72)	42 (69)		43 (70)	43 (70)		43 (72)	40 (67)		42 (70)	41 (68)		40 (67)	43 (72)	

Low, below median; high, above median.

Table IV. Multivariate analysis of prognostic factors for survival and progression-free survival (PFS).

	Survival			PFS		
	HR	95% CI	Wald/p-value	HR	95% CI	Wald/p-value
ER/PgR status						
Negative	1			1		
Positive	0.38	0.22-0.64	<0.001	0.37	0.22-0.63	<0.001
No. of organs involved						
1	1			1		
2	1.07	0.56-2.02	0.840	1.96	1.11-3.47	0.020
≥3	2.51	1.27-4.96	0.008	4.21	2.23-7.94	<0.001
Adjuvant HT						
No				1		
Yes				3.14	1.91-5.17	<0.001
Visceral metastasis						
No	1					
Yes	2.53	1.34-4.78	0.004			
eNOS mRNA						
Low	1			1		
High	0.49	0.29-0.82	0.007	0.64	0.41-1.02	0.058
THBS-1 mRNA						
Low	1			1		
High	1.84	1.11-3.05	0.018	1.73	1.11-2.69	0.016

action. By contrast, eNOS, which is mostly expressed in endothelial cells, pericytes and mesenchymal cells surrounding tumor vessels, synthesizes lower levels of NO, which promote cell proliferation and angiogenesis. As mentioned above, bone marrow-derived myeloid cells play a significant role in tumor angiogenesis. Moreover, trans-differentiation of some of these cells to endothelial cells cannot be excluded (14). However, the role of myeloid cell eNOS in angiogenesis is largely unknown.

To our knowledge, the present study is the first where blood *eNOS* mRNA levels were measured in breast cancer patients treated with chemotherapy. *eNOS* mRNA expression appeared lower in the blood of patients in comparison to healthy controls; however, this statistically significant difference was not considered of biological relevance, based on the results presented for the corresponding inter-assay variability trial. Of note, however, high *eNOS* mRNA levels were found to be of favorable prognostic significance, irrespectively of established clinical parameters, such as hormone receptor status, number of metastatic sites and involvement of visceral organs. Notably, low eNOS immunohistochemical expression has been found to be associated with adverse prognostic features (23) and poor survival (24). Moreover, Choi *et al* reported poor survival in breast cancer patients bearing polymorphisms associated with low *eNOS* activity (25,26). Also, as in our study (trend for significance shown in Table III), other investigators (23,27) found higher *eNOS* mRNA expression in hormone receptor-positive tumors. NO findings are more difficult to explain. Surprisingly, plasma NO levels were significantly lower in patients than in healthy controls, although the absolute difference between the two groups was small. By contrast, plasma NO levels were positively correlated with the number of metastatic sites. As mentioned above, it seems that NO is mostly produced in cancer cells by iNOS. Most investigators found an adverse prognostic significance for iNOS expression (28-30) and nitrotyrosine tumor staining (which reflects intracellular NO levels) (21), whereas there is no direct evidence from the literature for the prognostic significance of plasma/serum NO levels (31,32). In the present study, high plasma NO levels predicted poor survival only in univariate analysis, apparently because of their direct correlation with the number of involved organs. On the other hand, it should be mentioned that plasma NO levels may also be influenced by several physiological conditions and, therefore may not reflect NO production by the tumor microenvironment. Additionally, there is some evidence that tumor cells may display various sensitivities to NO (21).

Equally complex is the role of thrombospondins in tumor progression. Although THBS-1 has been generally recognized as an antiangiogenic molecule, there is some evidence that its expression is important for cancer cell adhesion to endothelial cells and for tumor invasion, as it interacts with cell adhesion molecules and modulates protease expression (33). Indeed, invasive ductal breast tumors express higher THBS-1 protein and mRNA than normal breast tissues. Ductal cancers express THBS-1 mainly in the surrounding stroma, while in lobular tumors it is present in the more invasive tumor cells (33). In accordance with these observations, Tuszynski *et al* and Byrne *et al* found higher blood levels of THBS-1 protein in breast cancer patients compared to healthy controls (34,35), while the present study showed that blood *THBS-1* mRNA levels are also elevated. Thrombospondins are secreted in blood by platelets and monocytes (36). We preferred to measure blood *THBS-1* mRNA, instead of the corresponding protein, in order

to avoid the case of false high levels of THBS-1 due to platelet activation during the handling of the blood samples.

Probably the most interesting finding of the present study was the independent adverse prognostic significance of blood *THBS-1* mRNA levels. Lawler *et al* (37) showed that breast tumors grow twice as fast in THBS-1-null mice. Urquidi *et al* (38) found that the inability of the NM-2C5 breast tumor cell line to metastasize may be attributed to the increased expression of *THBS-1*, whereas Yee *et al* (39) observed that THBS-1-null polyomavirus middle T antigen (Pyt) transgenic mice displayed faster rates of mammary tumor growth and angiogenesis, but lower number of pulmonary metastatic sites. To our knowledge, the prognostic significance of *THBS-1* mRNA expression in breast cancer has never been confirmed (40,41). On the contrary, in other tumors several studies reported a favorable prognostic value of *THBS-1* expression (42,43), while others suggested a negative impact on survival (44,45). Certain investigators have linked increased blood THBS-1 protein expression with an aggressive phenotype in breast (35) and colon cancer (46). It could not be excluded, however, that circulating tumor cells activate THBS-1 secretion by platelets. Moreover, Zaslavsky *et al* (47) recently demonstrated that *THBS-1* expression is up-regulated in the platelets of tumor-bearing mice. Nevertheless, whichever its source, circulating blood tTHBS-1 may play a significant role in the co-ordination of tumor metastasis.

Although an antiangiogenic activity of weekly D was not demonstrated in this study, some interesting observations could be made. This was the first study to measure *VEGF-A*, *eNOS* and *THBS-1* mRNA expression in the peripheral blood, in order to directly monitor the transcriptional activation of these genes in blood cells in a homogeneously-treated metastatic breast cancer patient cohort. It was found that patients with relatively low *eNOS* and high *THBS-1* mRNA expression lived significantly longer, while this finding was independent from established clinical prognostic factors. Although their significance is not clear, these observations add a piece in the puzzle for a better understanding of the angiogenic process.

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