Determination of Interleukin-4, -5, -6, -8 and -13 in Serum of Patients with Breast Cancer Before Treatment and its Correlation to Circulating Tumor Cells

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Abstract. *Background/Aim: Circulating tumor cells (CTCs) in women with breast cancer are an indication of prognosis before starting systemic treatment. The aim of this study was the evaluation of cytokine profiles as marker for CTC involvement. Materials and Methods: The analysis of CTCs, the time of blood sampling and the methodology were prospectively designed. There were two groups of patients: 100 women with a positive result for presence of CTCs and 100 women negative for CTCs. These groups were matched into pairs by tumor factors and survival/death. A multi-array ELISA was used to screen T-helper cell (Th) 2 cytokines. The results were analyzed by Spearman correlation coefficient and Mann–Whitney U-test. Results: In patients who were CTC-negative, expression of interleukin-8 (IL-8) and IL-13 was increased (p=0.017 and p=0.045, respectively) if they were negative for progesterone receptor. In patients who died from their tumor, correlation between hormone receptor negativity and an increase in IL-4 was found. IL-5 was increased in patients with lymph node-positive and human epidermal growth factor receptor 2 (HER2)-positive disease (p=0.042). Moreover IL-4 was increased in patients with progesterone receptor-positive and estrogen receptor-negative status (p=0.024). Furthermore, the level of IL-6 was increased in patients with tumor grade G3 without progesterone receptor expression. Conclusion: Th2 cytokines are significantly modified in patients who are CTC-negative and progesterone receptor-positive. We suppose that an increase of IL-4 depends on hormone receptor status. In literature, a correlation between IL-4 and resistance to apoptosis is described. We suspect that IL-4 is responsible for the poor outcome of these cases.*

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The detection of circulating tumor cells (CTCs) is an independent prognostic factor for progression-free and overall survival for patients with metastatic and newly diagnosed breast cancer (1). The presence of CTCs is associated with poor disease-free survival (DFS), distant DFS, breast cancer-specific survival, and overall survival (2). The repeated detection of CTCs can help to evaluate the success of treatment in patients with breast cancer (3). The role of CTCs in patients with breast cancer is being analyzed in the SUCCESS study. This is a trial to differentiate the prognostic significance associated with reduced survival of patients with CTCs in early breast cancer before starting systemic adjuvant treatment and after adjuvant treatment in a large patient cohort (2). The intention of our study was the evaluation of cytokine profiles as an indicator for CTC involvement in patients with breast cancer. The involvement of the lymphatic system, especially lymphocytes, can play a major role in the progression of breast cancer (4). T-Lymphocytes and their cytokines interact with tumor cells and can influence on the prognosis (5). Through their tumor-promoting or tumor-suppressive properties, cytokines can influence the progression of cancer. Some cytokines of the Th2 subgroup [interleukin (IL-)6, and IL-10] found at higher levels in patients with cancer are associated with worse prognosis in terms of overall and DFS (6). Therefore we wanted to analyze such cytokines, especially to determine their levels in patients with breast cancer.

We used the serum of patients with breast cancer who took part in the SUCCESS study. Data on CTC involvement,

histopathological grading, lymph-node status, hormone-receptor type, TNM classification and survival of patients with breast cancer *vs*. those who died of their disease were collected.

The aim of this study was the investigation of a panel of Th2 cytokines in a group of patients with breast cancer diagnosed with CTCs (n=100) and in a control group negative for CTCs (n=100).

Materials and Methods

Study design. All patients who participated were women with breast cancer (stages pT1-T4, pN0 – N3, M0) who took part in the phase I SUCCESS study. In the SUCCESS study, which was a prospective, randomized adjuvant study in which the following therapy regimes were compared: three cycles of fluorouracil– epirubicin–cyclophosphamide (FEC; 500/100/500 mg/m^2) followed by three cycles of docetaxel (100 mg/m^2) every 3 weeks *vs*. three cycles of FEC followed by three cycles of gemcitabine (1,000 mg/m^2 days 1 and 8) with docetaxel (75 mg/m^2) every 3 weeks. After completing chemotherapy, patients were randomized to receive either 2 years or 5 years of therapy with the *bis*phosphonate zoledronate. Women with hormone receptor-positive tumors received endocrine therapy. The questions of research corresponding on CTC analysis, blood sampling time points and the methodology, were prospectively created. As a scientific objective of the study protocol, the prognostic value of CTCs was characterized. The SUCCESS study was managed in agreement with the Declaration of Helsinki and it was permitted by 37 German ethical boards (the leading ethical board was the LMU, Munich). Blood sampling for CTC enumeration was carried out for 2,090 patients after the primary tumor was completely removed by surgery and before starting adjuvant chemotherapy. In 64 cases, study participation was not possible because of test failures or the time interval between blood collection and sample preparation was too long (>96 h). There was a follow-up analysis for a subgroup of 1,492 patients after chemotherapy and before the start of endocrine or bisphosphonate therapy (see homepage: http://www.success-studie.de).

Patients. A total of 200 patients of the SUCCESS study were included in our study and they were divided into two groups: One collective with 100 patients who were CTC-positive and the second group with 100 patients who were CTC-negative. These groups were matched into pairs by histopathological grading, lymph-node status, hormone-receptor type, TNM classification and survival versus tumor-related death. Of all these 200 patients, 160 were still alive at the last monitoring after the end of therapy and 40 patients had died during therapy due to their tumor. The collective included 98 patients with a tumor of grade G2 and 102 patients with a tumor of grade G3. Patients with a tumor of grade G1 were not included. The stage of the tumor was classified according to the TNM classification (WHO System) (7). Patients were matched in agreement with the criteria. Histopathological grading of the tumors was categorized according to the Bloom and Richardsen system (8).

Collection of blood samples and detection of CTCs. The method was carried out as described by the SUCCESS Study group (2) using the CellSearch System (Janssen Diagnostics, South Raritan, NJ, USA). After peripheral blood was drawn into three CellSave tubes (30 ml), the examination was arranged at the central laboratory at the University of Munich. It was necessary to examine the blood samples within 96 hours of collection. In brief, identification and enumeration of CTCs were achieved using a CellTracks Analyzer II (Janssen Diagnostics). CTCs were defined as nucleated cells lacking CD45 and expressing cytokeratin. In the case of positive samples, two independent investigators made the evaluation. All samples with a minimum of one CTC per 30 ml of blood were indexed as being CTC-positive. Blood samples from 84 individuals without clinical evidence of malignant disease were processed blind and used as a negative control. In four cases of these negative controls (4.9%), there were cells that fit the definition of epithelial cells and which could be interpreted as CTCs (one control with one epithelial cell, two controls with two, and one control with three epithelial cells).

Measurement of cytokines. For screening the blood serum samples for the Th2 cytokines IL-4, IL-5, IL-6, IL-8 and IL-13, a recently developed multicytokine/chemokine ELISA array (Meso Scale Discovery®, Rockville, MD, USA) was used to screen for the Th2 cytokines: IL-4, IL-5, IL-6, IL-8 and IL-13. In our case, anti-species MULTI-ARRAY 96-well plates for the development of a sandwich immunoassay were used. The 10 spot MULTI-SPOT plates were useful to immobilize a primary capture antibody against the specific protein of interest specific for each cytokine (IL-4, IL-5, IL-6, IL-8 and IL-13). We added standards and samples to the appropriate wells. Firstly we added the blood serum, calibrator and control then incubated plates at room temperature with shaking for 2 hours. We then removed the excess sample from each well with wash buffer, and added antibody conjugated with electrochemiluminescent labels over the course of two incubation periods. After a second incubation with shaking (time was different for each test), wash buffer was used to remove the entire unbound enzymes and MSD Read Buffer was added to produce the appropriate chemical environment for electrochemiluminescence. The plate was then loaded into an MSD instrument (MESO QuickPlex SQ 120) to measure the intensity of the emitted light as a quantitative measure of the amount of the protein of interest that was present in the sample (9, 10) (see www.mesoscale.com) by comparison with a standard curve for each assay.

Statistical analysis. For statistical analysis, SPSS 22.0 (SPSS Inc., IBM, Chicago, IL- USA) was used. The relationship between each cytokine of the Th2 group (IL-4, IL-5, IL-6, IL-8 and IL-13) and each factor studied was determined using the non-parametric Spearman correlation coefficient (*p*-values reported). All the statistically significant results by Spearman correlation coefficient were furthermore confirmed with the non-parametric Mann–Whitney *U*-rank sum test. Variables were examined by using box-plot analysis. All statistical tests were considered significant at $p<0.05$.

Results

CTC-positive vs. CTC-negative status. CTC-negative patients who were progesterone receptor-negative had a higher level of IL-8 in contrast to patients positive for progesterone receptor (Spearman correlation *p*-value=0.017, Mann–Whitney *U*-test $p=0.017$; Figure 1a).

The same effect was shown for higher levels of IL-13 in patients who were CTC-negative: In the group without

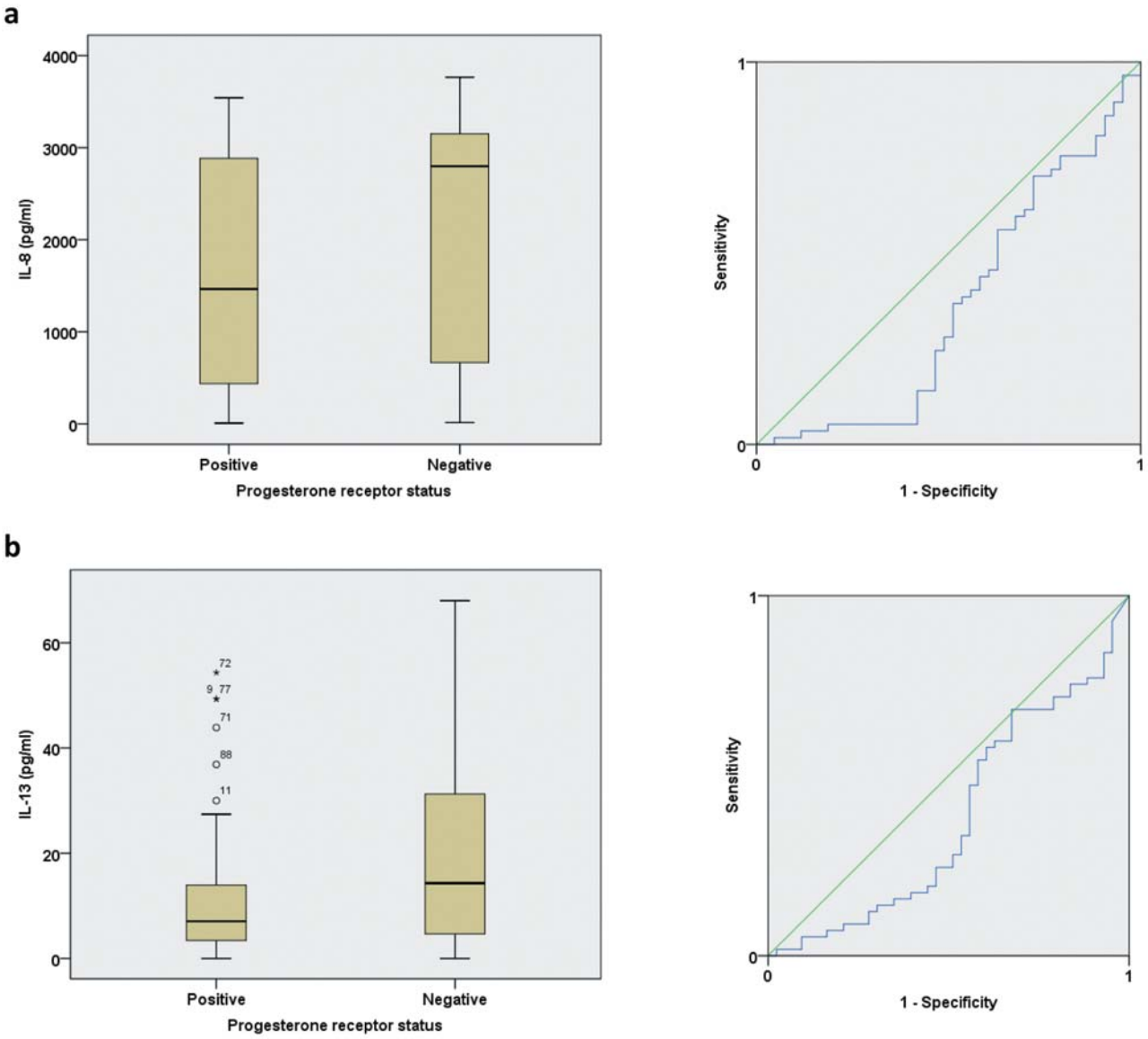

Figure 1. *Box-plot analysis of interleukin (IL)-8 (a) and IL-13 (b) expression in serum of patients with breast cancer negative for circulating tumor cells (CTCs) according to progesterone receptor status. The range between the 25th and 75th percentiles is represented by boxes with a horizontal line at the median. The bars show the 5th and 95th percentiles. Circles indicate values more than 1.5 box lengths. Asterisks indicate values (marked with a number) more than 3.0 box lengths from the 75th percentile. We identified significantly enhanced expression of IL-8 in patients with no CTCs and progesterone receptor-negative breast cancer compared to those who had progesterone receptor-positive disease (p=0.017) (a, left panel). Receiver operator curve analysis of sensitivity versus specificity of the ELISAs used was performed and gave area under the curve values of 0.361 (a, right panel) and 0.383 (b, right panel), respectively.*

expression of progesterone receptor, IL-13 was increased (Spearman correlation p=0045, Mann–Whitney U-test p=0.045; Figure 1b).

Regarding the patient group with breast cancer with the presence of CTCs, there was no significant difference in the statistical analysis relating to levels of Th2 cytokines (IL-4, IL-5, IL-6, IL-8 and IL-13).

Survival vs. death. In the group of patients who died, the box-plot analysis revealed that patients with progesterone receptor-negative status had a higher level of IL-4 than patients with progesterone receptor-positive status (Spearman correlation p=0.015, Mann–Whitney U-test p=0.017; Figure 2a).

The same pattern was found in patients negative for estrogen receptor expression: box-plot analysis indicated a higher level

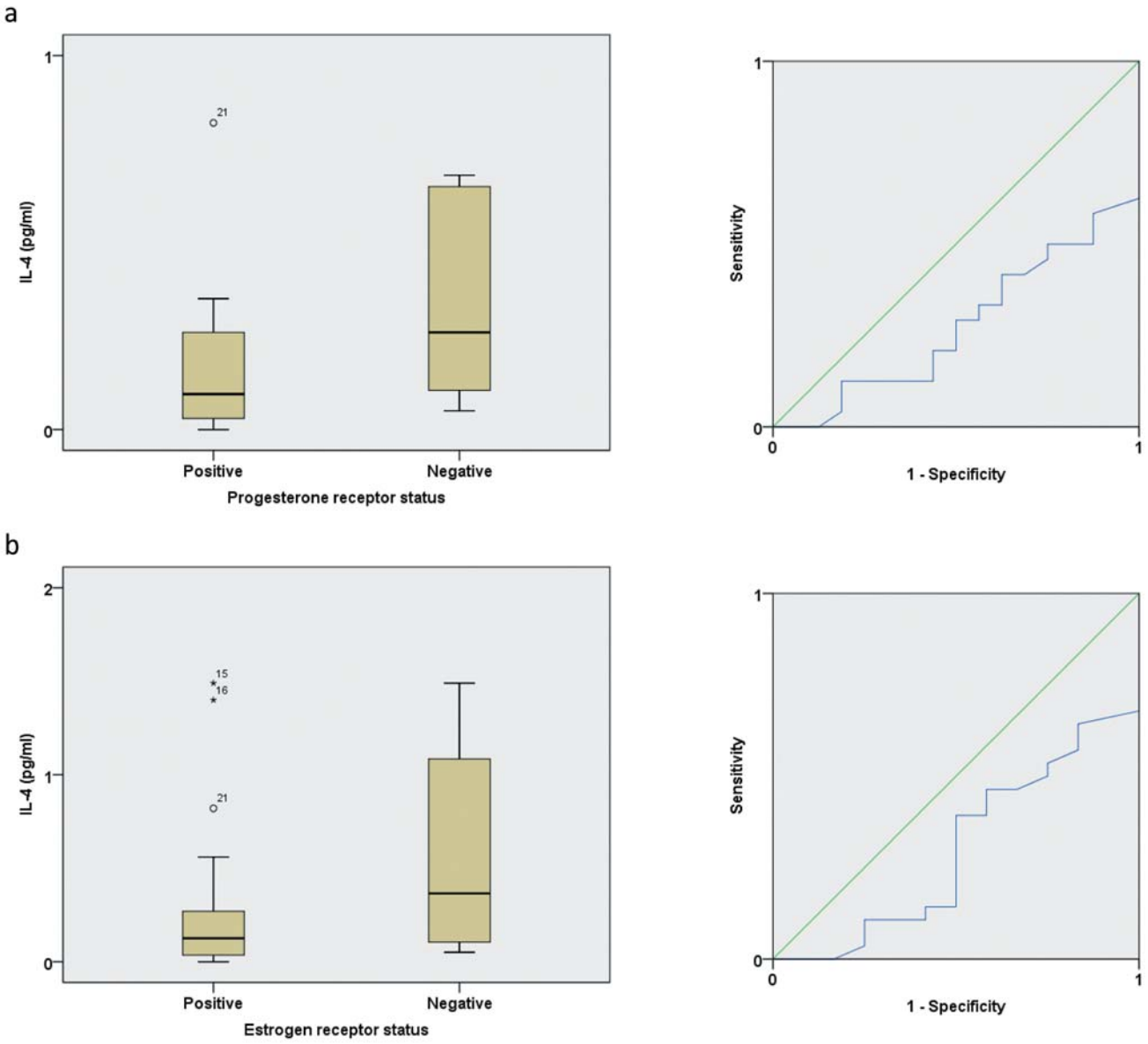

Figure 2. *Box-plot analysis of interleukin (IL)-4 expression in serum of patients with breast cancer who died from their disease (regardless of circulating tumor cell status) according to progesterone (a) and estrogen (b) receptor status. The range between the 25th and 75th percentiles is represented by boxes with a horizontal line at the median. The bars show the 5th and 95th percentiles. Circles indicate values more than 1.5 box lengths. Asterisks indicate values (marked with a number) more than 3.0 box lengths from the 75th percentile. We identified significantly enhanced expression of IL-4 in the collective who died with progesterone receptor-negative breast cancer compared to those with progesterone receptor-positive disease (p=0.017) (a, left panel), and in those who died with estrogen receptor-negative disease compared with those with estrogen receptor-positive disease (p=0.046) (b, left panel). Receiver operator curve analysis of sensitivity versus specificity of the ELISA used was performed and gave area under the curve values of 0.276 (a, right panel) and 0.299 (b, right panel), respectively.*

of IL-4 in comparison with patients positive for estrogen receptor (Spearman correlation p=0.045, Mann–Whitney U-test p=0.046; Figure 2b).

Regarding the patient group with breast cancer who were still alive, there was no significant difference in statistical analysis relating to Th2-cytokine levels (IL-4, IL-5, IL-6, IL-8 and IL-13).

Grade G2 vs. G3 tumor. The collective with grade G3 tumor was associated with statistically significant results based on

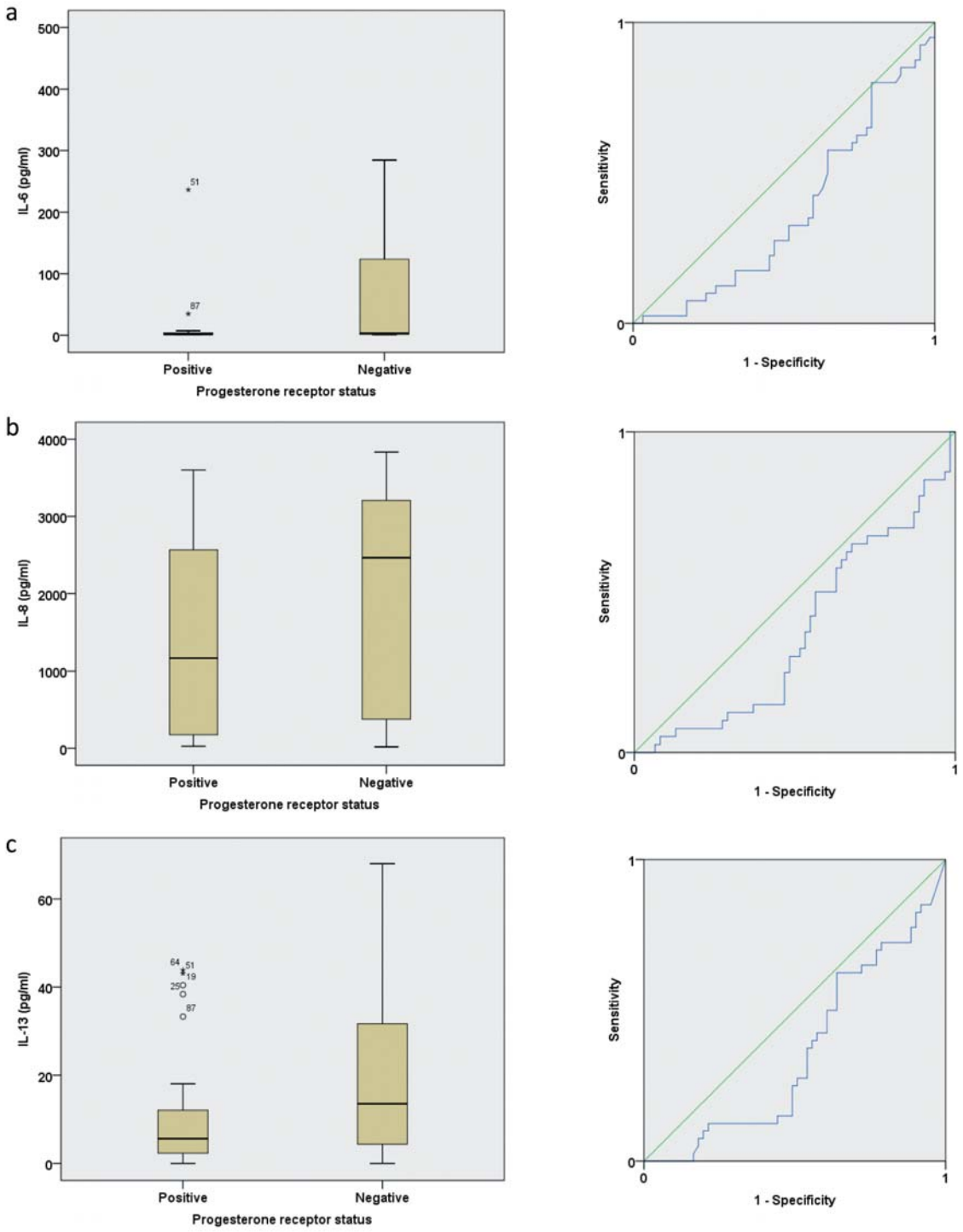

Figure 3. *Box plot analysis of interleukin (IL)-6, IL-8 and IL-13 (left panels of a, b and c, respectively) in sera of patients with G3 breast cancer (regardless of circulating tumor cell status). The range between the 25th and 75th percentiles is represented by boxes with a horizontal line at the median. The bars show the 5th and 95th percentiles. Circles indicate values more than 1.5 box lengths. Asterisks indicate values (marked with a number) more than 3.0 box lengths from the 75th percentile. In addition, receiver operator curve (ROC) analysis of sensitivity versus specificity of the ELISAs used was performed and the area under the curve (AUC) value was derived (a-c, right panels). We identified significantly enhanced IL-6 release in the G3 patient collective with progesterone receptor-negative breast cancer compared to patients with progesterone receptor-positive disease (p=0.027) (a, left panel), with an AUC in ROC analysis of 0.370 (a, right panel). Similarly, IL-8 (b) and IL-13 (c) release were also significantly greater in patients with G3 progesterone receptor-negative breast cancer compared to their counterparts with progesterone receptor-positive disease (p=0.033 and p=0.019, respectively), with AUCs of 0.375 and 0.361, respectively (b and c, right panels).*

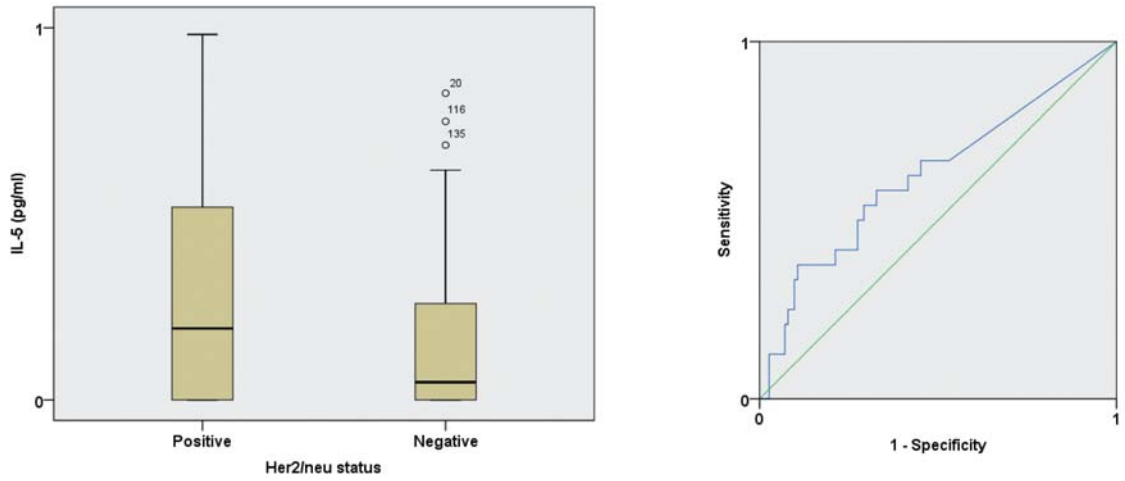

Figure 4. *Box plot analysis of interleukin (IL)-5 expression in sera of patients with breast cancer with lymph node metastasis (regardless of circulating tumor cell status). The range between the 25th and 75th percentiles is represented by boxes with a horizontal line at the median. The bars show the 5th and 95th percentiles. Circles indicate values more than 1.5 box lengths. Asterisks indicate values (marked with a number) more than 3.0 box lengths from the 75th percentile. We identified significantly enhanced IL-5 release in patients with lymph node metastasis and HER2/NEU-positive breast cancer compared to their counterparts with HER2/NEU-negative breast cancer (p=0.043) (left panel). Receiver operator curve analysis of sensitivity versus specificity of the ELISA used gave an area under the curve of 0.626 (right panel).*

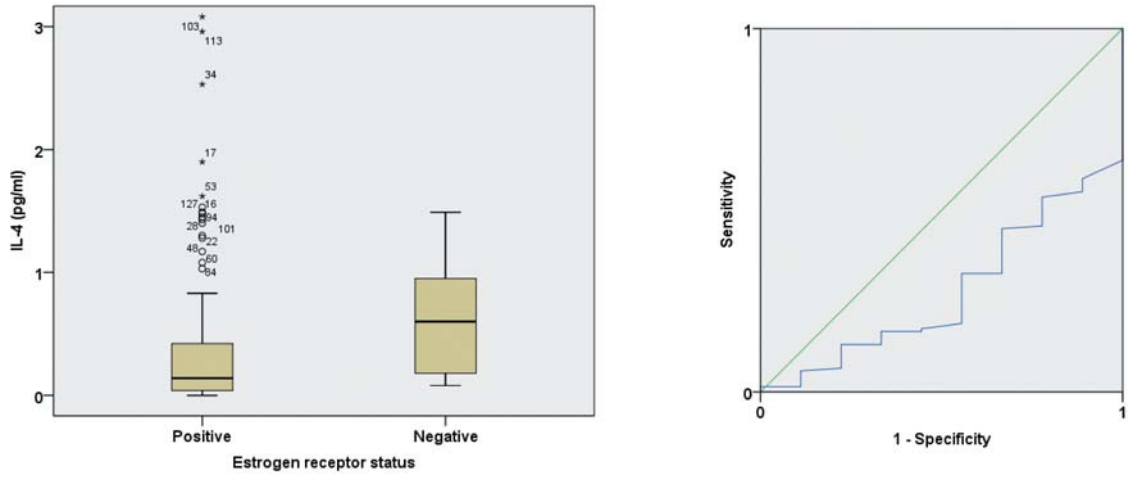

Figure 5. *Box plot analysis of interleukin (IL)-4 expression in sera of patients with progesterone receptor-positive breast cancer (regardless of circulating tumor cell status). The range between the 25th and 75th percentiles is represented by boxes with a horizontal line at the median. The bars show the 5th and 95th percentiles. Circles indicate values more than 1.5 box lengths. Asterisks indicate values (marked with a number) more than 3.0 box lengths from the 75th percentile. We identified significantly enhanced IL-4 release in patients with progesterone receptor-positive, estrogen receptor-negative breast cancer compared to their counterparts with estrogen receptor-positive disease (p=0.025) (left panel). Receiver operator curve analysis of sensitivity versus specificity of the ELISA used gave an area under the curve of 0.277 (right panel).*

hormone receptor status and cytokine levels: in progesterone receptor-negative cases, levels of IL-6, IL-8 and IL-13 were increased in comparison to patients with progesterone receptor-positive cases (IL-6: Spearman correlation p=0.026, Mann–Whitney *U*-test p=0.027; IL-8: Spearman correlation p=0.032, Mann–Whitney U-test p=0.033; IL-13: Spearman correlation p=0.018, Mann–Whitney U-test p=0.019; Figure 3).

Regarding the patient group with G2 grade tumor, there was no significant difference in the statistical analysis relating to Th2-cytokine levels (IL-4, IL-5, IL-6, IL-8 and IL-13).

Lymph node involvement vs. no lymph node involvement. For the collective with disease-positive lymph nodes, the box-plot analysis disclosed that those who were HER2/neu receptor-positive disease had higher levels of IL-5 in comparison to those who were HER2/neu receptor-negative (Spearman correlation $p=0.042$, Mann–Whitney *U*-test $p=0.043$; Figure 4a).

Regarding the group of patients without lymph node involvement, no significant difference in the statistical analysis relating to Th2 cytokine levels (IL-4, IL-5, IL-6, IL-8 and IL-13) was found.

Hormone receptor status. Patients who were progesterone receptor-positive and estrogen receptor-negative had high levels of IL-4 (Spearman correlation $p=0.024$, Mann–Whitney *U*-test $p=0.025$; Figure 5a).

However, in patients with both hormone receptor-negative and HER2/neu-negative tumor, no significant difference in statistical analysis relating to Th2 cytokine levels (IL-4, IL-5, IL-6, IL-8 and IL-13) was detected.

Discussion

The background of this study was an investigation using the serum of patients who participated in the SUCCESS I study. The levels of cytokines of the Th2 group were monitored depending on specific breast cancer criteria such as hormone receptor status, lymph node involvement, grading, CTC involvement and other criteria. In a specific number of patients, statistically significant variations of the cytokine levels were found.

Regarding IL-4, we found that its level increased in patients who died and for whom the progesterone receptor or the estrogen receptor was negative. The same effect was shown in the patient group with at least one positive hormone receptor: we found that the level of IL-4 was high when estrogen receptor expression was negative. In all these cases, if the tumor criteria showed a high level of IL-4, a poor prognosis was described for breast cancer (11). In literature, IL-4 is described in several reports to be involved in the pathogenesis of cancer or development of local metastasis, especially in the case of colorectal cancer (12, 13). Another finding was the involvement of increasing IL-4 in increasing tumor cell resistance to apoptosis (14). Regarding the results in our study, with a higher level of IL-4 in patients we expected a poor prognosis (hormone receptor-negative); we suspect that an increase of IL-4 is responsible for the poor outcome in these cases amongst others. A poorer outcome could be interpreted as a higher risk of metastasis and the involvement of resistance to apoptosis. Furthermore, the correlation of IL-4 and a negative outcome is supported by increased IL-4 levels in our collective of patients who ultimately died.

Patients in our collective with lymph node involvement and with expression of HER2/neu receptor had a significantly higher level of IL-5 than patients with HER2/neu-negative status. Breast cancer with lymph node involvement and with expression of the HER2/neu receptor has a poor prognosis (11). In the literature, we found only little information on the role of IL-5 concerning cancer. On the other hand, in bladder cancer, it was shown that an increased level of IL-5 enhanced the migration and invasion of bladder cancer cells *via* extracellular-signal-regulated kinase 1/2-mediated matrix metallopeptidase 9/nuclear factor κ-light-chain-enhancer of activated B-cells/activator protein 1 pathway (15).

In our collective, higher IL-5 was associated with poor prognosis, hence we suspect that IL-5 modifies the invasion of cancer or the development of metastasis in a negative manner.

Another investigated cytokine was IL-6, which was found at a higher level in patients with a tumor grade of G3 and who were negative for progesterone receptor. In literature, the pro inflammatory cytokine IL-6 is to be held responsible for tumor growth and differentiation in prostate cancer. IL-6 is also described as having a proliferative and anti-apoptotic effect (16). Furthermore, the serum IL-6 level is a potential biomarker for predicting disease progression in colorectal cancer (17). In our study, IL-6 was increased in patients with a poor prognosis because of their tumor grading and receptor status. As seen in the literature, IL-6 is associated with anti-apoptotic effect which is likely responsible for poorer outcome in our cases.

The level of IL-8 was statistically significantly increased in patients who were CTC-negative and without expression of progesterone receptor. In cases with a tumor grade G3 and negative for progesterone receptor, we also found an increased IL-8 level. A correlation is implied between IL-8 and neo-vascularization, which would encourage metastatic spread (18, 19). Hence increasing IL-8 is most likely responsible for the increased risk of metastatic spread in these cases. Therefore, IL-8 in patients CTC-negative and negative for progesterone receptor might be involved in local invasiveness through neovascularization. Furthermore an increase of IL-8 in our patient collective with G3 progesterone receptor-negative tumor supports a less favorable outcome.

Levels of IL-13 were increased in CTC-negative patients who were progesterone receptor-negative. The other collective with a high level of IL-13 was characterized by G3 tumor and no expression of progesterone receptor. Due to the fact that IL-13 is increased in hormone receptor-negative patients with G3 tumor, we expect that high expression of IL-13 to be associated with a poorer prognosis (11). In literature IL-13 is described to be involved in negatively modulating the development of effective Th1 immunity (20). In human pancreatic cancer, IL-13 exerts autocrine growth-promoting effects and its expression correlates with a propensity for lymph node metastases (21). Therefore, we may conclude that an increased IL-13 level is related to hormone receptor negativity and poorly graded

tumors but on the other hand also responsible for lymph node metastasis and immunosuppression.

In summary, increased levels of Th2 cytokines seem to be associated with a poor prognosis in breast cancer. Measurement and interpretation of Th2 cytokines in breast cancer could help develop new prognostic parameters or therapeutic strategies.

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