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Angiogenesis- and Hypoxia-Associated Proteins as Early Indicators of the Outcome in Patients with Metastatic Breast Cancer Given First-Line Bevacizumab-Based Therapy

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Abstract

Purpose: We examined whether pretreatment levels of angiogenesis- or hypoxia-related proteins and their changes after one cycle of first-line bevacizumab-based therapy were associated with response, PFS, or OS in patients with metastatic breast cancer.

Experimental Design: We included 181 patients enrolled in the phase II ATX trial evaluating first-line paclitaxel and bevacizumab without or with capecitabine (NTR1348). Plasma samples were analyzed for VEGF-A, soluble VEGFR2 (sVEGFR2), angiopoietin 2 (ANG2), soluble TIE2 (sTIE2), IL6, IL8, and carbonic anhydrase 9 (CA9). Baseline serum CA15-3 was documented. HR was adjusted for confounding factors. Where appropriate, an optimal cut-off value defining a high and a low group was determined with Martingale residuals.

Results: At baseline, multiple proteins were significantly associated with PFS (ANG2, IL6, IL8, CA9, CA15-3) and OS (ANG2, sTIE2, IL6, IL8, CA9, CA15-3). After one cycle, VEGF-A, ANG2, sTIE2, and IL8 significantly decreased, while sVEGFR2 and CA9 significantly increased. The relative change in sVEGFR2 ($P = 0.01$) and IL8 ($P = 0.001$) was associated with response. Defining optimal cut-off, patients with a high CA9 rise (>2.9%) had better PFS (HR 0.45) and OS (HR 0.54) than those with low/no rise.

Conclusions: Multiple angiogenesis- or hypoxia-related proteins were prognostic for PFS and OS. Molecular agents targeting these proteins might be beneficial in patients with high levels. Changes in IL8 or sVEGFR2 levels at second cycle appear predictive for response. Changes in CA9 levels during bevacizumab-based therapy for prediction of PFS and OS merit further study. *Clin Cancer Res; 22(7); 1611–20. ©2016 AACR.*

Introduction

Angiogenesis is an important process required for tumor growth, progression, and metastatic spread. Inhibition of new blood vessel formation is valuable to impede tumorigenesis and has led to the development of various antiangiogenic agents. Among them is bevacizumab (Genentech), a humanized monoclonal antibody, that exerts its antiangiogenic activity by capturing circulating VEGF-A. VEGF-A is an important growth factor promoting tumor angiogenesis through its major receptor, VEGFR2; inhibition results in reduced endothelial cell proliferation, migration, and survival leading to diminished formation of new tumor vasculature (1).

Bevacizumab combined with chemotherapy has broadened the treatment landscape of different solid tumors demonstrating increased clinical activity compared with chemotherapy alone. In metastatic breast cancer (MBC), clinical data from the pivotal E2100 phase III trial have shown a higher response rate as well as an improvement in progression-free survival (PFS) when

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Translational Relevance

Measurement of circulating proteins might indicate which metastatic breast cancer patients have clinical benefit from first-line bevacizumab-based therapy. We analyzed plasma samples collected at baseline and found that multiple proteins related to angiogenesis or hypoxia had prognostic significance. We also assessed protein levels after one treatment cycle. The magnitude of several protein changes was associated with tumor response and survival outcome. Our results point toward potential new protein drug targets. Moreover, changes in circulating proteins during bevacizumab-based therapy may have potential to timely predict efficacy from treatment.

bevacizumab was added to first-line chemotherapy with paclitaxel (2). The favorable outcome for patients treated with first-line taxane and bevacizumab has been confirmed in subsequent randomized phase III trials, albeit the magnitude of PFS gain was relatively modest (3, 4). Bevacizumab combined with an anthracycline or capecitabine has also resulted in higher clinical activity than chemotherapy alone (4). Thus far, no improvement in survival has been demonstrated with the addition of bevacizumab to first-line chemotherapy in MBC. Currently, the initial optimism about bevacizumab has been tempered by the recognition of variation in clinical activity among patients. Therefore, recent attention has been devoted to a search for biomarkers to predict tumor response or drug resistance, thereby providing the opportunity to refine patient selection for anti-VEGF therapy (5).

A number of molecular mechanisms have been postulated by which tumors may be refractory to anti-VEGF therapy or may rapidly develop resistance (6, 7). Alternative proangiogenic signaling routes have been acknowledged to sustain the complex process of tumor angiogenesis and to compensate for VEGF-A blockade. Several proteins are known to exert direct or indirect proangiogenic effects. Among them is the angiopoietins/TIE2 receptor signaling pathway, which is an important regulator in vessel remodeling and maturation (8). Increasing data indicate that angiopoietin 2 (ANG2) can function as a TIE2 agonist, particularly when overexpressed and/or in the absence of ANG1. In angiogenic tissues, such as cancer, endothelial cells secrete high levels of ANG2, which promote angiogenesis in concert with other proangiogenic factors, namely VEGF-A (9). Rigamonti and colleagues (9) have shown in a mouse model of pancreatic cancer that induction of resistance to VEGFR2 blockade resulted in revascularization and progression with concomitant upregulation of ANG2 and TIE2. The adaptive enforcement of ANG2/TIE2 signaling could be reversed by neutralization of ANG2. The proinflammatory interleukins IL6 and IL8 have also been implicated in the regulation of angiogenesis (10). IL6 exerts its proangiogenic activity predominantly through STAT3 signaling leading to VEGF transcription, endothelial cell proliferation, and migration. In several tumor models a role of IL6 has been described in tumor angiogenesis as well as in failure with antiangiogenic therapy (10). IL8 promotes an angiogenic response by binding to its receptors CXCR1 and 2 expressed by endothelial cells and may activate VEGFR2 in endothelial cells (11). Another protein to mention is MUC1, a hyperglycosylated transmembrane protein expressed on normal secretory epithelial cells, while 90% of carcinomas show aberrant MUC1 expression (12, 13). Preclinical models have demonstrated that MUC1 overexpression can stimulate VEGF synthesis and secretion through activation of the AKT signaling pathway (12) and can contribute to hypoxia-driven angiogenesis (13).

Hypoxia is a hallmark of growing tumors and can induce a spectrum of adaptive processes, among which is increased angiogenesis (14). The hypoxia-induced transcriptional program is activated by the hypoxia-inducible factors (HIF), mainly from stabilization of HIF1α protein. Proteins encoded by genes with HIF-responsive elements in their regulatory regions execute the adaptive responses to hypoxia, among which are VEGF-A and carbonic anhydrase 9 (CA9). CA9 is a transmembrane enzyme involved in essential physiologic processes including pH regulation and ion transport. CA9 distribution in tumor tissues corresponds with its HIF-governed regulation (14).

Given the complex regulation of blood vessel formation in tumors due to differential activation of pathways, assessment of multiple angiogenesis-associated proteins that are released in the circulation may provide a more comprehensive view of the angiogenic state. Moreover, treatment-related changes in the levels of these proteins have been postulated as indicators of response or may even indicate adaptive resistance to antiangiogenic therapies (5). In the current study in patients with HER2-negative MBC receiving first-line bevacizumab-based therapy, we measured plasma levels of circulating VEGF-A and soluble VEGFR2 (sVEGFR2), ANG2, and soluble TIE2 (sTIE2), IL6, IL8, and CA9 at baseline before treatment and following one treatment cycle to investigate whether changes in these protein levels might be associated with therapeutic efficacy and/or disease outcome. We also studied the concentrations of these proteins at baseline, including CA15-3 for MUC1, for their potential prognostic value.

Materials and Methods

Patients and study design

In this biomarker study, patients with HER2-negative MBC were included, who were previously enrolled in a randomized, multicenter, open-label, phase II BOOG 2006-06 ATX trial (Trial registration ID: NTR1348). Study details and clinical findings have been described in detail in ref. (15). In brief, 312 patients were randomized (1:1) to the first-line standard regime of paclitaxel (90 mg/m^2 i.v. on days 1, 8, 15) and bevacizumab (10 mg/m^2 i.v. on days 1, 8) every 4 weeks for 6 cycles, followed by bevacizumab (15 mg/m^2 i.v. on day 1) every 3 weeks afterwards (AT) or to the investigational regime of paclitaxel (90 mg/m^2 i.v. on days 1, 8), bevacizumab (15 mg/m^2 i.v. on day 1), and capecitabine (825 mg/kg orally twice daily on days 1–14) every 3 weeks for 8 cycles, followed by the same dose of bevacizumab and capecitabine every 3 weeks afterwards (ATX). Treatment was continued until disease progression, unmanageable toxicities or withdrawal of consent.

Prospective collection of plasma samples was part of the planned translational research. The protocol was approved by the institutional review board of all participating sites and was conducted in accordance with the Declaration of Helsinki. Separate informed consent from patients was received prior to participation in the biomarker study. Consenting to the biomarker study was not a requirement for trial participation. This study is reported according to the REporting recommendations

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for tumor MARKer for prognostic studies (REMARK) criteria as applicable (16).

Plasma sample collection

Blood was obtained by venipuncture at baseline and immediately before treatment cycle two (henceforth denoted as C2D1) in an EDTA tube. Within 30 minutes after collection, blood was centrifuged at 3,000 rpm for 10 minutes at 4°C and plasma was stored in aliquots at −80°C until analysis.

Protein measurement

Quantikine ELISA kits were used to measure plasma concentrations of unbound VEGF-A_{165}, sVEFGR2, ANG2, sTIE2, and CA9 according to the manufacturer's protocols (R&D Systems; catalog numbers DVE00 and SVE00, SVR200 and DVR200, DANG20 and SANG20, DTE200 and DCA900, respectively). In brief, plasma samples were dispensed into antibody-precoated 96-well plates and incubation followed for 2 hours. The samples were then aspirated and the plates were repeatedly washed followed by incubation with horseradish peroxidase–conjugated secondary antibody for 2 hours. After repeated washing steps, the plates were incubated with tetramethylbenzidine in hydrogen peroxide substrate solution for 30 minutes. The reaction was stopped by addition of 50 μL 2N sulfuric acid. The optical density per well was measured at 450 nm corrected for optical imperfections of the plates with a microplate spectrofluorometer (Biotek Instruments).

IL6 and IL8 were measured using a V-plex array from Meso Scale Discovery (catalog number K15049D). In brief, plasma samples loaded onto 96-well plates were incubated for 2 hours. Following aspiration and repeated washing steps, the plates were incubated with detection antibody for 2 hours. Washing steps were repeated and read buffer was added. The plates were read immediately on the Meso Scale Discovery instrument (SECTOR Imager 2400).

All described procedures were performed at room temperature. The plasma samples were measured in duplicate and blinded to clinical outcome. Duplicate samples were randomly allocated onto each plate. Plasma protein concentrations were interpolated from the average calibration curve prepared by serial dilution of the stock solution. The average concentration of the duplicates was used for data analysis. Baseline serum CA15-3 concentrations were measured in routine clinical care and were retrieved from the case report forms.

Study endpoints and evaluation

Protein levels at baseline and their changes on C2D1 were tested for a possible relation with the following efficacy outcomes: response, PFS, and overall survival (OS). Response rate was defined as the percentage of complete and partial response (CR + PR) according to Response Evaluation Criteria in Solid Tumors (RECIST version 1.0; ref. 17). Best documented response was used for patients remaining on study at the time of database closure. PFS was defined as the time from randomization to first documented disease progression or death from any cause. Patients receiving nonprotocol treatment prior to progression were censored at the first date of nonprotocol treatment. OS was defined as the time from randomization to death from any cause. Patients not experiencing an event were censored at the date of last follow-up.

Statistical analysis

Results were expressed as median with range, actual value, or percentage. Comparison between protein data (continuous variables) and categorical variables was carried out using Mann–Whitney *U* test or Kruskal–Wallis test (in case of more than two-group comparisons). Spearman's correlation test was used for correlation analysis between two proteins. The relative change in protein levels, expressed as percentage change from baseline to C2D1, was analyzed using Wilcoxon signed-rank test.

Cox proportional hazards model was used to estimate hazard ratio (HR) and related 95% confidence interval (95% CI) for progression or death. Clinical characteristics were analyzed for a potential association with PFS or OS by univariate Cox proportional hazards model and significant ($P < 0.05$) or considered clinically relevant factors were included in the multivariable analysis. Each protein was then evaluated as a log10-transformed continuous covariate to estimate the HR while adjusting for clinical prognostic factors. For analysis of protein changes, a constant value was added to allow log10 transformation of any negative values. An HR > 1 indicates worse outcome, whereas HR < 1 indicates improved outcome. Violation of the proportional hazard assumption was checked for each covariate in each model. The functional form of the relationship between log10-transformed protein data and clinical outcome was examined using Martingale residual plot (18) with lowess smooth, which displays excess progression or death (*y*-axis) over the whole range of protein levels (*x*-axis). In case of a nonlinear form of a covariate, the optimal cut-off value was estimated by using maximally selected rank statistics (19). On the basis of Martingale residuals, the CA9 changes were divided into a high and low group for OS analysis. Given the exploratory nature of analyses, *P* values without correction for multiplicity of testing were reported. Statistical analyses were performed using R version 3.0.3 and SPSS version 20.

Results

Biomarker population

A number of 181 patients with at least one measurement of protein level formed the biomarker population. Sample size for each protein was the following: VEGF-A (baseline, $n = 173$; C2D1, $n = 142$), sVEGFR2 (baseline, $n = 180$; C2D1, $n = 149$), ANG2 (baseline, $n = 173$; C2D1, $n = 142$), sTIE2 (baseline, $n = 173$; C2D1, $n = 142$), IL6 (baseline, $n = 180$; C2D1, $n = 149$), IL8 (baseline, $n = 180$; C2D1, $n = 149$), CA9 (baseline, $n = 179$; C2D1, $n = 148$), and CA15-3 (baseline, $n = 174$). Among proteins, VEGF-A levels were below the lower limit of detection in four samples at baseline (one in AT and three in ATX) and in 85 samples on C2D1 (46 in AT and 39 in ATX), but no significant differences were found between treatment arms ($P = 0.70$). Baseline patient and tumor characteristics of the biomarker population were generally similar to those of the parental trial population (Table 1).

At the cut-off date (April 2013) for data analysis, the median follow-up period was 47 months. A total of 160 patients (89%) had progressive disease, whereas 152 patients (84%) had died. Median PFS was 9.6 months (95% CI, 8.6–10.7) and median OS was 24.2 months (95% CI, 22.3–26.0). Tumor response evaluation was available in 172 patients (95%). Nine patients had no evaluation of tumor response due to toxicity ($n = 5$), intercurrent death ($n = 3$) or other reason ($n = 1$) and were excluded from response analysis. The response rate (CR + PR)

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Table 1. Patient characteristics

	Parental trial population ($n = 312$) *n* (%)	Biomarker population ($n = 181$) *n* (%)
Age, years		
Median (range)	57 (32–76)	57 (32–76)
<55 years	136 (43)	83 (46)
≥55 years	176 (56)	98 (54)
Baseline ECOG performance status		
0	163 (52)	96 (53)
1	149 (48)	85 (47)
Hormone receptor status		
Positive	265 (85)	159 (88)
Negative	47 (15)	22 (12)
HER2-negative disease	309 (99)	181 (100)
Measurable disease		
Yes	268 (86)	162 (90)
No	44 (14)	19 (10)
Disease-free interval		
≤24 months	102 (33)	59 (33)
>24 months[a]	210 (67)	122 (67)
Distant metastases at initial diagnosis		
Yes	42 (13)	25 (14)
No	270 (87)	156 (86)
Sites of metastatic disease		
Lung metastases	94 (30)	61 (34)
Liver metastases	180 (58)	112 (62)
Bone-only disease	25 (8)	10 (6)
No. of metastatic sites		
1	51 (16)	23 (13)
2	123 (40)	64 (35)
≥3	137 (44)	94 (52)
Prior hormonal therapy		
Primary breast cancer	157 (50)	94 (52)
Metastatic breast cancer	151 (48)	88 (49)
Prior (neo)adjuvant chemotherapy	178 (57)	103 (57)

[a]Disease-free interval was defined as the period from primary breast cancer diagnosis to first relapse.

was 69.2% ($n = 119$). Upon combination of both treatment regimes, the clinical outcome of the biomarker population did not differ from that of the parental trial population (Supplementary Table S1).

A total of 94 patients were assigned to receive AT and 87 patients received ATX. Response rate was not significantly different between both treatment regimes in this subset of patients (66.7 vs. 67.8%, $P = 0.25$). Median PFS was significantly longer in ATX compared with AT (11.3 vs. 8.5 months, log-rank $P < 0.001$). Median OS was similar in AT- and ATX-treated patients (23.9 vs. 24.3 months, log-rank $P = 0.84$).

The following clinical characteristics were examined for association with PFS or OS: first-line therapy (AT or ATX), age (<55 or ≥55 years), Eastern Cooperative Oncology Group Performance Status Scale (ECOG PS, 0 or 1), disease-free interval from primary diagnosis until first proof of metastases (≤24 or >24 months), hormone receptor status (negative or positive), measurable disease (yes or no), and bone-only disease (yes or no). Of the clinical characteristics, first-line therapy (HR for AT vs. ATX = 1.9; 95% CI, 1.4–2.6) and ECOG PS (HR 0 vs. 1 = 0.71; 95% CI, 0.52–0.97) were significantly associated with PFS, while hormone receptor status was associated with OS (HR negative vs. positive = 2.0; 95% CI, 1.3–3.3). These clinical variables were included in the multivariate Cox proportional hazards models.

Protein levels at baseline and clinicopathologic characteristics

The associations between baseline protein levels and patient characteristics are shown in Supplementary Table S2. Lower baseline ANG2 ($P = 0.007$), sTIE2 ($P = 0.001$), IL8 ($P = 0.007$), and CA15-3 ($P = 0.023$) were measured in patients with ECOG PS of 0. High baseline ANG2 ($P = 0.025$), sTIE2 ($P = 0.001$), and CA15-3 ($P < 0.001$) were associated with hormone receptor–positive breast cancer rather than triple-negative breast cancer, while a significantly higher baseline IL8 was noted in patients with nonmeasurable metastatic spread ($P = 0.027$). Moreover, baseline VEGF-A was significantly higher in patients with visceral metastases ($P = 0.028$) and significantly different among the number of metastatic sites ($P = 0.017$); those with a high number of metastatic sites had highest levels. Finally, IL6 and IL8 were significantly different among the three percentile groups of baseline CA15-3 levels ($P = 0.007$ and $P = 0.009$, respectively); highest levels were detected in the >75th percentile group.

Next, we explored a possible relation between protein levels at baseline. Spearman analysis revealed several significant, but mostly weak to moderate correlations with $\rho = 0.43$ as highest value (Supplementary Table S3). Baseline VEGF-A was positively correlated with baseline IL6, IL8, and CA15-3, but not with its receptor sVEGFR2. There was an inverse correlation between baseline sVEGFR2 and CA15-3. Baseline ANG2 level was positively correlated with its receptor sTIE2 as well as with IL6, IL8, and CA9. Baseline sTIE2 was positively correlated with IL6, IL8, CA9, and CA15-3. Finally, other positive correlations were found between baseline IL6 and IL8, CA9, CA15-3, between baseline IL8 and CA9, CA15-3, and between baseline CA9 and CA15-3.

Protein levels at baseline and clinical outcome

In the biomarker population the response rates were not significantly different between both treatment regimes because of which all patients were combined for the response association analysis. None of the baseline protein levels were associated with response (data not shown).

As a continuous covariate and adjusted for first-line therapy and ECOG PS in the multivariate Cox proportional hazards model, high baseline levels of ANG2, IL6, IL8, CA9, and CA15-3 were significantly associated with poor PFS (Table 2). After adjustment for hormone receptor status prognostic for OS, high baseline levels of ANG2, IL6, IL8, CA9, and CA15-3 were similarly associated with poor OS (Table 2), whereas high baseline levels of sTIE2 emerged as a prognostic factor for poor OS.

Table 2. Baseline protein levels as continuous covariate and estimated HR for progression and death

Proteins	PFS[a] HR (95% CI)	OS[b] HR (95% CI)
VEGF-A	0.86 (0.62–1.18)	0.94 (0.65–1.34)
sVEFGR2	0.57 (0.11–2.94)	0.39 (0.07–2.33)
ANG2	**2.54 (1.15–5.62)**	**3.79 (1.69–8.50)**
sTIE2	2.94 (0.85–10.13)	**4.23 (1.13–15.80)**
IL6	**1.49 (1.02–2.19)**	**2.03 (1.39–2.97)**
IL8	**1.95 (1.31–2.91)**	**2.83 (1.86–4.31)**
CA9	**1.75 (1.16–2.62)**	**2.04 (1.35–3.11)**
CA15-3	**1.44 (1.10–1.89)**	**1.33 (1.00–1.77)**

NOTE: HR corresponds to one unit increase in the log-transformed covariate; significant HRs are shown in bold.
[a]Hazard ratio adjusted for ECOG PS and first-line treatment.
[b]Hazard ratio adjusted for hormone receptor status.

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Table 3. Plasma protein changes from baseline to C2D1

Proteins	No. of patients	LOD	Baseline median (range)	C2D1 median (range)	Percent change from baseline	*P* value[a]
VEGF-A (pg/mL)[b]	142	9 pg/mL	100.5 (<LOD – 4,678)	8.3 (<LOD – 75.5)	−92.7	<0.001
sVEGFR2 (pg/mL)	149	4.6 pg/mL	8,724 (5,359–14,730)	9,128 (4,670–14,780)	5.1	<0.001
ANG2 (pg/mL)	142	8.29 pg/mL	2,922 (700–31,745)	2,340 (833–9,461)	−18.1	<0.001
sTIE2 (ng/mL)	142	14 pg/mL	25.2 (14.1–120)	22.5 (12.3–68.7)	−10.5	<0.001
IL6 (pg/mL)	149	0.06 pg/mL	1.29 (0.21–34.8)	1.27 (0.12–121)	−14.1	0.055
IL8 (pg/mL)	149	0.04 pg/mL	10.2 (0.82–215.7)	9.3 (2.0–97.5)	−11.0	0.021
CA9 (pg/mL)	148	2.28 pg/mL	74.6 (14.5–1,700)	98.2 (16.6–1,001)	31.0	<0.001

Abbreviations: LOD, limit of detection.

[a]Wilcoxon signed-rank test.

[b]VEGF-A levels were below the lower limit of detection in four baseline samples (one in AT and three in ATX) and in 85 C2D1 samples (46 in AT and 39 in ATX).

Changes in protein levels after short-term bevacizumab-based therapy

Changes in protein levels from baseline to C2D1 were analyzed to identify proteins potentially modulated by bevacizumab-based chemotherapy. CA15-3 levels were not consistently available on C2D1. Analysis of paired plasma samples revealed significant changes in all protein levels, with exception of IL6 (Table 3). As expected, unbound VEGF-A decreased significantly with a median change of −92.7%. Levels of ANG2, sTIE2, and IL8 also significantly decreased from baseline to C2D1, whereas there was a significant rise of sVEGFR2 and CA9 on C2D1. Plasma protein changes for the various categories of patient and tumor characteristics are given in Supplementary Table S4. Comparison between both treatment regimes revealed that the extent of changes was grossly similar for most proteins including VEGF-A, sVEGFR2, ANG2, sTIE2, and CA9 (Supplementary Table S5). Changes in IL6 and IL8 were; however, only significant in patients treated with ATX.

Spearman analysis revealed that a change in ANG2 was positively correlated with changes in sTIE2 and in IL8 (Supplementary Table S6). A positive correlation was also observed between changes in IL6 and IL8 as well as between changes in IL8 and CA9. A change in sVEGFR2 was inversely correlated with a change in IL6. Again, correlations were only weak to moderate with $\rho = 0.45$ as highest value.

Changes in plasma protein levels and clinical outcome

The magnitude of changes in sVEGFR2 and IL8 was associated with response to treatment. For sVEGFR2, a relatively higher rise was observed in responders compared to nonresponders (median relative change 6.5% vs. 2.1%, $P = 0.01$, Fig. 1A). A relative decrease in IL8 levels was measured in responders, whereas a relative increase was noted in nonresponders (median relative change −19.4% vs. 22.3%, $P = 0.001$, Fig. 1B). Although IL8 changes were more pronounced in ATX- than in AT-treated patients (Supplementary Table S5), similar significant associations were found. For AT the median relative change was −10.7% versus 22.3% in responders versus nonresponders ($P = 0.025$) and for ATX the median relative change was −30.4% versus 15.7% in responders versus nonresponders ($P = 0.022$).

Among protein changes from baseline to C2D1, only a change in CA9 level was associated with PFS and OS (Table 4). Patients with a high increase of CA9 on C2D1 had a better PFS (HR 0.44; 95% CI, 0.23–0.86). A similar relation was found for OS (HR 0.57; 95% CI, 0.30–1.07), albeit not significant.

Martingale residual plot indicated a nonlinear form of the relationship between CA9 change and OS (Supplementary Fig. S1A). Hence, we determined 2.9% as the optimal cut-off value of

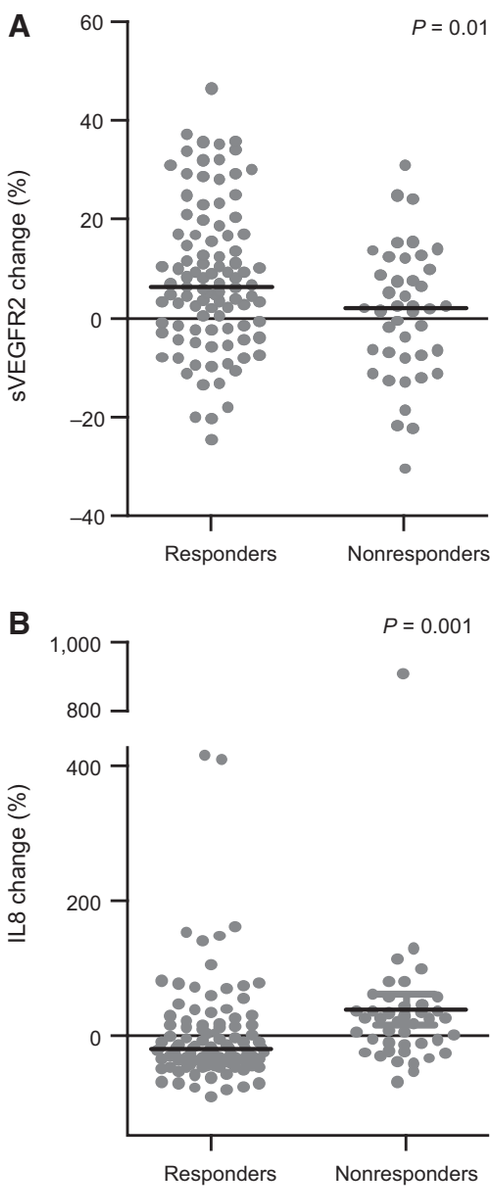

Figure 1.
A, changes in levels of sVEGFR2 and tumor response. B, changes in levels of IL8 and tumor response.

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Table 4. Protein level changes as continuous covariate and estimated HR for progression and death

Protein change	PFS HR (95% CI)[a]	OS HR (95% CI)[b]
sVEFGR2	0.79 (0.04–17.62)	0.61 (0.03–14.33)
ANG2	0.40 (0.03–4.71)	0.33 (0.02–4.75)
sTIE2	0.70 (0.37–1.31)	0.56 (0.28–1.13)
IL6	0.40 (0.03–4.71)	0.33 (0.02–4.75)
IL8	0.70 (0.37–1.31)	0.56 (0.28–1.13)
CA9	**0.44 (0.23–0.86)**	0.57 (0.30–1.07)

NOTE: HR corresponds to one unit increase in the log-transformed covariate; significant hazard ratio is shown in bold.
[a]HR adjusted for ECOG PS and first-line treatment.
[b]HR adjusted for hormone receptor status.

CA9 change that allowed separation of two subgroups with the largest difference in OS in the present population (Supplementary Fig. S1B). This cut-off value was used to define patients with a high CA9 rise (>cut-off) or a low/no CA9 rise (≤cut-off, Supplementary Fig. S1C). A high CA9 rise was associated with better OS compared with a low/no CA9 rise [26.6 months (95% CI, 23.2–30.1) vs. 19.3 months (95% CI, 14.1–24.4), log-rank $P < 0.001$; Fig. 2A]. According to the multivariate Cox proportional hazards model for OS adjusted for hormone receptor status, a high CA9 rise was associated with improved OS (HR 0.54; 95% CI, 0.37–0.79). Using the same cut-off value, a high CA9 rise was also associated with better PFS compared with a low/no CA9 rise [11.3 months (95% CI, 9.0–11.6) vs. 8.1 months (95% CI, 7.8–8.5), log-rank $P < 0.001$; Fig. 2B] with estimated HR of 0.45 (95% CI, 0.31–0.67) when including first-line treatment and ECOG PS in the multivariate Cox proportional hazards model.

Discussion

We have recently concluded the randomized ATX trial (15) in HER2-negative MBC patients designed to evaluate capecitabine added to first-line bevacizumab and paclitaxel versus bevacizumab and paclitaxel, according to the schedule as reported by Miller and colleagues (2) We have demonstrated that capecitabine added to bevacizumab and paclitaxel significantly improved PFS, response rate, and response duration, albeit not OS. In search for biomarkers useful in the context of bevacizumab-based therapy, we measured a series of circulating proteins involved in alternative proangiogenic pathways or hypoxia at baseline and on C2D1. Several proteins at baseline or their early changes during treatment were associated with the risk of poor clinical outcome. Our findings might be useful for the identification of an appropriate subset of patients that may benefit from inhibition of alternative routes involved in angiogenesis and tumor growth.

In our study, all patients received bevacizumab in addition to chemotherapy. Baseline VEGF-A_{165} or sVEGFR2 levels had no prognostic value for PFS and OS, while patients with high levels had no superior benefit from bevacizumab-based therapy than those with low levels. In previous reports, increased circulating VEGF-A levels at baseline have been identified as indicators of poor prognosis in patients with different types of solid tumors in randomized trials investigating the usefulness of bevacizumab (20–22). Biomarker analysis in the AVADO trial comparing docetaxel without or with bevacizumab in patients with HER2-negative MBC has demonstrated that a high baseline VEGF-A level (assayed preferentially for isoforms shorter than VEGF-A_{165}) was associated with short PFS, while treatment benefit from adding bevacizumab was better in patients with high levels (22). In the AVEREL trial in HER2-positive MBC on trastuzumab and docetaxel without or with bevacizumab, Gianni and colleagues (20) have also reported that a high baseline VEGF-A level was prognostic for short PFS, while patients with high levels had more pronounced benefit from bevacizumab than those with low levels. A recent large-scale meta-analysis of four phase III trials reaffirmed the prognostic value of baseline VEGF-A levels in nonbevacizumab-treated patients with advanced colorectal cancer, non–small cell lung cancer and renal cell cancer, but the predictive analysis revealed

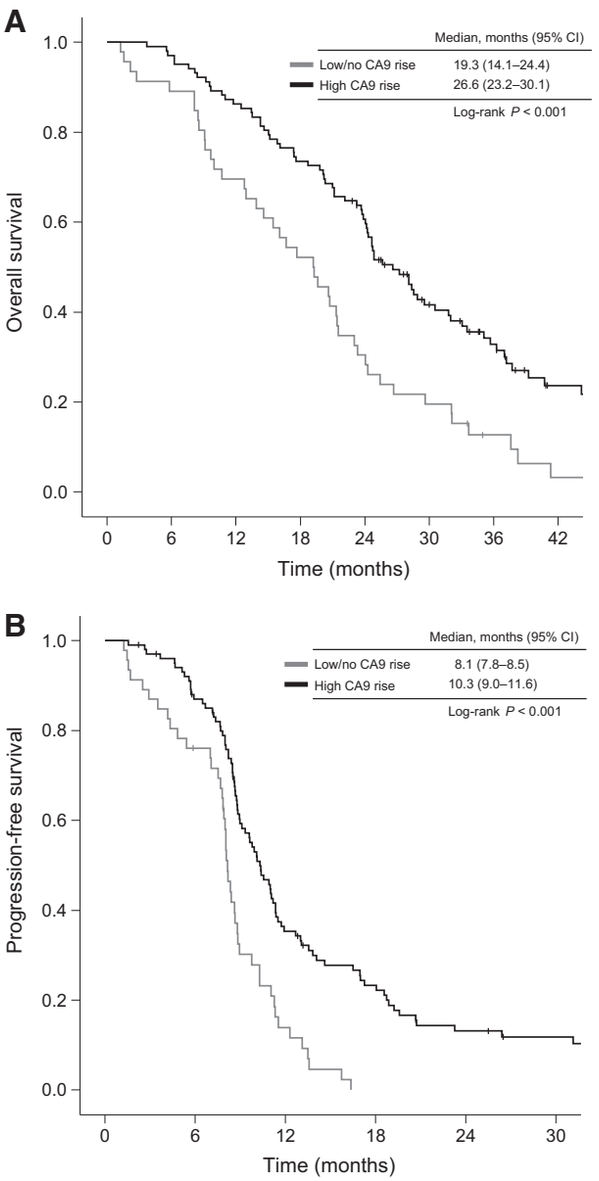

Figure 2.
A, Kaplan–Meier estimate of OS between a high CA9 rise and low/no CA9 rise using an optimal cut-off value. B, Kaplan–Meier estimate of PFS between a high CA9 rise and low/no CA9 rise using an optimal cut-off value.

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no significant treatment effect of bevacizumab between patients with high or low VEGF-A levels (21). For further testing of the predictive value of high baseline VEGF-A for bevacizumab benefit, measurement of the short VEGF-A_{121} isoform seems to be more promising (5). With regard to baseline sVEGFR2 levels, most studies have not demonstrated prognostic value, while a few reports suggested predictive potential. In the AVADO trial, a high baseline level of sVEGFR2 was predictive for PFS benefit from bevacizumab and docetaxel compared with docetaxel alone (22). Prospective clinical trials have been initiated to test the predictive value of high VEGF-A or sVEGFR2 levels for possible benefit from bevacizumab, such as the MERiDiAN trial in HER2-negative patients stratifying for plasma short VEGF-A isoforms at the start of treatment (5).

In the multivariate Cox proportional hazards model, we demonstrated that high baseline levels of ANG2, IL6, and IL8 were significantly associated with poor PFS and including sTIE2 also for OS indicating that these proteins convey prognostic information beyond those reflected by clinical characteristics. Coexistence of high baseline levels of proangiogenic proteins may therefore be indicative of a subset of carcinomas with a particularly aggressive nature. High baseline levels of ANG2 have been associated earlier with a poor clinical outcome in patients receiving a bevacizumab-containing regime (23–25). Daly and colleagues (26) have shown in a human tumor xenograft model that ANG2 is a TIE2 agonist and may thereby limit the antivascular effects of VEGF inhibition. In this respect, it may be hypothesized that patients with high ANG2 levels have limited benefit from bevacizumab added to chemotherapy as observed in our patients. The findings regarding IL6 and IL8 have not yet been documented in bevacizumab-treated patients. IL6 and IL8 are particularly well known for their immunomodulating function, but a substantial body of evidence indicates an emerging role in tumor angiogenesis (10, 11). IL6 may promote tumor angiogenesis through upregulation of VEGF-A via the STAT3 signaling pathway (27). Interestingly, a weak relationship between IL6 and VEGF-A was present in our correlative analysis at baseline (Supplementary Table S3). High circulating levels of IL6 have been correlated with advancing stage and worse survival in several malignancies (10). Circulating IL8 also reflects tumor burden and stage, and its levels correlate with OS (11, 28). We here report not only that IL6 and IL8 levels were prognostic for PFS and OS, but it was also apparent that patients with high IL6 or IL8 levels had no particular benefit from bevacizumab-based therapy.

Already at the start of cycle 2 of bevacizumab-based therapy, levels of VEGF-A, sVEGFR2, ANG2, sTIE2, and IL8 had significantly changed compared with baseline concentrations. Reactive responses in circulating angiogenesis-associated proteins during anti-VEGF therapy are different between bevacizumab and VEGFR tyrosine kinase inhibitors (TKI). As anticipated for bevacizumab, there was a pronounced decrease in unbound VEGF-A (−92.7%) to a near undetectable level in all patients indicating adequate VEGF-A inhibition. In contrast, increased VEGF-A levels can be expected during treatment with TKIs, as reported for sunitinib (29), sorafenib (30) and cediranib (31, 32). We measured a reactive increase in sVEGFR2 levels on C2D1, in agreement with reports from small cohorts of patients during bevacizumab-based therapy (33–35), whereas decreased sVEGFR2 levels have been observed in patients receiving sorafenib (30) or cediranib (31, 32). Ebos and colleagues (36) have demonstrated that VEGFR2 downregulation *in vitro* resulted in decreased release of VEGFR2 in conditioned media. For bevacizumab, we hypothesize that the increase in sVEGFR2 might result from shedding due to the near absence of VEGF-A to stimulate its receptor. The relative decrease in ANG2 (−18.1%) and sTIE2 (−10.5%) levels has also been described for sunitinib (29), sorafenib (30) and cediranib (31, 32). It thus appears that blockade of the VEGF/VEGFR signaling route inhibits ANG2/TIE2 activation promoting tumor vessel normalization by ANG1 (37). Among other proteins, a decrease in IL6 (−14.1%) and IL8 levels (−11%) was noted, which, to our knowledge, has not yet been reported for bevacizumab-based therapy. In the study of Batchelor and colleagues (31), cediranib with chemoradiation in glioblastoma patients led to an increase in IL6 and IL8 levels, but a similar increase was observed in patients on chemoradiation alone. As a conclusion, the pattern of change for a particular angiogenesis-associated protein in the circulation appears to depend on the type of angiogenesis-inhibiting therapy.

Here, we observed that a relatively larger increase in sVEGFR2 level as well as a decrease in IL8 level was associated with clinical response. As mentioned above, increased sVEGFR2 may be caused by prevention of ligand binding. As bevacizumab alone hardly affects tumor size (38), substantial reduction of disease by cytotoxic agents might also contribute to the acute release of sVEGFR2. In contrast to bevacizumab-based therapy, a decrease in sVEGFR2 was associated with improved tumor blood perfusion in glioblastoma patients receiving cediranib and chemoradiation, while such patients experienced longer survival (31). For IL8, clinical data suggest that decreasing levels may likely reflect early tumor shrinkage during treatment. Earlier, it has been reported that IL8 levels dropped rapidly as measured within 5 to 7 days after surgical tumor removal (28). In addition, circulating IL8 levels in melanoma patients monitored during treatment with BRAF inhibitors were low at the time of clinical response and showed a substantial rise at disease progression (28). Further studies are warranted to determine whether sVEGFR2 and specifically IL8 may be useful as an early biomarker in the prediction of efficacy from a particular type of treatment.

Hypoxia represents another key resistance mechanism to anti-angiogenic therapy mediating the production of proangiogenic factors and recruitment of vascular progenitor cells to the tumor microenvironment, which modulate the tumor vasculature (6). CA9 expression in tumor and stromal tissue can be considered as a marker of hypoxia (14). Ectodomain shedding of CA9 mainly occurs by hypoxic cancer cells and leads to an increase in circulating levels (39). Therefore, the use of circulating CA9 may provide a noninvasive means of assessing tumor hypoxia. Our analysis indicated that a high baseline level of CA9 was indicative of impaired PFS and OS. Poor OS outcome has also been observed in patients with other advanced solid tumor types with high blood CA9 levels (40–42). Of importance, a treatment-induced increase of CA9 in our patients was associated with improved PFS and OS. Although this may seem counterintuitive, increased intratumoral CA9 expression in patients with advanced renal cell carcinoma treated with sunitinib for 18 weeks has also been associated with better OS (43). An increased level in CA9 has also been observed in glioblastoma patients during treatment with cediranib and chemoradiation or chemoradiation alone (31). The molecular mechanism of the CA9 increase associated with improved clinical outcome in our patients might be clarified in the following way. Both bevacizumab and the TKI sunitinib have been demonstrated to induce hypoxia as a result from decreased tumor

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perfusion (44). Dynamic contrast (DC) MRI has demonstrated that the magnitude of reduction in transfer constants by VEGF(R) inhibitors, such as K^{trans} (composite of vascular permeability and endothelial surface area), is correlated with the attainment of stable or better disease outcome (45). Although we did not carry out DC-MRI, it is likely that patients with benefit from bevacizumab-based therapy had decreased transfer constants to such an extent that this leads to strong chronic hypoxia resulting in an excessive release of CA9 on C2D1. Furthermore, both chemotherapy and radiation may promote induction of HIF1α protein expression and stabilization resulting in transcriptional activity (46, 47). Lastly, induction of CA9 overexpression in human colon cancer cells appeared to be associated with necrosis and apoptosis in spheroids as well as in human tumor xenografts (48). In all, we hypothesize that the increase in CA9 is not only explained by the inhibition of perfusion, but also by treatment-induced necrosis and apoptosis. It would be interesting to carry out comparative analysis of DC-MRI, tumor CA9 immunohistochemistry and plasma CA9 monitoring at corresponding time points in patients before and during anti-VEGF(R) therapy to determine whether circulating CA9 is useful as an early biomarker to predict outcome.

Tumor marker antigen CA15-3, which corresponds to an immunodominant epitope in the extracellular portion of the membrane bound MUC1, is shed into the bloodstream. CA15-3, when elevated, can be used to monitor the course of disease in patients with MBC (49). In the current study, higher baseline 15-3 levels were detected in patients with higher IL6 and IL8 levels, likely reflecting extensive disease. Similar to IL6 and IL8, baseline CA15-3 level was also independently associated with PFS and OS. In our study, CA15-3 was not routinely measured on C2D1. It is, however, known that CA15-3 is not useful as biomarker when assessed within 3 to 4 weeks of treatment, as paradoxical increases explained by tumor cell lysis can occur in a considerable number of patients (49, 50).

The design of our study contains some limitations. First, we did not carry out serial measurements of proteins, as we were interested in early changes that might predict outcome in bevaicimab-based therapy. Our observations preclude insight in the patterns of changes among patients during the course of the disease. Second, accurate measurements of total tumor burden in advanced breast cancer are not possible, but we have included commonly used prognostic factors in the multivariate Cox proportional hazards model. Lastly, we cannot exclude the possibility of a nonspecific, chemotherapy-induced effect on the changes in the levels of proteins at cycle 2. In this respect, it can be mentioned that addition of capecitabine to AT led to a more pronounced decrease in IL6 and IL8 (Supplementary Table S5). As mentioned above, IL8 can decrease upon reduction of tumor burden (28). Our results illustrate the complexity of the interpretation of the effects of bevacizumab-based therapy, as not only damage to blood vessels, but also to tumor-associated stromal cells as well as cancer cells will influence the release of these proteins. The question on the magnitude of bevacizumab-induced effects on circulating proteins in chemosensitive solid tumor types should be addressed in a randomized trial.

In conclusion of this explorative analysis of the ATX trial, high baseline levels of several proangiogenic (ANG2, IL6, and IL8) and hypoxia-related (CA9) proteins were associated with poor clinical outcome. With the increasing availability of molecular-targeted agents entering the clinic that selectively inhibit ANG2 (51), IL6 (10, 52), IL8 (11), CA9 (14), patients with high levels of these proteins are particular candidates for testing regimes incorporating these drugs. During bevacizumab-based therapy, a relatively larger increase in sVEGFR2 levels and a relative decrease in IL8 levels were associated with clinical response, while a relative increase in CA9 levels was associated with improved PFS and OS. As all our MBC patients received bevacizumab, it remains to be investigated whether these protein changes are specific for the regimes given and can be used as a biomarker for early prediction of treatment efficacy.

Disclosure of Potential Conflicts of Interest

S.W. Lam reports receiving commercial research grants from Center for Translational Molecular Medicine (CTMM-MAMMOTH), and Roche. E. Boven reports receiving commercial research grants from Novartis and Roche. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.W. Lam, N.M. Nota, H. van Tinteren, E. Boven
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Angiogenesis- and Hypoxia-Associated Proteins as Early Indicators of the Outcome in Patients with Metastatic Breast Cancer Given First-Line Bevacizumab-Based Therapy

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