

Changes in the Circulating Plasma Levels of VEGF and VEGF-D after Adjuvant Chemotherapy in Patients with Breast Cancer and 1 to 3 Positive Lymph Nodes

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Abstract. *Background:* The goal of the present study was to investigate the changes in concentration of the important lymph-angiogenesis factors vascular endothelium-derived growth factor (VEGF) and VEGF-D under adjuvant chemotherapy. *Materials and Methods:* The blood plasma of a total of 142 patients with breast carcinoma and with 1 to 3 affected lymph nodes was investigated, using the quantitative sandwich enzyme immunoassay technique, prior to and following chemotherapy, within the framework of a randomized phase III study: the patients received either conventional or dose-intensified chemotherapy. *Results:* In general, there was a significant reduction in VEGF levels after chemotherapy only in patients with large tumors (T3) ($p=0.043$). There was also an almost significant reduction in patients with an overexpression of *c-erbB-2* (Dako Score +3, $p=0.052$). In contrast, the clearest reduction in VEGF-D occurred in patients with a positive hormone receptor status ($p=0.04$) or in patients with a low expression of *c-erbB-2* (Dako Score +1, $p=0.05$). A significant effect of chemotherapy on VEGF-D was

determined only in patients who had a baseline level that was above the normal (conventional treatment $p=0.005$; dose-intensified treatment $p=0.004$). *Conclusion:* Both VEGF and VEGF-D levels changed after chemotherapy, depending on the patient and tumor characteristics. With respect to changes in the plasma levels of VEGF and VEGF-D, there were no significant differences between dose-intensified and conventional chemotherapy.

Angiogenesis is essential for the growth of tumors and the spread of metastases (1-3). Among the decisive factors regulating angiogenesis and lymph-angiogenesis are a number of hormones and cytokines. In association with hypoxia, they form a multi-complex network that directs the patho-mechanism of neo-vascularization and plays an important role in tumor progression.

VEGF (vascular endothelium-derived growth factor) is one of the most important stimulators of angiogenesis, which binds to the cell surface at two specific tyrosine-kinase receptors, [VEGFR-1:Flt-1 (fms-like tyrosine kinase-1); VEGFR-2 (KDR-kinase domain region)], as well as at an additional co-receptor (neuropilin, NP-1) (4-6). Differential exon splicing of the VEGF gene results in 6 main mRNA species which code for 6 secreted isoforms (VEGF₁₂₁, 145, 148, 165, 189, 206) (7). In tissue the dominant form is VEGF₁₆₅, while VEGF₁₂₁ and VEGF₁₆₅ are the isoforms mainly detectable in the serum or plasma.

VEGF-D, a member of the VEGF family, binds to VEGFR-2 and VEGFR-3(Flt-4), which are located on

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vascular and lymphatic endothelial cells and, therefore, plays a role in both angiogenesis and lymph-angiogenesis (8-10). Recently published retrospective studies have shown that an increase in VEGF is an independent prognostic factor in the relapse-free survival and overall survival of patients with breast cancer (11-16). It was demonstrated that, independently of any effect on lymph nodes, an increase in the blood or tumor cytosol values of VEGF correlated significantly with reduced overall survival.

To date, only immunohistological analyses of the prognostic value of VEGF-D in breast cancer have been published (17, 18). The goal of our study was the investigation of VEGF and VEGF-D under adjuvant chemotherapy conditions within the framework of a randomized phase III study (NOGGO Trial-Nordostdeutsche Gesellschaft für Gynäkologische Onkologie [Northern German Society for Gynecological Oncology]) in patients with 1 to 3 positive lymph nodes and their correlation with already existing valid prognostic factors. For the first time, changes in circulating angiogenic factors under dose-intensified chemotherapy were evaluated.

Materials and Methods

Patient characteristics and study design. During the period from 2000 to 2003, the plasma parameters of 142 patients with stage IIa to stage IIIa breast cancer and 1 to 3 positive lymph nodes were investigated prior to and following adjuvant chemotherapy, within the framework of a prospective, randomized phase III study (NOGGO Trial). The patients were randomly assigned to one of two arms: arm A received 4 cycles of epirubicin and cyclophosphamide (90/600 mg/m², d1, q21), followed by 4 cycles of paclitaxel (175 mg/m², d1, q21), while arm B received a dose-intensified treatment with 4 cycles of epirubicin (120 mg/m², d1, q14) followed by 4 cycles of paclitaxel (175 mg/m², d1, q14), with obligatory granulocyte colony-stimulating factor support. There were 73 patients in arm A and 69 in arm B.

The median age of the patients was 54 years (range 25 - 72 years). At the time of randomization of the 142 patients in the study, 59 were pre-menopausal and 81 post-menopausal. An evaluation of the hormone-receptor status showed 109 patients to be hormone-receptor-positive as opposed to 32 patients who were estrogen- and progesterone-receptor-negative (Table I). (The numbers given for the different patient groups in Table I are those used for statistical analysis, after exclusion of missing or obviously erroneous data.)

Blood samples. During the investigation of routine parameters, additional blood samples were taken as EDTA plasma after randomization of the patients for the study, before the beginning and after the end of the adjuvant chemotherapy. After centrifugation, the plasma samples were stored at -80°C. The Ethics Commission of the Charite, Germany, gave separate approval for these additional investigations within the framework of a companion scientific program of the above-named study. All participating patients gave their written approval for this additional investigation.

ELISA tests for VEGF and VEGF-D. For evaluation of the VEGF values, commercially available quantitative immunoassay kits for human VEGF₁₆₅ and VEGF-D were used (quantitative, human VEGF and VEGF-D; R&D Systems, Minneapolis, MN, USA; Quantikine®). All measurements were determined twice. The minimal detectable value was less than 9 pg/ml for VEGF and 42 pg/ml for VEGF-D. Our intrassay/interassay precision (%) was 4.5/7.0 for VEGF at a concentration of 235/250 pg/ml and 4.2/7.2 for VEGF-D at a concentration of 970/956 pg/ml. For healthy women, the mean for VEGF, as given by the manufacturer of the immunoassay kits, is 61 pg/ml, with the normal range being 9-115 pg/ml. For VEGF-D, the normal range is 91-437 pg/ml, with a mean of 208 pg/ml.

Statistical analysis. The Wilcoxon test, as a non-parametric test for two matched random samples, was used to evaluate the influence of chemotherapy on changes in VEGF and VEGF-D in the subcollectives defined by conventional prognostic factors (e.g., premenopausal/post-menopausal). Cross tables were set up and the asymptotic χ^2 test according to Pearson, as well as the exact χ^2 test according to Fisher, were used to determine group changes (the group of decreasing values as opposed to the group of increasing values after chemotherapy).

To evaluate the significance of the conventional prognostic factors with respect to the VEGF and VEGF-D values before and after chemotherapy, the non-parametric Kruskal-Wallis test for independent samples was used. To determine a possible significant correlation between VEGF and VEGF-D, the Spearman test was used. In addition, all the statistical tests were carried out excluding unchanged values (unchanged values: $\pm 10\%$ of the starting value after chemotherapy). All of the *p*'s were two-tailed and the 0.05 level was considered statistically significant.

Results

General analysis of the change in the plasma levels of VEGF and VEGF-D. The median concentration of VEGF for the 142 patients prior to the start of chemotherapy was 409 pg/ml and for VEGF-D was 616 pg/ml (the VEGF range was 9-2590 pg/ml and the VEGF-D range was 60-2168 pg/ml). The median values following adjuvant chemotherapy were 402 pg/ml for VEGF and 447 pg/ml for VEGF-D (ranges after therapy were 9-2194 pg/ml and 42-2550 pg/ml for VEGF and VEGF-D, respectively) (Table I). Taking all patients together, the pre- and post-chemotherapy VEGF values were almost identical; on the other hand, the VEGF-D values showed a reduction after chemotherapy which was just short of the statistical significance level $p=0.05$ (Wilcoxon matched-pairs test - *p*-values VEGF / VEGF-D: =0.618 / 0.067). Within the VEGF group, 130 patients had a change of more than 10%: in 62 patients, there was an increase after chemotherapy, while in 68 patients there was a decrease (the remaining 12 patients had changes of less than 10%). Within the VEGF-D group, the values increased by more than 10% after chemotherapy in 52 patients, fell by more than 10% in 70 patients and were unchanged, i.e., within the $\pm 10\%$ range, in the remaining 20 cases.

Table I. VEGF and VEGF-D in relation to patient and tumor characteristics.

	No.	VEGF*		p-value	No.	VEGF-D*		p-value
		Pre-/Post-chemo				Pre-/Post-chemo		
All patients	142	409/402		0.618	142	616/447		0.067
Changes >10%	130	422/402		0.655	122	626/440		0.076
Elevated values (>10%)	62	253/584		0.001	52	664/1166		0.0001
Decreased values (>10%)	68	585/317		0.001	70	1165/492		0.0001
Unchanged values (\pm 10%)	12	308/321			20	508/519		
Menopausal status								
Pre-menopausal	59	362/329		0.351	59	632/638		0.850
Post-menopausal	81	416/433		0.969	81	587/368		0.054
Grading								
G1	12	325/220		0.480	12	739/293		0.117
G2	77	435/433		0.733	77	584/531		0.947
G3	42	287/322		0.396	42	638/363		0.076
Tumor size								
pT1	66	403/341		0.271	66	586/482		0.373
pT2	63	394/410		0.443	63	694/437		0.120
pT3	5	917/411		0.043	5	1365/378		0.225
ER/PR status								
Positive	109	396/410		0.638	109	657/486		0.040
Negative	32	441/364		0.668	32	456/304		0.793
c-erbB-2 status								
Dako score negative	81	396/411		0.577	81	518/371		0.540
Dako score:+1	21	362/276		0.958	21	558/442		0.050
Dako score:+2	11	507/500		0.859	11	720/526		0.790
Dako score:+3	29	541/352		0.052	29	701/655		0.117
Treatment								
Conventional (arm A)	73	446/393		0.207	73	600/492		0.308
Dose-intensified (arm B)	69	362/396		0.504	69	654/444		0.103

*For all values the median is given in pg/ml.

Where the total number in a category was less than 142, the corresponding information was either missing or obviously erroneous for certain patients and therefore not taken into consideration.

In the evaluation with respect to patient and tumor characteristics, with regard to menopausal status there was no significant change in VEGF levels after chemotherapy (either in the pre-menopausal or the post-menopausal subcollective). However, for post-menopausal women there was a reduction in the median value of VEGF-D from 587 to 368 pg/ml, just short of statistical significance ($p=0.054$). Regarding the different tumor grading groups, there were no significant changes in the plasma levels of VEGF or VEGF-D. There was a statistically significant reduction ($p=0.043$) in VEGF after chemotherapy for pT3 tumors (>5 cm), although the number of cases in this category was small ($n=5$). For patients with positive estrogen- or progesterone-receptor status, there was a statistically significant reduction in VEGF-D ($p=0.040$). With regard to c-erbB-2 status, there was a reduction for patients with a Dako score of +3 ($n=29$, $p=0.052$), which almost reached statistical significance; for VEGF-D, however, there was a significant change only in

the group with a Dako score of +1 ($n=21$, $p=0.050$). For the two therapy arms with standard and dose-intensified treatment, there were no significant changes in plasma values (Table I). When the patients who showed no changes (*i.e.*, with a change less than 10%) were excluded, statistical analysis showed no additional significant changes.

Changes in VEGF and VEGF-D levels (baseline values above the normal range). Prior to chemotherapy, 121 patients had VEGF baseline values above the normal range (Table II). Of these patients, 46 showed an increase and 65 a decrease in VEGF values after chemotherapy, with 10 patients showing no change (less than 10% change). The VEGF-D values prior to chemotherapy were above the upper limit of the normal range in 94 patients. There was a statistically significant decrease in this group as a whole (elevated values $n=23$, decreased values $n=60$, $p<0.001$) and, compared with the entire population, there

Table II. Patients with VEGF and VEGF-D levels above the normal range (VEGF > 115 and/or VEGF-D > 437 pg/ml) prior to chemotherapy in relation to patient and tumor characteristics..

	No.	VEGF*		p-value	No.	VEGF-D*		p-value
		Pre-/Post-chemo				Pre-/Post-chemo		
Patients	121	470/452		0.164	94	810/523		<0.001
Elevated values (>10%)	46	347/751		0.001	23	742/1067		<0.001
Decreased values (>10%)	65	605/322		0.001	60	880/346		<0.001
Unchanged values (within ±10%)	10	405/418			11	958/897		
Menopausal status								
Pre-menopausal	48	480/422		0.119	42	809/786		0.943
Post-menopausal	71	462/493		0.492	50	810/408		<0.001
Grading								
G1	10	392/220		0.169	8	853/293		0.036
G2	67	470/523		0.428	50	777/649		0.121
G3	33	447/411		0.979	29	951/492		0.004
Tumor size								
pT1	54	503/458		0.053	44	736/511		0.04
pT2	54	413/423		0.853	44	880/532		0.017
pT3	5	917/411		0.043	3	1491/378		0.109
ER/PR status								
Positive	95	462/428		0.231	76	880/523		<0.001
Negative	25	509/523		0.265	17	672/492		0.177
c-erbB-2 status								
Dako score negative	68	447/465		0.621	46	814/504		0.005
Dako score:+1	18	441/393		0.845	14	761/563		0.026
Dako score:+2	9	658/590		0.515	8	992/466		0.208
Dako score:+3	26	579/501		0.055	26	737/657		0.058
Treatment								
Conventional (arm A)	62	560/506		0.06	49	800/533		0.005
Dose-intensified (arm B)	58	401/427		0.960	61	810/506		0.004

*For all values the median is given in pg/ml.

Where the total number in a category was less than 121 for VEGF and 94 for VEGF-D, the corresponding information was either missing or obviously erroneous for certain patients and therefore not taken into consideration.

was an even clearer reduction of VEGF-D levels in post-menopausal women from 810 pg/ml to 408 pg/ml ($p<0.001$). With respect to tumor grading, there was also a statistically significant decrease in VEGF-D for G3 tumors ($p=0.004$). In the analysis of the various tumor sizes, an almost significant reduction in VEGF ($p=0.053$) was found for the tumor size pT1 (≤ 20 mm). The reduction in VEGF-D levels reached statistical significance for tumor sizes pT1 ($p=0.04$) and pT2 (≤ 50 mm, $p=0.017$) and there was also a reduction in these levels for tumors larger than 5 cm (pT3). This result should, however, be considered in the context of the small number of cases ($n=3$). The statistical significance of the reduction in VEGF-D values in those patients with positive hormone-receptor status was even clearer when compared with the same effect in the total population ($p<0.001$). For the c-erbB-2 status, there was a significant reduction in VEGF-D levels in patients with a negative c-erbB-2 status. The statistical

analysis of the two therapy modalities within these collectives above the normal range showed a statistically significant reduction in the VEGF-D values in both arms (arm A: $p=0.005$ / arm B: $p=0.004$).

Comparison between increasing and decreasing values of VEGF and VEGF-D. The analysis of the menopausal status indicated that, for patients with a baseline VEGF-D value above the normal range ($n=92$), significantly more post-menopausal patients showed a reduction in values ($n=39/78.0\%$) than did pre-menopausal women ($n=19/45.2\%$). On the other hand, a further increase in values was found in 16 (38.1%) pre-menopausal as opposed to 7 (14.0%) post-menopausal patients (Pearson χ^2 test, $p=0.004$). With respect to the grouped changes (increase/decrease in VEGF/VEGF-D by more than 10%), no further statistically significant changes were determined between the subcollectives defined by the conventional prognostic factors (Table III).

Table III. VEGF and VEGF-D (change >10% increase or decrease) in relation to patient and tumor characteristics.

	No.	VEGF*		p-value	No.	VEGF-D*		p-value
		Pre-/Post-chemo				Pre-/Post-chemo		
All patients	142	409/402		0.618	142	616/447		0.067
Changes >10%	130	422/402		0.655	122	626/440		0.076
Elevated values (>10%)	62	253/584		0.001	52	664/1166		0.0001
Decreased values (>10%)	68	585/317		0.001	70	1165/492		0.0001
Unchanged values (within $\pm 10\%$)	12	308/321			20	508/519		
°Menopausal status								
Pre-menopausal	54	404/335		0.320	48	633/647		0.943
Post-menopausal	74	422/443		0.968	72	610/371		0.054
°Grading								
G1	12	325/220		0.480	11	747/341		0.091
G2	68	451/465		0.802	63	600/593		0.913
G3	39	273/321		0.395	38	638/351		0.070
°Tumor size								
pT1	62	403/341		0.252	56	586/461		0.434
pT2	56	398/424		0.357	55	720/378		0.132
pT3	5	917/411		0.043	5	1365/378		0.225
°ER/PR status								
Positive	99	407/411		0.680	96	654/437		0.054
Negative	30	441/364		0.673	25	526/442		0.904
°c-erbB-2 status								
Dako score negative	74	384/420		0.548	70	586/376		0.508
Dako score: +1	19	362/276		0.968	17	558/341		0.049
Dako score: +2	11	507/500		0.859	10	637/511		0.878
Dako score: +3	26	560/420		0.058	25	699/542		0.115
°Treatment								
Conventional (arm A)	67	462/393		0.214	61	600/437		0.249
Dose-intensified (arm B)	63	362/411		0.485	61	683/451		0.144

*For all values the median is given in pg/ml.

°All values outside the range of 10% were taken into account.

Where the total number in a category was less than 130, the corresponding information was either missing or obviously erroneous for certain patients and therefore not taken into consideration.

Correlation between VEGF and VEGF-D. Changes in the pre- and post-therapy concentrations of VEGF and VEGF-D correlated significantly according to the Spearman test ($p < 0.001$): a reduction in VEGF correlated positively with a reduction in VEGF-D. Further analysis showed that patients who had a baseline VEGF value that was above normal did not simultaneously have a VEGF-D value above normal.

Evaluation of VEGF and VEGF-D with respect to patient and tumor characteristics. Analysis of the baseline values prior to the onset of chemotherapy showed significantly increased VEGF values with tumor size (VEGF_{pT3} > VEGF_{pT1/pT2}) (Kruskal Wallis test, $p = 0.039$). Comparable results were also obtained in the partial analyses with regard to VEGF and VEGF-D levels above the normal range prior to the start of chemotherapy ($p = 0.04$ and $p = 0.01$, respectively). Clearly increased VEGF-D baseline values were shown for hormone-

receptor-positive patients as compared to hormone-receptor-negative patients ($p = 0.026$) (Table I).

Discussion

Recently published data have shown that dose-intensified, adjuvant chemotherapy offers a survival advantage for patients with breast cancer and positive lymph nodes. (19, 20). Based on the Norton-Simon hypothesis, the idea of minimizing the growth of tumors during the treatment pause (non-exponential Gompertzian growth kinetics) by means of dose-intensification, in the form of shorter intervals between the cycles, was developed (21, 22). An additional increase in overall survival was achieved by the introduction of the taxanes into adjuvant therapy (23, 24). The present study focussed on the influence of these chemotherapy regimens within the framework of a randomized phase III study on the

angiogenesis and lymph-angiogenesis factors (VEGF, VEGF-D) circulating in the plasma.

In our patient population, all of whom had 1 to 3 positive lymph nodes (n=142), 85.2% (n=121) showed an increase in VEGF levels and 66.2% (n=94) showed an increase in VEGF-D levels after excision of the tumor. This post-excision increase in VEGF in patients with a positive lymph node status corroborates the results obtained by Wu *et al.* (15). Currently, there are no reported comparable plasma values for VEGF-D. An immunohistochemical examination of 36 invasive breast ductal carcinomas found the frequency of VEGF-D expression to be significantly higher in node-positive patients (25). The immunohistochemical analyses carried out by Nakamura *et al.* (17) showed that the risk of a relapse was increased by a factor of 4 in patients with a positive VEGF-D test result. Our results indicated that the chemotherapy in both arms had a stronger influence in reducing VEGF-D in plasma compared with VEGF; this reduction was statistically significant in patients who already had had an increased plasma level before treatment began. In the general analysis, the reduction in VEGF was clearest in the case of an overexpression of c-erbB-2 (Dako Score +3) or in the presence of a large tumor. On the other hand, VEGF-D was clearly reduced in post-menopausal, hormone-receptor-positive patients, as well as in those patients who overexpressed c-erbB-2 with a Dako score of +1. In the analysis of patients with increased starting levels of VEGF (Table II), the total variations (Table I) were confirmed. For VEGF-D (with baseline levels above the normal range) there was, in addition, a significant reduction for patients with G1 and G3 tumors. Furthermore, there was a significant decrease in VEGF-D values in patients with T1 and T2 tumors, as well as in c-erbB-2-negative patients.

Using immunochemistry on tumor material, Ludovini *et al.* (26) obtained VEGF expression in only 28% of their 228 patients. Fifty of their patients were lymph node-negative. No significant correlations between immunochemically-determined VEGF values and the relapse-free and overall survival rates were obtained. In a recently published prospective study into the efficacy of docetaxel plus epirubicin combination chemotherapy in patients with metastatic breast cancer, the VEGF levels were measured in the pretreatment serum by enzyme immunoassay. Elevated VEGF levels were not found to be correlated with the response rate or survival (27). Manders *et al.* (28) investigated the predictive value of cytosolic levels of VEGF in 1127 breast cancer patients and concluded that the tumor levels of VEGF do not predict the efficacy of adjuvant systemic therapy in primary invasive breast cancer. In contrast, Linderholm *et al.* (11) showed that increased cytosolic values of VEGF₁₆₅, quantified by immunoassay, correlated with a significantly reduced overall survival rate after adjuvant chemotherapy. Foekens *et al.* (13) obtained

similar results and showed that an increased tumor tissue level of VEGF was significantly correlated with a poorer response to therapy.

No correlation between the overexpression of c-erbB-2 and an increased level of VEGF, as shown immunohistochemically in the studies of Yang *et al.* (29) and by means of an immunoassay performed on the tumor cytosol reported by Linderholm *et al.* (30), was determined in the plasma of our patient population. It is true that increased VEGF and VEGF-D values corresponded to increasing c-erbB-2 expression (see Table I), but only for VEGF-D did this attain statistical significance ($p=0.015$).

In contrast with the results obtained by previous studies, (28, 31, 32), the values for VEGF were not increased in our patient population with negative hormone-receptor status. On the other hand, the VEGF-D baseline values in our patients with a negative hormone-receptor status were significantly lower in comparison to those with a positive hormone-receptor status ($p=0.026$). Coradini *et al.* (33) determined that the combination of a negative hormone-receptor status and increased intra-tumor VEGF values correlated significantly with an increased rate of relapse. In the study by Manders *et al.* (31), patients with a positive hormone-receptor status and low VEGF levels showed the highest response rate to endocrine therapy. Similarly, Rydén *et al.* (34) detected a beneficial effect of tamoxifen in terms of relapse-free survival among post-menopausal breast cancer patients with estrogen-receptor-positive tumors and negative VEGF expression. In contrast, Coradini *et al.* (33) reported that the VEGF level and a positive hormone-receptor status had no prognostic relevance.

In conclusion, this study presents the first data on the influence of adjuvant dose-intensified chemotherapy as compared to conventional chemotherapy on the circulating plasma levels of VEGF and VEGF-D, with respect to conventional prognostic factors. Either VEGF or VEGF-D was changed significantly with respect to the menopausal status, tumor size, the hormone-receptor status and the c-erbB-2 expression. According to Colleoni *et al.* and Lissoni *et al.* (35-37), patients experiencing a reduction in VEGF levels after chemotherapy had an improved response to therapy. Time will tell whether our patients, whose values decreased after chemotherapy by more than 10%, will benefit more from the therapy than those patients whose VEGF and VEGF-D plasma levels remained unchanged or increased on therapy.

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