# Tamoxifen and Aromatase Inhibitors Differentially Affect Vascular Endothelial Growth Factor and Endostatin Levels in Women with Breast Cancer

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**Abstract**

**Purpose:** Circulating and cellular proangiogenic and antiangiogenic proteins such as vascular endothelial growth factor (VEGF) and endostatin contribute to the local angiogenic balance. We explored the effects of tamoxifen and aromatase inhibitors on concentrations of VEGF and endostatin in plasma, serum, and platelet releasate (induced by platelet activation).

**Experimental Design:** VEGF and endostatin concentrations were measured with a quantitative immunoassay before and after 1 to 5 weeks of treatment in 30 women with breast cancer treated with either tamoxifen ($n = 14$) or aromatase inhibitors ($n = 16$). Platelet activation was induced by a thrombin receptor agonist.

**Results:** Tamoxifen therapy resulted in an increase in platelet releasate concentrations of VEGF ($P = 0.01$) but no change in plasma VEGF. In contrast, aromatase inhibitor therapy did not affect serum, plasma, or platelet releasate VEGF. In univariate analysis, aspirin use attenuated the tamoxifen-associated increase in VEGF in the platelet releasate and decreased serum levels of VEGF ($P = 0.03$).

Aromatase inhibitor therapy resulted in a decrease in serum endostatin concentrations ($P = 0.04$), whereas plasma concentrations of endostatin tended to be higher during treatment with aromatase inhibitors ($P = 0.06$). Tamoxifen therapy resulted in no change in serum or plasma endostatin concentrations. Platelet releasate concentrations of endostatin did not change with either treatment. Interindividual variability was noted among both aromatase inhibitor– and tamoxifen-treated patients.

**Conclusions:** Tamoxifen and aromatase inhibitor therapy affect VEGF and endostatin levels and likely contribute to the angiogenic balance in breast cancer patients. Aspirin decreased the proangiogenic effects of tamoxifen, suggesting that antiplatelet and/or antiangiogenic therapy might improve the effectiveness of tamoxifen in women with breast cancer.

Angiogenesis is an essential requirement for breast tumor growth and metastasis. In breast cancer, tumor-induced angiogenesis is first evident at the preinvasive stage of ductal carcinoma *in situ* (1). Tumor angiogenesis is recognized as an important therapeutic target in breast cancer, and antiangiogenic agents have recently shown benefit in clinical trials (2–4).

Vascular endothelial growth factor (VEGF) and endostatin have been identified as potent proangiogenic and antiangiogenic proteins involved in modulating tumor growth and progression. Overexpression of VEGF by breast cancer has been associated with worsened clinical outcome and response to chemotherapy and hormonal therapy (5, 6). Elevated serum VEGF levels have been detected in breast cancer patients and significantly correlated with high intratumoral microvessel density (7), which is an independent prognostic factor for breast cancer survival (8). There is also a linear relationship between breast cancer progression and platelet accumulation of VEGF (9). In contrast to VEGF, endostatin potently inhibits the vascularization and growth of tumors (10). High preoperative plasma endostatin levels have been correlated with decreased intratumoral microvessel density in women with breast cancer (11). Additionally, plasma endostatin levels have been correlated with relapse-free survival in a small series of patients with breast cancer (12).

Platelets contribute to the balance of tumor-associated angiogenesis through release of both stimulators and inhibitors of angiogenesis (13). Angiogenic proteins are released during the process of platelet activation, and platelet activation and deposition are seen in the tumor microenvironment (14). At least 30 proangiogenic and antiangiogenic proteins are contained in the platelet, including VEGF and endostatin. For proangiogenesis proteins such as VEGF, the platelet pool represents 80% to 90% of total intravascular protein content (15). Recently, α and β forms of the estrogen receptor have

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been found on the platelet membrane (16, 17). Limited studies have found that estradiol as well as tamoxifen metabolites may enhance stimulus-mediated platelet aggregation, suggesting that occupancy of the estrogen receptor may influence the release of intraplatelet proteins, such as VEGF and endostatin, when platelets are stimulated in the tumor microenvironment (18).

For patients with hormonally responsive tumors such as breast cancer, endocrine therapy has undergone limited study with regard to angiogenesis. Estradiol has been recognized as angiogenic and estrogen effects may be mediated by induction of VEGF (19). Whereas tamoxifen leads to decreased VEGF transcription in breast cancer cells, tamoxifen use has been associated with higher plasma and platelet-derived VEGF levels (20–22). Assessments of VEGF levels in tamoxifen-treated patients have been complicated by endometrial hyperplasia that is often seen with the drug (23). VEGF expression in breast cells lines is the only available data using aromatase inhibitors (24). There are no reports on the effect of aromatase inhibitors on VEGF or endostatin levels in patients.

Given the importance of angiogenesis in breast cancer, we determined the comparative effects of the two most extensively used classes of endocrine therapy, the selective endocrine receptor modulators (tamoxifen) and the aromatase inhibitors (anastrozole, letrozole, and exemestane), in women with noninvasive (ductal carcinoma *in situ*) and invasive breast cancer (stage I-III) after initiation of adjuvant treatment. The goal of our study was to determine the effect of these commonly used adjuvant therapies on three pools of VEGF and endostatin: (*a*) plasma concentration reflecting circulating protein content, (*b*) serum concentration reflecting total intravascular protein content (plasma + intraplatelet), and (*c*) platelet releasate reflecting the protein content released by platelets during activation. One of the principal mechanisms of platelet activation is by thrombin generated in the tumor microenvironment. Because of the importance of thrombin, we used the thrombin receptor activating peptide (TRAP) that binds to protease-activated receptor 1 (PAR1) to activate platelets and then measured the platelet releasate. Given that platelet aggregation is stimulated by estrogen and that platelets contain functionally active estrogen receptors, we hypothesized that tamoxifen and aromatase inhibitors would differentially affect stimulus-mediated platelet release of VEGF and endostatin.

## Materials and Methods

Thirty women with a diagnosis of noninvasive (to include high-risk women and ductal carcinoma *in situ*) or invasive breast cancer (stage I-III), who were candidates for the use of tamoxifen or aromatase inhibitors as adjuvant endocrine therapy, were enrolled in this single center study. Because no clear differences in clinical outcomes are known with the three available third-generation aromatase inhibitors (anastrozole, exemestane, and letrozole), they were considered as a group for statistical purposes.

To minimize potential confounding factors of prior therapy on the study markers, a predefined interval was determined for each therapeutic modality and time until study entry as follows: surgery, minimum of 28 d from last surgical procedure; chemotherapy, minimum of 28 d from last chemotherapeutic agent administered; radiation therapy, minimum of 7 d from last radiation treatment; prior endocrine therapy, minimum of 30 d since last dose. These intervals were chosen based on limited literature about the effect of these modalities on platelet activation and function. Patients unable to meet the predefined interval time between last therapeutic modality and study entry were excluded.

Patients receiving chronic aspirin therapy (for any indication) were not excluded from enrollment. Chronic aspirin therapy was defined as aspirin therapy at any dose taken >4 d/wk for longer than 1 mo. Intermittent aspirin users were asked to abstain from aspirin use during the study period. Fourteen days must have passed from last aspirin use to study entry in intermittent aspirin users. Patients unable to abstain from intermittent aspirin use and those using heparin (unfractionated or low molecular weight), warfarin, or alternate platelet aggregation inhibitors were excluded. The study was approved by the institutional review board of University of Vermont and written informed consent meeting all federal, state, and institutional guidelines was obtained from all patients.

***Sample collection.*** Venous blood samples were collected before initiation of endocrine therapy and 1 to 5 wk following the initiation of therapy. Samples were collected into vacutainer tubes supplemented with 0.5 mL of 3.2% sodium citrate (for plasma), 0.5 mL of 3.2% sodium citrate with subsequent addition of 25 μmol/L (final) TRAP-14 (SFLLRNPNDKYEPF; Calbiochem, Inc.), or no anticoagulant (serum). Plasma samples were mixed for 30 s, separated by centrifugation (3,000 × *g* for 10 min), and stored at -80°C. Serum samples were incubated at room temperature for 45 min, separated by centrifugation (2,000 × *g* for 15 min), and stored at -80°C. Sample collection was standardized for all subjects to minimize unintended platelet activation during phlebotomy. Parallel samples were run for routine hematologic counts in an automated blood Coulter counter (Beckman Coulter).

***Enzyme immunoassay.*** VEGF and endostatin levels were measured with a quantitative sandwich enzyme immunoassay (Quantikine human VEGF kit and Quantikine human Endostatin kit, R&D Systems, Inc.) according to the manufacturer's instructions. All measurements were done in duplicate. Mean values were used as the final concentrations. Serum and platelet releasate concentrations of VEGF and endostatin were corrected for platelet count based on the method of Adams et al. (20), where platelet-derived VEGF and endostatin were calculated and expressed per platelet number, taking into account platelet count and plasma volume.

***Statistical analysis.*** Data are presented as mean ± SD. Student's paired *t* test was used to compare pretreatment and posttreatment differences in serum, platelet releasate, and plasma VEGF and endostatin levels for tamoxifen and aromatase inhibitors. Based on a SD in VEGF levels of 100 pg/mL as shown in the work of Adams et al. (20), 30 subjects provided an 80% power to detect an 18% difference in VEGF levels before and after endocrine therapy, with an $\alpha$ error of 0.05. The two-sample *t* test was used to compare posttreatment differences in VEGF and endostatin levels after initiation of tamoxifen compared with aromatase inhibitors. The Fisher exact test and Kruskal-Wallis test were used to compare binary and ordered demographic variables, respectively, among the two groups.

Patients ≤50 y of age who reported their menopausal status as perimenopausal were considered premenopausal for statistical purposes. Patients >50 y of age who reported their menopausal status as perimenopausal were considered postmenopausal for statistical purposes.

Correlations within each endocrine therapy group were sought using Spearman's method. $P < 0.05$ was considered statistically significant for all measures. All calculations were done on commercially available statistical packages [Minitab release 14 (Minitab, Inc.) and SAS System for Windows, version 9.1.3].

To examine pretreatment to posttreatment group differences in VEGF and endostatin levels, a repeated-measures multivariate analysis of covariance was done by using VEGF and endostatin levels pretreatment and posttreatment as the repeated measures and group (tamoxifen versus aromatase inhibitors) as the between-subject factor. Age,

**Table 1.** Characteristics of patients included in the study

| | No. patients (%) | | *P* |
|---|---|---|---|
| | Tamoxifen (*n* = 14) | AI (*n* = 16) | |
| Age, y | | | |
| Median | 51 | 54.5 | 0.077 (Kruskal-Wallis test) |
| Range | 45-65 | 41-70 | |
| 40-50 | 7 (50) | 3 (19) | |
| 51-59 | 5 (36) | 8 (50) | |
| ≥60 | 2 (14) | 5 (31) | |
| Menopausal status | | | |
| Premenopausal | 6 (43) | 0 (0) | 0.005 (Fisher's exact test) |
| Postmenopausal | 8 (57) | 16 (100) | |
| Study diagnosis | | | |
| Invasive | 8 (57) | 15 (94) | 0.031 (Fisher's exact test) |
| Noninvasive | 6 (43) | 1 (6) | |
| Lymph node status | | | |
| Negative | 8 (57) | 8 (50) | 0.317 (Kruskal-Wallis test) |
| 1-3 positive nodes | 1 (7) | 5 (31) | |
| ≥4 positive nodes | 1 (7) | 2 (13) | |
| Not assessed | 4 (29) | 1 (6) | |
| Prior chemotherapy | | | |
| Yes | 4 (29) | 8 (50) | 0.284(Fisher's exact test) |
| No | 10 (71) | 8 (50) | |
| Chronic aspirin therapy | | | |
| Yes | 5 (36) | 2 (12) | 0.204 (Fisher's exact test) |
| No | 9 (64) | 14 (88) | |
| Mean duration of endocrine therapy (± SD), d | 19 ± 7 | 19 ± 10 | 0.99 |

Abbreviation: AI, aromatase inhibitors.

menopausal status, duration of tamoxifen or aromatase inhibitor therapy, prior chemotherapy use, and chronic aspirin use were used as nuisance covariates in this analysis. Age and duration of endocrine therapy were examined as continuous variables; prior chemotherapy use and chronic aspirin use were examined as binary variables; and menopausal status was examined as a categorical variable with the following categories: premenopausal women treated with tamoxifen, postmenopausal women treated with tamoxifen, and postmenopausal women treated with aromatase inhibitors.

## Results

***Demographics.*** The demographic characteristics of the 30 women initiating therapy with tamoxifen and aromatase inhibitors are shown in Table 1. Six of 14 (43%) women on tamoxifen were postmenopausal whereas all women on aromatase inhibitors were postmenopausal. Four of 12 (33%) women in the tamoxifen group had received prior chemotherapy whereas 67% of women on aromatase inhibitors received prior chemotherapy. Thirty-six percent of women in the tamoxifen group were on chronic aspirin therapy. Two of 16 (13%) women on aromatase inhibitors were on chronic aspirin therapy. The duration of endocrine therapy at the time of follow-up was similar in both groups ($P = 0.99$).

***Serum VEGF and endostatin levels before and during treatment.*** To investigate whether treatment with endocrine therapy affects serum levels of VEGF and endostatin, we compared VEGF and endostatin levels before and during treatment in women initiating tamoxifen or aromatase inhibitors (Fig. 1). Serum concentrations of VEGF (per $10^6$ platelets) were similar before treatment among both groups (tamoxifen, 0.739 ± 0.499 pg/mL; aromatase inhibitors, 0.740 ± 0.557 pg/mL; $P = 0.99$). No change in serum VEGF levels was seen after treatment with tamoxifen or aromatase inhibitors (tamoxifen, 0.625 ± 0.425 pg/mL, $P = 0.08$ versus baseline; aromatase inhibitors, 0.658 ± 0.392 pg/mL, $P = 0.35$ versus baseline) although a trend toward a decrease in VEGF levels in the tamoxifen treatment group was noted.

Because aspirin therapy may affect platelet activation (as seen in serum samples), we evaluated the effect of endocrine therapy in the subgroup of women using chronic aspirin therapy. Women who used aspirin and were treated with tamoxifen exhibited a decrease in serum VEGF from pretreatment (0.578 ± 0.401 pg/mL) to on-treatment (0.431 ± 0.411 pg/mL; $P = 0.03$; Fig. 1B). No change in serum VEGF was seen in tamoxifen-treated women not on aspirin ($P = 0.33$) or women treated with aromatase inhibitors with or without concurrent aspirin use.

In contrast to VEGF, serum endostatin (per $10^3$ platelets) decreased during treatment with aromatase inhibitors (0.152 ± 0.191 ng/mL; $P = 0.04$; Fig. 2). Serum endostatin levels did not change during treatment with tamoxifen (0.213 ± 0.224 ng/mL; $P = 0.41$). Baseline serum endostatin levels were similar in women who were treated with tamoxifen (0.174 ± 0.219 ng/mL) and aromatase inhibitors (0.216 ± 0.152 ng/mL; $P = 0.55$). The use or nonuse of aspirin therapy did not influence the levels of endostatin in either group.

***Platelet releasate content of VEGF and endostatin (induced by TRAP).*** Because thrombin-mediated release of angiogenic proteins likely occurs in the tumor microenvironment, we examined thrombin receptor–mediated release of VEGF and endostatin during treatment with tamoxifen and

aromatase inhibitors. TRAP-induced platelet release of VEGF (per $10^6$ platelets) was similar at baseline in women treated with tamoxifen (0.533 ± 0.451 pg/mL) and aromatase inhibitors (0.369 ± 0.273 pg/mL; $P = 0.23$). During tamoxifen treatment, TRAP-induced platelet release of VEGF was increased (0.774 ± 0.568 pg/mL; $P = 0.01$; Fig. 3). No difference in TRAP-mediated platelet VEGF release was observed after initiation of treatment with aromatase inhibitors (0.462 ± 0.445 pg/mL; $P = 0.45$).

The effect of aspirin use on thrombin-induced activation was subsequently assessed in univariate analysis. Women not on aspirin therapy ($n = 9$) showed an increase in VEGF levels from baseline (0.631 ± 0.498 pg/mL) to treatment (0.885 ± 0.529 pg/mL; $P = 0.04$). There was no change among women taking aspirin ($P = 0.21$). Aspirin did not alter TRAP-induced platelet release of VEGF during treatment with aromatase inhibitors (0.462 ± 0.445 pg/mL; $P = 0.45$). Treatment with tamoxifen or aromatase inhibitors did not alter TRAP-induced release of endostatin (per $10^3$ platelets). In addition, no between-group differences were apparent at baseline and during therapy in either treatment group ($P > 0.16$).

Based on recent reports of VEGF and endostatin counter-regulation through the PAR1 receptor, we analyzed TRAP-induced release of VEGF and endostatin by platelets at baseline in women before treatment with tamoxifen. We found a negative correlation between TRAP-induced release of VEGF and endostatin at baseline in women before treatment with tamoxifen (Spearman's correlation, $r = -0.54$; $P = 0.05$). This counterregulation disappeared after initiation of treatment with tamoxifen ($r = 0.22$; $P = 0.45$), suggesting that tamoxifen therapy influenced platelet activation pathways that mediate VEGF and endostatin protein release. No correlation was observed between TRAP-induced platelet release of VEGF and endostatin at baseline or during treatment with aromatase inhibitors ($P > 0.07$).

***Serum compared with TRAP-induced platelet release of VEGF and endostatin.*** Platelet content of VEGF from serum samples was greater than TRAP-induced release of VEGF before initiation of tamoxifen ($P = 0.04$), suggesting that only a portion of the total platelet content of VEGF was released despite high concentrations of the agonist TRAP. During treatment with tamoxifen, however, total serum VEGF was the same as that released by TRAP stimulation ($P = 0.24$). Serum VEGF was greater than TRAP-induced release both pretreatment and during treatment with aromatase inhibitors ($P = 0.03$). Serum endostatin release was significantly higher than TRAP-induced release by platelets both before and during treatment with either tamoxifen or aromatase inhibitors ($P < 0.007$).

Using multivariate analysis, age was associated with higher serum VEGF ($P = 0.03$) and TRAP-induced platelet VEGF levels ($P = 0.04$) in women on aromatase inhibitors; however, age had no effect on these levels in tamoxifen-treated women ($P > 0.05$). There was no effect of menopausal status, duration of therapy, or prior chemotherapy use on serum or TRAP-mediated platelet release of VEGF or endostatin. The effect of aspirin use on platelet content and release of VEGF and endostatin observed with univariate analysis was not observed with multivariate analysis ($P > 0.09$).

Because all platelet activation–dependent levels (serum and platelet releasate samples) of VEGF and endostatin were

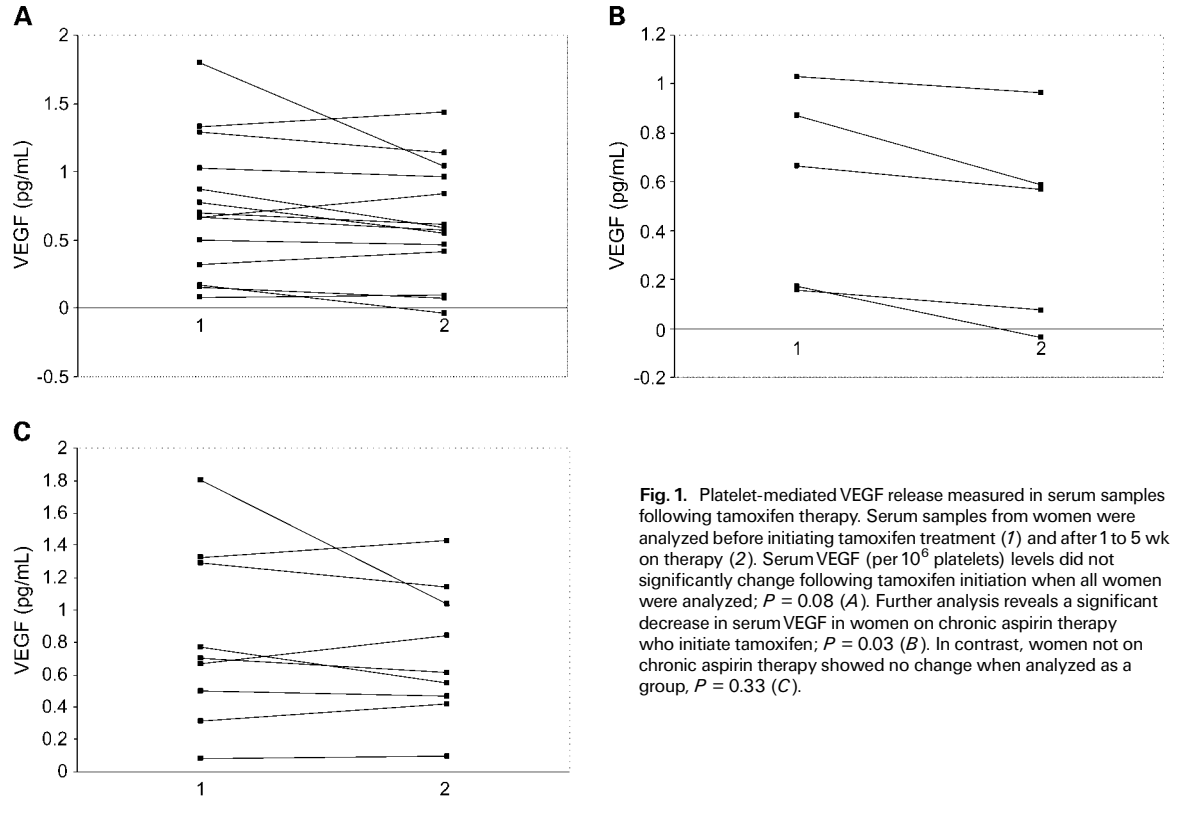


**Fig. 1.** Platelet-mediated VEGF release measured in serum samples following tamoxifen therapy. Serum samples from women were analyzed before initiating tamoxifen treatment (*1*) and after 1 to 5 wk on therapy (*2*). Serum VEGF (per $10^6$ platelets) levels did not significantly change following tamoxifen initiation when all women were analyzed; $P = 0.08$ (*A*). Further analysis reveals a significant decrease in serum VEGF in women on chronic aspirin therapy who initiate tamoxifen; $P = 0.03$ (*B*). In contrast, women not on chronic aspirin therapy showed no change when analyzed as a group, $P = 0.33$ (*C*).

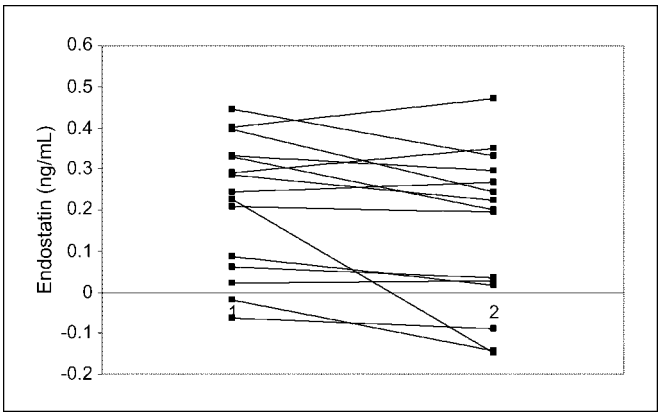


**Fig. 2.** Serum endostatin levels decrease following initiation of aromatase inhibitor therapy. Endostatin levels were analyzed before (*1*) and following 1 to 5 wk (*2*) of therapy. The decrease in endostatin levels [mean, 0.174 ± 0.219 ng/mL (before therapy) and 0.152 ± 0.191 ng/mL (on therapy); $P = 0.04$] was not seen in the tamoxifen-treated group. Data are expressed as endostatin per $10^3$ platelets.

corrected for platelet count, we determined the effect of endocrine therapy on total platelet number to avoid underestimating or overestimating total protein levels based on this method of reporting. Initiation of endocrine therapy did not affect total platelet counts when pretreatment and posttreatment levels for each group ($P = 0.12$ for tamoxifen; $P = 0.19$ for aromatase inhibitors) were compared. No differences in platelet counts at baseline and at follow-up between treatment groups ($P > 0.18$) were seen. No differences in platelet counts at baseline or at follow-up among women who were on chronic aspirin therapy compared with non–aspirin users for either treatment cohort ($P > 0.40$) were seen.

***Plasma VEGF and endostatin concentrations before and during endocrine therapy.*** Plasma protein levels are indicative of circulating protein levels that are independent of platelet activation, a distinction that makes these levels unique from serum and TRAP1-mediated platelet release levels and of interest in our analysis. Plasma VEGF levels did not change during treatment with tamoxifen (33.67 ± 11.14 pg/mL; $P = 0.11$) or aromatase inhibitors (37.72 ± 11.07 pg/mL; $P = 0.80$). A between-group comparison revealed no difference in plasma VEGF levels before initiation of tamoxifen (40.13 ± 15.75 pg/mL) and aromatase inhibitors (36.93 ± 11.70 pg/mL; $P = 0.53$) and no difference on treatment ($P = 0.33$).

When plasma endostatin levels were assessed before initiation of therapy, no differences were found between the tamoxifen (95.51 ± 27.15 ng/mL) and aromatase inhibitor (104.52 ± 43.84 ng/mL) groups ($P = 0.51$). Like VEGF, plasma endostatin levels did not change during treatment with tamoxifen (86.60 ± 26.86 ng/mL; $P = 0.26$) or aromatase inhibitors (116.12 ± 51.54 ng/mL; $P = 0.17$). A trend toward higher plasma endostatin levels was seen when comparing aromatase inhibitor– and tamoxifen-treated women ($P = 0.057$; Fig. 4).

Multivariate analysis showed that in women treated with aromatase inhibitors, prior chemotherapy was associated with decreased plasma VEGF levels ($P = 0.08$), and the lack of prior chemotherapy use was associated with decreased plasma endostatin levels ($P = 0.04$). For women treated with tamoxifen, prior chemotherapy did not have an effect on plasma VEGF levels ($P = 0.65$) and the lack of prior chemotherapy use did not have an effect on plasma endostatin levels ($P = 0.55$). No other significant associations were seen in multivariate analysis.

## Discussion

Whereas targeted antiangiogenesis therapies have come to the forefront as new strategies for the treatment of breast cancer, little is known about the effect of current hormonal therapies on the angiogenic balance. We have shown an effect of tamoxifen and aromatase inhibitors on VEGF and endostatin protein levels shortly after initiation of treatment. A short duration of treatment (1-5 weeks) was chosen to maximize effects associated with platelet-related contributions to VEGF and endostatin levels and minimize the known potential effects of tamoxifen-induced endometrial hyperplasia in postmenopausal women, which can affect the interpretation of VEGF levels.

Unique to our study is the characterization of agonist-induced platelet release of VEGF and endostatin. Platelet activation has been shown to modulate endostatin and VEGF release and current evidence supports a clinical link between breast cancer and platelet activation (25). Breast cancer cell lines show an interaction between tumor cells and platelets via the activated platelet protein marker P-selectin (26), and breast cancer patients, as compared with healthy matched controls, have increased levels of soluble P-selectin (27).

Serum levels of VEGF and endostatin have been most commonly used to measure the available vascular pool of proteins because they reflect both intraplatelet and plasma protein levels. TRAP, an agonist of the thrombin receptor PAR1, was chosen to assess platelet release of VEGF and endostatin because of the prominent role of thrombin in platelet activation *in vivo* and the known central role for thrombin in the tumor microenvironment (28). This assessment offers a unique mechanism-based assessment of the vascular protein pool that is distinct from serum. Serum samples rely on contact activation of coagulation and *in vitro*

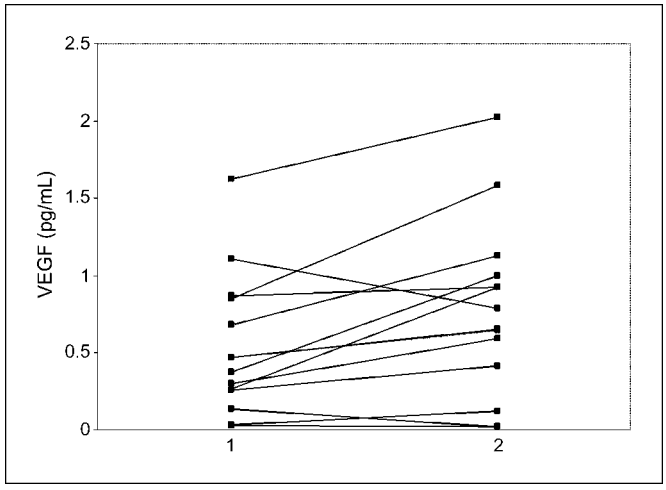


**Fig. 3.** TRAP-induced release of VEGF is increased following the initiation of tamoxifen therapy. Stimulation of platelets with the thrombin receptor agonist TRAP induces release of VEGF via the thrombin receptor PAR1. *Ex vivo* stimulated samples were analyzed before (*1*) and following 1 to 5 wk (*2*) of tamoxifen therapy. Increases in VEGF (per $10^6$ platelets) were shown in all patients in the group with the exception of 3.

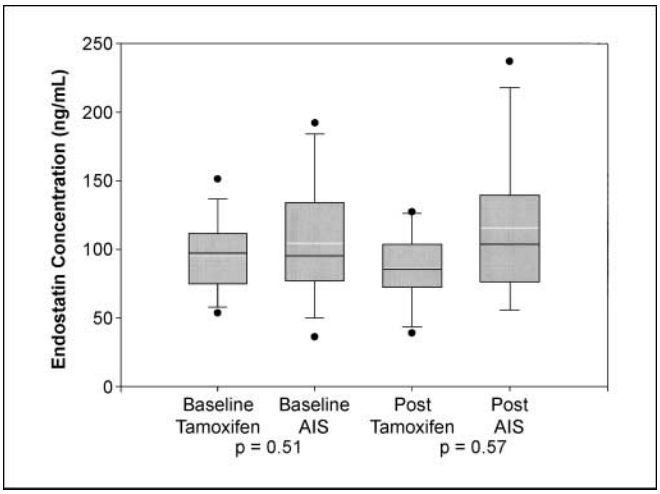


**Fig. 4.** Plasma endostatin levels in tamoxifen- and aromatase inhibitor – treated women. Baseline (before endocrine therapy) and on-therapy endostatin levels were analyzed. Whereas no difference was seen within each group following initiation of endocrine therapy, a between-group comparison reveals an increase in endostatin levels in aromatase inhibitor – treated women (versus tamoxifen). Mean levels are indicated in white within the box plots.

clot generation to activate platelets; this *in vitro* contact activation system does not play a role in platelet activation *in vivo* and the TRAP method we used may be a more realistic assessment of platelet VEGF release *in vivo*.

We hypothesized that tamoxifen and aromatase inhibitors may have different effects on platelet-mediated VEGF and endostatin release based on the recent demonstration of the α and β forms of the estrogen receptor on the platelet membrane (16, 17). Whereas conflicting results have been reported for the role of estrogen in the regulation of platelet function, binding of the estrogen receptor by 17β-estradiol has been shown to result in an increase in thrombin-induced platelet aggregation and suggests that such binding may increase intraplatelet protein release of VEGF and endostatin in the tumor microenvironment (18).

Our findings show that treatment with tamoxifen increases thrombin receptor – mediated VEGF release when platelets are exposed to a PAR1 agonist. These results are consistent with *in vitro* studies showing an increase in stimulus-mediated platelet aggregation when washed platelets are exposed to tamoxifen metabolites (29). Tamoxifen has also been shown to decrease platelet intracellular calcium concentrations with a limited correlation of this observation with increased aggregation *in vivo* (30). Interestingly, lower estrogen concentrations (from aromatase inhibitor therapy) did not affect platelet-mediated VEGF release. The reasons for this observation are unclear but the short time interval of treatment may have precluded seeing such an effect. The ability of tamoxifen to act as a "proestrogen" on the platelet surface is analogous to its effects in the endometrium and will need confirmation in further studies.

In contrast to VEGF, thrombin receptor – mediated endostatin release was not affected by tamoxifen or aromatase inhibitor therapy. This result is consistent with recent reports of PAR1-mediated increases in VEGF release without concomitant changes in endostatin release in *ex vivo* platelet-rich plasma experiments (31, 32). The differential regulation of VEGF and endostatin is also supported by our observation of a negative correlation of these proteins before initiation of tamoxifen therapy. During treatment with tamoxifen, this differential regulation was lost, further suggesting that tamoxifen affects platelet activation responses.

We found that tamoxifen treatment did not change plasma VEGF levels and only decreased serum VEGF levels in women on chronic aspirin therapy (with no change in women not on aspirin therapy). This result is in contrast to an earlier study by Adams et al. (20) who found higher plasma, serum, and theoretical platelet VEGF levels among women with breast cancer in remission treated with tamoxifen as compared with non – tamoxifen users. Multivariate analysis failed to reveal any influencing variables in that study although aspirin use was not reported and patients on adjuvant chemotherapy were included (our study excluded concomitant chemotherapy use due to its potential effect on angiogenic protein levels). The investigators reported longer durations of tamoxifen treatment, which can result in tamoxifen-induced hyperplasia of the endometrium. In a separate study, patients with increased endometrial thickness after 3 months of adjuvant tamoxifen therapy showed increased serum VEGF levels and women with normal endometrial thickness showed a decrease in serum VEGF after tamoxifen therapy (23).

We also note that whereas tamoxifen and aromatase inhibitors affected several angiogenic protein measures presented in this study, not all measures were affected by therapy. This was not an unanticipated result as others have found, for example, that serum and plasma levels of VEGF do not correlate (33). This observation has led to controversy and an unresolved question about the most important predictive measure of angiogenesis and prognosis. Verhuel and Pinedo (34) have hypothesized that both serum and plasma VEGF levels are important as markers of tumor angiogenesis. It is for this reason that we included multiple measures of proangiogenic and antiangiogenic proteins in our study. The introduction of TRAP-induced platelet release of VEGF is novel to our study. Given the mechanisms of platelet-induced activation relevant to tumors, we would hypothesize that TRAP-induced platelet releasate might be the most relevant, but we have not yet proved this in a clinical outcome study. Further research will be needed to determine which of these measurements, if any, will best predict treatment outcome.

Additionally, we still do not understand whether changes in proangiogenic proteins (like VEGF) or changes in antiangiogenic proteins (like endostatin) are more influential in determining the angiogenic balance and influencing clinical outcome. We believe that additional studies such as ours that report multiple circulating angiogenic protein levels in cancer patients will aid in this determination in future studies.

Our conclusions are limited to short-term treatment effects of endocrine therapy, and longer-term treatment studies are needed. Numerous studies have shown correlations between circulating VEGF and endostatin levels and tumor progression. It is intriguing to hypothesize that differences observed in our study may, in part, explain differences in clinical outcome between tamoxifen- and aromatase inhibitor – treated women, but our sample size was too small and duration of treatment too short to assess these clinical end points. In addition, variability in response to drug was seen within our study population. Features predictive of response included aspirin

use and age; however, not all patient and tumor characteristics were analyzed due to the small sample. Larger studies will be needed to identify and refine predictors of response in this population.

Our data suggest that antiplatelet therapy may affect angiogenic protein levels in women treated with endocrine therapy. Antiangiogenesis therapy and/or antiplatelet therapy may be of particular benefit in women treated with tamoxifen. An understanding of the effect of currently used breast cancer therapies on angiogenesis will likely aid in our ability to combine antiangiogenic and antiplatelet therapies with existing regimens to improve clinical outcome.

## Disclosure of Potential Conflicts of Interest

C.E. Holmes has a relationship with Breast Cancer Research Foundation.

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