Abstract. Background: A soluble fragment of the epidermal growth factor receptor (EGFR) extracellular domain (sEGFR) can be detected in the serum of cancer patients, but the role of sEGFR is still unclear. Materials and Methods: Blood samples from patients receiving chemotherapy for metastatic breast cancer were collected before (n=101) and after 3 courses of therapy (n=39). Levels of sEGFR and serum HER-2/neu extracellular domain (ECD) were determined by standardized ELISA. Results: A higher percentage of cancer patients (15%) showed sEGFR values below 45ng/mL compared with control subjects (3%, p<0.001). Patients with sEGFR levels below 45 ng/mL showed a trend towards shorter overall survival (median 11.7 versus 15.4 months, p=0.08), which was more pronounced in patients with estrogen receptor-positive primary tumors (median 9.6 versus 15.4 months, p=0.022). Patients with low sEGFR and elevated serum HER-2/neu ECD (>15 ng/mL) also showed a shorter overall survival than those with normal values for both parameters (7.1 versus 15.4 months, p=0.03). Again, this difference was higher in patients with estrogen receptor-positive tumors (4.6 versus 15.4 month, p<0.0001). During treatment, a decrease of sEGFR levels occurred in 74.4% of the patients (p=0.014). Conclusion: Low sEGFR levels in patients with metastatic breast cancer are associated with a shorter overall survival, particularly in patients with estrogen receptor-positive tumors. Chemotherapy frequently induces a decrease of sEGFR. The combined determination of sEGFR and serum HER-2/neu ECD also delivers relevant information. These findings suggest that the sEGFR status in metastatic breast cancer could be of clinical relevance.

The epidermal growth factor receptor (EGFR) oncogene encodes a transmembrane tyrosine kinase receptor. The EGFR is a prototypic member in a family of 4 receptors that shows extensive homology with the HER-2/neu oncogene product. Today, EGFR, like HER-2/neu, represents one of the promising molecular targets for cancer therapy (1-4), but the role of EGFR as a predictive and/or prognostic marker is still controversial. Variable rates of EGFR expression or overexpression were reported in primary breast cancers (5-8). However, the published studies used different methods to measure EGFR levels. Some studies in patients with primary breast cancer suggested that EGFR expression is a marker of a more aggressive type of breast cancer (5-7). The overexpression of EGFR and its homologous receptor HER-2/neu was also described to be associated with resistance to endocrine therapies (9-13). For HER-2/neu, it was demonstrated that patients showing gene amplification of the primary tumor
or increased HER-2/neu serum levels at the time of metastatic disease showed an increased response to taxane-based chemotherapy in the metastatic setting (14, 15). For EGFR, no such results have been published to date. In addition, little is known about the expression of EGFR in metastatic lesions of breast cancer patients and the possible differences in expression pattern between primary tumors and metastatic sites.

A soluble fragment of the EGFR protein (sEGFR) can be detected in the serum or plasma of patients with breast cancer and other solid tumors since a 110 kDa portion is shed from the full-length 170 kDa protein (16). It was described that patients with solid tumors showed decreased levels of sEGFR protein compared with healthy control subjects (16, 17), while other authors found increased levels (18) or no difference between healthy subjects and patients (3, 19, 20). Thus, the biological background of this observation, the range of normal values and the possible clinical implications of sEGFR measurements still remain unclear. Nevertheless, sEGFR determinations are of interest since serum levels might reflect the biology of the tumor in the metastatic setting when therapy is administered. In addition, repeated examinations for sEGFR during the course of treatment are possible (21) and might help to monitor treatment response, as indicated by one publication examining patients with lung cancer (22).

It was demonstrated that determinations of serum HER-2/neu extracellular domain (ECD) delivered clinically relevant information (13, 23-27). The EGFR can interact with other members of the HER growth factor family like HER-2/neu (28) and, therefore, the combined determination of both serum sEGFR and HER-2/neu ECD might deliver additional biological information, as suggested by data from a previous report that used the combined determination of EGFR and HER-2/neu status by immunohistochemistry from primary tumors (29).

In breast cancer patients, the CA 15.3 glycoprotein is used for the monitoring of therapy response and as a parameter for tumor burden (30). However, it remains unclear whether altered serum levels of sEGFR are correlated with increased levels of serum or plasma tumor markers like CA 15.3.

EGFR seems to play a role in the biology of progression and is a therapeutic target for therapy in breast cancer and other solid tumors. Interactions with HER-2/neu and other members of the HER-family of tyrosine kinase receptors occur. The optimal method for EGFR assessment is not yet clear. Limited information is available both about the biology and the potential of sEGFR as a prognostic and predictive marker and about the changes of sEGFR during the course of therapy. Therefore, the prognostic impact and predictive role of sEGFR for response to chemotherapy in our defined group of breast cancer patients was examined. In addition, sEGFR levels were examined during the course of treatment to evaluate the potential of this marker to monitor therapy response. The possible correlation between sEGFR and HER-2/neu ECD serum levels was also investigated. Our findings indicated a biological relevance and a potential clinical application of sEGFR in metastatic breast cancer.

Materials and Methods

Patients. Pretreatment sera were obtained from 101 patients who participated in a randomized clinical multicenter trial on first-line chemotherapy for metastatic breast cancer (31). These patients were randomly assigned to receive either epirubicin and paclitaxel, 60/175 mg/m² (ET) or epirubicin and cyclophosphamide, 60/600 mg/m² (EC) every 21 days (maximum of 10 courses). The response was evaluated every 3 courses and classified according to the UICC criteria. The median age of the patients was 56 years (range 33 – 73 years). Eighty percent had visceral disease. With regard to the primary tumor characteristics, 45% of the tumors were undifferentiated (grade 3), 35% of the patients were estrogen receptor-negative and 38% of the patients had adjuvant chemotherapy, 4.9% with anthracyclines. The median follow-up of the study population was 8.9 months (0.5 – 36 months).

Serum was collected before therapy (n=101) and after 3 courses of therapy in 39 patients. Healthy female blood donors (n=30) were compared with the whole patient population and also with 30 age-matched patients to verify the normal values of sEGFR.

Quantitative analysis of sEGFR levels. Serum aliquots were assayed for the extracellular binding domain level of EGFR by a sandwich quantitative ELISA using a mouse monoclonal capture antibody against the extracellular portion of the EGFR precoated onto a microtiter plate and an alkaline phosphatase-labelled mouse monoclonal antibody to detect specific human EGFR, according to the manufacturer’s instructions (Oncogene Science, Bayer Corporation, Cambridge, UK) (17). All of the samples and standards were assayed in duplicate. One hundred microliters of the standards and samples diluted at 1:50 with sample diluent, respectively, were added to the microtiter wells and incubated for 1.5 hours at 37°C. After washing, 100 μL of alkaline phosphatase-labelled mouse monoclonal detector EGFR antibody was added for 30 min at room temperature. After several washes to remove any unbound antibody-enzyme reagent, a substrate solution (containing substrate) was added for 1 h at room temperature. One hundred microliters of stop solution were added to each well. Colorimetric quantification was performed using a multireader spectrophotometer at 650 nm. A standard curve was created by plotting the mean absorbance of each standard versus the concentrations of sEGFR. The results were expressed in nanograms per milliliter. For each analysis, control samples of known concentration with low, mid and high levels were used as internal quality control; the intra- and inter-assay coefficients of variation were below 6% for sEGFR. Laboratory and clinical data were collected independently.

Quantitative analysis of serum HER-2/neu ECD levels. Serum HER-2/neu ECD was quantified by a commercially available ELISA (Oncogene Science, part of Bayer Diagnostics, Cambridge, USA). In brief, serum samples were diluted 1:50 with sample diluting
buffer containing bovine serum albumin and sodium azide. The diluted samples were dispensed into 96-well plates coated with the monoclonal capture antibody (NB3) and incubated for 3 h at 37°C. The wells were washed and the detection antibody (TA1) was added. The plates were incubated for 60 min at 37°C, washed and then further incubated with anti-rabbit immunoglobulin/horseradish peroxidase conjugate for 30 min at room temperature. After washing, the substrate (o-phenylenediamine) was added for 45 min at room temperature. The reaction was stopped with 2.5 N H2SO4 and absorbance was read at 490 nm by an automated plate-reader (TiterScan, Labsystems, Finland). The HER-2/neu ECD concentration was estimated from the standard curve. Each sample, standard and control was analyzed in duplicate. Inter-assay and intra-assay coefficients of variation were less than 10%.

**Determination of the tumor marker CA15.3.** The concentration of the tumor marker CA15.3 was determined in sera from patient samples using a standard ELISA (IMx CA15.3 second generation by Abbott Laboratories, Chicago, IL, USA). Values above 30 kU/L were considered to be increased.

**Statistical methods.** Progression-free interval and overall survival were estimated with the method of Kaplan-Meier. Significances of differences in progression-free interval and overall survival in relation to serum sEGFR and/or serum HER-2/neu ECD levels were calculated using the Log-rank test. Group differences were examined with the Kruskall-Wallis test (>2 groups) or Mann-Whitney U-test (2 independent groups) as well as Wilcoxon test (2 paired groups). The correlation between immunohistochemistry and sEGFR concentrations was calculated using the Spearman-correlation coefficient. Cross-tables were analyzed with the χ² test, or the Fisher’s exact test if low numbers were examined. Unless otherwise stated, an error of less than 5% probability (p≤0.05) was considered as statistically significant. All p-values correspond to two-sided tests. For statistical analyses, SPSS software (version 12.0) was used. The reference interval was calculated with Medcalc software (version 8.0).

**Results**

**sEGFR levels in breast cancer patients and in healthy individuals; correlation of sEGFR levels with patient and tumor characteristics.** For verification of the normal range, sEGFR levels were determined in 30 healthy female blood donors. The sEGFR concentrations in this control group showed a median of 60.31 ng/mL and were above 45 ng/mL in all except one subject (range 41.63 to 76.46 ng/mL). Compared with healthy controls (n=1, 3%) a higher percentage of breast cancer patients (n=15, 15%) showed decreased concentrations of sEGFR (<45 ng/mL) (p<0.001; Mann-Whitney) (Figure 1). However, the overall sEGFR levels of patients (32 to 112.72 ng/mL, median 51.78 ng/mL) and healthy controls were in the same range. The comparison of the control cohort with a sample of 30 age-matched patients showed distributions in a similar range and a difference was detected (p<0.005, data not shown). Estimation for the upper 95% percentile of the control cohort resulted in a right-sided reference value of 48 ng/mL (95%CI: 44.3 – 51.8).

**Correlation of sEGFR levels with concentrations of serum HER-2/neu ECD and the tumor marker CA 15.3.** Interactions between EGFR and HER-2/neu occur and therapeutic strategies targeting both receptors are in clinical development. Therefore, the correlation between the soluble forms of these growth factor receptors was examined in serum. No correlation was found between the sEGFR levels before the initiation of therapy and number of metastatic sites, location of the metastatic sites or tumor grade or stage of the primary tumor. Also, no difference was observed between the sEGFR levels in patients on comparing different age groups (above and below 55 years). The only significant correlation was the tendency towards lower sEGFR levels in those patients with estrogen receptor-positive primary tumors (p<0.05; Mann-Whitney, data not shown).

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**Correlation of sEGFR levels with concentrations of serum HER-2/neu ECD and the tumor marker CA 15.3.** Interactions between EGFR and HER-2/neu occur and therapeutic strategies targeting both receptors are in clinical development. Therefore, the correlation between the soluble forms of these growth factor receptors was examined in serum. No correlation was observed between serum levels of HER-2/neu ECD (above 15 ng/mL) and those of sEGFR (r=0.134, p=0.18; Rank Spearman). Patients with elevated HER-2/neu ECD showed a median sEGFR concentration of 53 ng/mL (range 38.5 – 112.7), while those with normal values showed a median concentration of 51.6 ng/mL (range 32 – 88.3).

In addition, no correlation was observed between serum levels of the tumor marker CA 15.3 and sEGFR levels. Patients with elevated CA 15.3 levels (above 30 kU/L) showed a median sEGFR concentration of 50.6 ng/mL (range 38.5 – 67.0), while those with normal values showed a median concentration of 49.9 ng/mL (range 40.3 – 64.4).
Correlation of sEGFR levels with survival. Low pretherapeutic sEGFR levels in patients with metastatic breast cancer were correlated with a shorter overall survival time. The median survival in the patient group with low sEGFR levels (n=15) was different from those with non-elevated levels (n=86, p=0.08; log-rank). When the prognostic impact of the sEGFR values was evaluated with other cut-off levels between 42 and 51 ng/mL, no relevant different impact concerning survival was observed (data not shown). In the group of patients with estrogen receptor-positive primary tumors, the negative impact of low sEGFR levels was statistically significant. Patients with low sEGFR levels had a median survival of 9.6 months (95% CI 4.6 - 14.7) compared with 15.4 months (95% CI 15.4 - 16.3 months) in those with non-elevated serum levels (p=0.022).
For patients with estrogen receptor-negative primary tumors and sEGFR levels above 45 ng/mL, the median survival was 16.8 months (95% CI 10.3 – 22.3). Due to the low number (n=3, with 1 censored case) of patients with low sEGFR values and negative estrogen receptor, no survival with a meaningful CI could be estimated (median 11.3 months).

With the combined analysis of sEGFR and serum HER-2/neu ECD in the entire patient cohort, those patients with low sEGFR and elevated serum HER-2/neu ECD (>15 ng/mL) had a significantly lower median survival (7.1 months, 95% CI 1.7 – 12.5) than patients showing normal EGFR and HER-2/neu ECD levels (15.4 months, 95% CI 11.4 – 19.4, p=0.03; log-rank) (Figure 2c). In patients with estrogen receptor-positive primary tumors, this difference was highly significant with a median survival of 4.6 months (95% CI 1.2 – 8) with low sEGFR and elevated serum HER-2/neu ECD, compared to 15.4 months (95% CI 12.9 – 17.9) with normal sEGFR and serum HER-2/neu ECD levels (p<0.0001; log-rank) (Figure 2d).

Patients with negative estrogen receptor status and normal sEGFR and HER-2/neu ECD levels showed a median survival of 12.9 months (95% CI 11.4 – 14.3). None of the patients with negative estrogen receptor status had low sEGFR and elevated serum HER-2/neu ECD concentrations.

Predictive value of sEGFR levels for response to therapy and progression-free interval. No significant correlation between the response to chemotherapy and sEGFR levels was observed. The median sEGFR level was 51.9 ng/mL in patients showing a complete or partial response, compared to 51.5 ng/mL or 54.4 ng/mL in those with stable or progressive disease, respectively.

For the entire patient group, no influence of pretherapeutic sEGFR levels on progression-free interval was observed (Figure 3a). However, a longer progression-free interval of 6.7 months (95% CI 2.5 – 9.2) was found if patients with decreased sEGFR levels were treated with ET compared to 3.4 months (95% CI 2.1 – 9.2) with EC. This difference reached borderline significance (p=0.07) and no such difference between the treatment arms was observed in patients with sEGFR levels above 45 ng/mL (Figure 3b).

Course of sEGFR levels during therapy. In 39 patients, we were able to study sEGFR levels during the course of treatment and in 36 response to treatment was evaluable. Regarding all patients, the sEGFR values decreased in 74.4% of the patients and were significantly lower after 3 cycles of therapy than before (p=0.014, Wilcoxon). No difference in the percentage of patients with decrease of sEGFR levels was observed between patients younger or older than 50 or patients younger or older than 55 years, respectively (data not shown).
Fifteen out of 16 patients (94%) with complete (CR) or partial remission (PR) showed a decrease of sEGFR levels after 3 courses of therapy, while only 1 patient (6%) showed an increase. In contrast, only 7 out of 20 patients (35%) with stable disease or progression had a decrease. With the number of patients examined, this difference was not significant ($p=0.1$, Fisher) (Table I, Figure 4).

A correlation between the change of sEGFR and serum HER-2/neu ECD levels after 3 cycles of therapy was observed. Only 2 out of 24 patients with decreasing serum HER-2/neu ECD showed increasing sEGFR, while 8 out of 15 patients with increasing serum HER-2/neu ECD also showed increasing sEGFR levels ($p<0.005$; Fisher) (Table II).

**Discussion**

sEGFR serum levels were studied in patients with metastatic breast cancer in the context of systemic chemotherapy. The patients were treated in a randomized clinical trial that compared 2 different chemotherapy regimens for the first-line treatment of metastatic breast cancer. The inclusion criteria of that trial led to a selection of high-risk patients with a high proportion of visceral metastasis. We observed a prognostic and predictive impact of serum HER-2/neu ECD in these patients in a previous study (14) and here we examined if sEGFR determinations in serum could also deliver relevant information. Also, we wanted to determine if the combined use of sEGFR and HER-2/neu ECD could be of clinical relevance.

Currently, the potentially most important indication for EGFR testing is the prediction of response to treatment with monoclonal antibodies or tyrosine kinase inhibitors directed against EGFR. However, in contrast to the treatment directed against HER-2/neu, no correlation between receptor expression in primary tumors and response to therapies has been observed to date, suggesting that evaluation of the primary tumor for expression of sEGFR protein is not suitable for the prediction of therapy response in the metastatic setting. Therefore, new strategies are required for this purpose.

The soluble fragment of the sEGFR can be detected in serum or plasma of patients since a 110 kDa portion is shed from the full-length 170 kDa protein (16). Little is known about the range of normal values and the possible clinical
patients from our study with decreased sEGFR levels as a prognostic factor in the context of metastatic breast cancer. Serum which might affect the ELISA results.

different splice variants of sEGFR may be released to the proliferative activity. Baron et al. (32) suggested that different splice variants of sEGFR may be released to the serum which might affect the ELISA results.

Little is known about the potential of EGFR as a prognostic factor in the context of metastatic breast cancer. Patients from our study with decreased sEGFR levels before initiation of therapy for metastatic breast cancer showed a strong trend \( p=0.08 \) towards a worse prognosis with regard to overall survival (Figure 2a). With regard to the subgroup of patients with estrogen receptor-positive primary tumors, the prognostic impact of low sEGFR serum values was significant (Figure 2b). This supports a link between EGFR and the estrogen receptor, as suggested by experimental and clinical data, and could also be of importance in the context of strategies that are aimed at overcoming endocrine resistance (33-35).

Strategies directed against both EGFR and HER-2/neu are in clinical development. Therefore, we were interested in whether the combined analysis of sEGFR and serum HER-2/neu ECD would deliver clinically-relevant information. For the entire patient cohort, it was found that patients with low sEGFR and elevated serum HER-2/neu ECD levels (>15 ng/mL) had a significantly lower median survival than patients with normal sEGFR and HER-2/neu ECD levels (Figure 2c). Again, in patients with estrogen receptor-positive primary tumors, this difference was more pronounced (Figure 2d). Although the number of patients was limited, combined analysis seemed to be superior over the use of sEGFR determination alone. In a study presented at the Annual Meeting of the American Society for Clinical Oncology in 2005, Leitzel and coworkers examined patients treated with endocrine therapy for metastatic breast cancer by a combined analysis of sEGFR and serum HER-2/neu ECD (36). Using the same assay system as our study, they found a cut-off for sEGFR of 44.1 ng/mL and a similar percentage of patients with decreased sEGFR levels (11%). They also stated that the combined analysis of sEGFR and HER-2/neu ECD would deliver clinically-relevant information.

Although we found an impact on overall survival, there was no influence of pretherapeutic sEGFR levels on progression-free interval in the entire patient group (Figure 3a), and only a slight difference of progression-free interval between paclitaxel-treated patients and EC-treated patients (Figure 3b). Therefore, it cannot be concluded from our data that decreased sEGFR levels reflect therapy resistance.

In our cohort, the sEGFR values were significantly lower after 3 courses of therapy than before. To our knowledge, this is the first report that has demonstrated a decrease of sEGFR levels in patients who underwent chemotherapy for metastatic breast cancer. With a different assay system, Perez and coworkers did not observe a change of sEGFR during the course of treatment in breast cancer patients (37). However, no direct comparison between the 2 assay systems is available and it can be speculated that the different epitope specificity of the antibodies used contributes to these conflicting results.

In our study, most patients with complete or partial remission showed a decrease of sEGFR levels after 3 courses of therapy, while in patients with stable disease or progression we found both increasing and decreasing sEGFR levels (Table I, Figure

<table>
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<th>Change of serum HER-2/neu ECD</th>
<th>No. of patients with decrease of sEGFR</th>
<th>No. of patients with increase of sEGFR</th>
<th>Total No. of patients</th>
</tr>
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<tbody>
<tr>
<td>Decrease</td>
<td>22</td>
<td>2</td>
<td>24</td>
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<tr>
<td>Increase</td>
<td>7</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>10</td>
<td>39</td>
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The relationship between changes in HER-2/neu ECD and sEGFR is significant \( p<0.005 \; \text{Fisher’s test}. \)

The implications of sEGFR measurements. We detected serum levels below 45 ng/mL in only 1 of 30 healthy female control subjects. Whereas our study was not designed to establish a normal range for the assay system used, this portion (3%) as well as the estimation of the 95%-percentile of 48.0 ng/mL (95% CI 44.3 – 51.8) are in agreement with the usage of 45 ng/mL as the 95% right-sided reference interval, as suggested by other authors as well in the assay system (17). Using a different assay system, one report suggested that sEGFR levels seemed to be associated with menopausal status (32). However, we did not observe any difference between the pretherapeutic sEGFR levels of different age groups or a correlation with age. Searching for the best cut-off level with regard to the prognostic impact, we found that the sEGFR serum concentration of 44 ng/mL was associated with the highest likelihood of discrimination (data not shown). Although the study was not designed to determine the cut-off in this way, these findings suggest that the recommended cut-off has a biological background.

No correlation of sEGFR levels with the tumor marker CA 15.3 as indicator of the tumor load was observed. It is possible that tissues other than the tumor tissue itself may produce sEGFR, so that the sEGFR levels are rather the result of a modified regulation, e.g., by paracrine or endocrine activity of the tumor cells, than merely surrogate of the tumor load. So far there is no conclusive explanation as to why sEGFR levels tend to be lower in cancer patients than in healthy individuals. Among different hypotheses, it is discussed that cancer cells with increased malignant potential show a decreased proteolytic cleavage of the EGFR extracellular domain, e.g., for maintenance of their proliferative activity. Baron et al. (32) suggested that different splice variants of sEGFR may be released to the serum which might affect the ELISA results.
4). Probably due to the number of patients in each group, this difference did not reach statistical significance. Our finding is in line with a study of Gregorc and coworkers, who found a correlation of a higher response rate associated with decrease in sEGFR levels during treatment in patients with lung cancer (22). Laflky and coworkers reported a significant decrease of sEGFR concentrations in patients with metastatic breast cancer during treatment with the aromatase inhibitor letrozole using a different assay system (21). They explained the drop of serum sEGFR by a decrease of serum estradiol, since they had previously shown that sEGFR values were lower in postmenopausal women than in premenopausal women (32).

We did not observe differences in sEGFR levels between women of premenopausal and postmenopausal age before therapy. Also, the proportion of patients with decreasing sEGFR levels during treatment in our patient cohort was similar for the 2 age groups. Our findings indicate that mechanisms other than the estradiol-decreasing effect of therapy caused the decrease of sEGFR. Finally, we observed a higher rate of decreasing sEGFR serum levels in patients with decreasing serum HER-2/neu ECD after 3 courses of therapy (Table II). This also supports that the change in sEGFR levels has a tumor-related biological background. However, the fact that decreasing sEGFR serum levels are associated with increased response to therapy while patients with low levels have an impaired prognosis can currently not be explained by a biological model.

In conclusion, we studied the role of sEGFR serum levels in patients with metastatic breast cancer in the context of systemic therapy and found a prognostic impact of pretherapeutic sEGFR serum levels. Patients with low sEGFR levels, particularly those with estrogen receptor-positive tumors, had a worse prognosis than those with high sEGFR levels. A decrease of sEGFR levels during treatment seemed to be associated with response to therapy and decrease in serum HER-2/neu ECD levels. These markers should be further examined in the context of new therapies directed against members of the EGFR family.

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