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**Influence of circulating tumour cells on the immune response of  
T-Lymphocytes and Angiogenic cytokines in sera of patients with the  
primary diagnosis of breast cancer before treatment**

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Meinem Papa und meinen Schwestern Janina und Josefina

In Andenken an meine Mama

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## **1. INTRODUCTION**

### **1.1. Epidemiology Breast Cancer**

Worldwide, breast cancer is still the most common malignant tumour diagnosed in women with an calculated 1.7 million new breast cancer cases and 522,000 breast cancer associated deaths in 2012 (1). To continue, invasive breast cancer remains to be considered one of the utmost challenges for specialists to regulate and additionally to improve the survival of patients (2). Nevertheless, the survival of breast cancer patients is strongly related to prognostic factors including tumour size, hormone-receptor-profile and manifestation of metastases (3). Even though the treatment options of breast cancer counting surgery, chemotherapy, aromatase inhibitors and hormone receptor modulators (4-6) have tremendously advanced over the former centuries, yet the constantly high mortality numbers especially due to tumour metastasis to the vital organs and lymph node remain (7). Mortality is predominantly correlated to distant metastatic growth (7, 8).

### **1.2. Circulating tumour cells and the SUCCESS Study Group**

Previous studies have established an association between poor prognosis and the detection of circulating tumour cells (CTCs) before the start of systemic treatment (9). Also referred to as 'liquid biopsy', this method of detection has established itself as an important device for assessing and observing the efficiency of treatments in patients with breast cancer. The technique can refer to molecular analysis of the tumour's genetic structures grounded on circulating genetic material in the peripheral blood originated from CTCs (10, 11). Furthermore, CTCs in the peripheral blood have been found to be a prognostic marker for reduced disease free survival (DFS), distant DFS, breast cancer-specific DFS and overall survival (OS) before the start of systemic treatment (9, 12-15). The technology for identifying CTCs has evolved from simple cell counting into innovative molecular subtyping (16) over the past years. This process has established a minimally invasive method in early carcinoma detection and furthermore in disease monitoring. CTCs are thought to originate from the primary tumour and even considered to obtain genetic heterogeneity during evolution, as a conclusion CTC values

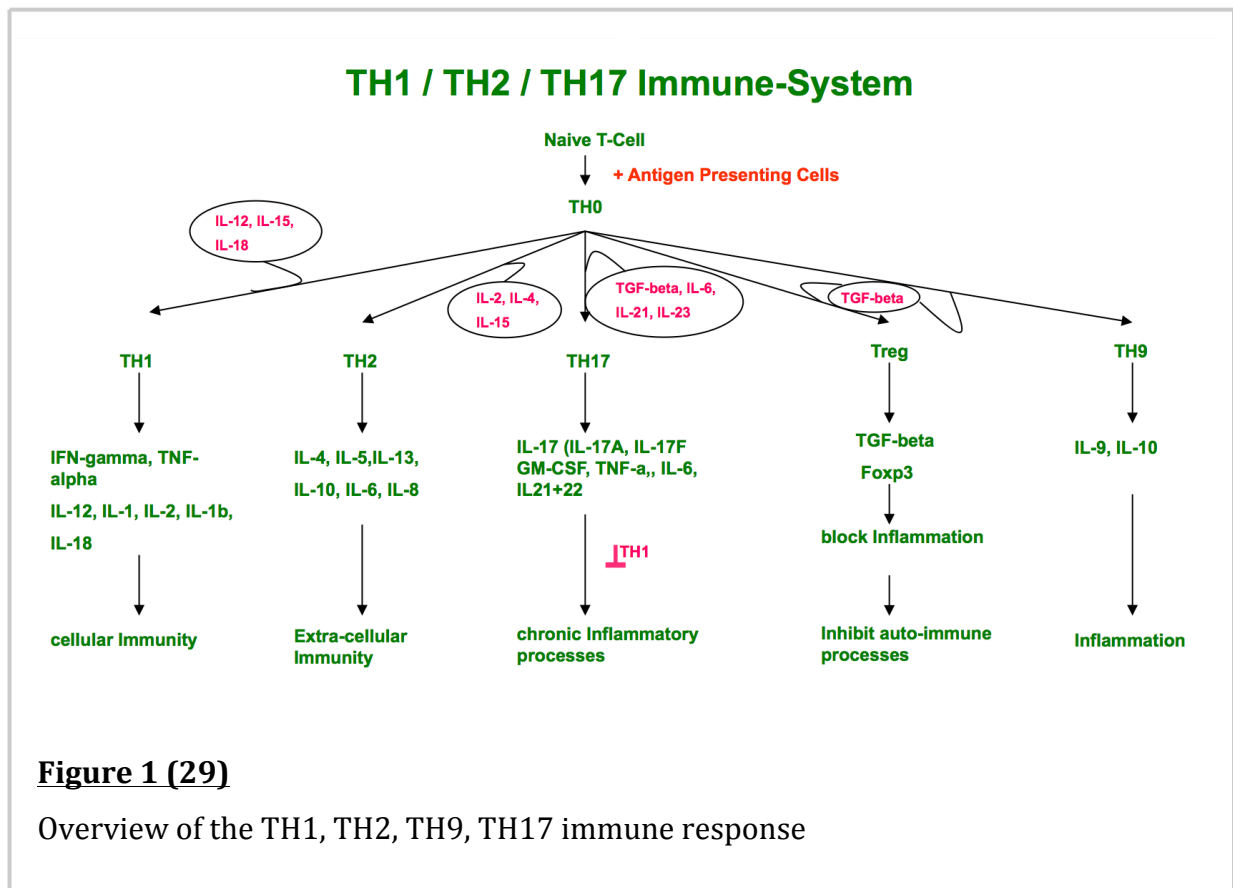
shortly after commence of treatment deliver complementary information regarding therapy response (15).

The SUCCESS study was one of the first trials to determine the role of CTCs in patients with breast cancer. The study specified the strong prognostic significance of CTCs being associated with a less favourable outcome, when detected in early breast cancer before the start of systemic adjuvant treatment and after adjuvant chemotherapy, in a large patient cohort (17).

### **1.3. Immune response of T-lymphocytes & Angiogenic cytokines**

The overall aim and intention of this study concerning Paper 1,2 and 3 was the evaluation of cytokines and the lymphatic system as an indicator for CTC involvement in patients with breast cancer. Having explained the significant and crucial role of CTCs in breast cancer patients, the determination of CTCs is however still a time consuming and high-priced method. Furthermore, it has become increasingly evident that not only the breast cancer cells itself, but also the microenvironment of the tumour including the immunological reaction plays a significant role in terms of tumour progression, metastasis formation and treatment response (18). In concern to the arising significance of the lymphatic system involvement and cytokine measurement in cancer therapy, the intention of this analysis was therefore the specific evaluation of cytokine profiles (Paper 2 & 3) and tumour-angiogenesis markers (Paper 1) in association to CTC involvement in patients with the primary diagnosis of breast cancer (19). The involvement of T-Lymphocytes and their cytokines similarly to vascular markers are proved to interact with tumour cells and show an impact on the patient's prognosis (20). T-lymphocytes and their distinct maturation to cytokines and chemokines are known to be a vital factor of the adaptive immune response (21). Through their tumour-promoting or tumour-suppressive properties on critical cell derivation, the arrangements of cytokines that stimulate helper T-lymphocyte maturation can furthermore through different interaction lead to the expression of both pro-inflammatory or anti-inflammatory cytokines (22-24). Through this specified function cytokines and chemokine's are able to stimulate the cancers progress. Their role has been described to be an important prognostic factor in the presence of breast cancer tumours (21).

Moreover, vascular tumour angiogenesis also acts as a vital component in the development and progression of breast cancer. Similar to the cytokine and chemokine pathway it is understood that angiogenesis in tumours is part of a multistep evolution involving the signalling between breast cancer cells and numerous cell varieties within the tumours microenvironment (3, 25). The close relationship displays how a range of proangiogenic cytokines yielded by overexpression of factors by the tumour on the other hand promote angiogenesis (26). Neoangiogenesis is defined as a process of new blood capillary development from pre-existing vessels, essentially in both embryonic and postnatal growth such as in the modification of numerous organ structures and specifically in tumour development and expansion (25). The progression of neo-vascularisation is referred to as the “angiogenic switch” (3, 27), where the balance amongst pro- and antiangiogenic cytokines and chemokines slender for proangiogenic markers (26). This clearly illustrates the interactions of the Immune system, describing progression to a growing vascularized tumour and finally to malignant behaviour (26-28).



The intention of this study concerning paper 1,2 and 3 was therefore to examine the distribution of T helper (TH) 1, TH2, TH17, Regulatory T cells (Treg) and TH9 cytokines of the T-lymphocyte immune response including angiogenesis markers and reveal the differences of cytokine levels in breast cancer patients of the SUCCESS study group with respect to CTC involvement, histopathological grading, lymph node status, hormone receptor type, TNM classification and survived breast cancer patients vs. deceased tumour associated patients. The assessment of certain cytokine profiles and furthermore the evaluation of their functions could help for a revised understanding of the illness. It could offer new clinical perspectives by enhancing personalised treatment opportunities in regards to the tumour phenotype, furthermore allow a profounder understanding of how immunological-related genes may influence breast carcinogenesis, evaluating the discrete risk of patients at the time of primary diagnosis and improve therapy observation.

### **1.3.1. Angiogenic cytokines (Paper 1)**

The cytokines belonging to the vascular endothelial growth factor (VEGF) family and their significant involvement in angiogenesis have been substance of major relevance. The VEGF family is so far known to include six related gene members being VEGF, VEGF-B, VEGF-C, VEGF-D, VEGF-F and placental growth factor (PIGF). They are described as regulators of angiogenesis or lymphangiogenesis or of both processes (3, 26, 30). Many studies have already focused on establishing a link of the precarious involvement of those markers with tumour evolution and have therefore become a promising focus in cancer therapy (3). It is understood that angiogenesis in carcinomas is part of a multistep evolution including the signalling between breast cancer cells and multiple other cell types within the tumours microenvironment (3, 25). It has been implicated that the mentioned markers bind with various affinity to one of the three tyrosine kinase receptors also recognised as vascular endothelial growth factor receptor VEGFR-1 (sFlt1), VEGFR-2 and VEGFR-3 (31-33), instructing a signalling cascade endorsing survival, progression and migration of carcinoma cells (3, 34). Enhanced VEGF levels implement a well-established indicator of unfortunate prognosis in tumour patients (35). On the other hand, PIGF and sFlt1 levels have predominantly been associated to preeclampsia, in regards to breast cancer these markers have so far been



associated with a lower risk of illness later in life (36, 37). Paper 1 therefore focuses on analyzing the distribution of the angiogenic markers sFlt1, PlGF, VEGF, VEGF-C and VEGF-D and disclose the differences of their expression in breast cancer patients of the SUCCESS study group in terms of CTC involvement, histo-pathological grading, lymph node metastasis, hormone receptor status, TNM classification and survived breast cancer patients vs. deceased tumour associated patients.

### **1.3.2. TH1 cytokines immune response (Paper 2)**

Cytokines belonging to the TH1 immune response are implied to play a key part in phagocytic and intracellular defence (22) and have been subject of numerous studies. Analyses have suggested specific TH1 expressions appear to correlate with TNM stage and lymph node involvement in breast cancer patients. Paper 2 focuses on the distribution for the TH1 cytokines: Interferon gamma (IFN- $\gamma$ ), Tumour necrosis factor alpha (TNF- $\alpha$ ), Interleukin (IL)-12, IL-1 $\alpha$ , IL-1 $\beta$ , IL-2 and IL-18 to evaluate profiles as marker for CTC involvement regarding specific breast cancer criteria as mentioned before, in hindsight to the implied cytokine manners stated above. For example, increased IFN- $\gamma$  values are associated with a positive outcome in breast cancer patients (38), while the existence of the IL-1 system was discovered to inversely correlate with local sex steroid receptor expression, stressing an enhanced malignant behaviour (39). IFN- $\gamma$  is even associated to a protective role against carcinomas by promoting apoptosis by enhancing cytotoxic T lymphocyte activity and expressing influence on p53 (38, 40). Presumed that IFN- $\gamma$  genetic polymorphisms might even be significantly related with a higher risk of breast cancer, its detection in correlation to CTCs is of major interest (41). IL1- $\alpha$  is a TH1 cytokine that on the other hand is hypothesized to derive from tumour cells itself (42). Implying that senescence increases in IL-1 $\alpha$  expression to generate a microenvironment that is beneficial to metastatic disease progression in cancer patients (42), suggesting an IL1- $\alpha$  involvement in circulation specific metastasis opposed to metastasis via the lymphatic system. Furthermore, it is suggested that IL-1 $\alpha$  expressed on malignant cells stimulate an anti-tumour immunity (43, 44). In contrast, IL-1 $\beta$ , originating from the microenvironment or the malignant breast carcinoma cells itself, triggers inflammation that endorses invasiveness and encourages tumour resolved

suppression (39, 43, 44). IL-12 on the other hand is supposed to play the most critical part for the induction of TH1 cytokines responses (22). Significantly enhanced IL-12p40 expression combined with significant increase in quantity of lymphocytes, CD4+, CD8+, Natural killer (NK) cells and C-reactive protein (CRP) has been stated for a clinical benefit, as opposed to tumour growth, predominantly in hormone responsive metastatic breast cancer(45). Furthermore, IL-12 is emphasised to express an anti-angiogenic effect and is implied to induce a T-cell based anti-tumour immune response efficient of eradicating disseminated cancer cells (46-48). IL-12 cytokines in intra-tumour treatment in combination with other cytokines in breast cancer patients induce infiltration by polymorphonuclear cells, dendritic cell antigen presentation and CD8+ T-cells resulting in tumour regression (46, 49). IL-12 local therapy has revealed in certain studies, stimulate specific antitumor T-cells in lymph nodes thus ensuing in a memory immune response (46, 48, 49).

### **1.3.3. TH2 cytokines immune response (Paper 3)**

Certain cytokines belonging to the TH2 immune response and found at increased levels in patients with carcinomas are correlated with inferior prognosis in terms of overall and DFS (50). Therefore, the investigation of a panel of TH2 cytokines including IL-4, IL-5, IL-6, IL-8 and IL-13 in a group of patients diagnosed with breast cancer in regards to the presence or absence of CTCs in hindsight to specific breast cancer criteria listed above is of major interest. To continue, literature has described the role of IL-4 to be part of the pathogenesis of cancer and expansion of local metastasis, particularly in colorectal cancer (51, 52). Enhanced IL-4 levels are emphasized to be responsible for increasing tumour cell resistance to apoptosis (53). To continue, the increased presence of IL-5 in bladder cancer was described to enhance the migration and invasion of cancer cells *via* extracellular-signal-regulated kinases (ERK) 1/2-mediated Matrix metalloproteinase 9 (MMP-9)/ nuclear factor  $\kappa$ -light-chain-enhancer of activated B cells (NF- $\kappa$ B)/ Activator protein 1 (AP-1) pathway (54). The pro inflammatory cytokine IL-6 on the other hand is held responsible for tumour progress and differentiation in prostate cancer by promoting a proliferative and anti-apoptotic effect (55). IL-6 level found in the sera of cancer patients is presumed to be a potential biomarker for predicting disease progression in colorectal cancer (56). Analyses furthermore suggest a

correlation between IL-8 and neo-vascularization (Paper 1&3) thus encouraging metastatic spread (57, 58). IL-13 on the other hand is described to negatively modulate the effective development of TH1 immune response (Paper 2&3) (59). IL-13 is implied to exert autocrine progress-promoting effects and its expression correlates with evolution of lymph node metastases in human pancreatic cancer (60). Measurement and interpretation of TH2 cytokines levels in breast cancer patients could thus assist to acquire new prognostic parameters and therapeutic strategies.

#### **1.4. Study outline and Patients included**

As described before, SUCCESS was a prospective, randomized adjuvant study comparing three cycles of *fluorouracil-epirubicin-cyclo-phosphamide* (FEC; 500/100/500mg/m<sup>2</sup>) followed by 3 cycles of *docetaxel* (100mg/m<sup>2</sup>) every 3 weeks vs. three cycles of FEC followed by 3 cycles of *gemcitabine* (1000mg/m<sup>2</sup> d1,8)-*docetaxel* (75 mg/m<sup>2</sup>) each 3 weeks. After the completion of chemotherapy, the patients were randomized a second time for receiving either 2 or 5 years of *zoledronate*. Hormone receptor-positive women moreover received suitable endocrine treatment. Suitable patients were defined as women with breast cancer (Lymph node positive subgroup and Lymph node negative subgroup with high risk trades including grade 3 tumour, hormone receptor negative, age under 35,  $\geq$  pT2) who willingly acknowledged to take part in the SUCCESS study (see homepage: <http://www.success-studie.de>). The study was conducted as defined by the SUCCESS Study group (17).

The patients included in this study concerning Paper 1,2 and 3 were 200 patients of the SUCCESS study and assigned into two groups: 100 Patients were CTC positive (group 1) and the other 100 Patients were CTC negative (group 2). These two groups were subsequently framed and investigated accordingly. Patients from respectively groups were matched into pairs of two according to histo-pathological grading, lymph node involvement, hormone receptor type, TNM classification and survived patients vs. deceased patient's tumour associated. Out of the 200 patient samples that were considered, a total of 160 patients were still alive at last observation after end of therapy and 40 patients had deceased during therapy tumour associated. The groups examined enclosed 98 patients graded G2 and 102 patients graded G3. Matching criteria of the 200

patient collective did not include patients graded G1. Tumour stage of the anamnestic diagnosis was classified according to the TNM-classification, which was conducted correspondingly to the WHO System (61). The matching of patients was accomplished in regards to the criteria at the time of primary diagnosis. The histo-pathological grading was classified fitting to the Bloom and Richardson system classification (62).

Furthermore, for the measurement of cytokines and chemokines (paper 1,2 and 3) a commercial ELISA was used to screen the blood serum samples. ELISA kits used were acquired by Meso Scale Discovery® (Rockville, USA). We used anti-species MULTI-ARRAY 96-well plates for the development of a sandwich immunoassay. The 10 spot multi-spot plates were pre-coated with capture antibodies on independent and well defined spots that enabled us to immobilize a primary capture antibody against our protein of interest - specific for one of each cytokine.

### **1.5. Overview on results and achievements**

The study revealed differences of cytokine levels in breast cancer patients of the SUCCESS study group with respect to CTC involvement, histopathological grading, lymph node status, hormone receptor type, TNM classification and survived breast cancer patients vs. deceased tumour associated patients.

To begin with, as described in Paper 1, the distribution of the angiogenic markers disclosed differences of their expression in breast cancer patients of the SUCCESS study group. Statistical significant correlation was proven for sFlt1 values regarding the CTC-status. CTC negative patients demonstrated enhanced sFlt1 expression contrasting to CTC positive breast cancer patients ( $p=0,0034$ ). Significant enhanced PlGF values were additionally disclosed in CTC negative patients compared to patients being CTC positive ( $p=0,043$ ). Amongst the alive patient collective, significant differences were found in sFlt1 ( $p=0,030$ ) and PlGF ( $p=0,025$ ) values regarding the CTC negative and CTC positive patient's collective. To continue, the collective with a G2 graded tumour revealed significantly increased sFlt1 expressions ( $p=0,041$ ) amongst the patients with no CTCs. The patient collective with no lymph node metastasis and CTC negativity furthermore indicated statistically significant enhanced sFlt1 values ( $p=0,039$ ).

To continue, also amongst the TH1 derived cytokines significant differences in the sera of breast cancer patients with and without circulating tumour cells were shown

(Paper 2). The CTC positive patient collective implied a significant correlation in terms of lymph node involvement concerning IL-1 $\alpha$  ( $p=0,043$ ). The CTC negative collective on the other hand showed a significant difference regarding progesterone receptor positive/negative patients in terms of IL-1 $\beta$  ( $0,029$ ). Furthermore, the living patient collective revealed significant differences in IL-12p40 levels in regards to lymph node involvement ( $p=0,041$ ) and triple negative hormone receptor breast cancer ( $p=0,043$ ). In contrast, deceased patients implied significant results amongst the oestrogen receptor positive/negative patient collective regarding IL-1 $\alpha$  ( $p=0,050$ ) and IL-1 $\beta$  ( $p=0,034$ ). IL-1 $\alpha$  levels furthermore indicated significant differences in terms of triple negative hormone receptor breast cancer ( $p=0,033$ ) within the deceased patient collective. Moreover, the collective graded G2 demonstrated significant results amongst patients with Her2/neu association regarding IFN- $\gamma$  levels ( $p=0,031$ ) and in regards to lymph node involvement concerning IL-1 $\alpha$  levels ( $p=0,014$ ). Tumours graded with G3 in contrast exposed a significant correlation amongst progesterone receptor positive/negative patients in terms of IL12p70 Levels ( $p=0,048$ ) and triple negative hormone receptor breast cancer regarding IL12p40 levels ( $p=0,033$ ).

Furthermore, analysis of the TH2 derived cytokines demonstrated significant levels in patients with breast cancer (Paper 3). The CTC-negative patient collective implied higher expression of IL-8 ( $p=0,017$ ) and IL-13 and ( $p=0,045$ ) being negative for progesterone receptor. In patients who died tumour associated, a correlation between hormone receptor negativity and enhanced IL-4 levels was implied. IL-4 levels were also increased in patients with progesterone receptor-positive and oestrogen receptor-negative status ( $p=0,024$ ). Moreover, IL-5 levels were enhanced in patients with lymph node-positive and human epidermal growth factor receptor 2 (HER2)-positive disease ( $p=0,042$ ). Furthermore, the IL-6 was significantly increased in patients with tumour grade G3 without progesterone receptor expression.

### **1.4 Contribution to Publications Included in this Thesis**

TV (Theresa Vilsmaier) performed the experiments for Publications 1, 2, 3 and made substantial contributions to acquisition and interpretation of data. TV majorly contributed to data analysis, completed the statistical work and drafted the manuscripts for the Publications 1, 2 and 3.

## 2. PUBLICATIONS INCLUDED IN THIS THESIS

### 2.1. Publication 1

*Title:*

Angiogenic cytokines and their influence on circulating tumour cells in sera of patients with the primary diagnosis of breast cancer before treatment

*Authors:*

Theresa Vilsmaier, Brigitte Rack, Wolfgang Janni, Udo Jeschke, Tobias Weissenbacher and SUCCESS Study Group

*Journal:*

BMC Cancer. 2016 Jul 27;16:547. doi: 10.1186/s12885-016-2612-7.

*Abstract:*

**Background:** Circulating tumour cells (CTCs) have been found to be a prognostic marker for reduced disease free survival, breast cancer-specific survival, and overall survival before the start of systemic treatment. In correspondence to the arising significance of CTC involvement in cancer therapy, the aim of this study was the evaluation of tumour-angiogenesis markers, which act as a crucial factor in the development and progression of breast cancer, in association to CTC involvement.

**Methods:** Patients sera chosen for this study were women with breast cancer of the phase I SUCCESS study. CTC analysis, the blood sampling time points, and the methodology were prospectively designed, and the prognostic value of the CTCs was defined as a scientific objective of the study protocol. 200 patients' sera were included in this study, 100 patients being CTC positive and 100 patients being CTC negative. One patient of each group was subsequently matched into pairs of two. Matching criteria were histo-pathological grading, lymph node metastasis, hormone receptor status, TNM classification and survived breast cancer patients vs. deceased tumour associated patients. A recently developed multi cytokine/chemokine array (Meso Scale Discovery ®, Rockville, USA) was used to screen the sera for the angiogenic markers: sFlt1, PlGF, VEGF, VEGF-C and VEGF-D. The vascular markers values were correlated to the

matching criteria and analysed with the Spearman correlation coefficient and the Mann-Whitney-U test.

**Results:** Statistical significant correlation was exposed for sFlt1 values in regard to the CTC-Status. CTC negative patients displayed increased sFlt1 expression opposed to CTC positive breast cancer patients ( $p=0.034$ ). Furthermore, significant enhanced PlGF values were also disclosed in CTC negative patients compared to patients being CTC positive ( $p=0.043$ ). Analysing the living patient collective that did not decrease breast cancer associated, these established significant differences in sFlt1 and PlGF values in regard to CTC negative and CTC positive patients. Both vascular markers showed enhanced expression in the CTC negative patient collective (sFlt1  $p=0.030$ , PlGF  $p=0.025$ ). To continue, the collective graded G2 showed significantly enhanced sFlt1 expressions amongst patients with no CTCs ( $p=0.041$ ). Moreover, the patient collective with no lymph node metastasis and CTC negativity indicated statistically significant increased sFlt1 values ( $p=0.039$ ).

**Conclusion:** A functional interaction of sFlt1 and PlGF was found, suggesting that their overexpression in tumour cells inhibits CTCs entering the peripheral blood, thus emphasising a significant anti-angiogenic effect, inhibiting tumour growth and metastasis. Furthermore, in regard to CTC negativity, sFlt1 and PlGF values may potentially serve as predictive markers.



## RESEARCH ARTICLE

## Open Access



# Angiogenic cytokines and their influence on circulating tumour cells in sera of patients with the primary diagnosis of breast cancer before treatment

Theresa Vilsmaier<sup>1</sup>, Brigitte Rack<sup>1</sup>, Wolfgang Janni<sup>2</sup>, Udo Jeschke<sup>1\*</sup> , Tobias Weissenbacher<sup>1</sup> and SUCCESS Study Group

## Abstract

**Background:** Circulating tumour cells (CTCs) have been found to be a prognostic marker for reduced disease free survival, breast cancer-specific survival, and overall survival before the start of systemic treatment.

**Methods:** A total of 200 patients' sera were included in this study, 100 patients being CTC positive and 100 patients being CTC negative. Matching criteria were histo-pathological grading, lymph node metastasis, hormone receptor status, TNM classification and survived breast cancer patients vs. deceased tumor associated patients. A multi cytokine/chemokine array was used to screen the sera for the angiogenic markers.

**Results:** Statistical significant correlation was exposed for sFlt1 values in regard to the CTC-Status. CTC negative patients displayed increased sFlt1 expression opposed to CTC positive breast cancer patients. Furthermore, significant enhanced PIGF values were also disclosed in CTC negative patients compared to patients being CTC positive. Analyzing the living patient collective we found significant differences in sFlt1 and PIGF values in regard to CTC negative and CTC positive patients.

**Conclusion:** Both vascular markers showed enhanced expression in the CTC negative patient collective. To continue, the collective graded G2 showed significantly enhanced sFlt1 expressions amongst patients with no CTCs. Moreover, the patient collective with no lymph node metastasis and CTC negativity indicated statistically significant increased sFlt1 values. A functional interaction of sFlt1 and PIGF was found, suggesting that their overexpression in tumour cells inhibits CTCs entering the peripheral blood. Furthermore, in regard to CTC negativity, sFlt1 and PIGF values may potentially serve as predictive markers.

**Trial registration:** The TRN of this study is NCT02181101 and the date of registration was the 4<sup>th</sup> of June 2014. The study was retrospectively registered.

**Keywords:** Breast cancer, Vascular markers, sFLT1, PIGF

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## Background

Worldwide, breast cancer is the most common tumour diagnosed in women with an estimated 1.7 million new breast cancer cases and 522,000 breast cancer deaths in 2012 [1]. Whereby, the survival of breast cancer patients is intensely associated with prognostic factors such as tumour size, hormone-receptor-profile and presence of metastases [2]. New approaches have also established a correlation between poor prognosis and the detection of Circulating tumour cells (CTCs) before the start of systemic treatment [3]. CTCs in the peripheral blood can be used as a prognostic marker for reduced disease free-, breast cancer specific-, and overall- survival before the start of systemic treatment [3–7]. The detection of CTCs shortly after commence of therapy even provide complementary information concerning treatment response [6]. The SUCCESS study was one of the first trials to indicate the strong prognostic importance, associated with a less favourable outcome, of CTCs in early breast cancer before commencing systemic adjuvant treatment and after adjuvant chemotherapy in a large patient cohort [8].

It is increasingly evident that not only the breast cancer cells itself, but also the microenvironment of the tumour plays a significant role in terms of tumour progression, metastasis formation and treatment response [9]. To continue, tumour angiogenesis acts as a crucial factor in the microenvironment in the development and progression of breast cancer. In correspondence to the arising significance of CTC involvement in cancer therapy, the aim of this study was the evaluation of tumour-angiogenesis markers in association to CTC involvement. Furthermore, vascular markers could act as indicators for the absence or presence of CTCs, as the determination of CTCs is a time intense and expensive technique.

Neo-angiogenesis, the process of new blood capillary formation from pre-existing vessels, acts as a fundamental part in both embryonic and postnatal development, in the remodelling of various organ structures, and in particular in tumour growth [10]. Its precarious involvement with tumour evolution and penetration has already become a promising focus in cancer therapy [2]. It is implied that angiogenesis in tumours is part of a multistep progression including the signalling between breast cancer cells and several cell types within the tumours microenvironment [2, 10]. A range of pro-angiogenic cytokines, which succumb an overexpression of factors by the tumour, induces Angiogenesis [11]. One of the best described is the vascular endothelial growth factor (VEGF). This process of neo-vascularisation is also referred to as the “angiogenic switch” [2, 12]. This describes the transition of tumour cells, where the balance between pro- and anti-angiogenic factors lean towards pro-angiogenic markers, designating a progression to an expanding vascularized tumour and eventually to malignant behaviour [11–13]. Consequently our intention was

to analyse the distribution of angiogenic markers: sFlt1, PlGF, VEGF, VEGF-C and VEGF-D and disclose the differences of their expression in breast cancer patients of the SUCCESS study group in terms of CTC involvement, histo-pathological grading, lymph node metastasis, hormone receptor status, TNM classification and survived breast cancer patients vs. deceased tumour associated patients. A Sandwich immunoassay ELISA and anti-species Multi-Array 96 well plates were used to screen the blood serum samples that enabled us to screen for all mentioned vascular markers in just one well at the same time.

The cytokines belonging to the vascular endothelial growth factor (VEGF) family and its important involvement in angiogenesis have been subject of major interest. The VEGF family includes six related gene members; VEGF, VEGF-B, VEGF-C, VEGF-D, VEGF-F and placental growth factor (PlGF) that are regulators of angiogenesis or lymphangiogenesis or of both processes [2, 11, 14]. To continue, the markers have been described to bind with diverse affinity to one of the three tyrosine kinase receptors known as vascular endothelial growth factor receptor VEGFR-1 (sFlt1), VEGFR-2 and VEGFR-3 [15–17], initiating a signalling cascade promoting survival, growth and migration of tumour cells [2, 18]. Increased levels of VEGF in tumour patients have been described as a well-established indicator of poor prognosis [19]. PlGF and sFlt1 on the other hand have been known to play a major role in preeclampsia, and even associated with a lower breast cancer risk later in life of those patients [20, 21].

In conclusion, the assessment of vascular tumour angiogenesis markers in relationship to CTC involvement and the expression of angiogenesis markers in terms of histo-pathological grading, lymph node involvement, hormone receptor status, TNM classification and survived breast cancer patients vs. deceased tumour associated patients, could found an advantage in regard to assessing the discrete risk of patients at the time of primary diagnosis. The assessment of the angiogenesis factors in patients with different phenotype breast cancer, could furthermore allow a profounder understanding of how angiogenesis-related genes may influence breast carcinogenesis, thus allowing an increased enhanced individualized treatment.

## Methods

### Study design and ethical board permission

Eligible patients were defined as women with breast cancer (stages pT1–T4, pN0–N3, M0) who accepted to participate in the phase I SUCCESS study ([www.success-studie.de](http://www.success-studie.de)). SUCCESS was a prospective, randomized adjuvant study comparing three cycles of fluorouracil-epirubicin-cyclo-phosphamide (FEC; 500/100/500 mg/m<sup>2</sup>) followed by 3 cycles of docetaxel (100 mg/m<sup>2</sup>) every 3 weeks vs. three cycles of FEC followed by 3 cycles of gemcitabine (1000 mg/m<sup>2</sup> d1,8)-docetaxel (75 mg/m<sup>2</sup>) each

3 weeks. After the completion of chemotherapy, the patients were furthermore randomized to receive either 2 or 5 years of zoledronate. Hormone receptor-positive women moreover received suitable endocrine treatment. The research questions related to CTC analysis, the blood sampling time points, and the methodology were prospectively designed, and the prognostic value of the CTCs was described as a scientific objective of the study protocol. The study was permitted by 37 German ethical boards (lead ethical board: LMU, Munich) and conducted in agreement with the Declaration of Helsinki.

Blood samples for CTC enumeration were collected from 2090 consecutive patients after complete resection of the primary tumour and before adjuvant chemotherapy after written informed consent was acquired. Nevertheless, sixty-four patients were disqualified because of test failure or a time intermission of more than 96 h between the blood collection and sample preparation. A follow-up evaluation after chemotherapy and before the beginning of endocrine or bisphosphonate treatment was available for a subgroup of 1492 patients (see home page: <http://www.success-studie.de>).

#### Patients

In this study 200 Patients of the SUCCESS study were incorporated and assigned into two groups: 100 Patients were CTC positive (Group 1. CTC Positive) and the other 100 Patients were CTC negative (Group 2. CTC Negative). These two groups were then framed and investigated correspondingly. Patients from respectively groups were then matched into pairs of two rendering to histo-pathological grading, lymph node involvement, hormone receptor type, TNM classification and survived patients vs. deceased patients breast cancer associated. The 200 patient samples that were investigated contained 160 patients that were still alive at last observation after end of therapy and 40 patients that had deceased during therapy tumour associated. Furthermore, the groups considered contained 98 patients graded G2 and 102 patients graded G3. Matching criteria of the 200 breast cancer patients did not allow patients graded G1. Tumour stage of the anamnestic diagnosis was categorised according to the TNM-classification, which was conducted correspondingly to the WHO System [22]. The matching of patients was executed according to the criteria at the time of primary diagnosis. The histo-pathological grading was classified conferring to the Bloom and Richardson system classification [23].

#### Collection of blood samples and Detection of CTCs

Method was conducted as defined by the SUCCESS Study group [8]. CTCs were examined using the CellSearch System (Veridex, Raritan, NJ). Peripheral blood was drawn into three CellSave tubes (3x10 mL – Serum

Vacutainer from BD Ref. Nr. 367896), sent at room temperature to the central laboratory at the University of Munich, and inspected within 96 h of collection. Consequently, the patient sera was frozen at  $-80^{\circ}\text{C}$  and seasoned in Nitrogen for long-term storage.

The patient blood samples were then centrifuged for 10 min at  $800 \times g$ . The plasma was removed, and a dilution buffer was added. This arrangement was overlaid on 6 mL of Histopaque (Sigma, Steinheim, Germany) and centrifuged for 10 min at  $400 \times g$ . Subsequently, 7.5 mL of this sample enclosing the buffy coat was treated on the CellTracks AutoPrep system using the CellSearch Epithelial Cell Kit (Veridex). After immuno-magnetic enrichment with an anti-Epcam antibody, the cells were marked with fluorescent anticytokeratin (CK8, 18, 19–phycoerythrin) and anti-CD45 antibodies (CD45–allophycocyan), and 4,6-diamidino-2-phenylindole dihydrochloride was used to classify the intact cells.

The identification, documentation and enumeration of CTCs were achieved using the CellTracks Analyzer II. CTCs were stated as nucleated cells lacking CD45 and expressing cytokeratin. Two independent investigators assessed all positive samples. The samples with a minimum of one CTC per 30 mL of blood were considered as CTC positive.

#### Measurement of cytokines

ELISA was performed with recently developed multi cytokine/chemokine arrays (Meso Scale Discovery®, Rockville, USA) to screen the blood serum samples for the vascular markers sFlt1, PlGF, VEGF, VEGF-C and VEGF-D. The immunoassays were commercially available. We used anti-species MULTI-ARRAY 96-well plates for the development of a sandwich immunoassay. Each assay in the panel was verified individually for the Specificity by running single calibrator with single detection antibodies. Non-specific binding levels were less than 0.5 % for all assays. The 10 spot MULTI-SPOT plates were pre-coated with capture antibodies on independent and well defined spots that allowed us to immobilize a primary capture antibody against our protein of interest - specific for one of each vascular marker. Standards and samples were added to the appropriate wells. A standard curve was furthermore run with each assay. We firstly added the blood serum, calibrator and control. After that we incubated at room temperature with shaking for 2 h. After eliminating excess samples from the well with wash buffer, we added a solution containing the detection (anti-target) antibody conjugated with electrochemiluminescent labels over the course of two incubation periods. During incubation time, where time slots differed in each test, the target present in the sample bound to the capture antibody immobilized on the working electrode surface by the anti-species antibody. Recruitment of the labelled detection antibody by the bound target completed the sandwich. After a second shaking

incubation period (time differed for each test) wash buffer was used to eliminate the entire unbound enzymes and a MSD Read Buffer was added to produce the suitable chemical environment for electrochemiluminescence. We then loaded the plate into an MSD instrument (MESO QuickPlex SQ 120) for examination where voltage applied to the plate electrodes caused the captured labels to emit light. The instrument calculated the intensity of the emitted light to present a quantitative measure of the amount of the protein of interest that was present in the sample [24, 25] (see homepage: [www.mesoscale.com](http://www.mesoscale.com)).

### Statistical analysis

Statistical analysis was implemented using SPSS 22.0 (SPSS Inc., IBM, Chicago, IL). The outcomes collected were recorded and inserted into the SPSS database in the implied manner. We evaluated the relationship between each vascular marker: sFlt1, PIGF, VEGF, VEGF-C and VEGF-D and each matching criteria (1. CTC-Positive vs. CTC-Negative, 2. Patient survived vs. Patient deceased, 3. Grading G2 vs. Grading G3, 4. Lymph node involvement vs. No lymph node involvement, 5. Triple positive vs. Triple negative 6. Progesterone receptor-positive vs. Progesterone receptor-negative, 7. Oestrogen receptor-positive vs. Oestrogen receptor-negative, 8. Her 2/neu receptor-positive vs. Her 2/neu receptor-negative) in the total patient collective and also regarding each matching criteria alone, by the use of the non-parametric Spearman correlation coefficient. Each parameter to be considered needed to have a  $p$  value  $< 0.50$ . Statistical significant results within the Spearman correlation coefficient were then additionally assessed with the non-parametric Mann-Whitney- $U$ -test. Moreover, variables were scrutinized by the use of Box-Plot analysis. All statistical tests were considered significant at  $p < 0.05$ .

## Results

### CTC positive vs. CTC negative

In the total patient collective, statistical significant differences were shown for sFlt1 values in regard to the CTC-Status. Box-plot analysis revealed that CTC negative patients exposed increased sFlt1 expression opposed to the CTC positive breast cancer patients that showed decreased sFlt1 values. The spearman correlation coefficient assessed the  $p$ -value of 0.034, additionally supported by the Mann-Whitney- $U$ -Test  $p = 0.034$ , proving a significant correlation between CTC-status and sFlt1. In addition, ROC analysis was performed, exposing an AUC value of 0.413 (see Fig. 1a).

Furthermore, a statistical significant correlation was found for PIGF values concerning the CTC-status. Box-plot analysis identified significant enhanced PIGF values in CTC negative patients compared to patients being CTC positive. The spearman correlation coefficient assessed the

$p$ -value of 0.043 which was moreover supported by the Mann-Whitney- $U$ -Test  $p = 0.043$ . ROC analysis implemented an AUC value of 0.417. (see Fig. 1b).

Nevertheless, the statistical analysis confirmed no significant correlation in terms of the CTC-Status regarding the vascular markers VEGF, VEGF-C, VEGF-D.

### Patient survived vs. patient deceased

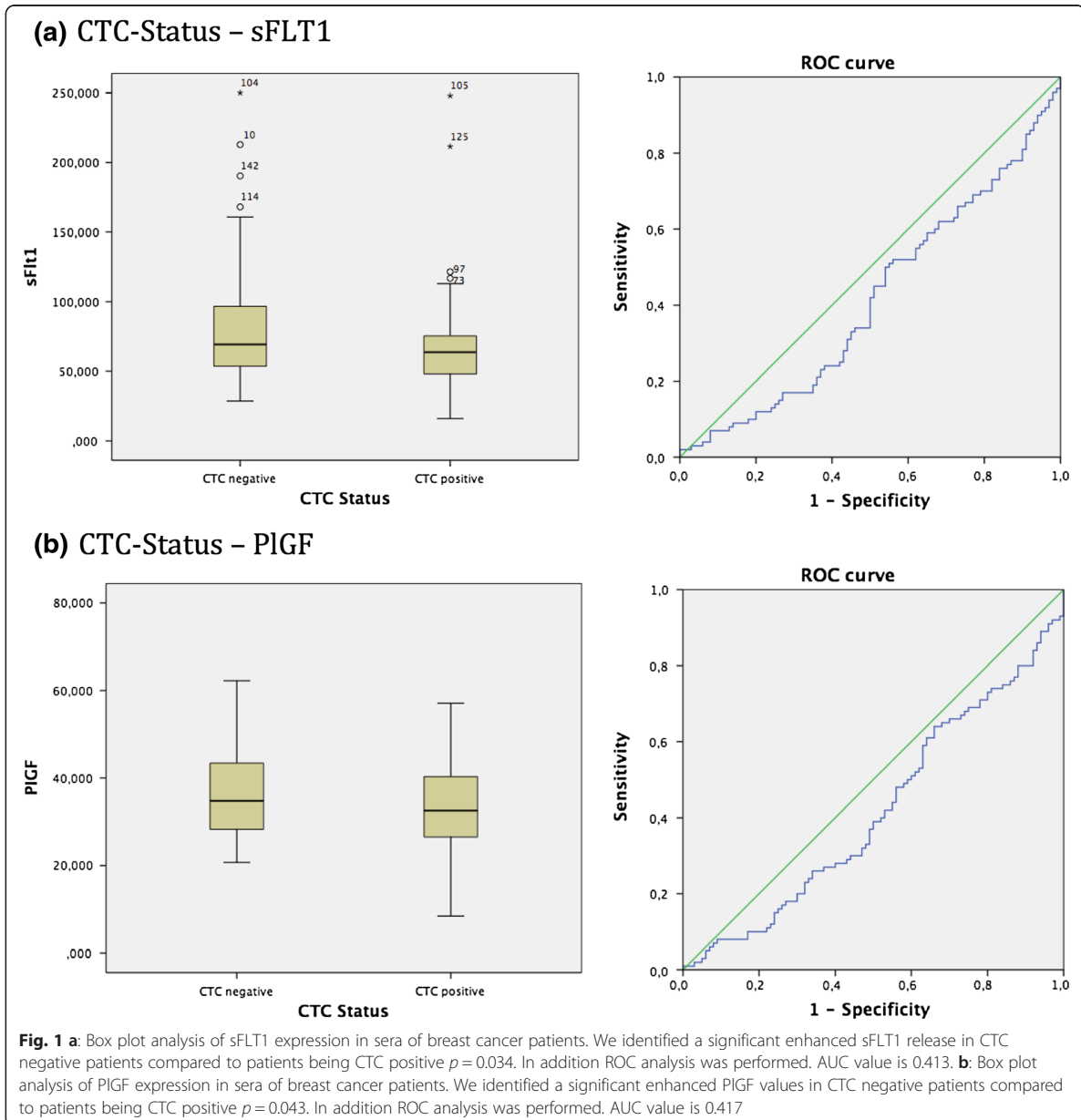
Analysing the patient collective who were still alive, and did not decrease breast cancer associated, these showed statistically significant differences between CTC negative and CTC positive patients in terms of the vascular marker sFlt1. The box-plot analysis revealed that the survived patients collective who were CTC negative display higher levels of sFlt1 compared to the reduced values of sFlt1 in the survived patients with the presence of CTCs. The spearman correlation coefficient assessed the  $p$ -value of 0.030 which was additionally supported by the Mann-Whitney- $U$ -Test  $p = 0.030$ . To continue, ROC analysis was performed, displaying an AUC value of 0.401 (see Fig. 2a).

Moreover, a statistical significant correlation was also proven for survived breast cancer patients being either CTC negative or CTC positive in respect to the vascular marker PIGF. The box-plot analysis disclosed that the survived patients collective who were CTC negative demonstrate increased PIGF expression in comparison to the decreased PIGF values in the survived patients being CTC positive. The spearman correlation coefficient evaluated the  $p$ -value of 0.025 which was furthermore reinforced by the Mann-Whitney- $U$ -Test  $p = 0.026$ . ROC analysis was executed, revealing an AUC value of 0.398 (see Fig. 2b).

However, the statistical analysis verified no significant correlation in the survived patient collective regarding the vascular markers VEGF, VEGF-C, VEGF-D. To conclude, statistical analysis also demonstrated no significant differences concerning the deceased patients who had died breast cancer associated, with and without the presence of CTCs, in respect to the vascular markers sFlt1, PIGF, VEGF, VEGF-C and VEGF-D.

### Lymph node involvement vs. no lymph node involvement

The patient collective with no lymph node metastasis indicated statistically significant differences between CTC negative and CTC positive breast cancer patients in regard to the vascular marker sFlt1. Box-plot analysis exposed that patients with no lymph node metastasis and CTC negativity demonstrated increased sFlt1 values in contrast to the reduced sFlt1 levels in patients with no lymph node metastasis and CTC positivity. The spearman correlation coefficient calculated the  $p$ -value of 0.039 which was furthermore sustained by the Mann-

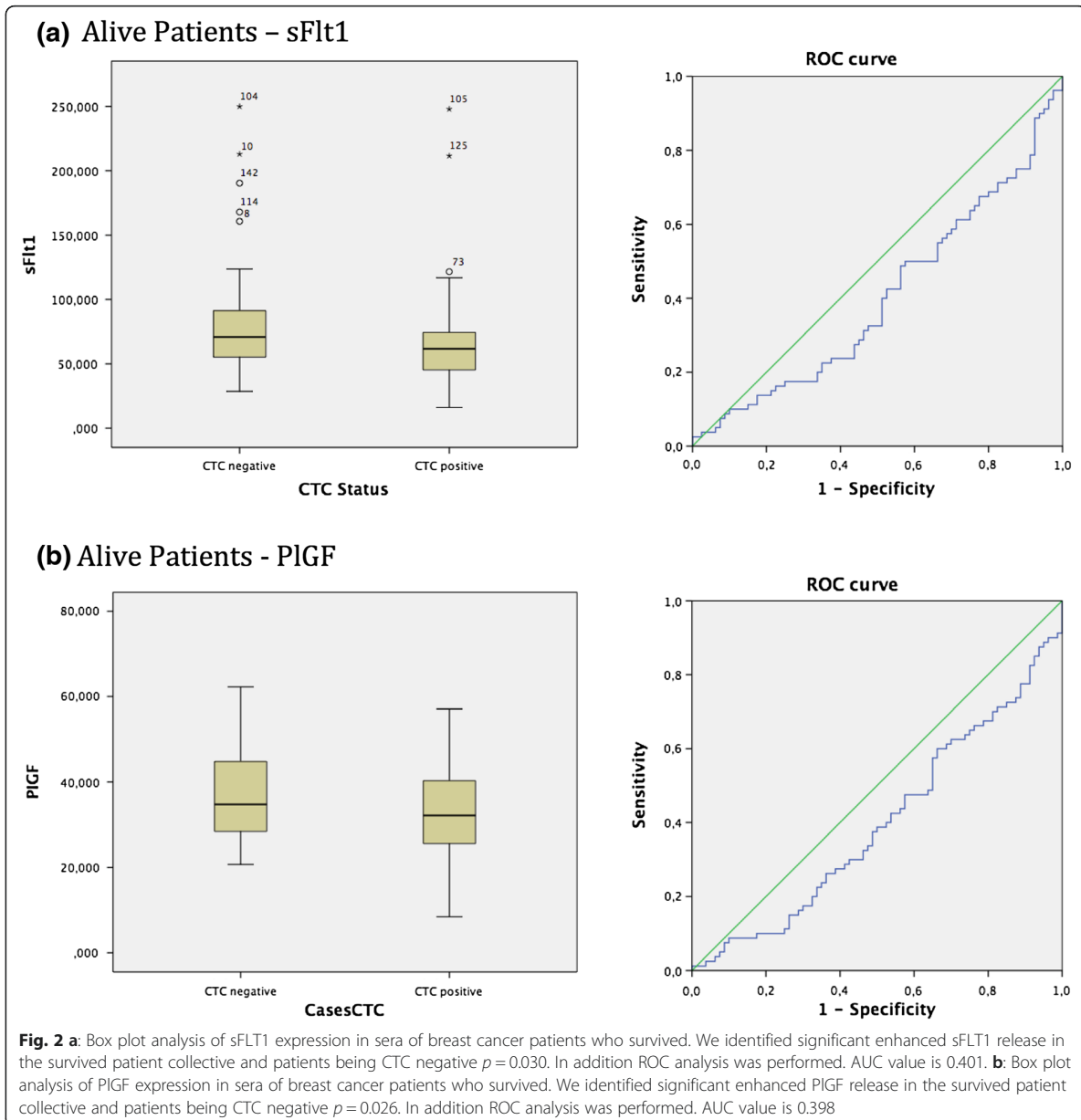


Whitney- $U$ -Test  $p = 0.041$ . ROC analysis assessed the AUC value of 0.350 (see Fig. 3a).

Nonetheless, statistical analysis demonstrated no significant correlation concerning patients without lymph node involvement in respect to the vascular markers PIGF, VEGF, VEGF-C and VEGF-D. Furthermore, patients with lymph node metastasis, with and without the presence of CTCs, displayed no significant difference in respect to the vascular markers sFlt1, PIGF, VEGF, VEGF-C and VEGF-D.

#### Grading G2 vs. Grading G3

The collective graded G2 showed significant correlations amongst patients with the presence or absence of CTCs in terms of the vascular marker sFlt1. The box-plot analysis identified that patients graded with a G2 breast cancer and furthermore being negative for CTCs display higher levels of sFlt1 in comparison to the decreased values of sFlt1 in G2 graded breast cancer with the presence of CTCs. The spearman correlation coefficient evaluated the  $p$ -value of 0.041 which was additionally



sustained by the Mann-Whitney- $U$ -Test  $p = 0.042$ . To continue, ROC analysis was performed, revealing an AUC value of 0.381 (see Fig. 4a). None of the other vascular markers tested revealed differences in expression patterns in terms of Grading.

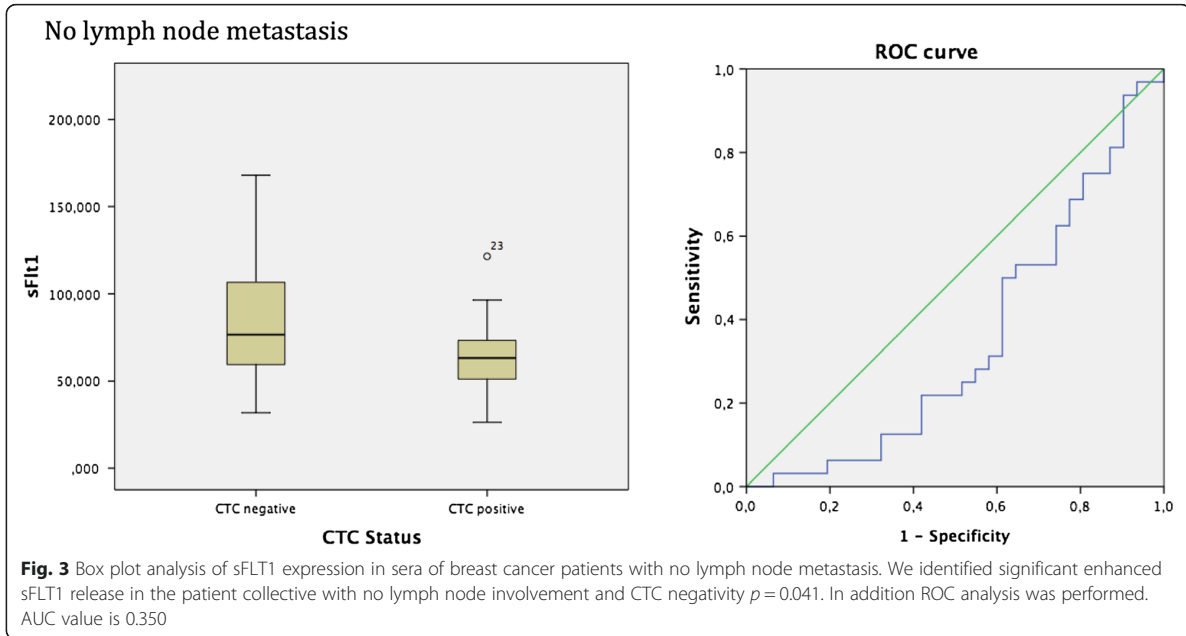
#### Hormone receptor type presence vs. absence

Furthermore, the statistical analysis also verified no significant correlation regarding the presence or absence of each single hormone receptor type; Progesterone receptor, oestrogen receptor, Her2/neu, in patients with breast

cancer with and without the presence of CTCs, with respect to the vascular markers sFlt1, PlGF, VEGF, VEGF-C and VEGF-D.

#### Triple positive vs. triple negative

Moreover, no statistically significant correlation could be demonstrated with the general comparison of a triple negative hormone receptor to a triple positive breast cancer with and without the presence of CTCs in terms of the vascular markers sFlt1, PlGF, VEGF, VEGF-C and VEGF-D.



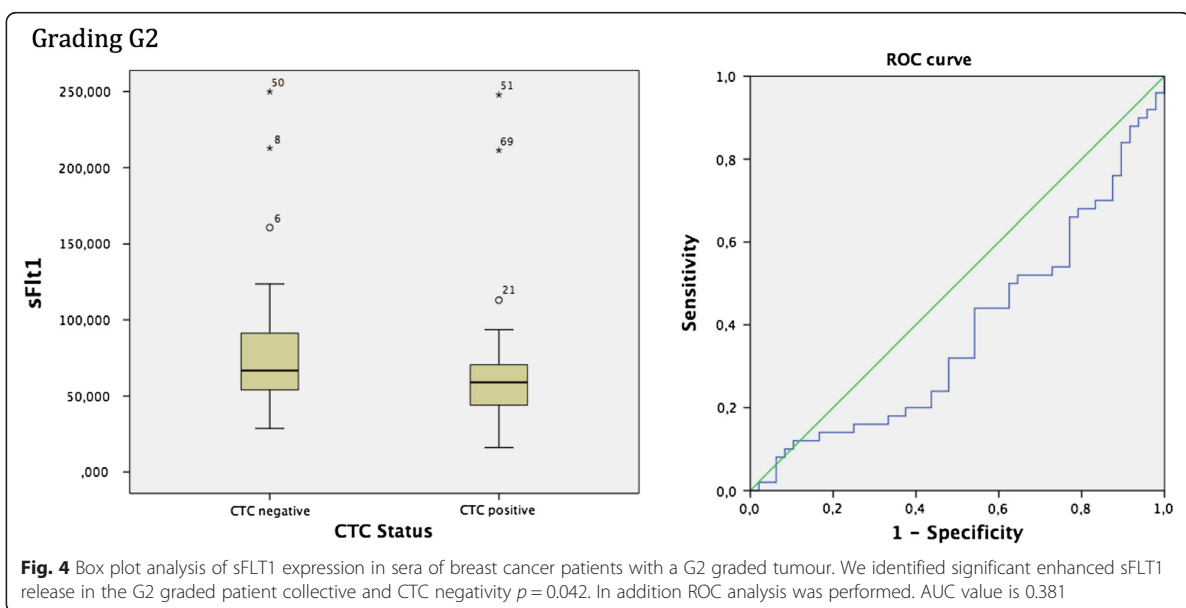
**Vascular marker correlation**

Significant correlation could be validated with the spearman correlation coefficient for the association between the vascular markers itself. The vascular markers examined were sFlt1, PIGF, VEGF, VEGF-C and VEGF-D. In regard to the total patient collective, with and without the presence of CTCs, significant correlations were found between sFlt1 and PIGF ( $p=0.000066$ ), sFlt1 and VEGF-C ( $p=0.022$ ), PIGF and VEGF ( $p=0.038$ ), VEGF

and VEGF-C ( $p=0.045$ ), VEGF-C and VEGF-D ( $p=0.0000001$ ) (see Fig. 5).

**Discussion**

Within this study we analysed the distribution of angiogenic markers: sFlt1, PIGF, VEGF, VEGF-C and VEGF-D and reveal the differences of their expression in the sera of breast cancer patients with and without circulating tumour cells. Significantly enhanced sFlt1 values were



Correlation								
			sFlt1	PlGF	VEGF	VEGF-C	VEGF-D	
Spearman-Rho	sFlt1	coefficient of correlation		.278**	.009	-.162*	-.083	
		Sig. (2-tail)		.000	.900	.022	.245	
		N		200	200	200	200	
	PlGF	coefficient of correlation		.278**		.147*	-.084	-.020
		Sig. (2-tail)		.000		.038	.235	.776
		N		200		200	200	200
	VEGF	coefficient of correlation		.009	.147*		.142*	-.019
		Sig. (2-tail)		.900	.038		.045	.790
		N		200	200		200	200
	VEGF-C	coefficient of correlation		-.162*	-.084	.142*		.363**
		Sig. (2-tail)		.022	.235	.045		.000
		N		200	200	200		200
	VEGF-D	coefficient of correlation		-.083	-.020	-.019	.363**	
		Sig. (2-tail)		.245	.776	.790	.000	
		N		200	200	200	200	

\*\* . Correlation is significant on the level 0.01 (2- tail).  
 \* . Correlation is significant on the level 0.05 (2- tail).

**Fig. 5** Spearman correlation of vascular markers: sFlt1, PlGF, VEGF, VEGF-C and VEGF-D. In regard to the total patient collective, significant correlation was found between: sFlt1 and PlGF ( $p = 0.000066$ ), sFlt1 and VEGF-C ( $p = 0.022$ ), PlGF and VEGF ( $p = 0.038$ ), VEGF and VEGF-C ( $p = 0.045$ ) and VEGF-C and VEGF-D ( $p = 0.0000001$ )

shown for the group of patients diagnosed with no CTCs. It is implied that sFlt1, which is the extra-cellular soluble domain of the VEGF receptor 1 (VEGFR1), traps VEGF thus acting as an important factor in the negative down regulation of angiogenesis [26, 27]. Furthermore, sFlt1 is described as an inhibiting factor of the pro-angiogenic VEGFR2 that acts as a positive signal conductor, whereas VEGFR-1 is a suppressor of VEGFR-2 signalling, consequently decreasing the neo-vascularisation stimulus [27, 28]. The tumour growth and the development of an invasive, aggressive and metastatic breast cancer are essentially reliant on the neo-vascularisation to provide blood supply for the nourishment and growth of the tumour. One can consequently suggest that the increased sFlt1 values in the CTC negative collective cause a significant disruption of tumour vascularisation, inhibiting CTCs being released into the peripheral blood. One could also suggest a significant additive inhibition delay in tumour growth, explaining and ensuing in the absence of CTCs in those patients.

This hypothesis is furthermore supported by increased levels of sFlt1 in the sera of the survived breast cancer patient collective and no CTC involvement. The ability of sFlt1 to bind and neutralize its target as it moves through the interstitial matrix is mostly described for one of the greatest powerful regulatory angiogenic agent VEGF [29, 30]. The role of sFlt1 has been previously shown to exercise a favourable outcome and advanced therapeutic effect in several tumour models [31]. Therefore, sFlt1 might have a major impact on anti-angiogenic activity by the requisitioning and neutralization of tumour secreted pro-angiogenic factors such as VEGF. This angiogenic inhibition might therefore contribute to

slower and decreased tumour growth, inhibiting circulation specific metastasis, thus enhancing survival and resulting in a favourable conduct of those breast cancer patients.

To continue, the group of patients with no lymph node metastasis and no CTC involvement in the peripheral blood also indicated significantly enhanced sFlt1 expression. It is hypothesized that sFlt1 inhibits endothelial cell proliferation and sprouting in the tumours microenvironment, resulting in a decrease of the total number of tumour blood vessels, as well as the number of perfused vessels, demonstrating a combined anti-tumour and anti-vascular effect [9, 28]. The tumours microenvironment in association to sFlt1 values might therefore benefit from its anti-angiogenic effects by successfully inhibiting lymphangiogenesis. One can consequently suggest that sFlt1 not only acts as an important factor in terms of down regulating neo-vascularisation hence decreasing tumour progression, but also influences lymph metastasis formation in favour of patient outcome.

Furthermore, in the sera of breast cancer patients with a G2 histologically graded tumour and CTC negativity, showed significantly enhanced sFlt1 expression. It is implicated that sFlt1 leads to a significant delay in tumour growth without altering the revascularization of ischemic peripheral tissue [26, 32]. Also, sFlt1 presumably does not directly control growth of the malignant tumour cells but is linked with a more favourable outcome through the restriction of tumour vascularization [33]. Therefore, significantly enhanced sFlt1 expressions in CTC negative patients indicate a correlation with medium differentiated (G2) breast cancer patients and resulting in a favourable breast cancer manner.



Similarly to the sFlt1 values pattern, significantly enhanced Placental growth factor (PlGF) values were shown for the group of patients diagnosed with no CTCs in contrast to CTC positive breast cancer patients. Furthermore, increased levels of PlGF were also proven in the sera of the survived breast cancer patient collective and no CTC involvement. sFlt1 and PlGF values therefore correlate in these patient collectives, both showing enhanced values.

PlGF also belongs to the VEGF family, which exclusively binds to the sFlt1 receptor (VEGFR1) [34]. The association between PlGF and sFlt1 has already been established, especially in terms of preeclampsia. Placental production of sFlt1 is increased during preeclampsia [35, 36], whereas PlGF and VEGF are decreased during active disease and several weeks before commencement of symptoms [37]. sFlt1 hereby acts as a potent anti-angiogenic factor, binding and neutralizing the pro-angiogenic proteins VEGF and PlGF, playing a key role in the inhibition of placental angiogenesis [38]. Nevertheless, the role of PlGF in terms of tumour angiogenesis and tumour growth remains controversial. Some studies claim that PlGF promotes tumour angiogenesis and tumour growth [39–41], although numerous other analyses indicated that overexpression of PlGF in tumour cells suppresses tumour neovascularization and growth [42–47]. Research implies that in addition to forming homodimers, PlGF and VEGF can also form heterodimers that contrary to prior evidence may be inactive and function as inhibitors of tumour angiogenesis [11, 43, 48]. Therefore, PlGF may negatively modulate VEGF-induced angiogenesis by formation of biologically inactive heterodimers [43, 46, 49]. It is furthermore implied that PlGF in tumours significantly normalizes tumour vessels against vascular leakage, whereas blocking sFlt1 leads to an increased vascular leakage, thus causing a less favourable outcome [47]. Our results in terms of enhanced PlGF values in the CTC negative patient collective and furthermore in the survived breast cancer patient collective and no CTC involvement supports the implication that tumour derived PlGF negatively modulates tumour angiogenesis and tumour growth [47]. This hypothesis is furthermore supported by the increased levels of sFlt1 in these patient collectives as it is presumed that PlGF stimulates the proliferation of cell types that express sFlt1 [50]. Therefore one can suggest that the tumour derived PlGF in our breast cancer patients suppresses tumour angiogenesis, tumour growth and metastasis by a probable mechanism including PlGF homodimers or PlGF–VEGF heterodimers, activating a negative neovascularisation feedback via sFlt1 activation. Moreover, this hypothesis is reinforced by the significant correlations amongst the vascular markers. Significant correlation between sFlt1 and PlGF emphasize their association as well as the significant correlation between

PlGF and VEGF, implying the presence of PlGF–VEGF heterodimers.

These findings demonstrate the functional interaction of sFlt1 and PlGF, suggesting that their overexpression in tumour cells inhibits CTCs entering the peripheral blood, thus emphasising a significant anti-angiogenic effect, inhibiting tumour growth and metastasis. Furthermore, in regard to CTC negativity, sFlt1 and PlGF values may potentially serve as predictive markers.

### Conclusion

Circulating tumour cells (CTCs) are a prognostic marker for reduced disease free survival, breast cancer–specific survival, and overall survival. CTC negative patients displayed increased sFlt1 expression opposed to CTC positive breast cancer patients. Furthermore, significant enhanced PlGF values were also disclosed in CTC negative patients compared to patients being CTC positive. In former studies, a functional interaction of sFlt1 and PlGF was found. Results of our study suggest that their overexpression in tumour cells inhibits CTCs entering the peripheral blood. Furthermore, in regard to CTC negativity, sFlt1 and PlGF values may potentially serve as predictive markers.

### Abbreviations

CTC, circulating tumour cells; sFlt1, soluble fms-like tyrosine kinase-1; PlGF, phosphatidylinositol-glycan biosynthesis class F protein; VEGF, vascular endothelial growth factor

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### Availability of data and materials

Information about the study is published on the homepage of the study (<http://www.success-studie.de>).

### Authors' contributions

TV performed the study and made substantial contributions to acquisition and interpretation of data. BR was involved in drafting the manuscript and revising it critically for important intellectual content. WJ made the conception of the Success study and was involved in revising the manuscript. UJ made conception and design of the specific study. TW was involved in acquisition of data, analysis and interpretation of data and has given final approval of the version to be published. All authors have read and approved the manuscript.

### Competing interests

The authors declare no conflict of interest.

### Consent for publication

All patients used for this study gave written informal consent for publication of the study results.

**Ethics approval and consent to participate**

The study was permitted by 37 German ethical boards (lead ethical board: LMU, Munich) and conducted in agreement with the Declaration of Helsinki. All patients used for this study gave written informal consent to participate.

**Studies involving animals**

Not applicable.

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## 2.2. Publication 2

*Title:*

Influence of circulating tumour cells on production of IL-1 $\alpha$ , IL-1 $\beta$  and IL-12 in sera of patients with the primary diagnosis of breast cancer before treatment

*Authors:*

Theresa Vilsmaier, Brigitte Rack, Alexander König, Klaus Friese, Wolfgang Janni, Udo Jeschke, Tobias Weissenbacher and SUCCESS Study Group

*Journal:*

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*Abstract:*

**Background:** Circulating tumour cells (CTCs) have been found to be a prognostic marker for reduced disease free survival (DFS), distant DFS, breast cancer-specific survival, and overall survival (OS) before the start of systemic treatment. Determination of CTCs with the CellSearch System (Veridex, Raritan, NJ) is a valuable but time consuming and costly method. Therefore the aim of this study was the evaluation of cytokine profiles as marker for CTC involvement.

**Methods:** Patients chosen for this study were defined as women with breast cancer who agreed to participate in the phase I SUCCESS study. CTC analysis, the blood sampling time points, and the methodology were prospectively designed, and the prognostic value of the CTCs was defined as a scientific objective of the study protocol. A total of 100 patients being positive for circulating tumour cells and additional 100 patients being negative for circulating tumour cells were matched into pairs of two. Matching criteria were histo-pathological grading, lymph node status, hormone receptor type, TNM classification and survived breast cancer patients vs. deceased tumour associated patients. Commercial ELISA was used to screen the blood serum samples for the TH1 cytokines: IFN- $\gamma$ , TNF- $\alpha$ , IL-12, IL-1 $\alpha$ , IL-1 $\beta$ , IL-2 and IL-18. The cytokine levels correlation to the matching criteria listed above was analysed with the Spearman correlation coefficient and the Mann-Whitney-U rank-sum test.

**Results:** CTC positive patient group indicated a significant difference in terms of lymph node involvement regarding IL-1 $\alpha$  ( $p=0,043$ ). The CTC negative collective exposed a

significant correlation regarding progesterone receptor positive/negative patients in terms of IL-1 $\beta$  (p=0,029). Furthermore, the living patient collective established significant differences in IL-12p40 levels in association to lymph node involvement (p=0,041) and triple negative hormone receptor breast cancer (p=0,043). Deceased patients on the other hand presented significant results within oestrogen receptor positive/negative patients in terms of IL-1 $\alpha$  (p=0,050) and IL-1 $\beta$  (p=0,034). Moreover, IL-1 $\alpha$  levels also indicated a significant correlation regarding triple negative hormone receptor breast cancer (p=0,033) within the deceased collective. To continue, the collective graded G2 showed significant correlations amongst patients with Her2/neu association regarding IFN- $\gamma$  levels (p=0,031) and in terms of lymph node involvement concerning IL-1 $\alpha$  levels (p=0,014). The patient group graded with G3 on the other hand revealed a significant correlation regarding progesterone receptor positive/negative patients in terms of IL12p70 Levels (p=0,048) and triple negative hormone receptor breast cancer with regard to IL12p40 levels (p=0,033).

**Conclusion:** Regarding CTC involvement we found significant differences in IL-1 $\alpha$  secretion in CTC positive patients. Increased values of this cytokine were found in sera of patients without lymph node involvement. Therefore we may speculate that IL-1a might be a marker for the release of tumour cells into the circulation and not in the lymphatic system. In addition, also Il-1b was found to correlate to CTC release. Only CTC negative patients showed a correlation of the progesterone receptor expression and Il-1b release. Therefore we conclude that as well as IL-1a as IL-1b are connected to CTC release of breast cancer patients.

## Influence of Circulating Tumour Cells on Production of IL-1 $\alpha$ , IL-1 $\beta$ and IL-12 in Sera of Patients with Primary Diagnosis of Breast Cancer Before Treatment

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**Abstract.** *Background:* Circulating tumour cells (CTCs) have been found to be a prognostic marker for reduced disease-free survival (DFS), distant DFS, breast cancer-specific survival, and overall survival (OS) before the start of systemic treatment. Determination of CTCs with the CellSearch System (Veridex, Raritan, NJ, USA) is a valuable but time-consuming and costly method. Therefore, the aim of this study was to evaluate cytokine profiles as a marker for CTC involvement. *Patients and Methods:* Patients chosen for this study were defined as women with breast cancer who agreed to participate in the phase I SUCCESS study. CTC analysis, the blood sampling time points, and the methodology were prospectively designed, and the prognostic value of the CTCs was defined as a scientific objective of the study protocol. A total of 100 patients positive for CTCs and an additional 100 patients negative for CTCs were matched into pairs. Matching criteria were histopathological grading, lymph node status, hormone receptor type, TNM classification and survival vs. tumour associated death. Commercial enzyme-linked immunosorbent assay (ELISA) was used to screen the blood serum samples for the Th1 cytokines: interferon gamma (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin 12 (IL-12), IL-1 $\alpha$ , IL-1 $\beta$ , IL-2 and IL-18. The correlation of cytokine levels to the matching criteria listed above were analyzed with the Spearman correlation coefficient and the Mann-Whitney-U rank-sum test. *Results:* The IL-1 $\alpha$  level was significantly lower in the CTC-positive patient group ( $p=0.043$ ) but significantly

higher in the CTC-negative progesterone receptor-positive collective ( $p=0.029$ ). Furthermore, in patients who survived, significantly higher IL-12p40 levels were found in those with lymph node involvement ( $p=0.041$ ) and those with triple-negative breast cancer ( $p=0.043$ ). Of patients who died, those with oestrogen receptor-negative disease had higher IL-1 $\alpha$  ( $p=0.050$ ) and higher IL-1 $\beta$  ( $p=0.034$ ) levels. Moreover, of those who died, those with triple-negative breast cancer had significantly higher IL-1 $\alpha$  levels ( $p=0.033$ ). In patients with grade 2 tumour, patients with HER2/neu expression had significantly higher IFN- $\gamma$  levels ( $p=0.031$ ) and those with no lymph node involvement had significantly higher IL-1 $\alpha$  levels ( $p=0.014$ ). In the collective with grade 3 tumour, patients with progesterone receptor-negative disease had significantly higher IL12p70 concentrations ( $p=0.048$ ), while those with triple-negative breast cancer had lower IL12p40 levels ( $p=0.033$ ). *Conclusion:* Regarding CTC involvement, we speculate that IL-1 $\alpha$  might be a marker for the release of tumour cells into the circulation and not into the lymphatic system. In addition, IL-1 $\alpha$  like IL-1 $\beta$  appears to be related to CTC release in patients with breast cancer.

Analyses with the highest level of evidence have established a correlation between poor prognosis and the detection of circulating tumour cells (CTCs) before the start of systemic treatment (1). CTCs in the peripheral blood have been found to be a prognostic marker for reduced disease-free survival (DFS), distant DFS, breast cancer-specific survival, and overall survival (OS) before the start of systemic treatment (1-5). Furthermore, CTC values shortly after commencing therapy provide complementary information concerning the treatment response (5). The SUCCESS study was one of the first trials to determine the strong prognostic association of CTCs with poorer survival in early breast cancer before the start of systemic adjuvant treatment and after adjuvant chemotherapy in a large patient cohort (6).

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*Key Words:* Breast cancer, IL-1 $\alpha$ , IL-1 $\beta$ , IL-12, circulating tumor cells.

Nevertheless, the determination of CTCs is time-consuming and costly. Given the increasing significance of cytokine measurement in cancer therapy, the aim of this study was the evaluation of cytokine profiles as a marker for CTC involvement.

T-Lymphocytes and their individual differentiation depending on cytokines and chemokines are a crucial component of the adaptive immune response, presumed to be an important prognostic factor in the presence of breast cancer tumours (7). The differential expression of cytokines that stimulate helper T-lymphocyte maturation can result in stimulation or suppression of critical cell derivation (8, 9). Different interactions lead to the expression of either pro-inflammatory or anti-inflammatory cytokines (10). Therefore our intention was to analyse the distribution of T-helper 1 (Th1), Th2, Th17, regulatory T-cells (Treg) and Th9 cytokines of the T-lymphocyte immune response and reveal differences in cytokine levels in patients with breast cancer of the SUCCESS study group. We studied this with respect to CTC involvement, histopathological grading, lymph node status, hormone receptor type, TNM classification and survival vs. tumour-associated death.

This article focuses on the expression of the Th1 cytokines: Interferon gamma (IFN- $\gamma$ ), tumour necrosis factor alpha (TNF- $\alpha$ ), interleukin 12 (IL-12), IL-1 $\alpha$ , IL-1 $\beta$ , IL-2 and IL-18. Th1 cytokines have been presumed to play a major role in phagocytic and intracellular defence (8). Analyses imply that patients with breast cancer present specific Th1 expressions that correlate with TNM stage and lymph node involvement. Higher IFN- $\gamma$  values, for instance, correlated with a positive outcome in patients with breast cancer (11), whereas the presence of the IL-1 system was found to correlate inversely with local sex steroid receptor expression, emphasizing a increased malignant behaviour (12). Moreover, IL-12 is assumed to be one of the most critical cytokines for the induction of Th1 responses (8).

The evaluation of TH1 cytokine profiles as a marker for CTC involvement could benefit assessment of the individual risk of patients at the time of primary diagnosis. Correlation of Th1 cytokines to CTC involvement could offer a new clinical prospective in terms of tumour phenotype in order to enhance individualized treatments.

## Materials and Methods

*SUCCESS study design.* SUCCESS was a prospective, randomized adjuvant study comparing three cycles of fluorouracil–epirubicin–cyclophosphamide (FEC; 500/100/500 mg/m<sup>2</sup>) followed by three cycles of docetaxel (100 mg/m<sup>2</sup>) every 3 weeks vs. three cycles of FEC followed by three cycles of gemcitabine (1,000 mg/m<sup>2</sup> d1,8) docetaxel (75 mg/m<sup>2</sup>) every 3 weeks. Following the completion of chemotherapy, the patients were further randomized to receive either 2 or 5 years of bisphosphonate therapy with zoledronate. Hormone receptor-positive women received suitable endocrine treatment.

Eligible patients were defined as women with breast cancer (stages pT1–T4, pN0–N3, M0) who were approved to participate in the phase I SUCCESS study ([www.success-studie.de](http://www.success-studie.de)).

The research questions associated with CTC analysis, the blood sampling time points, and the methodology were prospectively designed, and the prognostic value of the CTCs was described as a scientific objective of the study protocol. The study was permitted by 37 German ethical boards (lead ethical board: LMU, Munich) and conducted in agreement with the Declaration of Helsinki.

*Blood sample collection for CTC enumeration.* Blood samples for CTC enumeration were taken from 2,090 consecutive patients after complete resection of the primary tumour and before adjuvant chemotherapy after written informed consent was acquired. Sixty-four patients were excluded because of test failure or a time interval of more than 96 h between the blood collection and sample preparation. A follow-up evaluation after chemotherapy and before the commencement of endocrine or bisphosphonate treatment was available for a subgroup of 1,492 patients (see homepage: <http://www.success-studie.de>).

The method was conducted as described by the SUCCESS study group (6). CTCs were investigated using the CellSearch System (Veridex, Raritan, NJ, USA). Peripheral blood was drawn into three CellSave tubes (30 ml), sent at room temperature to the central laboratory at the University of Munich, and examined within 96 hours of collection.

The patient samples were then centrifuged for 10 minutes at 800 × g. The plasma was removed, and a dilution buffer was added. This combination was overlaid on 6 ml of Histopaque (Sigma, Steinheim, Germany) and centrifuged for 10 minutes at 400 × g. Subsequently, 7.5 ml of this sample enclosing the buffy coat was treated on the CellTracks AutoPrep system using the CellSearch Epithelial Cell Kit (Veridex). After immuno-magnetic enrichment with an antibody to epithelial cell adhesion molecule (EpCAM), the cells were marked with fluorescent anti-cytokeratin (CK8,18,19–phycoerythrin) and anti-CD45 (CD45–allophycocyan) antibodies, and 4,6-diamidino-2-phenyl-indole-dihydrochloride was used to identify the intact cells.

*Patients included.* In this study, 200 patients of the SUCCESS study were included and assigned into two groups: 100 Patients were CTC-positive (group 1) and the other 100 patients were CTC-negative (group 2). These two groups were then framed and investigated accordingly. Patients from the respective groups were then matched into pairs of two according to histopathological grading, lymph node involvement, hormone receptor type, TNM classification and survival vs. tumour associated death. Out of 200 patient samples that were investigated, 160 patients were still alive at the last observation after the end of therapy and 40 patients had died from their tumour during therapy. The groups investigated included 98 patients with tumour graded G2 and 102 patients graded G3. Matching criteria of the 200 patient collective did not allow patients with grade G1. Tumour stage of the anamnestic diagnosis was classified according to the TNM classification, which was conducted accordingly to the WHO system (13). The matching of patients was performed according to the criteria at the time of primary diagnosis. The histopathological grading was classified according to the Bloom and Richardson system classification (14).

*Detection of CTCs and cytokine determination.* The identification and enumeration of CTCs were achieved using CellTracks Analyzer

II. CTCs were defined as nucleated cells lacking CD45 and expressing cytokeratin. All positive samples were assessed by two independent investigators. Samples with a minimum of one CTC per 30 ml of blood were regarded as CTC-positive.

The blood from 84 persons with no clinical evidence of malignant disease was processed blinded and used as a negative control. Four of these negative controls (4.9%) contained cells that fit the definition of epithelial cells and which could be interpreted as CTCs (one control had one, two controls had two, and one control had three epithelial cells). For the measurement of cytokines a commercial enzyme-linked immunosorbent assay (ELISA) was used to screen the blood serum samples for the Th1 cytokines: IFN- $\gamma$ , TNF- $\alpha$ , IL-12, IL-1 $\alpha$ , IL-1 $\beta$ , IL-2 and IL-18. The ELISA kits used were acquired by Meso Scale Discovery® (Rockville, MD, USA). We used anti-species multi-array 96-well plates for the development of a sandwich immunoassay (see Figure 1). The 10 spot Multi Spot plates were pre-coated with capture antibodies on independent and well defined spots that enabled us to immobilize a primary capture antibody against the protein of interest - specific for each cytokine (IFN- $\gamma$ , TNF- $\alpha$ , IL-12, IL-1 $\alpha$ , IL-1 $\beta$ , IL-2 and IL-18). Standards and samples were also added to the appropriate wells. A standard curve was run with each assay. We firstly added the blood serum, calibrator and control then incubated plates at room temperature with shaking for 2 h. After removing excess sample from the well with wash buffer, we added the solution containing the detection (anti-target) antibody conjugated with electrochemiluminescent labels over the course of two incubation periods. During incubation, where time slots differed in each test, the target present in the sample bound to the capture antibody immobilized on the working electrode surface by the anti-species antibody. Recruitment of the labelled detection antibody by the bound target completed the sandwich. After a second shaking and incubation (time differed for each test), wash buffer was used to remove the entire unbound enzymes and an MSD Read Buffer was added to produce the appropriate chemical environment for electrochemiluminescence. We then loaded the plate into an MSD instrument (MESO QuickPlex SQ 120) for analysis where voltage applied to the plate electrodes caused the captured labels to emit light. The instrument measured the intensity of the emitted light to present a quantitative measure of the amount of the protein of interest that was present in the sample (15, 16) (see homepage: www.mesoscale.com).

**Statistical analysis.** Statistical analysis was accomplished using SPSS 22.0 (IBM Corp., Armonk, NY, USA).

We evaluated the relationship between each cytokine of the Th1 group (IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-12 and IL-18) and each matching criterion (CTC-positive *vs.* CTC-negative; survival *vs.* death; grade 2 *vs.* grade 3; lymph node involvement *vs.* no lymph node involvement; triple hormone receptor-positive *vs.* triple hormone receptor-negative; progesterone receptor-positive *vs.* progesterone receptor-negative; oestrogen receptor-positive *vs.* oestrogen receptor-negative; HER2/neu receptor-positive *vs.* HER2/neu receptor-negative) by using the non-parametric Spearman correlation coefficient. Each parameter to be considered was required to have a *p*-value of less than 0.05. Statistically significant results for the Spearman correlation coefficient were then assessed with the non-parametric Mann-Whitney-U rank-sum test. Moreover, variables were examined by the use of box-plot analysis. All statistical tests were considered significant at *p*<0.05.

## Results

**CTC-positive *vs.* CTC-negative patients.** In the CTC-positive patient group, patients with no lymph node involvement had high levels of IL-1 $\alpha$ , whereas those with lymph node involvement expressed low levels of IL-1 $\alpha$  (Spearman correlation coefficient *p*=0.042, Mann-Whitney *U*-test *p*=0.043; Figure 1a).

On the other hand, analysis of the CTC-negative collective showed that patients with progesterone receptor-negative disease had higher levels of IL-1 $\beta$  in comparison to those with progesterone receptor-positive disease (Spearman correlation coefficient *p*=0.028, Mann-Whitney *U*-test *p*=0.029; Figure 1b).

However, statistical analysis found no significant difference according to the presence or absence of CTCs in patients with breast cancer in regard to the levels of IFN- $\gamma$ , TNF- $\alpha$ , IL-2 and IL-18.

**Patient survival.** The box plot analysis of the collective of patients who remained alive revealed that patients with lymph node involvement also had higher levels of IL-12p40. (Spearman correlation coefficient *p*=0.041, Mann-Whitney *U*-test *p*=0.041; Figure 2a). Statistically significant correlations were also proven for patients with triple hormone receptor-negative compared to -positive breast cancer, with the former displaying significantly higher levels of IL-12p40. (Spearman correlation coefficient *p*=0.042, Mann-Whitney *U*-test *p*=0.043; see Figure 2b).

In comparison, in the group of patients who died, patients with oestrogen receptor-negative tumour had higher levels of IL-1 $\alpha$  and IL-1 $\beta$  (Spearman correlation coefficient *p*=0.049, Mann-Whitney *U*-test *p*=0.050 for IL-1 $\alpha$ ; Spearman correlation coefficient *p*=0.032, Mann-Whitney *U*-test *p*=0.034 for IL-1 $\beta$ ; Figure 3b and c respectively). A significant correlation was also found for patients with triple hormone receptor-negative breast cancer having higher levels of IL-1 $\alpha$ . (Spearman correlation coefficient *p*=0.031, Mann-Whitney *U*-test *p*=0.033; Figure 3a).

Statistical examination confirmed no significant difference regarding the survival or death of patients with breast cancer in regard to the levels of IFN- $\gamma$ , TNF- $\alpha$ , IL-2, and IL-18.

**Grading 2 *vs.* grade 3 tumour.** In the collective with grade 2 tumour, patients with no lymph node involvement had higher levels of IL-1 $\alpha$  (Spearman correlation coefficient *p*=0.013, Mann-Whitney *U*-test *p*=0.014; Figure 4a). Patients with grade 2 HER2/neu-positive breast cancer also had higher levels of IFN- $\gamma$  (Spearman correlation coefficient *p*=0.030, Mann-Whitney *U*-test *p*=0.031; Figure 4b).

In patients with grade 3 tumours, those with progesterone receptor-negative disease had higher levels



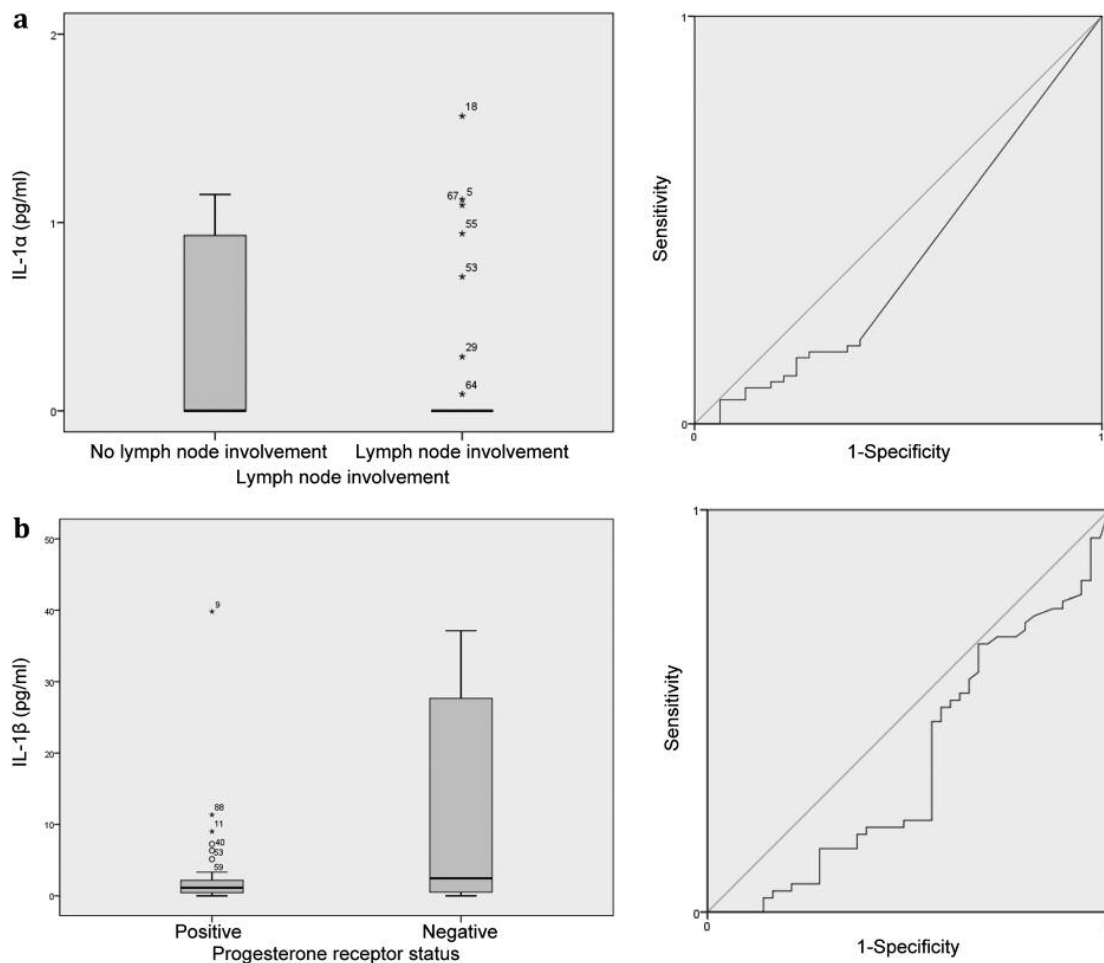


Figure 1. *a*: Left panel: Box plot analysis of interleukin 1-alpha (IL-1 $\alpha$ ) expression in sera of patients with circulating tumour cell (CTC)-positive breast cancer. IL-1 $\alpha$  release was significantly higher in patients without lymph node metastasis compared to patients with lymph node involvement ( $p=0.043$ ). Right panel: Receiver operator curve analysis of sensitivity versus specificity gave an area under the curve (AUC) of 0.402. *b*: Left panel: Box plot analysis of interleukin 1 beta (IL-1 $\beta$ ) expression and patients with CTC-negative disease. IL-1 $\beta$  release was significantly higher in those with progesterone receptor-negative compared to those with progesterone receptor-positive breast cancer ( $p=0.029$ ). Right panel: Receiver operator curve analysis of sensitivity versus specificity gave an AUC of 0.372. Boxes indicate the 25th and 75th percentiles, with a horizontal line at the median and bars display the 5th and 95th percentiles. Circles specify values more than 1.5 box lengths. Asterisks specify values (marked with a number) more than 3.0 box lengths from the 75th percentile.

of IL-12p70 (Spearman correlation coefficient  $p=0.048$ ; Mann-Whitney  $U$ -test  $p=0.048$ ; Figure 5a). Patients with grade 3 tumours had a significantly higher IL-12p40 release in those with triple hormone receptor-positive status compared to those with triple-negative disease (Spearman correlation coefficient  $p=0.032$ ; Mann-Whitney  $U$ -test  $p=0.033$ ; Figure 5b). None of the other cytokines tested revealed differences in expression patterns in terms of tumour grading.

*Lymph node involvement.* Statistical analysis demonstrated no significant difference concerning lymph node involvement or no lymph node involvement in patients with breast cancer with and without the presence of CTCs in respect to the cytokine levels of IFN- $\gamma$ , TNF- $\alpha$ , IL-12, IL-1 $\alpha$ , IL-1 $\beta$ , IL-2 and IL-18.

*Hormone receptor type presence vs. absence.* Furthermore, the statistical analysis also verified no significant difference regarding the presence or absence of single hormone receptor

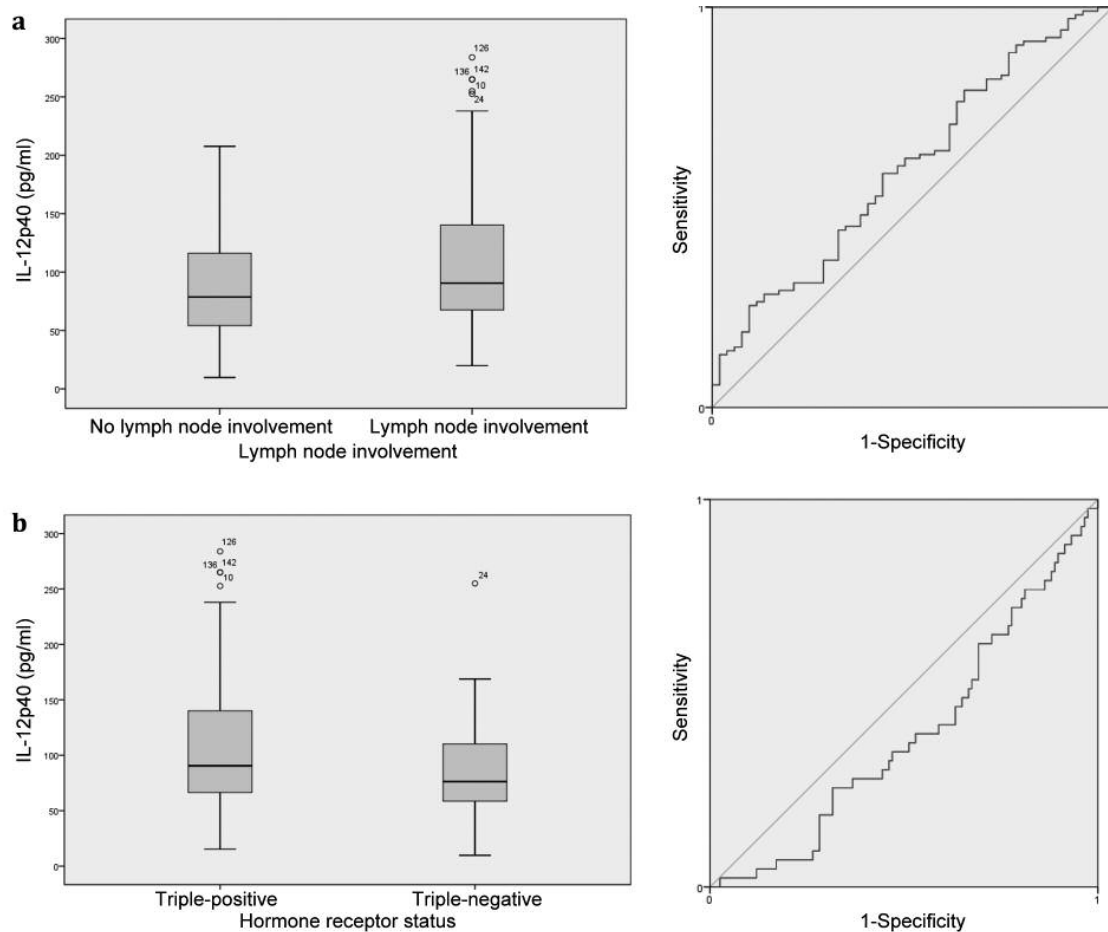


Figure 2. Box plot analysis of interleukin 12p40 (IL-12p40) expression in sera of patients who remained alive [regardless of circulating tumour cell (CTC) status] according to lymph node involvement (a) and hormone receptor status (b). IL-12p40 release was significantly higher in patients who remained alive and had lymph node metastasis compared to patients without lymph node involvement ( $p=0.041$ ) (a, left panel), and in patients with triple-positive hormone receptor status compared to those with triple-negative hormone receptor disease ( $p=0.043$ ) (b, left panel). Receiver operator curve analysis of sensitivity versus specificity gave an area under the curve of 0.599 (a, right panel) and 0.395 (b, right panel) respectively. Boxes indicate the 25th and 75th percentiles, with a horizontal line at the median and bars display the 5th and 95th percentiles. Circles specify values more than 1.5 box lengths. Asterisks specify values (marked with a number) more than 3.0 box lengths from the 75th percentile.

types (progesterone receptor, oestrogen receptor, HER2/neu) in patients with breast cancer with and without the presence of CTCs in regards to the levels of IFN- $\gamma$ , TNF- $\alpha$ , IL-12, IL-1 $\alpha$ , IL-1 $\beta$ , IL-2 and IL-18.

*Triple positive vs. triple negative.* No statistically significant correlation was demonstrated with the general comparison of triple-negative to triple-positive breast cancer with and without the presence of CTCs in respect to the cytokine levels of IFN- $\gamma$ , TNF- $\alpha$ , IL-12, IL-1 $\alpha$ , IL-1 $\beta$ , IL-2 and IL-18.

## Discussion

In this study, we described Th1-derived cytokines in the sera of patients with breast cancer with and without CTCs. The group of patients diagnosed with CTCs and no lymph node involvement had significantly enhanced IL-1 $\alpha$  levels. IL-1 $\alpha$  is a Th1 cytokine derived from tumour cells. It is hypothesized that cell senescence increases IL-1 $\alpha$  expression and creates a microenvironment that is conducive to metastatic disease progression in patients with cancer (17). Therefore IL-1 $\alpha$  might be involved in

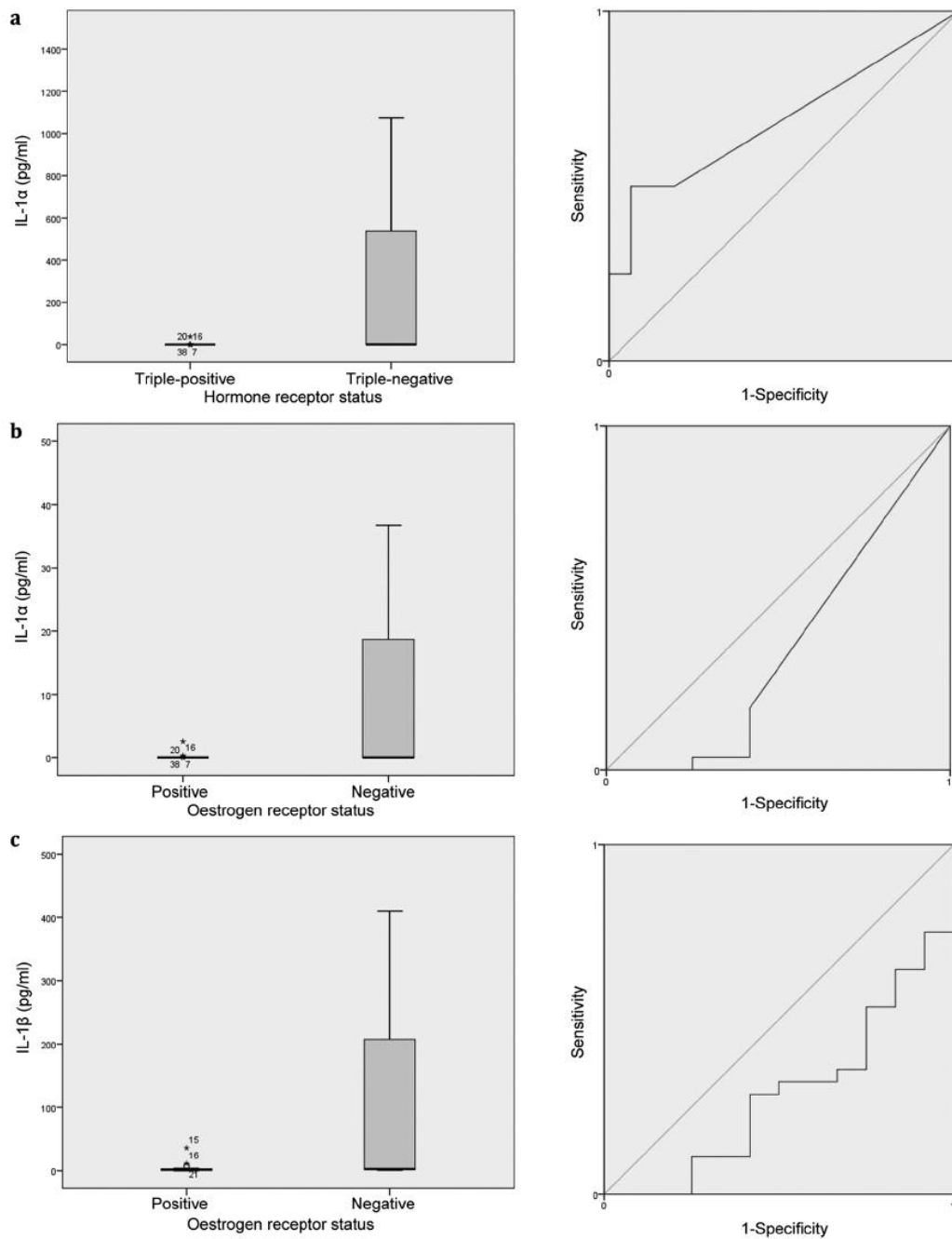


Figure 3. a: Box plot analysis of interleukin 1-alpha ( $IL-1\alpha$ ) expression in sera of patients who died from their disease [regardless of circulating tumour cell (CTC) status] according to triple hormone receptor status (a) and oestrogen receptor status (b) and of  $IL-1\beta$  according to oestrogen receptor status (c). Significantly higher  $IL-1\alpha$  release was found in patients who died with triple-negative hormone receptor status compared to patients with triple-positive hormone receptor ( $p=0.031$ ) (a, left panel), and in those with oestrogen receptor-negative compared to those with oestrogen receptor-positive cancer ( $p=0.049$ ) (b, left panel). Similarly, we identified significantly increased  $IL-1\beta$  release in those with oestrogen receptor-negative compared to those with oestrogen receptor-positive cancer ( $p=0.034$ ) (c, left panel). Receiver operator curve analysis of sensitivity versus specificity gave an area under the curve of 0.688, 0.350 and 0.286 (right panel, a, b and c, respectively). Boxes indicate the 25th and 75th percentiles, with a horizontal line at the median and bars display the 5th and 95th percentiles. Circles specify values more than 1.5 box lengths. Asterisks specify values (marked with a number) more than 3.0 box lengths from the 75th percentile.

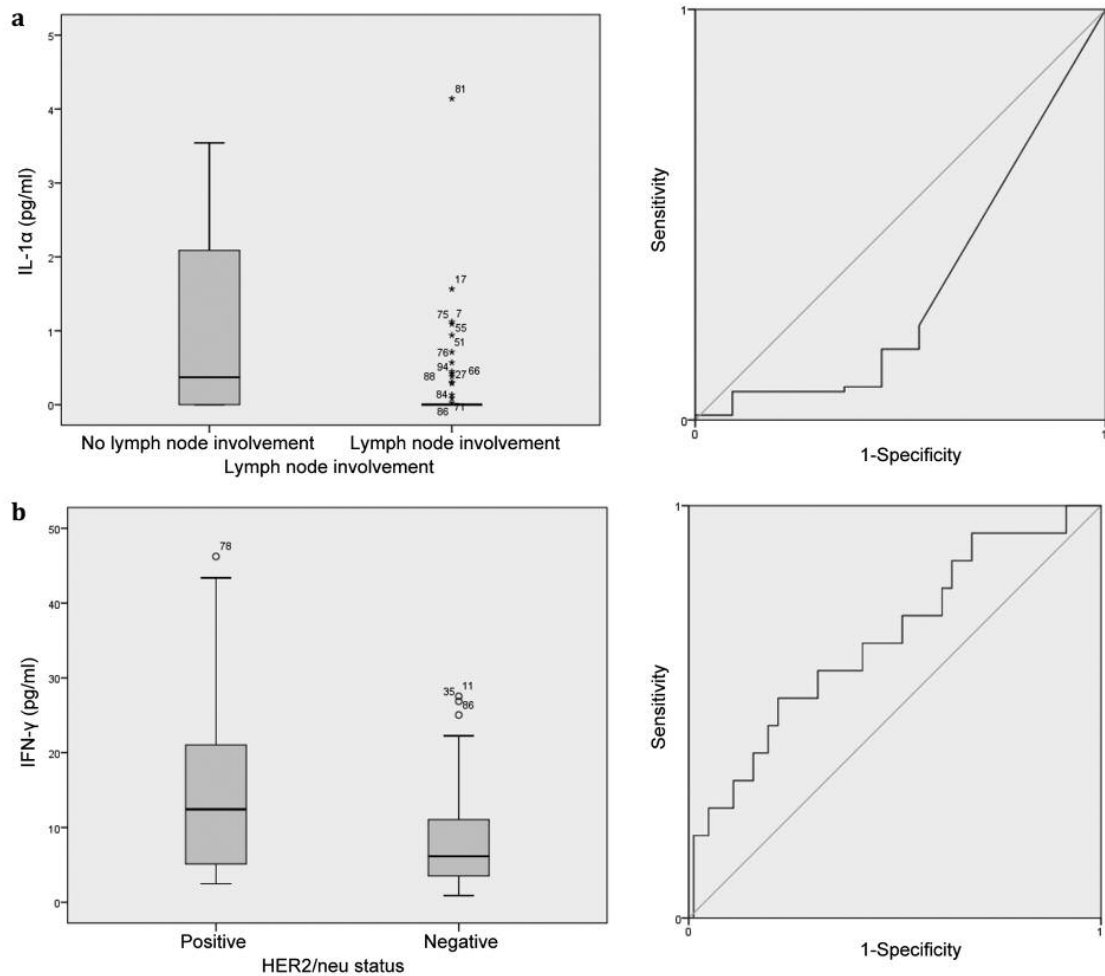


Figure 4. Box plot analysis of interleukin 1-alpha (IL-1 $\alpha$ ) (a) and interferon gamma (IFN- $\gamma$ ) (b) expression in sera of patients with grade 2 (G2) tumour [regardless of circulating tumour cell (CTC) status]. IL-1 $\alpha$  release was significantly higher in the G2 patient collective with no lymph node metastasis compared to patients with lymph node involvement ( $p=0.013$ ) (a, left panel). Significantly enhanced IFN- $\gamma$  release was shown in the G2 patient collective with HER2/neu receptor-positive compared to HER2/neu receptor-negative breast cancer ( $p=0.030$ ) (b, left panel). Boxes indicate the 25th and 75th percentiles, with a horizontal line at the median and bars display the 5th and 95th percentiles. Circles specify values more than 1.5 box lengths. Asterisks specify values (marked with a number) more than 3.0 box lengths from the 75th percentile. Receiver operator curve analysis of sensitivity versus specificity gave an area under the curve of 0.322 (a, right panel) and 0.676 (b, right panel), respectively.

circulation-specific metastasis and not metastasis *via* the lymphatic system. This hypothesis is furthermore supported by increased levels of IL-1 $\alpha$  in patients with grade 2 tumours and no lymph node involvement. These findings identify IL-1 $\alpha$  as a crucial factor for the ability of senescent cells to generate a tissue microenvironment that stimulates cancer expansion (18). The abnormal increased expression of IL-1 $\alpha$  in less differentiated breast cancer cells with no lymph metastasis might be responsible for local invasiveness and malignant behaviour.

Significantly enhanced IL-1 $\alpha$  levels were also found in patients with triple hormone receptor-negative breast cancer who died from their disease. Furthermore, increased IL-1 $\alpha$  and IL-1 $\beta$  levels were shown for patients who died from oestrogen receptor-negative breast cancer. It is conjectured that IL-1 $\alpha$  expressed on malignant cells stimulates antitumour immunity, while IL-1 $\beta$  that originates from the microenvironment or malignant breast cancer cells activates inflammation that increases invasiveness and induces tumour suppression (12, 19, 20). Therefore, one can suggest that

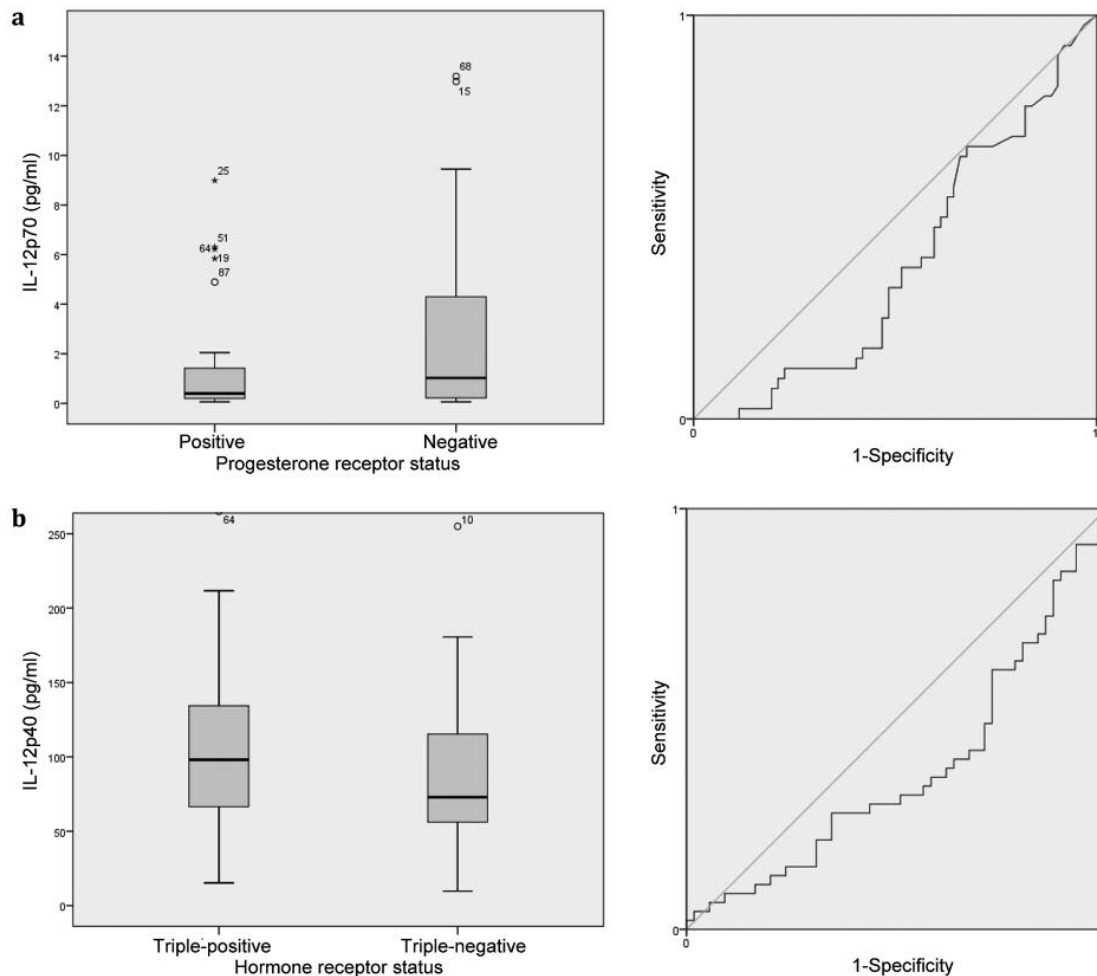


Figure 5. a: Box plot analysis of interleukin 12p70 (IL-12p70) (a) and IL-12p40 expression in sera of patients with grade 3 (G3) tumour (regardless of circulating tumour cell (CTC) status). IL-12p70 release was significantly higher in patients with progesterone receptor-negative compared to those with progesterone receptor-positive breast cancer ( $p=0.048$ ) (a, left panel). IL-12p40 release was significantly higher in those with triple-positive compared to patients with triple-negative breast cancer ( $p=0.032$ ) (b, left panel). Boxes indicate the 25th and 75th percentiles, with a horizontal line at the median and bars display the 5th and 95th percentiles. Circles specify values more than 1.5 box lengths. Asterisks specify values (marked with a number) more than 3.0 box lengths from the 75th percentile. Receiver operator curve analysis of sensitivity versus specificity gave an area under the curve of 0.384 (a, right panel) and 0.377 (b, right panel), respectively.

increased expression of IL-1 $\alpha$  and IL-1 $\beta$  in patients with a triple-negative hormone status who died, especially in terms of negative oestrogen receptor status, is potentially responsible for the severity of tumour malignancy and local invasiveness, ultimately resulting in the death of those patients. We can consequently also infer that the manipulation of IL-1 $\alpha$  and IL-1 $\beta$  in malignant cells or in the tumour microenvironment could offer new approaches in terms of cancer therapy for patients with triple-negative breast cancer. Furthermore, significantly enhanced IL-1 $\beta$

expression in patients diagnosed as having progesterone receptor-negative breast cancer not having CTCs might indicate the involvement of IL-1 $\beta$  in local invasiveness rather than circulation, leading to less favourable outcome.

Regardless of CTC status, in patients who survived, we identified significantly enhanced IL-12p40 release in combination with a triple-positive hormone receptor status and lymph node metastasis. It is hypothesized that IL-12 has an anti-angiogenic effect and the ability to induce a T-cell-based antitumour immune response capable of eliminating

disseminated cancer cells (21, 22). It has already been implied that intratumoural treatment of patients with breast cancer with IL-12 in combination with other cytokines leads to infiltration by polymorphonuclear cells, dendritic cell antigen presentation and CD8<sup>+</sup> T-cells, with consequent tumour regression. Furthermore, it has been shown that local IL-12 therapy stimulates specific antitumor T-cells in lymph nodes resulting in a memory immune response (23), and is even capable of eradicating disseminated tumour cells (20, 24). An enhanced IL-12p40 level in this collective of survivors with triple-positive hormone receptor status therefore indicates a natural advantage, most probably responsible for local apoptosis, eradicating distant disease and thus enhancing survival. However, in our study, patients with lymph node metastasis, indicating a highly malignant phenotype, nevertheless survived. The increased IL-12p40 expression in these patients emphasises antitumour immune response in distant metastatic lesions in lymph nodes, increasing a favourable outcome. Moreover, we identified significantly enhanced IL-12p40 release in the patient collective with grade 3 triple-positive breast cancer. A pattern of increased IL-12p40 expression together with a significant increase in the total number of lymphocytes, CD4+, CD8+, natural killer cells and C-reactive protein has been described to be of clinical benefit, as opposed to progression, in hormone-responsive metastatic breast cancer (25). One can suppose an association exists between triple-positive breast cancer and enhanced IL-12p40 expression, furthermore responsible for counteracting the malignant invasiveness of a poorly differentiated tumour. Moreover, the significantly enhanced IL-12p70 release in patients with grade 3 progesterone receptor-negative disease suggests a direct association between IL-12p70 and the absence of progesterone receptor in poorly differentiated tumours.

Patients with grade 2 HER2/neu receptor-positive breast cancer, regardless of CTC status, had a significantly higher IFN- $\gamma$  expression. IFN- $\gamma$  is purportedly responsible for promoting apoptosis by enhancing cytotoxic T-lymphocyte activity and influencing p53 expression, associated with a protective role against cancer (11, 26). It is furthermore presumed that IFN- $\gamma$  genetic polymorphisms might even be significantly related to a higher risk of breast cancer (27). Therefore, significantly enhanced IFN- $\gamma$  expression in patients with less differentiated (grade 2) and HER2/neu receptor-positive breast cancer may enhance tumour apoptosis, resulting in a more favourable outcome.

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### 2.3. Publication 3

*Title:*

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

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*Journal:*

Anticancer Res. 2016 Jun;36(6):3123-30.

*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.

## 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.

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,

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*Key Words:* Breast cancer, IL-4, IL-5, IL-6, IL-8, IL-13, circulating tumor cells.

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<sup>2</sup>) followed by three cycles of docetaxel (100 mg/m<sup>2</sup>) every 3 weeks vs. three cycles of FEC followed by three cycles of gemcitabine (1,000 mg/m<sup>2</sup> days 1 and 8) with docetaxel (75 mg/m<sup>2</sup>) every 3 weeks. After completing chemotherapy, patients were randomized to receive either 2 years or 5 years of therapy with the bisphosphonate 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 Richarlsen 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](http://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

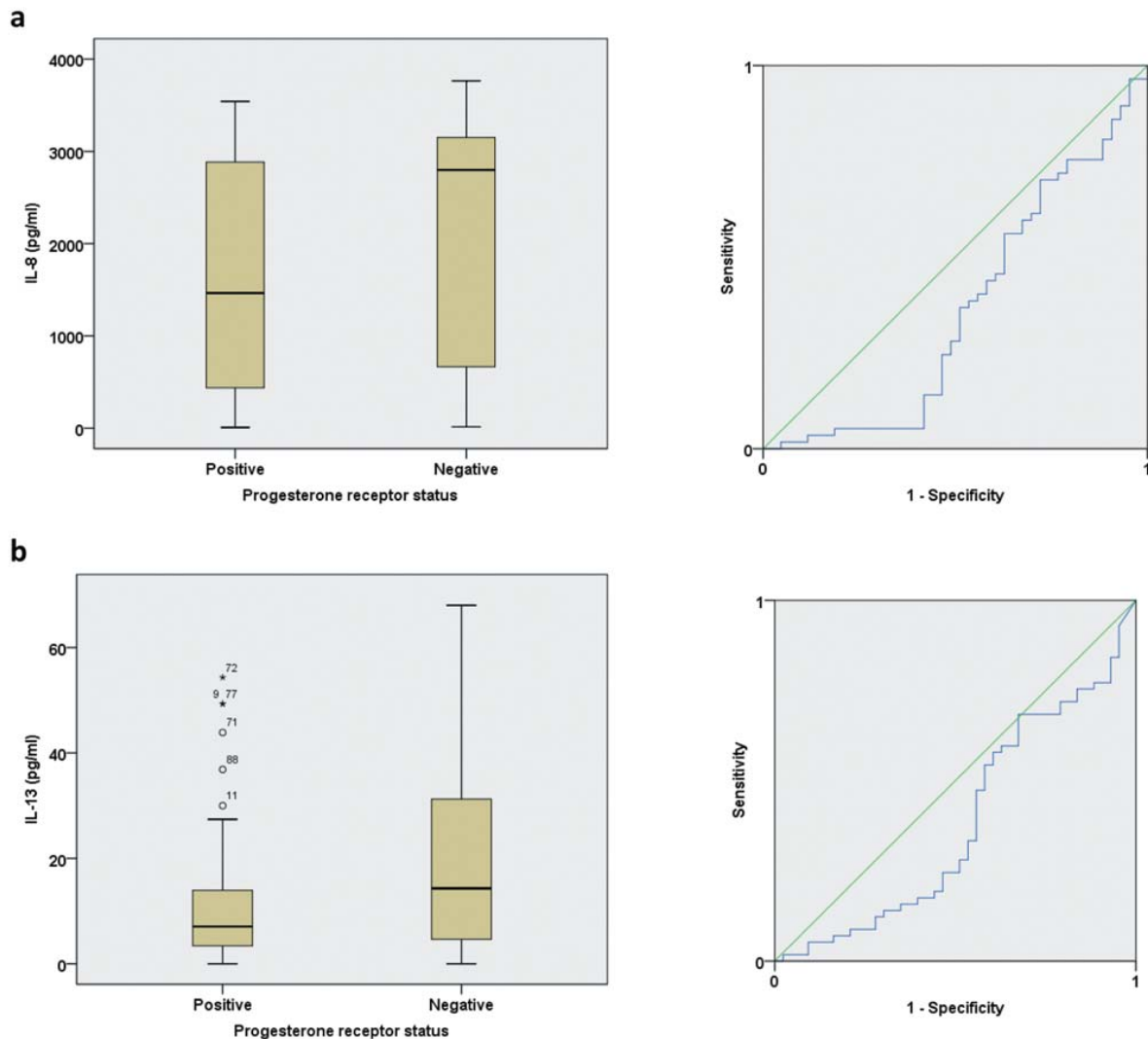


Figure 1. Box-plot analysis of interleukin (IL)-8 (a) and IL-13 (b) expression in serum of patients with breast cancer negative for circulating tumor cells (CTCs) according to progesterone receptor status. The range between the 25th and 75th percentiles is represented by boxes with a horizontal line 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  $p=0.045$ , 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

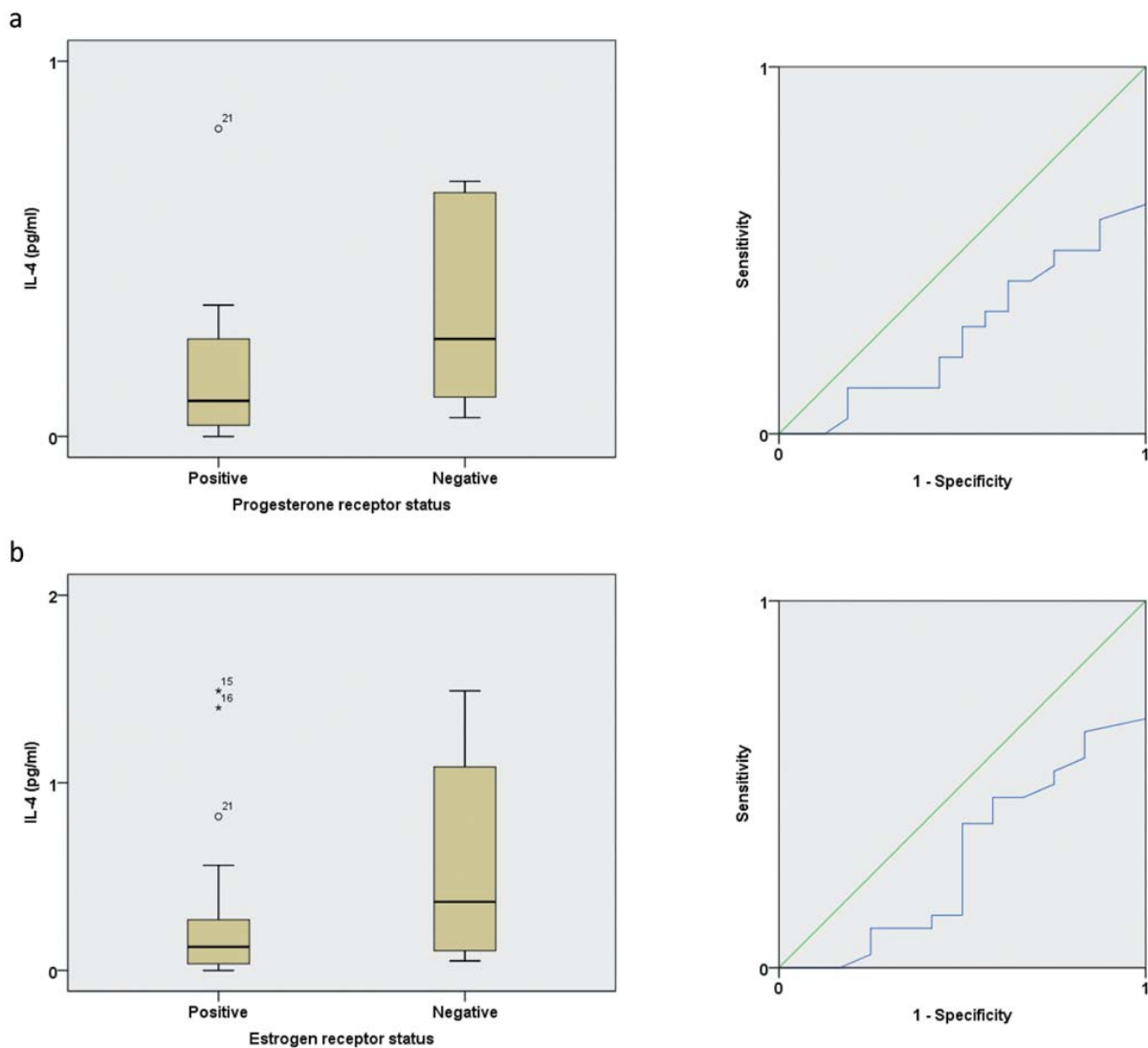


Figure 2. Box-plot analysis of interleukin (IL)-4 expression in serum of patients with breast cancer who died from their disease (regardless of circulating tumor cell status) according to progesterone (a) and estrogen (b) receptor status. The range between the 25th and 75th percentiles is represented 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

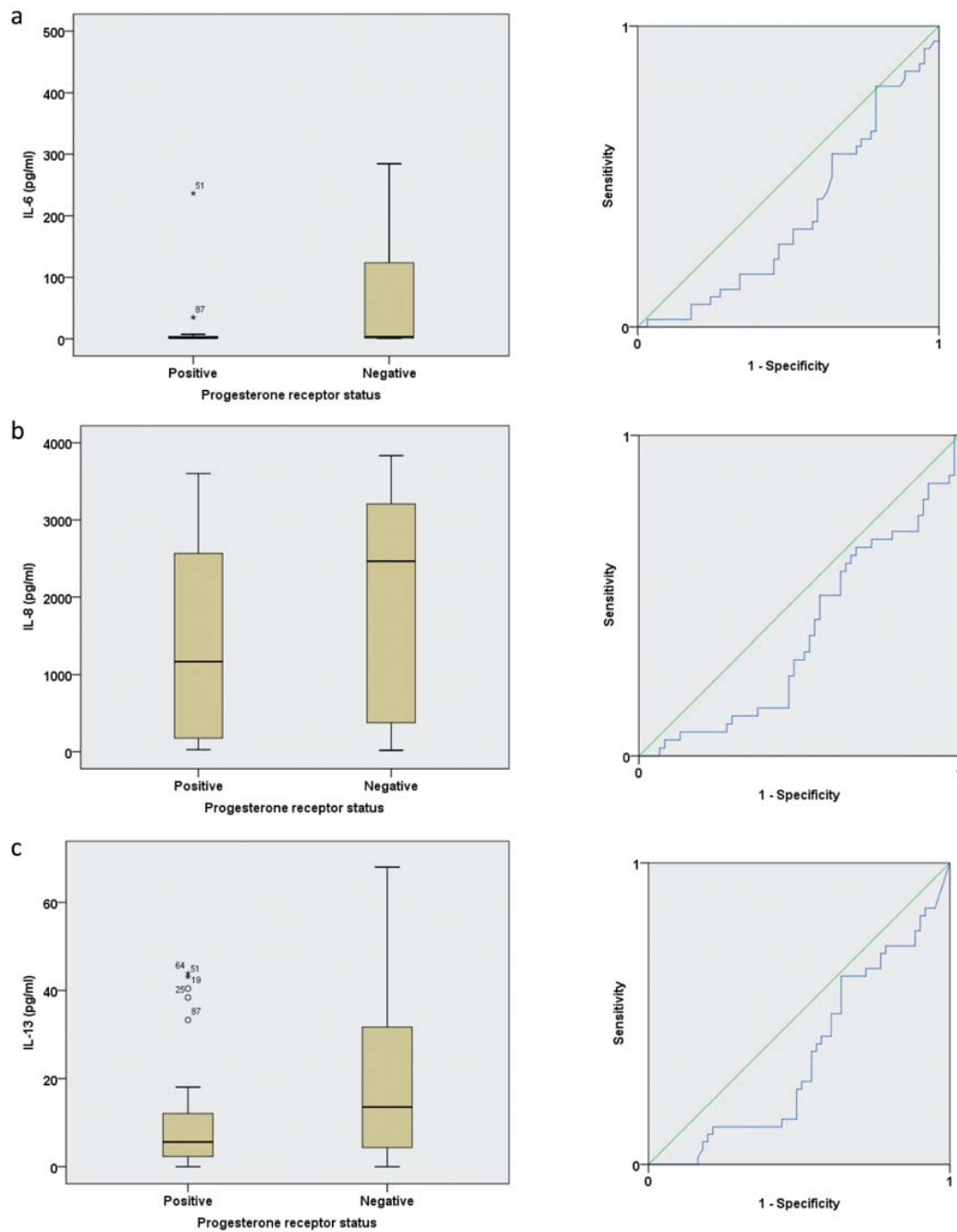


Figure 3. Box plot analysis of interleukin (IL)-6, IL-8 and IL-13 (left panels of a, b and c, respectively) in sera of patients with G3 breast cancer (regardless of circulating tumor cell status). The range between the 25th and 75th percentiles is represented by boxes with a horizontal line at the median. 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).

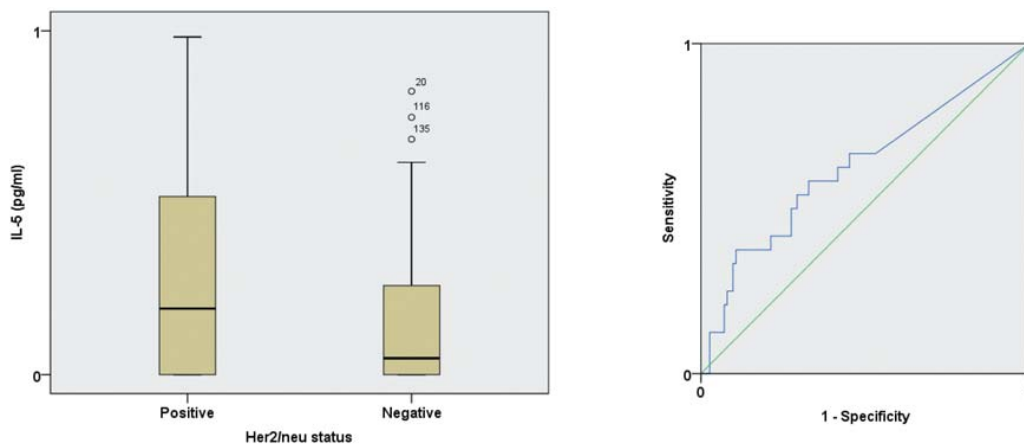


Figure 4. Box plot analysis of interleukin (IL)-5 expression in sera of patients with breast cancer with lymph node metastasis (regardless of circulating tumor cell status). The range between the 25th and 75th percentiles is represented by boxes with a horizontal line at the median. The bars show the 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).

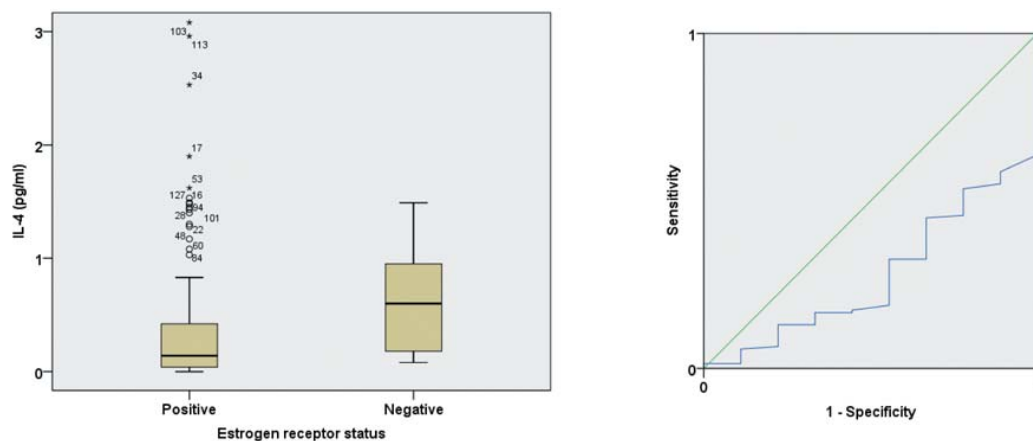


Figure 5. Box plot analysis of interleukin (IL)-4 expression in sera of patients with progesterone receptor-positive breast cancer (regardless of circulating tumor cell status). The range between the 25th and 75th percentiles is represented by boxes with a horizontal line at the median. The bars show 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 metalloproteinase 9/nuclear factor  $\kappa$ -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 neovascularization, 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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### 3. SUMMARY

Within this thesis we thoroughly describe the significant results of TH1 and TH2 derived cytokines of the T-lymphocyte immune response including angiogenesis markers and disclose the differences of cytokine levels in breast cancer patients of the SUCCESS study group with respect to CTC involvement in hindsight to specific breast cancer criteria. Firstly, sFlt1 values being significantly enhanced in those patients who survived, with no lymph node involvement, G2 graded tumour, and altogether negative for CTCs suggests the anti-vascular cytokine inhibits CTCs being released into the peripheral blood signifying an additive inhibition delay in tumour growth, triggering a significant disruption of tumour vascularization (63), consequently enhancing survival (18, 64-66). To continue, significantly enhanced PlGF values were revealed for the CTC negative patient collective and in the sera of survived patients with no CTC involvement, supporting the implication that tumour derived PlGF negatively modulates tumour angiogenesis and tumour progress (66). A functional interaction of sFlt1 and PlGF was found, suggesting that their combined overexpression in tumour cells inhibits CTCs entering the peripheral blood. Furthermore, highlighting only the most significant results amongst the TH1 derived cytokine, significant enhanced IL-1 $\alpha$  secretion in CTC positive patients were revealed. Increased values of this cytokine were also found in sera of patients without lymph node involvement. Thus, implying that IL-1 $\alpha$  can be a marker for the release of tumour cells into the circulation but not in the lymphatic system (42, 67). IL-1 $\beta$  on the other hand emphasised that only CTC negative patients showed a correlation of the progesterone receptor expression and IL-1 $\beta$  release (39, 43, 44). Concluding, IL-1 $\alpha$  and IL-1 $\beta$  are associated to CTC release in breast cancer patients. Similarly, the TH2 cytokines exposed significantly modified values in patients who are CTC negative and showed progesterone receptor expression, suggesting that an increase of IL-4 is reliant on hormone receptor status. The correlation between IL-4 and resistance to apoptosis can be endorsed and be held responsible for the poor outcome of these cases (51-53, 68). In total, increased levels of TH2 cytokines appear to be related with an unfortunate prognosis in breast cancer. The cytokine profiles revealed help for a profounder understanding of the illness and how immunological-related genes affect breast carcinogenesis. Measurement and interpretation of cytokine profiles in breast cancer could help assess the discrete risk of patients at the time of primary diagnosis and even improve therapy observation and strategy.

#### 4. ZUSSAMENFASSUNG

Diese Dissertation beschreibt signifikante Unterschiede des Vorkommens von TH1, TH2 und Angiogenese abgeleiteten Zytokinen bei Brustkrebspatientinnen der SUCCESS Studie in Hinblick auf CTC Beteiligung in Relation zu spezifischen Brustkrebskriterien. Der sFlt1 Marker zeigt erhöhte Werte in der Gruppe der überlebenden Brustkrebspatientinnen sowie bei Patientinnen ohne Lymphknotenbeteiligung oder mit G2 Tumoren. All die Gruppen waren allesamt negativ für CTCs. Dies impliziert das sFlt1, als antivaskulärer Trigger, eine Tumor-Vaskularisation verhindert (63), das Tumorwachstum hemmt und folglich das Überleben verbessert (18, 64-66). Erhöhte PIGF Werte sind bei CTC-negativen und überlebenden Patientinnen ohne CTC-Beteiligung nachgewiesen. Schlussfolgernd befürwortet dies die These, dass vom Tumor sezerniertes PIGF die Angiogenese sowie die Tumor-Progression negativ beeinflusst (66). Die nachgewiesene Wechselwirkung zwischen sFlt1 und PIGF lässt vermuten, dass deren Überexpression in Tumorzellen die Freisetzung von CTCs in das periphere Blutsystem abwendet. Eines der hoch signifikanten Ergebnisse in der Gruppe der TH1 abgeleiteten Zytokine ist die gesteigerte IL-1 $\alpha$ -Sekretion bei CTC-positiven Patientinnen sowie bei Patientinnen ohne Lymphknotenbeteiligung. Dies deutet darauf hin, dass die Präsenz von IL-1 $\alpha$  für die Freisetzung von Tumorzellen in den Kreislauf verantwortlich sein könnte, ein Progress in das lymphatische System jedoch verhindert (42, 67). Eine erhöhte IL-1 $\beta$  Konzentration kann bei CTC-negativen Patientinnen und vorhandener Progesteron Rezeptor-Expression nachgewiesen werden. Zusammenfassend spielen IL-1 $\alpha$  und IL-1 $\beta$  eine bedeutende Rolle bei der Freisetzung von CTCs in das periphere Blutsystem. In ähnlicher Weise zeigten TH2-Zytokine signifikant veränderte Werte bei CTC-negativen Patientinnen und der Progesteron Rezeptor-Expression. Ein Anstieg von IL-4 scheint demzufolge auf den Hormonrezeptor-Status angewiesen zu sein. IL-4 korreliert mit einer Apoptoseresistenz und kann demnach für das schlechte Ergebnis in diesen Fällen verantwortlich gemacht werden (51-53, 68). Erhöhte TH2-Konzentrationen bei Brustkrebspatientinnen scheinen mit einer ungünstigen Prognose in Zusammenhang zu stehen. Die beschriebenen Zytokinprofile erlauben ein verbessertes Verständnis für Brustkrebs und verdeutlichen wie immunologisch relevante Gene die Karzinogenese beeinflussen. Die Messung und Interpretation von Zytokinprofilen könnte in der Zukunft von Nutzen sein, um das Risiko von Patientinnen zu dem Zeitpunkt der Erstdiagnose abzuschätzen und die Therapieoptionen anzupassen.

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To my mum, your love showed me the way...

## 6. EIDESSTATTLICHE VERSICHERUNG

### Eidesstattliche Versicherung

Vilsmaier, Theresa

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Name, Vorname

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

**Influence of circulating tumour cells on the immune response of T-Lymphocytes and Angiogenic cytokines in sera of patients with the primary diagnosis of breast cancer before treatment**

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, 09.08.2018

Theresa Vilsmaier

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Ort, Datum

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Unterschrift Doktorandin

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## 8. ABBREVIATIONS

<b>CTC</b>	Circulating tumour cells
<b>sFlt1</b>	Soluble fms-like tyrosine kinase-1
<b>PIGF</b>	Phosphatidylinositol-glycan biosynthesis class F protein
<b>VEGF</b>	Vascular endothelial growth factor
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>TH1</b>	T helper 1
<b>TH2</b>	T helper 2
<b>TH17</b>	T helper 17
<b>Treg</b>	Regulatory T cells
<b>TH9</b>	T helper 9
<b>IFN-<math>\gamma</math></b>	Interferon gamma
<b>TNF-<math>\alpha</math></b>	Tumour necrosis factor alpha
<b>IL-12</b>	Interleukin 12
<b>IL-1<math>\alpha</math></b>	Interleukin 1 alpha
<b>IL-1<math>\beta</math></b>	Interleukin 1 beta
<b>IL-2</b>	Interleukin 2
<b>IL-18</b>	Interleukin 18
<b>DFS</b>	Disease-free survival
<b>OS</b>	Overall survival
<b>EpCAM</b>	Epithelial cell adhesion molecule
<b>AUC</b>	Area under the curve
<b>FEC</b>	Fluorouracil-epirubicin-cyclo-phosphamide
<b>CRP</b>	C-reactive protein
<b>NK</b>	Natural killer
<b>HER2</b>	Human epidermal growth factor receptor 2

