Cell Oncol.
DOI 10.1007/s13402-015-0264-2

Simultaneous detection of circulating immunological parameters and tumor biomarkers in early stage breast cancer patients during adjuvant chemotherapy

B. Rovati[1] · S. Mariucci[1] · S. Delfanti[1] · D. Grasso[1] · C. Tinelli[2] · C. Torre[3] · M. De Amici[3] · P. Pedrazzoli[1]

Accepted: 22 December 2015

Abstract
Background Chemotherapy-induced immune suppression has mainly been studied in patients with advanced cancer, but the influence of chemotherapy on the immune system in early stage cancer patients has so far not been studied systematically. The aim of the present study was to monitor the immune system during anthracycline- and taxane-based adjuvant chemotherapy in early stage breast cancer patients, to assess the impact of circulating tumor cells on selected immune parameters and to reveal putative angiogenic effects of circulating endothelial cells.
Methods Peripheral blood samples from 20 early stage breast cancer patients were analyzed using a flow cytometric multicolor of antibodies to enumerate lymphocyte and dendritic cell subsets, as well as endothelial and tumor cells. An enzyme-linked immunosorbent assay (ELISA) was used to measure the levels of various serological factors.
Results During chemotherapy, all immunological parameters and angiogenesis surrogate biomarkers showed significant decreases. The numbers of circulating tumor cells showed significant inverse correlations with the numbers of T helper cells, a lymphocyte subset directly related to effective anti-tumor responses. Reduced T helper cell numbers may contribute to systemic immunosuppression and, as such, the activation of dormant tumor cells.
Conclusions From our results we conclude that adjuvant chemotherapy suppresses immune function in early stage breast cancer patients. In addition, we conclude that the presence of circulating tumor cells, defined as pan-cytokeratin^{+}, CD326^{+}, CD45^{-} cells, may serve as an important indicator of a patient's immune status. Further investigations are needed to firmly define circulating tumor cells as a predictor for the success of breast cancer adjuvant chemotherapy.

Keywords Adjuvant breast cancer chemotherapy · Cytokine · Lymphocyte subsets · Dendritic cells · Endothelial cells · Tumor cells

1 Introduction

Tumors and chemotherapy (CT) are known to significantly alter immune responses [1, 2], but surprisingly few studies have been reported on how cancer stages may affect immune responses during CT. Recent studies on therapy responses in breast cancer (BC) have focused primarily on the relevance of molecular or genetic biomarkers, while little attention has been paid to the significance of cellular immune responses to clinical outcome [3], despite the fact that BC patients have a lower baseline immune response than healthy individuals [4–6]. There is also evidence indicating that low lymphocyte counts predict a greater likelihood of disease recurrence in patients with early stage BC [7], as also poorer disease-free and overall survival rates in metastatic BC patients [8, 9].

In cancer patients subjected to CT, the clinical immunodeficiency is primarily related to T cell depletion, associated with

Electronic supplementary material The online version of this article (doi:10.1007/s13402-015-0264-2) contains supplementary material, which is available to authorized users.

✉ B. Rovati
b.rovati@smatteo.pv.it

[1] SC Oncologia e Laboratorio di Citofluorimetria, e Terapie Cellulari, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

[2] Servizio di Biometria e Statistica Medica, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

[3] SC Pediatria, Laboratorio di Immuno Allergologia, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy

Published online: 14 January 2016

inhibition of the ability of dendritic cells (DCs) to induce both primary and secondary T and B cell responses [10]. Natural killer (NK) cells constitute an important component of innate immunity, able to limit viremia and to mediate spontaneous killing of various tumor cells, even before the adaptive immune system is activated [11]. Complete restoration of immunocompetence following anti-neoplastic therapy implies the progressive recovery of various cell subpopulations, which is a complex process that is dependent on the type, the dose, the scheduling and the interactions of the drugs used [10].

In recently reported studies evidence has been obtained for immunological suppression of tumor cells in a dormant state [12]. The mechanisms underlying the activation of dormant tumor cells and metastatic disease are, however, not fully understood. Besides genetic and immunological factors [13, 14], angiogenesis may also trigger the activation of dormant tumor cells [15–18]. Angiogenesis is an essential step in tumor growth and metastasis and may be measured via circulating endothelial cells (CECs) and bone marrow-derived circulating endothelial progenitor cells (CEPs) [19]. It has amply been shown that angiogenesis is regulated by chemokines, a group of cytokines involved in the migration of leukocytes that display pleiotropic immunologic effects in addition to promoting the proliferation of tumor cells and mediating organ-specific metastasis. In particular, it has been found that chemokines and their receptors play important roles in BC development. As yet, however, few reports have focused on changes in chemokine concentrations during BC chemotherapy, especially adjuvant chemotherapy [20, 21].

The aims of the present study were (1) to monitor in early stage BC patients the influence of anthracycline- and taxane-based adjuvant chemotherapy both on the immune system, by evaluating the distribution of lymphocyte subsets, and on circulating DCs including their subsets and some serological factors selected for their relevance to BC, (2) to analyze the relationship between circulating tumor cells (CTCs) and the quantitative distribution of lymphocyte subsets, in order to gain further insight into possible tumor-induced immune suppression and (3) to evaluate angiogenesis by changes in surrogate biomarkers of CECs and CEPs before and during chemotherapy.

2 Materials and methods

2.1 Study population

In total 20 early stage breast cancer (BC) patients aged 34–71 years (median age 53.5 years), referred to the Medical Oncology Unit of the IRCCS Foundation Policlinico San Matteo, were consecutively enrolled in this study from March 2012 to September 2013. Inclusion criteria were: (a) women 18 years old or older with stage I to III BC and receiving adjuvant chemotherapy, based on clinical-pathological characteristics, (b) absence of relevant comorbidities and (c) ability to sign informed consent prior to entering the study. Exclusion criteria were: (a) pregnancy or breastfeeding and (b) known HIV positivity. Eighteen women completed the study and two women withdrew from the study for family and personal reasons. Each patient provided written informed consent before entering the study and the study was conducted following approval by the Ethical Committee and Study Protocols Review Board of the IRCCS Foundation Policlinico San Matteo. All procedures used in this study are in agreement with the 1975 Helsinki Declaration [22]. A summary of the characteristics of the study population is listed in Table 1.

Table 1 Clinical-pathological characteristics of study population

Characteristics	Numbers	Percentage
Total patients	20	100
Age (years)		
Median	53.5	–
Range	34–71	–
Menopausal status		
Premenopausal	8	40
Postmenopausal	12	60
Tumor size (mm)		
pT1 ≤ 20	8	40
pT2 > 20 ≤ 50	11	55
pT3 > 50	1	5
Nodal status		
Node-negative	7	35
Node-positive	13	65
Histological type		
Ductal	15	75
Lobular	1	5
Mixed	4	20
Grading		
II	6	30
III	14	70
Estrogen Receptor (ER) status		
Negative	12	60
Positive	8	40
Progesterone receptor (PR) status		
Negative	13	65
Positive	7	35
Human epidermal growth factor 2 (HER2) expression		
Negative	14	70
Positive	6	30
Proliferative index Ki67		
Negative (<15 %)	2	10
Positive (>15 %)	18	90

2.2 Treatment schedule

The chemotherapy regimen applied consisted of four cycles of AC (adriamycin 60 mg/m^2 plus cyclophosphamide 600 mg/m^2) administered on day 1 every 3 weeks, followed by 12 weekly administrations of paclitaxel (80 mg/m^2). Antiemetic, corticosteroids and histamine receptor blockers were administered according to the institutional guidelines. Prophylactic use of pegfilgrastim, a PEGylated form of the recombinant human granulocyte colony-stimulating factor (GCSF), was adopted in six patients during the second cycle of adriamycin and cyclophosphamide (AC)-based therapy, after evidence of a G4 neutropenia episode at the nadir of the first cycle. No GCSF was used in the paclitaxel-based treatment. This schedule was adopted in our department at the time of the study in patients with a recent diagnosis of breast cancer that, based on current international guidelines, required anthracycline- and taxane-based chemotherapy. Human epidermal growth factor receptor 2 (HER2) positive patients received trastuzumab (Herceptin®) with weekly administrations in conjunction with paclitaxel. This treatment was continued every 3 weeks at the end of the chemotherapy cycle, thereby completing 1 year of chemotherapy. According to their clinical-pathological characteristics, patients received hormone therapy (based on menopausal status) and/or complementary radiotherapy at the end of the chemotherapy program, as required by international guidelines.

2.3 Study design

All cellular populations were evaluated by flow cytometry (FCM, see below) at the start of the study (baseline) to establish correlations with the tumor stage, histological grade, lymph node status, hormone receptor status, HER2 status, menopausal status, age and tumor size. Furthermore, each cellular parameter was analyzed during administration of the adjuvant therapy. Finally, the following serological factors were tested using an enzyme-linked immunosorbent analysis (ELISA) assay, at baseline and before the administration of the 1st and 12th cycle of taxane-based therapy: human vascular endothelial growth factor (VEGF), human stromal cell-derived factor 1 alpha (CXCL12/SDF-1α) and human epidermal growth factor receptor 2 (sp185/HER2). Immunophenotype profiles of lymphocyte and DC subsets from 31 to 19 healthy females (HFs), respectively, selected in order to exclude comorbidities that could modulate the immune system [23, 24], were used as references. The percentage and absolute number of CECs with their apoptotic fraction (CEC-APO) and CEPs in a group of 11 HFs, selected as previously reported [25], were used as references. All control groups consisted of age-matched HFs.

2.4 Flow cytometry (FCM)

Peripheral blood samples were collected in 4 ml Vacutainer (Becton Dickinson, Basel, Switzerland) tubes, containing liquid tripotassium ethylene diamine tetra-acetic acid (K3EDTA) as an anticoagulant, and processed within 2 h after collection. As is routine in our laboratory [23–25], flow cytometric analyses were carried out on whole blood without any enrichment procedure to avoid enrichment artefacts. A panel of monoclonal antibodies (MoAbs), including anti-CD45 to identify hematopoietic cells, in conjunction with appropriate analysis gates, was used to enumerate the different cellular populations. Blood erythrocytes were removed by adding 1 ml lysis solution (VersaLyse, Immunotech Beckman Coulter, Marseille, France) to 100 μl whole blood samples and incubation for 20 min at room temperature (RT) in the dark. Evaluation of nucleated cells from the whole blood samples was performed using a Navios flow cytometer (Beckman Coulter, USA). FCM data were analysed using the Kaluza flow cytometry analysis v1.1 software package. The precision, accuracy and stability of the cell counts were verified using international quality controls purchased from the United Kingdom National External Quality Assessment Scheme (UK NEQAS LI, Sheffield, UK) [26].

2.5 Multi-color staining and FCM of lymphocyte and DC subsets

The following mouse anti-human conjugated MoAbs (Beckman Coulter, USA) were used for lymphocyte subset detection: fluorescein isothiocyanate (FITC)-labelled anti-CD4 (clone 13B8.2) and anti-CD45RO (clone UCHL1), phycoerythrin (PE)-labelled anti-CD25 (clone B1.49.9) and anti-CD45RA (clone 2H4LDH11LDB9), phycoerythrin-texas red (ECD)-labelled anti-CD16 (clone 3G8) and anti-CD20 (clone B9E9), phycoerythrin cyanin-5.5 (clone PC5.5)-labelled anti-CD19 (clone J3.119) and anti-CD56 (clone N901), phycoerythrin cyanin-7(PC7)-labelled anti-HLA-DR (clone Immu357) and anti-CD38 (clone LSI98-4-3), allophycocyanin (APC)-labelled anti-CD3 (clone UCHT1) and anti-CD4 (clone 13B8.2), APC alexa fluor 700-labelled anti-CD8 (clone B9.11) and anti-CD23 (clone 9P25) and APC alexa fluor 750-labelled anti-CD45 (clone J.33). Mouse anti-human conjugated MoAbs (Beckman Coulter, USA) were used for DC subset identification: FITC-labelled anti-CD3 (clone UCHT1), anti-CD16 (clone 3G8), anti-CD19(clone 89B), anti-CD20 (clone H299), anti-CD14 (clone RM052), anti-CD34 (clone S81), anti-CD56 (clone N901) and anti-CD11b (clone 94), APC-labelled anti-HLA-DR (clone Immu357), PE-labelled anti-CD11c (clone BU15) and anti-CD123 (clone SSDCLY107D2) and APC alexa fluor 750-labelled anti-CD45 (clone J.33). As gating strategy, we applied a gate in the CD45 versus side scatter (SSC) dot plot on CD45-positive

events to identify leucocyte populations. The gating strategies applied for the lymphocyte and DC subsets are shown in Figs. 1 and 2, respectively.

2.6 Multi-color staining and FCM of CECs and CEPs

Six-color FCM was applied to measure CECs, including their apoptotic fractions, and CEPs using a procedure reported by Mariucci et al. [25] and the following panel of MoAbs: FITC-labelled anti-CD106, (clone 51-10C9; BD Pharmingen™, San Jose, California, USA), PE-labelled anti-CD146 (clone P1H12; BD Pharmingen™, San Jose, California, USA), PC7-labelled anti-CD34 (clone S81; Beckman Coulter, USA), APC-labelled anti-CD133 (clone AC133; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and APC alexa fluor 750-labelled anti-CD45 (clone J.33; Beckman Coulter, USA). To quantify apoptotic CECs an Annexin V kit (Bender Medsystems, Boehringer Ingelheim) was used. In order to discriminate viable and necrotic cells, 7-amino-actinomycin D (7-AAD Viability Dye; Beckman Coulter, Miami, USA) was added to each tube before FCM analysis. To exclude haematopoietic cells from CECs and CEPs, a gate in the CD45 versus side scatter (SSC) dot plot on CD45-negative events was applied. The gating strategy applied for CECs including their subsets and CEPs is shown in Fig. 3.

2.7 Multi-color staining and FCM of CTCs

For the measurements of CTCs by FCM, a method from Hristozova et al. [27] was applied with some modifications. The following panel of MoAbs was used: PE-labelled anti-CD326 (EpCAM) (clone HEA-125; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), FITC-labelled anti-Pan-Cytokeratin (PANCK) (clone CK3-6H5; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and APC alexa fluor 750-labelled anti-CD45 (clone J.33; Beckman Coulter, USA). The cells were fixed and permeabilized using a Fix&Perm kit (An Der Grub Bio Research GmbH, Wien, Austria) according

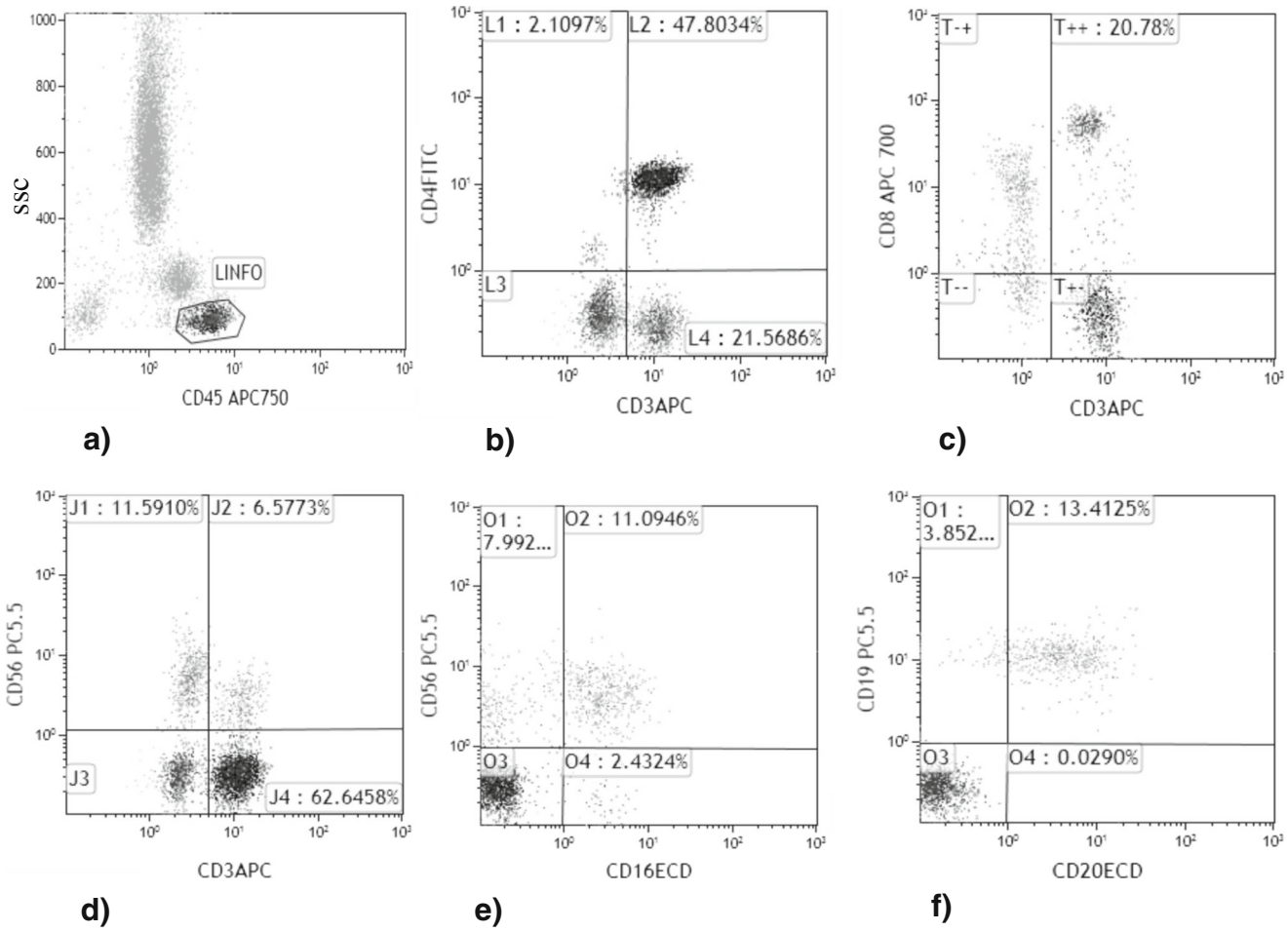

Fig. 1 Flow cytometric dot plot panels showing representative data relative to the gating strategy used for the identification of circulating lymphocyte subsets. Panel **a** analysis gate on lymphocytes identified by APC Alexa 750 labelled anti-CD45 versus side scatter (SSC); panels **b** and **c** T cell subsets identified by anti-CD3-APC and CD4-FITC for CD4 and CD3-APC and CD8-APCAlexa 700 for CD8; panels **d** and **e** NK subsets identified by anti-CD3-APC and CD56-PC5.5 and CD16-ECD and CD56-PC5.5; panel **f** B cell subset identified by anti-CD20-ECD and CD19-PC5.5

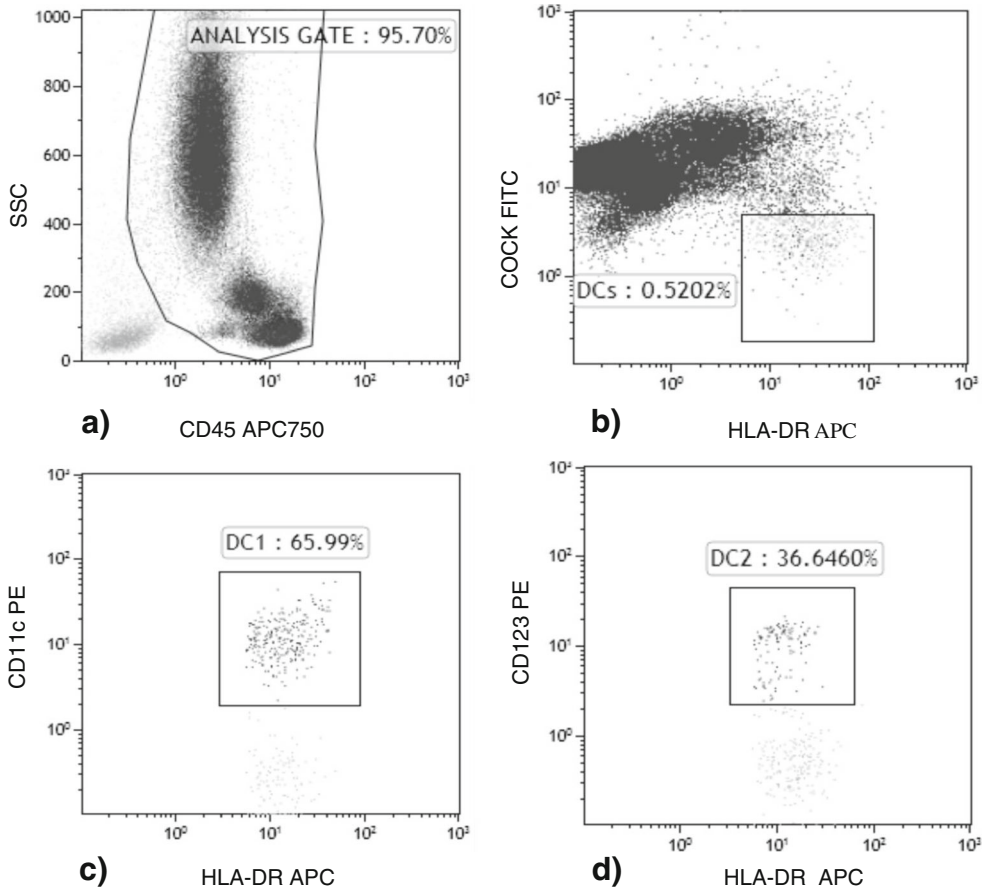

Fig. 2 Flow cytometric dot plot panels showing representative data relative to the gating strategy for the identification of circulating dendritic cells (DCs) and their subsets, with the lineage-negative analytical procedure. Panel **a** leukocyte analysis region applied to APC 750 labelled anti-CD45 versus side scatter (SSC) for exclusion of debris. Panel **b** DC cell population after labelling with a cocktail of FITC-conjugated MoAbs recognizing lineage-negative associated antigens (see text, paragraph 2.5) and APC labelled anti-HLA-DR MoAb. Panels **c** and **d** DC subsets identified by anti-HLA-DR-APC and CD11c-PE for DC1 and HLA-DR-APC and CD123-PE for DC2

to the manufacture's instructions. During the permeabilization step, an anti-PANCK antibody was added to the permeabilization buffer in a final volume of 100 μl. In each sample 5×10^5 to 1×10^6 cellular events were counted. Circulating tumor cells (CTC) were defined as $EpCAM^+$, $PANCK^+$, $CD45^-$. A blood sample was considered CTC-positive when at least one $EpCAM^+$, $PANCK^+$, $CD45^-$ cell was detected. Viable and necrotic cells were differentiated through 7-AAD staining. The gating strategy applied for CTC detection is shown in Fig. 4.

The efficiency of the method was tested using 15 patients with metastatic BC as clinical "positive controls" based on results from previous studies [28]. In addition, 18 peripheral blood samples from healthy volunteers to which different numbers (5 to 1,000) of SKBR3 or MCF7 breast cancer-derived cells were added, were used in parallel as a second positive control group. As negative controls, fresh blood samples were collected from 20 randomly-selected, age-matched healthy females.

2.8 Serological factor analyses

All serological samples were collected, assayed and determined in the same session to avoid inter-assay variation and to detect changes over time. Human VEGF, CXCL12/SDF-1α and sp185/HER2 immunoassays were performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, for VEGF and CXCL12/SDF-1α; Cusabio Biotech, Wuhan, China, for sp185/HER2) employing a quantitative sandwich enzyme procedure. Human VEGF and CXCL12/SDF-1α are expressed in pg/ml and human sp185/HER2 in ng/ml. The minimum detectable dose (MDD) of VEGF, using the Calibrator Diluent RD6U, is typically less than 9.0 pg/ml and the range of sample values in serum is 62–707 pg/ml. For SDF-1α 41 assays were evaluated and its minimum detectable dose was estimated to range from 1.0 to 47 pg/ml, while the mean MDD was 18 pg/ml and the range of sample values in EDTA plasma was 1,360–2,900 pg/ml. In both cases the MDD was determined by adding two standard deviations to the mean optical density value of twenty zero-standard replicates and calculating the corresponding concentrations. The minimum detectable dose of human sp185/HER2 is typically less than 0.04 ng/ml and the detection range is 0.156–10 ng/ml. The sensitivity of this assay, or lower limit detection (LLD), was defined as the lowest protein concentration that could be differentiated from zero. The LLD was determined from the mean of 20 zero-standard replicates plus three standard deviations.

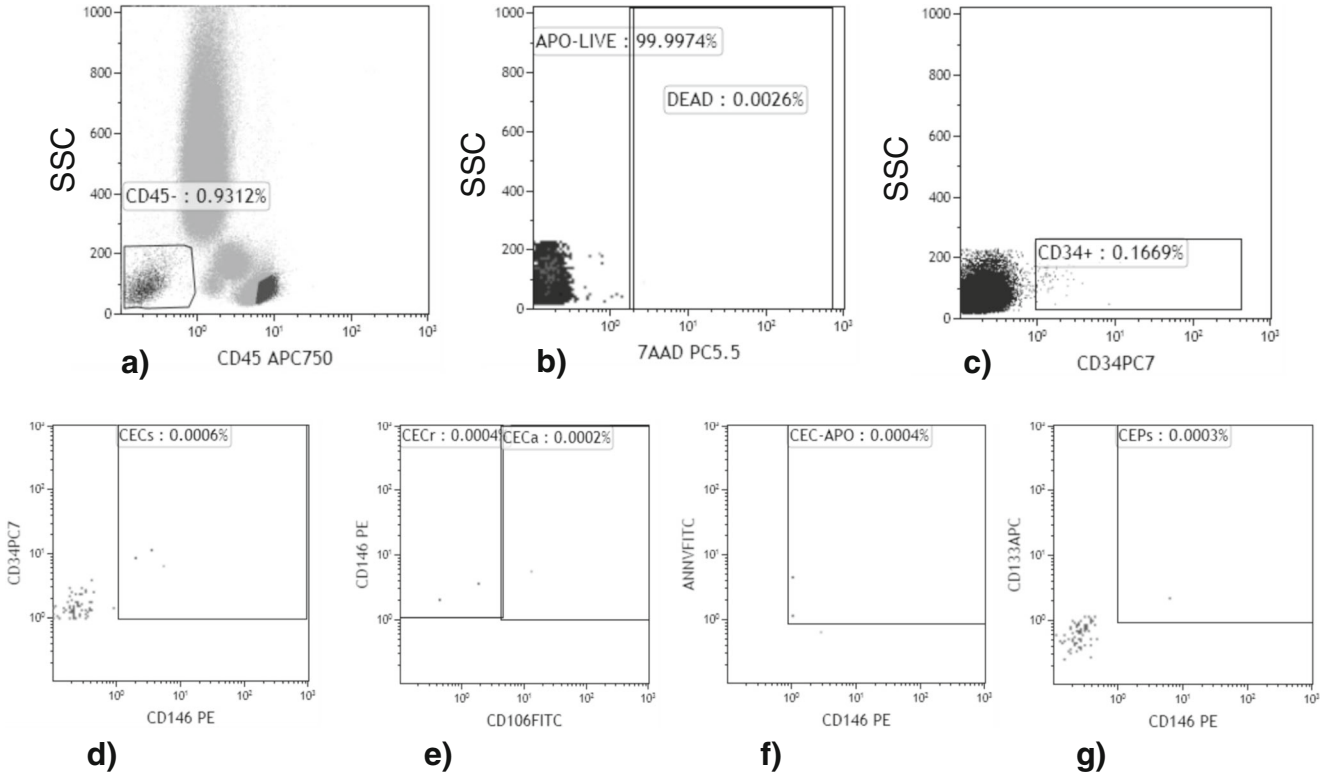

Fig. 3 Flow cytometric dot plot panels showing representative pictures relative to the gating strategy utilized for identification of circulating endothelial cells (CECs) both resting and activated (rCECs and aCECs) with their apoptotic fractions (CEC-APO) and circulating progenitor endothelial cells (CEPs). Panel **a** gate analysis used to exclude hematopoietic cells expressing the CD45 antigen versus side scatter (SSC). Panel **b** gate to distinguishing live from dead cells by 7AAD staining. Panel **c**, **d**, **e**, **f** and **g** sequential steps determining CECs (with rCEC and aCEC subsets), CEC-APO and CEPs

2.9 Statistical analyses

The cellular populations were normally distributed (Shapiro-Wilk test) and the results were, therefore, expressed as mean values ± SD. Linear regression models for repeated measures were used to compare quantitative variables among the baseline, AC-therapy and taxane-therapy groups. *T*-test for independent data and χ^2 statistics or Fisher's exact test, as appropriate, were applied to compare differences between healthy females and patient groups. $P < 0.05$ was considered statistically significant. All tests were two-sided. Data analyses were performed using the STATA statistical package (version 13; Stata Corporation, College Station, 2013, Texas, USA).

3 Results

At baseline, few significant correlations were observed between tumor stage, histological grade, lymph node status, hormone receptor status, HER2 status, menopausal status, age and tumor size (data not shown). Next, cellular populations were analyzed after the administration of the therapy, i.e., after adriamycin and cyclophosphamide (AC)-based therapy and at the end of taxane-based therapy. Finally, serological factors were tested at baseline and before the administration of the 1st and 12th cycle of taxane-based therapy. Eighteen women (90 %) completed the study and two women (10 %) withdrew from the study for family and personal reasons. In six patients (33 %) with a neutropenia episode, pegfilgrastim was administered as a prophylactic. Currently, the disease has progressed in two women (10 %). Baseline data were compared with those from a group of age-matched healthy females (HF). The clinical-pathological characteristics of the study population, at the time of the initial investigation, are listed in detail in Table 1.

3.1 Comparison between baseline patients and healthy females

Baseline means, percentages and absolute values for lymphocyte subsets and for DCs and CECs, including their subsets, were compared with those in a HF population. At baseline, the total lymphocyte numbers in BC patients were similar to those of the healthy controls, although statistically significant increases in percentages and absolute numbers of NK $CD16^+$ ($p = 0.032$; 0.048), NK $CD3^+$-$CD56^+$ ($p = 0.0004$, $p = 0.0008$) and double positive $CD4^+$-$CD8^+$ T cells ($p = 0.0004$, $p = 0.0014$) were observed. Also the $CD16^+$-$CD56^+$ NK subset showed an increase,

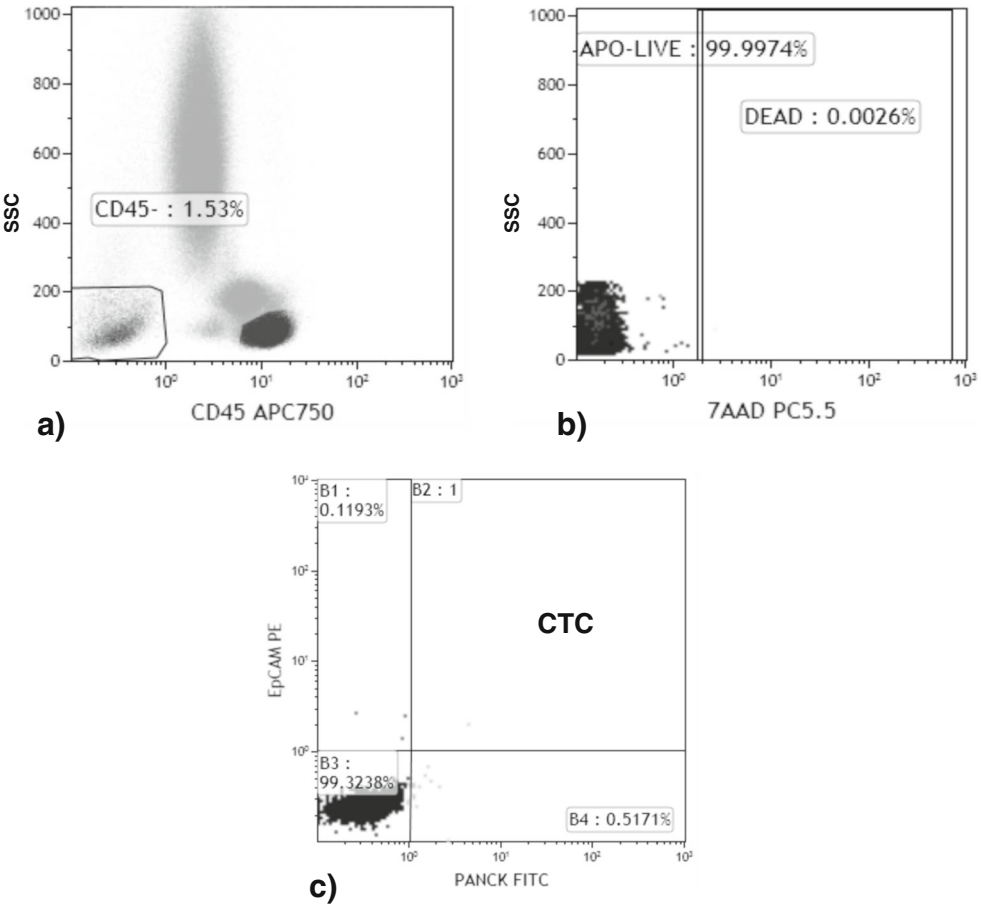

Fig. 4 Flow cytometric dot plot panels showing representative pictures relative to the gating strategy used for the identification of circulating tumor cells (CTCs). Panel **a** gate analysis used to exclude hematopoietic cells expressing the CD45 antigen versus side scatter (SSC). Panel **b** gate to distinguish live from dead cells by 7AAD staining. Panel **c** CTCs identified by anti-EpCAM-PE and anti- Pan-Cytokeratin (PANCK) FITC

but this increase was not significant ($p = 0.2261$). The activated T and B lymphocyte subsets ($CD4^+$-$CD25^+$ and $CD19^+$-$CD23^+$) also showed statistically significant increases, in both percentages and absolute numbers ($p < 0.0001$, $p < 0.0001$ and $p = 0.0001$, $p = 0.0001$). Early B lymphocyte subset ($CD19^+$-$CD38^+$) cells were significantly decreased, in both percentage and absolute number ($p = 0.0292$, $p = 0.0290$). As for changes in DC subsets and DC1:DC2 ratios, significant increases in percentages and absolute numbers were evident, whereas the DC2 subset was significantly decreased in both percentage and absolute number. Comparisons of mean values, with *p*-values, of immunological cell subsets in patients at baseline versus HF (percentages and absolute numbers) are summarized in Fig. 5 and Table 2, respectively. Finally, we found that circulating endothelial subsets (CECs), including their apoptotic fraction, and progenitor endothelial cells (CEPs) did not show any statistically significant changes in BC patients at baseline (data not shown).

3.2 Modification of cellular parameters during adjuvant treatment

Mean values with *p*-values of T and B lymphocytes and NK and DC cell subsets after AC-based therapy and at the end of taxane-based therapy, versus baseline (percentages and absolute numbers), are summarized in Fig. 6 and Table 3, respectively.

3.2.1 T lymphocyte subsets

After AC-based therapy, we observed significant decreases in the numbers of leucocytes ($p < 0.0001$) and total lymphocytes ($p < 0.0001$) and in the absolute numbers of mature ($CD3^+$) T cells ($p < 0.0001$), double-positive ($CD4^+$-$CD8^+$) cells ($p = 0.038$) and in the percentages and absolute numbers of T helper ($CD3^+$-$CD4^+$) cells ($p = 0.011$, $p < 0.0001$). In contrast, we found that the percentage of cytotoxic ($CD3^+$-$CD8^+$) T cells significantly increased ($p = 0.038$). Among the $CD4^+$ subsets, the percentage and absolute number of T naïve ($CD4^+$-$CD45RA^+$) cells were found to be decreased ($p = 0.04$, $p < 0.0001$). In contrast, the percentage of T memory ($CD4^+$-$CD45RO^+$) cells was found to be increased ($p = 0.04$), which in turn led to a significant increase in the $CD45RO^+$:$CD45RA^+$ ratio ($p = 0.041$), while their absolute number exhibited a significant decrease ($p = 0.005$). Finally, the activated ($CD4^+$-$CD25^+$) T cell subset showed a significant decrease in absolute number ($p = 0.011$). At the end of taxane-based therapy leucocytes, total lymphocytes and each T lymphocyte subset, except the percentage of T memory cells which recovered to

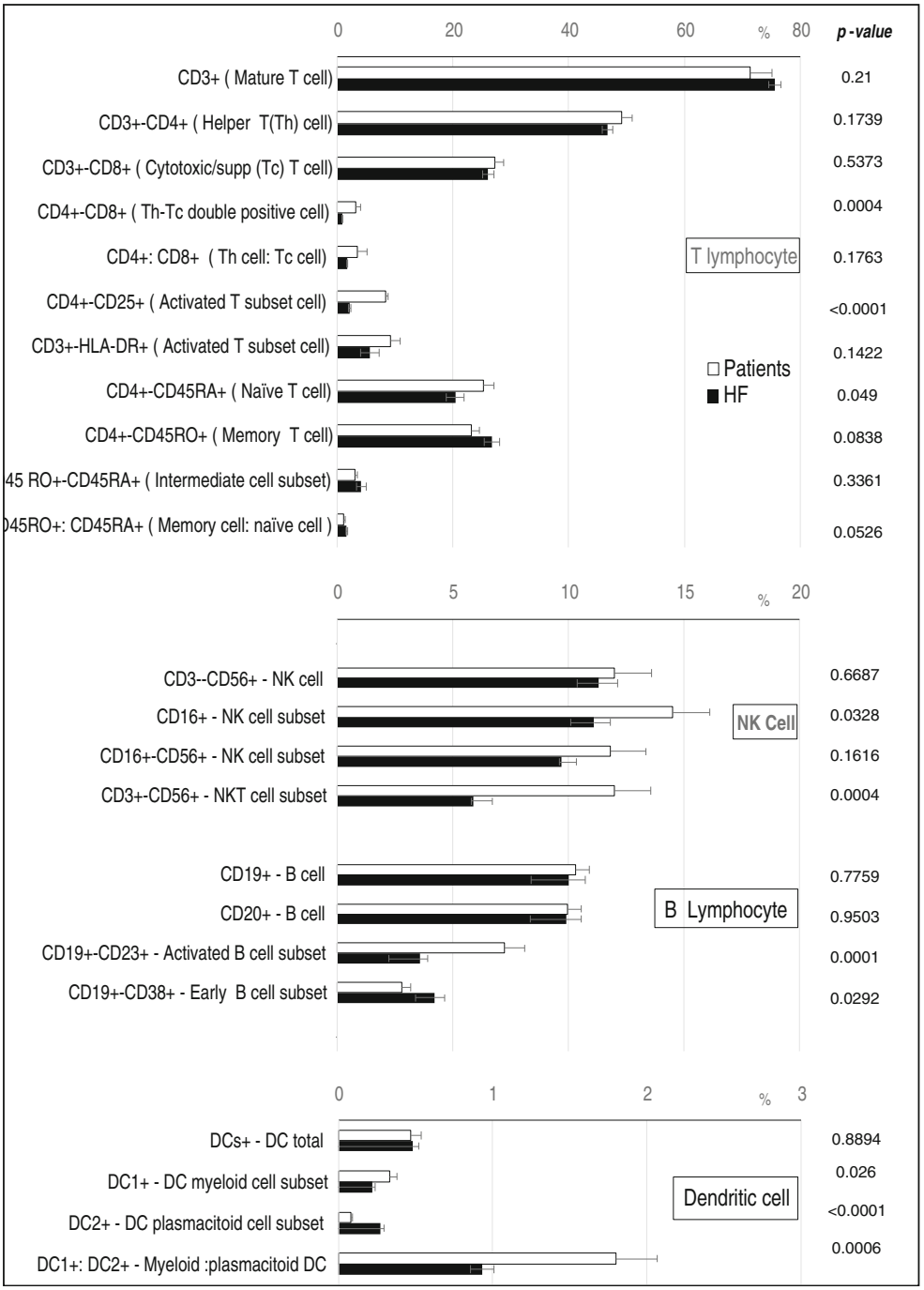

Fig. 5 Mean percentage levels of biomarker lymphocyte and DC subsets among baseline patients versus healthy females (HF)

baseline values, exhibited the same trend as seen in the AC-based therapy, in both percentages and absolute numbers.

3.2.2 B lymphocyte subsets

After AC-based therapy, significant decreases in absolute numbers and percentages of the $CD19^+$ and $CD20^+$ B cell subsets (p <0.0001, p <0.0001 and p <0.0001, p <0.0001), the activated $CD19^+$-$CD23^+$ B cell subset (p <0.0001, p <0.0001) and the early $CD19^+$-$CD38^+$ B cell subset ($p = 0.002$, $p = 0.001$), were observed. The same shifts, in both percentages and absolute numbers, were observed at the end of the taxane-based therapy for each B cell subset.

3.2.3 NK cells subsets

Despite the lymphopenia, which followed AC-based therapy, the NK cell numbers did not significantly decrease and, as a consequence, significant increases in the percentages of NK subsets, i.e., $CD16^+$ ($p = 0.043$), $CD16^+$-$CD56^+$ ($p = 0.048$),

Table 2 Comparison of cellular immunological profiles among baseline patients and healthy females

Parameter	Description	Healthy females (n°31) Mean (sd) cells/μl	Patients (n°20) Mean (sd) cells/μl	*p*-Value
Leucocyte	White cells	6485(1284)	6877(1537)	0.3295
Lymphocyte	White cells subset	2241(339)	2190(489)	0.6616
T lymphocyte				
$CD3^+$	Mature T cell	1713(305)	1585(507)	0.2649
$CD3^+$-$CD4^+$	Helper T(Th) cell	942(331)	1061(257)	0.1792
$CD3^+$-$CD8^+$	Cytotoxic/supp (Tc) T cell	558(152)	601(223)	0.4162
$CD4^+$-$CD8^+$	Th-Tc double positive cell	20(12)	73(86)	0.0014
$CD4^+$-$CD25^+$	Activated T subset cell	48(26)	184(61)	<0.0001
$CD3^+$-HLA-DR^+	Activated T subset cell	127(212)	198(151)	0.2003
$CD4^+$-$CD45RA^+$	Naïve T cell	457(216)	527(149)	0.2115
$CD4^+$-$CD45RO^+$	Memory T cell	598(198)	512(206)	0.1424
CD45 RO^+-$CD45RA^+$	Intermediate cell subset	102(102)	65(42)	0.1310
NK Cell				
$CD3^-$-$CD56^+$	NK cell	238(137)	266(168)	0.5176
$CD16^+$	NK cell subset	248(102)	326(173)	0.0480
$CD16^+$-$CD56^+$	NK cell subset	217(86)	260(164)	0.2261
$CD3^+$-$CD56^+$	NKT cell subset	134(97)	266(168)	0.0008
B Lymphocyte				
$CD19^+$	B cell	222(85)	225(81)	0.9008
$CD20^+$	B cell	220(83)	216(74)	0.8617
CD19-$CD23^+$	Activated B cell subset	79(40)	151(78)	0.0001
CD19-$CD38^+$	Early B cell subset	92(52)	61(41)	0.0290
Dendritic cell (DC)				
DCs^+	DC total	31(12)	30(23)	0.8395
$DC1^+$	DC myeloid cell subset	14(7)	21(18)	0.0561
$DC2^+$	DC plasmacitoid cell subset	17(8)	5(3)	<0.0001

$CD3^-$-$CD56^+$ ($p=0.036$) and $CD3^+$-$CD56^+$ ($p=0.026$) cells, were observed. At the end of the 12th cycle of taxane-based therapy, the percentages of the respective NK cell subsets had increased further.

3.2.4 DCs and their subsets

After AC-based therapy, a significant decrease was observed in the total dendritic cell (DC) number ($p=0.016$) and the percentages and absolute numbers of DC subsets, i.e., DC1 ($p=0.012$, $p=0.02$) and DC2 ($p=0.02$, $p=0.017$) and of the DC1:DC2 ratio ($p=0.045$). After taxane-based therapy, the total DCs ($p<0.0001$, $p<0.0001$) and their subsets (DC1 and DC2) showed significant decreases in percentages and absolute numbers ($p=0.001$, $p<0.0001$ and $p<0.0001$, $p<0.0001$).

3.2.5 CECs and their subsets

After AC-based treatment, the total CECs showed a non-significant decrease in both percentage and absolute number, while significant decreases in the rCEC ($p=0.048$) and CEC-APO subsets were observed in both percentages and absolute numbers ($p=0.032$, $p=0.004$). At the end of 12th cycle of taxane-based therapy the total CECs, including resting and apoptotic subsets, showed a significant decrease in the absolute number ($p=0.036$, $p=0.047$, $p=0.003$). After AC- and taxane-based therapy, the CEP numbers did not show any significant changes from the baseline values. Mean values, with *p*-values, of CECs and their subsets and CEPs, after AC-based therapy and at the end of taxane-based therapy, versus baseline both in percentages and absolute numbers, are listed in Tables 4 and 5, respectively.

3.3 Modification of serological parameters during adjuvant treatment

VEGF, SDF-1α and HER2 serum levels were assessed by ELISA, both at baseline and during adjuvant treatment. At baseline, the VEGF and SDF-1α mean values were within the normal reference ranges, while the HER2 mean values showed an increase compared to the reference range. After

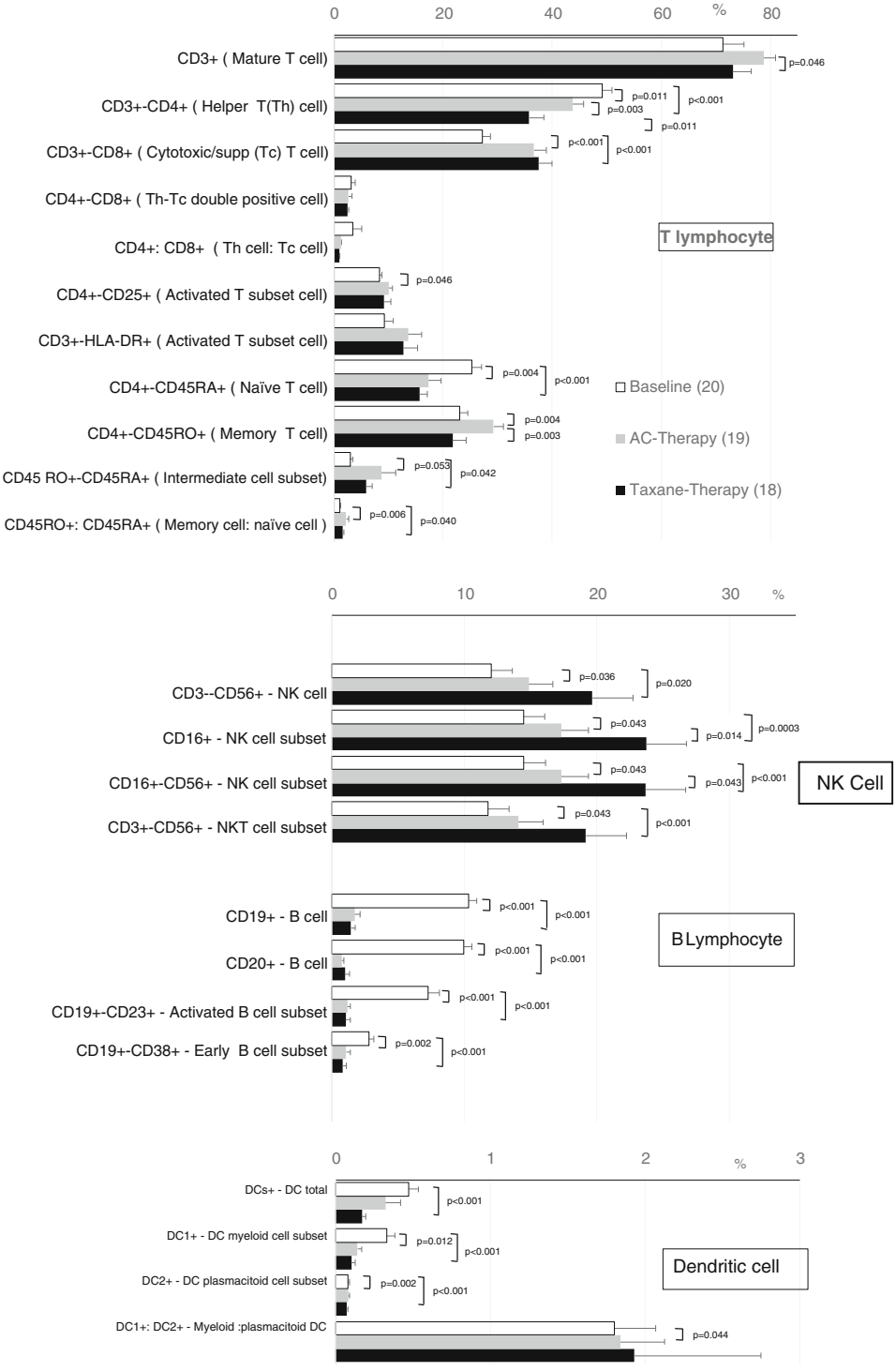

Fig. 6 Mean percentage levels of biomarker lymphocyte and DC subsets among baseline patients versus anthracycline-(AC) and taxane-based treatment

AC-based therapy the VEGF level showed a significant increase ($p = 0.011$), while the SDF-1α and HER2 levels showed significant decreases ($p = 0.001$ and $p = 0.004$), respectively, compared to baseline values. At the end of the 12th cycle of taxane-based therapy, the VEGF level again exhibited a significant increase ($p = 0.037$), while the SDF-1α and HER2 levels were found to decrease again, although this decrease was significant only for SDF-1α ($p = 0.003$). The serum cytokine data, with their mean values (± SD) and p-values, after AC-based and at the end of taxane-based therapy versus baseline, are summarized in Table 6. Additionally, we analyzed relationships between VEGF levels and the absolute numbers of CECs and their subsets and CEPs, but no direct correlations were detected.

Table 3 Comparison of cellular immunological profiles among anthracycline- (AC) and taxane-based therapy versus baseline

Parameter	Description	Baseline (n.20) Mean (sd) cells/μl	AC-Therapy (n.19) Mean (sd) cells/μl	*p*-Value	Taxane Therapy (n.18) Mean (sd) cells/μl	*p*-Value
Leucocyte	White cells	6877(1537)	5862(2596)	<0.0001	4820(1191)	<0.0001
Lymphocyte	White cells subset	2190(489)	1353(444)	<0.0001	1230(318)	<0.0001
T lymphocyte						
$CD3^+$	Mature T cell	1585(507)	1067(395)	<0.0001	913(340)	<0.0001
$CD3^+$-$CD4^+$	Helper T(Th) cell	1061(257)	589(222)	<0.0001	444(205)	<0.0001
$CD3^+$-$CD8^+$	Cytotoxic/supp (Tc) T	601(223)	509(231)	0.054	473(190)	0.002
$CD4^+$-$CD8^+$	cell Th-Tc double positive cell	73(86)	38(44)	0.038	30(18)	0.014
$CD4^+$-$CD25^+$	Activated T subset cell	184(61)	135(60)	0.011	102(47)	<0.0001
$CD3^+$-HLA-DR^+	Activated T subset cell	198(151)	198(216)	0.990	164(149)	0.407
$CD4^+$-$CD45RA^+$	Naïve T cell	527(149)	226(177)	<0.0001	182(84)	<0.0001
$CD4^+$-$CD45RO^+$	Memory T cell	512(206)	397(172)	0.005	275(171)	0.005
$CD45RO^+$-$CD45RA^+$	Intermediate cell subset	65(42)	120(198)	0.265	72(58)	0.706
NK Cell						
$CD3^-$-$CD56^+$	NK cell	266(168)	198(117)	0.043	231(151)	0.179
$CD16^+$	NK cell subset	326(173)	225(121)	0.003	278(154)	0.075
$CD16^+$-$CD56^+$	NK cell subset	260(164)	186(117)	0.011	226(153)	0.161
$CD3^+$-$CD56^+$	NKT cell subset	266(168)	155(134)	0.696	145(89)	0.504
B Lymphocyte						
$CD19^+$	B cell	225(81)	23(27)	<0.0001	16(20)	<0.0001
$CD20^+$	B cell	216(74)	10(12)	<0.0001	13(20)	<0.0001
$CD19^+$-$CD23^+$	Activated B cell subset	151(78)	13(11)	<0.0001	12(19)	<0.0001
$CD19^+$-$CD38^+$	Early B cell subset	61(41)	14(23)	0.001	10(14)	<0.0001
Dendritic cell (DC)						
DCs^+	DC total	30(23)	13(11)	0.016	8(6)	<0.0001
$DC1^+$	DC myeloid cell subset	21(18)	8(8)	0.02	5(5)	0.001
$DC2^+$	DC plasmacitoid cell subset	5(3)	5(3)	0.017	3(2)	<0.0001

3.4 Relationship between CTCs and immune parameters

We found that during adjuvant chemotherapy the circulating tumor cells (CTCs) showed no significant correlations with any of the peripheral immunological cell populations, except for an inverse correlation with the absolute numbers of T helper ($CD3^+$-$CD4^+$) cells ($p = 0.03$), the percentages and absolute numbers of the $CD45RO^+$-$CD45RA^+$ T cell intermediate subset ($p = 0.01$ and $p = 0.02$) and the DC1: DC2 ratio ($p = 0.03$). Comparisons of CTCs in the AC-based and taxane-based treatment groups versus baseline groups are listed in Table 7.

4 Discussion

The measurement of immunoregulatory cell levels in peripheral blood is important for the clinical management of cancer and for therapeutic approaches that may affect the immunological status of the patient. Numerous reports have indicated that both the presence of a tumor and the application of chemotherapy may impair immune responses [1, 2]. As of yet, most reports have focused primarily on immune changes in advanced stage cancer patients [29–33], while the immune status of early stage cancer patients has been less well studied. Here, we have monitored early breast cancer (BC) patients before and during adjuvant CT administration, using different factors selected for their relevance to BC: circulating lymphocyte and DC subsets, serological factors (VEGF, SDF-1α and HER2), circulating tumor cells (CTCs), circulating endothelial cells (CECs) and their subsets and bone marrow-derived circulating endothelial progenitor cells (CEPs). In addition, all cellular populations and serological factors were evaluated at the start of the study (baseline) to establish correlations with clinical-pathological parameters. However, few significant correlations were observed. It is important to note here that the majority of the patients included in this study was estrogen receptor (ER) negative. The patients enrolled in this study were diagnosed with early stage BC, and thus candidates for adjuvant chemotherapy. Among hormone-responsive BC carrying patients with positive prognostic factors, except ER-positive cases with high Ki67 and/or HER2 positivity

Table 4 Comparison of circulating endothelial cells (CECs) with their subsets (resting (CECr), activated (CECa) and apoptotic cells (CEC-APO) and circulating progenitors cells (CEPs) at baseline patients versus AC-based therapy

Parameter	Description	Percentage count		*p*-Value	Absolute count		*p*-Value
		Baseline (n.20) Mean (sd)	AC-Therapy (n.19) Mean (sd)		Baseline (n.20) Mean (sd)	AC-Therapy (n.19) Mean (sd)	
CECs	$CD146^+CD34^+CD133^-CD45^-$	0.0002 ± 0.0002	0.0002 ± 0.0001	0.257	18 ± 14	11 ± 13	0.166
CECr	$CD146^+CD34^+CD133^-CD106^-CD45^-$	0.0002 ± 0.0002	0.0001 ± 0.0001	0.207	16 ± 15	7 ± 7	0.048
CECa	$CD146^+CD34^+CD133CD106^+CD45^-$	0.0002 ± 0.0005	0.00003 ± 0.0001	0.625	1.4 ± 3.6	3 ± 13	0.464
CEPs	$CD146^+CD34^+CD133^+CD45^-$	0.0004 ± 0.0001	0.00004 ± 0.0001	0.861	1.9 ± 5	2 ± 5	0.815
CEC-APO	$CD146^+CD34^+CD133^-AnnV^+CD45$-	0.0001 ± 0.0001	0.00009 ± 0.0001	0.032	11 ± 10	4 ± 6	0.004

(Luminal B), the majority is currently not subjected to adjuvant chemotherapy according to international guidelines (12th St Gallen International Breast Cancer Conference, 2011). Our results may, therefore, be particularly reflective of the ER-negative subgroup of BC patients.

4.1 Lymphocyte subsets in early stage breast cancer before and after adjuvant chemotherapy

Patients with advanced tumors usually present with complex immune system dysfunctions, but the mechanisms underlying this phenomenon are as yet not completely understood [34]. Lymphopenia is frequently observed in patients with advanced cancers, before and during the administration of chemotherapy [35–38]. However, it remains unclear whether lymphopenia within specific lymphocyte subsets influences long term outcome, in particular progression-free survival (PFS) and overall survival (OS), in patients with solid tumors or lymphomas [39]. Compared to healthy controls, our early stage BC patients did not exhibit lymphopenia or significant changes in the percentages and absolute numbers of T and B cell subsets, except for a significant increase in $CD4^+CD8^+$ double positive T cells, activated T and B cells and NK cell subsets. Therefore, at presentation our BC population, in contrast to previous reports [4–6, 30, 40], does not show a deficit in the immune parameters studied, but rather an activation of T and B cell subsets and an increase in NK cells, an important component of innate immunity, able to limit viremia and to mediate spontaneous killing of various tumor cells, even before the adaptive immune system is activated [10].

Chemotherapy (CT) is considered to be a major cause of immune deficiency in cancer patients, but the impact of cytotoxic CT on the immune system has as yet not been fully elucidated. Also, little information is available on the actual impact of antineoplastic drugs such anthracyclines on the immune system [41]. In a preliminary study Mackall et al. [42] reported immunosuppressive effects of cytotoxic CT, such as decreases in T total and T helper cell subsets, following standard adjuvant CT and radiotherapy in BC patients. Significant reductions in the absolute numbers of T helper cells were also reported by Sewell et al. [43] in BC patients treated with standard CT, and by Sara et al. [44] in patients with solid tumors treated with CT. Schroeder et al. [45] reported specific alterations in T cell populations in patients with BC, such as a reduction in the absolute T cell number, but not in the $CD4^+$:$CD8^+$ ratio. Melichar et al. [46] observed some changes in lymphocyte subsets in a cohort of BC patients that were suggestive of T cell activation. Our group previously noted little impact of a topotecan-based CT on lymphocyte subsets in either naïve or pre-treated ovarian cancer patients [47]. Similarly, Collovà et al. [41] found no significant impairment of the immune system in BC patients during intensive CT programs with PEG support, with a rapid restoration of most immune competent cell populations.

Table 5 Comparison of circulating endothelial cells (CECs) with their subsets (resting (CECr), activated (CECa) and apoptotic cells (CEC-APO) and circulating progenitors cells (CEPs) at baseline patients versus taxane-based therapy

Parameter	Description	Percentage count		*p*-Value	Absolute count		*p*-Value
		Baseline (n.20) Mean (sd)	Taxane-therapy (n.18) Mean (sd)		Baseline (n.20) Mean (sd)	Taxane-therapy (n.18) Mean (sd)	
CECs	$CD146^+CD34^+CD133^-CD45^-$	0.0002 ± 0.0002	0.0002 ± 0.0002	0.459	18 ± 14	8 ± 9	0.036
CECr	$CD146^+CD34^+CD133^-CD106^-CD45^-$	0.0002 ± 0.0002	0.0001 ± 0.0002	0.318	16 ± 15	7 ± 9	0.047
CECa	$CD146^+CD34^+CD133CD106^+CD45^-$	0.0002 ± 0.0005	0.00003 ± 0.0001	0.591	1.4 ± 3.6	1.05 ± 2.99	0.743
CEPs	$CD146^+CD34^+CD133^+CD45^-$	0.0004 ± 0.0001	0.00005 ± 0.0001	0.782	1.9 ± 5	2.88 ± 9.67	0.737
CEC-APO	$CD146^+CD34^+CD133^-AnnV^+CD45$-	0.0001 ± 0.0001	0.0001 ± 0.0002	0.384	11 ± 10	2.66 ± 5.12	0.003

Table 6 Comparison of serum cytokine profiles among anthracycline- (AC) and taxane-based therapy versus baseline

Parameter	Description	Baseline Mean(sd) (n.20)	AC-Therapy Mean(sd) (n.19)	*p*-Value	Taxane-Therapy Mean(sd) (n.18)	*p*-Value
Human VEGF	human Vascular Endothelial Growth Factor	404 ± 199	745 ± 512	0.011	771 ± 684	0.037
Human CXCL12/SDF-1α	human Stromal cell-Derived Factor 1 alpha	2245 ± 364	1841 ± 337	0.001	1891 ± 300	0.003
sp185/HER2	human Epidermal Growth Factor Receptor 2	11.5 ± 2.24	9.43 ± 1.86	0.004	10.1 ± 2.69	0.116

In the present study we found, after AC-based therapy of early stage BC patients, significant decreases in the absolute numbers of leucocytes and total lymphocytes compared to baseline levels. Consequently, the absolute numbers of all T and B cell subset showed significant decreases, although the absolute numbers of NK subsets did not significantly decrease. In agreement with literature data [10, 40–47], we found that the percentages of T cells, T helper cells including their T naïve subset, and B lymphocyte subsets exhibited significant decreases after AC-based therapy. In contrast, the NK subsets, and the T cytotoxic and T memory subsets showed significant increases, suggesting a partial preservation of the immune competence. At the end of taxane-based therapy, the same trend was observed for the lymphocyte subsets, both in percentages and absolute numbers. Taken together, we conclude that in these patients anthracycline-based CT may be the major cause of the observed immune deficiency, which persisted through to the end of the therapeutic program. It is important to emphasize here that the immunodeficiency observed was partial and only concerned the adaptive immunity, while the innate immune system was not affected. Taxane has previously been reported to exert immunostimulatory effects that are supposed to be implicated in antitumor activity [48]. These findings may have important clinical implications for the design of immunotherapeutic approaches.

4.2 DC subsets in early stage breast cancer before and after adjuvant chemotherapy

Dendritic cells (DCs), the most powerful antigen-presenting cells, play a fundamental role in the induction of antitumor immune responses [49]. Peripheral blood DCs can be divided in two distinct subsets: myeloid and plasmacytoid DCs, based on their origins, phenotypic features and functions. Myeloid DCs are effective T cell stimulators that can induce tumor-specific immune responses, whereas the function of plasmacytoid DCs is as yet uncertain [50]. Adequate numbers and activities of these cells are crucial for eliciting effective antitumor effects and for the efficacy of cancer immunotherapies. Previous studies have reported that DCs isolated from cancer patients may exhibit both quantitative and functional deficiencies [49–53]. Gabrilovich et al. [51] observed e.g. reduced DC numbers in peripheral blood samples from patients with various malignancies. Della Bella et al. [52] reported that within a group of invasive breast cancer patients, no correlation could be found between DC count and cancer stage. In our baseline population we observed, compared to healthy controls, no statistically significant changes in total DCs, but we found a significant increase in the percentages and the absolute numbers of the DC1 subsets and the DC1: DC2 ratios, with the DC2 subset showing a significant decrease in percentage and absolute number. An increase in the myeloid DC subset may potentiate the tumor-specific T cells response, as reflected here by increases in the activated T and B cell subsets. Indeed, suppression of DC differentiation represents another important immune escape mechanism that has been encountered in some cancer patients [53]. Furthermore, DCs are now known to be potent stimulators of NK cell activation through the action of several cytokines. This may explain the persistence of the NK cells seen here [10]. At the end of the AC-based therapy, a significant decrease was observed in the percentages and absolute numbers of DC1 subsets and DC1:DC2 ratios, while the total DCs decreased only in absolute numbers. Finally, at the end of the 12th cycle of taxane-based therapy, DCs and their subsets showed significant decreases in percentages and absolute numbers. As previously observed in lymphocyte subsets and DCs, our results suggest

Table 7 Comparison of circulating tumor cells (CTC) among anthracycline- (AC) and taxane-based therapy versus baseline

Parameter	Description	Baseline (n.20) Mean (sd)	AC-Therapy (n.19) Mean (sd)	*p*-Value	Taxane Therapy (n.18) Mean (sd)	*p*-Value
nCTC*	$CD326^{+}PANCK^{+}CD45^{-}$	0.70(0.92)	0.73(0.80)	0.915	0.77(1.06)	0.825

**n.CTC* absolute number/3.75 ml, *PANCK* Pan-Cytokeratin

that in early stage BC patients anthracycline-based CT may be the major cause of immune deficiency, whereas taxane-based therapy may have played a relatively minor role.

4.3 Serological factors before and after adjuvant chemotherapy

In addition to the cellular immunological profiles, also the serological profiles of VEGF, SDF-1α and HER2 were evaluated before and during AC-based and taxane-based chemotherapy. VEGF is predominantly responsible for angiogenic signalling in endothelial cells [54, 55]. In a previous study it has been reported that the plasma level of VEGF may correlate with the CEC numbers [56]. Furstemberger et al. [57] reported that elevated CEC levels decreased significantly after anthracycline- and taxane-based therapy, but that these levels did not reach the level of the healthy controls. In our study population, the mean serum VEGF levels observed at baseline were within reference ranges, while during adjuvant-based therapy the VEGF levels were found to be significantly increased, although we did not detect a direct correlation between VEGF levels and CEC counts. The latter may be due to the fact that CEC numbers in tumor patients may be influenced by various additional factors, such as the localization of the tumor, the extent of tumor vascularisation and the proliferation status of the CECs. Additionally, preclinical data suggest a role of VEGF and SDF-1α in the homing and neoangiogenesis of metastases, including a synergistic hypoxia-dependent pro-angiogenic effect of VEGF and SDF-1α [58]. SDF-1α, also known as CXCL12, is the only known ligand for the CXC chemokine receptor 4 (CXCR4). This receptor plays a role in cellular motility, adhesion, chemotaxis, angiogenesis and metastasis. Though involved in many biological processes, the SDF-1-CXCR4 signalling axis has been shown to play an important role in BC [59, 60]. CXCR4 has been found to be over-expressed in both primary invasive and *in situ* ductal carcinomas, suggesting an important role for the SDF-1-CXCR4 axis at several stages of the disease. The exact impact of CXCR4 signalling on primary BC development, however, remains to be defined [61, 62]. In our study population, the serum SDF-1α mean values at baseline were within the normal range, whereas during adjuvant-based therapy a significant decrease, compared to baseline values, was observed.

Furthermore, we measured serum levels of the human epidermal growth factor receptor-2 (HER2), a receptor that has been found to play an important role in BC [63, 64]. Ample clinical studies have confirmed its prognostic significance in metastatic BC [65–70]. In patients with locally-advanced disease, the serum HER2 level has prognostic significance, and its increase has been associated with increased sensitivities to anthracycline-based CT and endocrine therapies. However, insufficient data are currently available on the value of serum HER2 testing in patients with early BC in order to allow a meaningful comparison with established BC prognostic factors, or to evaluate its potential for a possible early diagnostic and prognostic screen in these patients [71]. In our study population, the baseline mean serum HER2 levels were found to be increased compared to the normal reference ranges. However, we did not observe a direct correlation between serum HER2 levels and tissue HER2 status, nor between serum HER2 levels and clinical-pathological parameters. We did find, however, that AC-treatment, but not taxane treatment, resulted in decreased serum HER2 levels.

4.4 Circulating tumor cells during adjuvant chemotherapy

The systemic immunosuppression observed during adjuvant chemotherapy may contribute to activation of dormant tumor cells [12, 72]. The occurrence of circulating tumor cells (CTCs) in peripheral blood of patients with metastatic BC may be associated with a poor prognosis [8, 9], but there are as yet few data on the importance of these cells in patients with non-metastatic disease. Here, we failed to observe any significant correlation between CTC positivity and other established prognostic markers such as tumor stage, lymph node status, hormone receptor status, HER2 status, menopausal status and tumor size. According to previous reports, however, the detection of one or more CTCs as a potential independent prognostic factor in non-metastatic BC patients, might be useful in identifying early-stage patients at risk of disease progression [73, 74]. The CTC numbers did not correlate significantly with the numbers of peripheral blood lymphocyte subsets, except for inverse correlations observed for the DC1:DC2 ratio and the intermediate cell subset, in both percentages and absolute numbers, and the T helper cell subset in absolute number. The latter subset is directly related to an effective anti-tumor response, since these cells not only support the activation and expansion of cytotoxic T lymphocytes, but they are also essential for T cell priming in order to generate an effective $CD8^+$ T cell memory. The T helper cell deficit observed may contribute to systemic immune suppression and activation of dormant tumor cells. Further studies should clarify the exact effect of CTCs on the adaptive immune system.

4.5 Circulating endothelial and progenitor cells before and after adjuvant chemotherapy

In addition to genetic factors [13, 14] and immunological status, angiogenesis [15, 16] is an essential step in tumor growth and metastasis [17, 18]. Angiogenesis may be quantified through the identification of bone marrow-derived circulating endothelial progenitor cells (CEPs) and mature circulating endothelial cells (CECs). Previously, elevated CEC levels were reported as an adverse prognostic indicator for survival in

different types of cancer, suggesting that CEC quantification could be useful in identifying patients who might benefit from anti-angiogenesis therapy [75, 76]. Since ample studies have indicated that CEC and CEP levels may correlate with tumor size and grading [77–82], we hypothesised that tumor-bearing patients might have higher CEC and CEP levels than healthy controls. However, no significant differences were found. Perhaps CEC and CEP levels depend not only on the presence of a tumor, but also on other factors such as vascular and lymphatic invasion. Although most patients who received adjuvant CT had no gross residual tumor, vascular or lymphatic invasion is possible and may explain the CEC and CEP kinetics. Previous reports have been aimed at investigating how CECs and CEPs may be affected by different chemotherapeutic agents. Certain agents, such as taxanes and fluorouracil, have been found to rapidly induce CEP mobilization and subsequent tumor homing, while other agents, such as gemcitabine, cisplatin and doxorubicin, fail to do so [83, 84]. Furstenberger et al. [56] analyzed the levels of CECs and CEPs before and after neoadjuvant chemotherapy in BC patients and found that, after two cycles of CT, the CEC levels decreased and the CEP levels increased. This phenomenon may be explained by CEPs behaving as progenitor cells that could be mobilized from the bone marrow by a regular dose of CT. Although CEPs will gradually differentiate into CECs, the next course of CT may destroy the cells that are in transition before they can fully differentiate into CECs. This may explain why the CEC levels decreased and the CEP levels increased during CT administration. Finally, many preclinical studies have indicated that CEPs may contribute to tumor growth [78, 79, 85], which raises the concern that CT may mobilize CEPs and trigger additional tumor growth. In our study population, we found that total CECs, including their resting and apoptotic subset levels, decreased during adjuvant CT, whereas the CEP levels showed no significant changes compared to baseline values. Therefore, our results indicate that CEC and CEP counts change during CT treatment and that these changes should be borne in mind in planning any future study using CECs and CEPs as surrogate markers for angiogenesis status during CT treatment.

5 Conclusions

From our data we conclude that adjuvant CT, especially AC-based therapy, may have an immunosuppressive effect on patients with early stage BC and that this effect may persist till the end of the CT program. The immunodeficit observed is, however, partial and concerns only the adaptive immunity, while the innate immunity is not affected. Moreover, the taxane program is known to exert immunostimulatory effects, implicated in antitumor activity. Although the CTCs showed few significant correlations with peripheral blood lymphocyte subsets, their assessment may yield important information on the immune status of patients with non-metastatic BC, a patient subset in which the role of CTCs is not yet clarified. Further studies are required to verify the influence of CTCs on the adaptive immune system. Finally, the changes in CEC and CEP levels observed during therapy provide interesting information on the role of angiogenesis in early stage BC. A limitation of our study is the relatively small sample size. Therefore, future investigations should be carried out on larger patient cohorts in order to firmly establish that the adaptive immune system of these patients can be suppressed by chemotherapy, and to elucidate its clinical significance and the factors influencing immune recovery in order to develop potential rational therapeutic interventions.

Acknowledgments We wish to thank all the patients and volunteers for taking part in the study, as well as the nursing and the medical staff of the Oncologic Unit at the Fondazione IRCCS Policlinico San Matteo, for their support in the acquisition of clinical samples. The authors thank Giorgia Testa and Sabrina Nigrisoli (SC Pediatria, Laboratorio di Immuno Allergologia, Fondazione IRCCS Policlinico S. Matteo, Pavia) for technical support and Paul Baines (Cardiff, UK) for English language support.

Compliance with ethical standards All procedures involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent Informed consent was obtained from all individual participants included in the study.

Funding This work was supported by the Fondazione IRCCS Policlinico San Matteo, Pavia (Hospital Research grant n° 08067611 to P. Pedrazzoli) and "*quota 5×1000 dell'imposta su reddito delle persone fisiche*" designed for health research.

References

1. R.V. Gardner, Long term hematopoietic damage after chemotherapy and cytokine. Front. Biosci. **4**, e47–e57 (1999)
2. R.G. Van der Most, A. Currie, B.W. Robinson, R.A. Lake, Cranking the immunologic engine with chemotherapy: using context to drive tumor antigen cross-presentation towards useful antitumor immunity. Cancer Res. **66**, 601–604 (2006)
3. D.H. Kang, M.T. Weaver, N.J. Park, B. Smith, T. McArdle, J. Carpenter, Significant impairment in immune recovery after cancer treatment. Nurs. Res. **58**, 105–114 (2009)
4. M.J. Campbell, J. Scott, H.T. Maecker, J.W. Park, L.J. Esserman, Immune dysfunction and micrometastases in women with breast cancer. Breast Cancer Res. Treat. **91**, 163–171 (2005)
5. I. Caras, A. Grigorescu, C. Stavaru, D.L. Radu, I. Mogos, G. Szegli, A. Salageanu, Evidence for immune defects in breast and lung

cancer patients. Cancer Immunol. Immunother. **53**, 1146–1152 (2004)
6. G. Konievic, I. Spuzic, Stage dependence of NK cell activity and its modulation by interleukin 2 in patients with breast cancer. Neoplasma **40**, 81–855 (1993)
7. C. Wiltschke, M. Krainer, A.C. Budinsky, A. Berger, C. Muller, R. Zeillinger, P. Speiser, E. Kubista, M. Eibl, C.C. Zeilinski, Reduced mitogenic stimulation of peripheral blood mononuclear cells as a prognostic parameter for the course of breast cancer: a prospective longitudinal study. Br. J. Cancer **71**, 1292–1296 (1995)
8. Y. Nieto, E.J. Shpall, I.K. McNiece, S. Nawaz, J. Beaudet, S. Rosinski, J. Pellom, V. Slat-Vasquez, P.A. McSweeney, S.I. Bearman, J. Murphy, R.B. Jones, Prognostic analysis of early lymphopcyte recovery in patients with advanced breast cancer receiving high-dose chemotherapy with an autologous hematopoietic progenitor cell transplant. Clin. Cancer Res. **10**, 5076–5086 (2004)
9. I.F. Porrata, J.N. Ingle, M.R. Litzow, S. Geyer, S.N. Markovic, Prolonged survival associated wit early lymphocyte recovery after autologous hematopoietic stem cell transplantation for patients with metastatic breast cancer. Bone Marrow Transplant. **28**, 865–871 (2001)
10. S. Mariucci, B. Rovati, M. Manzoni, M.G. Della Porta, G. Comolli, S. Delfanti, M. Danova, Lymphocyte subpopulation and dendritic cell phenotyping during antineoplastic therapy in human solid tumors. Clin. Exp. Med. **11**, 199–210 (2011)
11. G. Ferlazzo, M. Pack, D. Thomas, C. Paludan, D. Schmid, T. Strowing, G. Bougras, V.A. Muller, L. Moretta, C. Munz, Distinct CT roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs. Proc. Natl. Acad. Sci. U. S. A. **101**, 16606–16611 (2004)
12. S. Zhao, Y. Liu, Q. Zhang, H. Li, M. Zhang, W. Ma, W. Zhao, J. Wang, M. Yang, The prognostic role of circulating tumor cells (CTCs) detected by RT-PCR in breast cancer. A meta-analysis of published literature. Breast Cancer Res. Treat. **130**, 809–816 (2011)
13. L.D. Wood, D.W. Parsons, S. Jones, J. Lin, T. Sjoblom, R.J. Leary, D. Shen, S.M. Boca, T. Barber, J. Ptak, N. Silliman, S. Szabo, Z. Dezso, V. Ustyanksky, T. Nikolskaya, Y. Nikolsky, R. Karchin, P.A. Wilson, J.S. Kaminker, Z. Zhang, R. Croshaw, J. Willis, D. Dawson, M. Shipitsin, J.K. Willson, S. Sukumar, K. Polyak, B.H. Park, C.L. Pethiyagoda, P.V. Pant, D.G. Ballinger, A.B. Sparks, J. Hartigan, D.R. Smith, E. Suh, N. Papadopoulos, P. Buckhaults, S.D. Markowitz, G. Parmigiani, K.W. Kinzler, V.E. Velculescu, B. Vogelstein, The genomic landscapes of human breast and colorectal cancers. Science **318**, 1108–1113 (2007)
14. C.A. Klein, D. Holzel, Systemic cancer progression and tumor dormancy: mathematical models meet single cell genomics. Cell Cycle **5**, 1788–1798 (2006)
15. S. North, M. Moenner, A. Bikfalvi, Recent developments in the regulation of the angiogenic switch by cellular stress factors in tumors. Cancer Lett. **218**, 1–14 (2005)
16. M. Relf, S. LeJeune, P.A. Scott, S. Fox, K. Smith, R. Leek, A. Moghaddam, R. Whitehouse, R. Bicknell, A.L. Harris, Expression of the angiogenic factors vascular endothelial cells growth factors, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor and pleiotrophin in human primary breast cancer and its relation to angiogenesis. Cancer Res. **57**, 963–969 (1997)
17. M. Campoli, S. Ferrone, A.H. Zea, P.C. Rodriguez, A.C. Ochoa, Mechanisms of tumor evasion. Cancer Treat. Res. **23**, 61–88 (2005)
18. H.R. Salih, V. Nüssler, Immune escape versus tumor tolerance: how do tumor evade immune surveillance? Eur. J. Med. Res. **6**, 323–632 (2001)
19. C.C. Lin, C.Y. Liu, M.J. Chen, T.E. Wang, C.H. Chu, H.Y. Wang, S.C. Shit, M.L. Hsu, T.C. Hsu, Y.J. Chen, Profiles of circulating endothelial cells and serum cytokines during adjuvant chemoradiation in rectal cancer patients. Clin. Transl. Oncol. **15**, 855–860 (2013)
20. E. Razis, K.T. Kalogeras, V. Kotoula, A.G. Eleftheraki, N. Nikitas, R. Kronenwett, E. Timotheadou, C. Christodoulou, D. Pectasides, H. Gogas, R.M. Wirtz, T. Makatsoris, D. Bafaloukos, G. Aravantinos, D. Televantou, N. Pavlidis, G. Fountzilas, Improved outcome of high-risk early HER2 positive breast cancer with high CXCL13-CXCR5 messenger RNA expression. Clin. Breast Cancer **12**, 183–193 (2012)
21. J. Wang, Q. He, Y.G. Shao, M. Ji, Chemokine fluctuate in the progression of primary breast cancer. Eur. Rev. Med. Farmacol. Sci. **17**, 596–608 (2013)
22. World Medical Association, Declaration of Helsinki: ethical principles for medical research involving human subjects. J. Postgrad. Med. **48**, 206–208 (2002)
23. B. Rovati, S. Mariucci, R. Poma, C. Tinelli, S. Delfanti, P. Pedrazzoli, An eight-colour flow cytometric method for the detection of reference values of lymphocyte subsets in selected healthy donors. Clin. Exp. Med. **14**, 249–259 (2014)
24. B. Rovati, S. Mariucci, M. Manzoni, K. Bencardino, M. Danova, Flow cytometric detection of circulating dendritics cells in healthy subjects. EJH **52**, 45–52 (2008)
25. S. Mariucci, B. Rovati, S. Chatzileontiadou, K. Bencardino, M. Manzoni, S. Delfanti, M. Danova, A six-colour flow cytometric method for simultaneous detection of cell phenotype and apoptosis of circulating endothelial cells. Scand. J. Clin. Lab. Invest. **69**, 433–438 (2009)
26. L. Whitby, W. Granger, I. Storie, K. Goodfellow, A. Sawle, J.T. Reilly, D. Barnett, Quality control of CD4+ T-lymphocyte enumeration : results from 9 years of the United Kingdom national external quality assessment scheme for immune monitoring (1993–2001). Cytometry **50**, 102–110 (2002)
27. T. Hristozova, R. Konschak, C. Stromberger, A. Fusi, Z. Liu, W. Weichert, A. Stenzinger, V. Budach, U. Keilholz, I. Tinhofer, The presence of circulating tumor cells (CTCs) correlates with lymph node metastasis in nonresectable squamous cell carcinoma of the head and neck region (SCCHN). Ann. Oncol. **22**, 1878–1885 (2011)
28. T.J. Molloy, A.J. Bosma, L.O. Baumbusch, M. Synnestvedt, E. Borgen, H.G. Russnes, E. Schlichting, L.J. van't Veer, B. Naume, The prognostic significance of tumor cell detection in the peripheral blood versus the bone marrow in 733 early-stage breast cancer patients. Breast Cancer Res. **3**, R61 (2011)
29. C. Jochems, J. Schlom, Tumor- infiltrating immune cells and prognosis: the potential link between conventional cancer therapy and immunity. Exp. Biol. Med. **236**, 567–279 (2011)
30. I. Poschke, D. Mougiakakos, R. Kiessling, Camouflage and sabotage: tumor escape from the immune system. Cancer Immunol. Immunother. **60**, 1161–1171 (2011)
31. J. de Boniface, I. Poschke, Y. Mao, R. Kiessling, Tumor-dependent down regulation of the Zeta chain in T cell is detectable in early breast cancer and correlates with immune cell function. Int. J. Cancer **131**, 129–139 (2012)
32. G.P. Dunn, L.J. Old, R.D. Schreiber, The immunobiology of cancer immunosurveillance and immunoediting. Immunity **21**, 137–148 (2004)
33. R. Kim, M. Emi, K. Tanabe, K. Arihiro, Tumor driven evolution of immunosuppressive networks during malignant progression. Cancer Res. **66**, 527–536 (2006)
34. S. Ferrari, F. Malugani, B. Rovati, C. Porta, A. Riccardi, M. Danova, Flow cytometric analysis of circulating dendritic cells subsets and intracellular cytokine production in advanced breast cancer patients. Oncol. Rep. **14**, 113–120 (2005)
35. M. Siddiqui, K. Ristow, S.N. Markovic, T.E. Witzig, T.M. Habermann, J.P. Colgan, D.J. Inwards, W.L. White, S.M. Ansell, I.N. Micallef, P.B. Johnston, T.G. Call, L.F. Porrata, Absolute

lymphocyte count predicts overall survival in follicular lymphomas. Br. J. Haematol. **134**, 596–601 (2006)
36. C. Borg, I. Ray-Coquard, I. Philip, G. Clapisson, N. Bendriss-Vermare, C. Menetrier-Caux, C. Sebban, P. Biron, J.Y. Blay, CD4 lymphopenia as a risk factor for febrile neutropenia and early death after cytotoxic chemotherapy in adult patients with cancer. Cancer **101**, 2675–2680 (2004)
37. I. Ray-Coquard, H. Ghesquiere, T. Bachelot, C. Borg, P. Biron, C. Sebban, A. LeCesne, F. Chauvin, J.Y. Blay, ELYPSE Study Group, Identification of patients at risk for early death after conventional chemotherapy in solid tumors and lymphomas. Br. J. Cancer **85**, 816–822 (2001)
38. Y. Oki, K. Yamamoto, H. Kato, Y. Kuwatsuka, H. Taji, Y. Kagami, Y. Morishima, Low absolute lymphocyte count is a poor prognostic marker in patients with diffuse large B-cell lymphoma and suggests patients' survival benefit from rituximab. Eur. J. Haematol. **81**, 1209–1215 (2008)
39. J. Péron, C. Cropet, O. Tredan, T. Bachelot, I. Ray-Coquard, G. Clapisson, S. Chabaud, I. Philip, C. Borg, P. Cassier, I. Labidi Galy, C. Sebban, D. Perol, P. Biron, C. Caux, C. Menetrier-Caux, J.Y. Blay, CD4 lymphopenia to identify and-of-life metastatic cancer patient. Eur. J. Cancer **49**, 1080–1089 (2013)
40. I. Poschke, J. De Boniface, Y. Mao, R. Kiessling, Tumor-induced changes in the phenotype of blood-derived and tumor-associated T cells of early stage breast cancer patients. Int. J. Cancer **131**, 1611–1620 (2012)
41. E. Collovà, B. Rovati, D. Grasso, K. Bencardino, M. Manzoni, M. Danova, Effect of peg-Filgrastim-supported dose-dense adjuvant chemotherapy on the peripheral blood leukocyte phenotype in breast cancer patients. Mol. Med. Rep. **2**, 85–88 (2009)
42. C.L. Mackall, T-cell immunodeficiency following cytotoxic antineoplastic therapy: a review. Stem Cells **18**, 10–18 (2000)
43. H.F. Sewell, C.F. Halbert, R.A. Robins, A. Galvin, S. Chan, R.W. Blamey, Chemotherapy induced differential changes in lymphocyte subsets and natural killer cell function in patients with advanced breast cancer disease. Int. J. Cancer **55**, 735–738 (1993)
44. E. Sara, A. Kotsakis, J. Souklekos, C. Kourousis, S. Kakolyris, E. Mavromanolakis, J. Vlachonicolis, V. Georgoulias, Post-chemotherapy lymphopoiesis in patients with solid tumors is characterized by CD4+ cell proliferation. Anticancer Res. **19**, 471–476 (1999)
45. W. Schroeder, A. Vering, M. Stegmuller, R. Strohmeier, Lymphocyte subsets in patients with ovarian and breast cancer. Eur. J. Gynaecol. **18**, 474–479 (1997)
46. B. Melichar, M. Touskova, J. Dvorak, P. Jandik, O. Kopecky, The peripheral blood leukocyte phenotype in patients with breast cancer: effect of doxorubicin/paclitaxel combination chemotherapy. Immunopharmacol. Immunotoxicol. **23**, 163–173 (2001)
47. S. Ferrari, B. Rovati, L. Cucca, C. Scarabelli, M. Presti, C. Beccaria, E. Collovà, C. Porta, M. Danova, Impact of the topotecan-based chemotherapy on the immune system of advanced ovarian cancer patients: an immunophenotypic study. Oncol. Rep. **9**, 1107–1113 (2002)
48. O.T. Chan, K.X. Yang, The immunological effects of taxanes. Cancer Immunol. Immunother. **49**, 181–185 (2000)
49. A. Pinzon-Charry, C.S.K. Ho, R. Laherty, T. Maxwell, D. Walker, R.A. Gardiner, L. O'Connor, C. Pyke, C. Schmidt, C. Furnival, J.A. López, A population of HLA-DR+ immature cells accumulates in the blood dendritic cells compartment of patients with different types of cancer. Neoplasia **7**, 1112–1122 (2005)
50. S.I. Labidi-Galy, V. Sisirak, P. Meeus, M. Gobert, I. Treilleux, A. Bajard, J.D. Combes, J. Faget, F. Mithieux, A. Cassignol, O. Tredan, I. Durand, C. Ménétrier-Caux, C. Caux, J.Y. Blay, I. Ray-Coquard, N. Bendriss-Vermare, Quantitative and functional alterations of plasmacytoid dendritic cells contribute to immune tolerance in ovarian cancer. Cancer Res. **71**, 5423–5434 (2011)
51. D.I. Gabrilovich, I.F. Ciernik, D. Carbone, Dendritic cells in antitumor immune responses. I defective antigen presentation in tumor-bearing hosts. Cell. Immunol. **170**, 101–110 (1996)
52. S. Della Bella, M. Gennaro, M. Vaccari, C. Ferraris, S. Nicola, A. Riva, M. Clerici, M. Greco, M.L. Villa, Altered maturation of peripheral blood dendritic cells in patients with breast cancer. Br. J. Cancer **89**, 1463–1472 (2003)
53. B. Almand, J.R. Resser, B. Lindman, S. Nadaf, J.I. Clark, E.D. Kwon, D.P. Carbone, D.I. Gabrilovich, Clinical significance of defective dendritic cell differentiation in cancer. Clin. Cancer Res. **6**, 1755–1766 (2000)
54. G. Absenger, J. Szkandera, M. Stotz, M. Pichler, T. Winder, T. Langsenlehner, U. Langsenlehner, H. Samonigg, W. Renner, A. Gerger, A common and functional gene variant in the vascular endothelial growth factor a predicts clinical outcome in early-stage breast cancer. Mol. Carcinog. **52**, E96–E102 (2013)
55. P. Mancuso, A. Burini, G. Pruneri, A. Goldhirsch, G. Martinelli, F. Bertolini, Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. Blood **97**, 3658–3661 (2001)
56. L.V. Beerpoot, N. Mehra, J.S. Vermaat, B.A. Zonnenberg, M.F. Gebbink, E.E. Voest, Increased levels of viable circulating endothelial cells are an indicator of progressive disease in cancer patients. Ann. Oncol. **15**, 139–145 (2004)
57. G. Furstenberger, R. von Moos, R. Lucas, B. Thürlimann, H.J. Senn, J. Hamacher, E.M. Boneberg, Circulating endothelial cells and angiogenic serum factor during neoadjuvant chemotherapy of primary breast cancer. Br. J. Cancer **94**, 524–531 (2006)
58. M.D. Groves, K.R. Hess, V.K. Puduvalli, H. Colman, C.A. Conrad, M.R. Gilbert, J. Weinberg, M. Cristofanilli, W.K. Yung, T.J. Liu, Biomarkers of disease: cerebrospinal fluid vascular endothelial growth factor (VEGF) and stromal cell derived factor (SDF)-1 levels in patients with neoplastic meningitis (NM) due to breast cancer, lung cancer and melanoma. J. Neurooncol. **94**, 229–234 (2009)
59. G. Lazennec, A. Richmond, Chemokines and chemokine receptors: new insights into cancer-related inflammation. Trends Mol. Med. **16**, 133–144 (2000)
60. L.V. Rodhes, M.R. Bratton, Y. Zhu, S.L. Tilghman, S.E. Muir, V.A. Salvo, C.R. Tate, S. Elliott, K.P. Nephew, B.M. Collins-Burow, M.E. Burow, Effects of SDF-1-CXCR4 signalling on microRNA expression and tumorigenesis in estrogen receptor-alpha (ER-α)-positive breast cancer cells. Exp. Cell Res. **317**, 2573–2581 (2011)
61. A. Muller, B. Homey, H. Soto, N. Ge, D. Catron, M.E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S.N. Wagner, J.L. Barrera, A. Mohar, E. Verástegui, A. Zlotnik, Involvement of chemokine receptors in breast cancer metastasis. Nature **410**, 50–56 (2001)
62. M.C. Smith, K.E. Luker, J.R. Garbow, J.L. Prior, E. Jackson, D. Piwnica-Worms, G.D. Luker, CXCR4 regulates growth of both primary and metastatic breast cancer. Cancer Res. **64**, 8604–8612 (2004)
63. V. Ludovini, S. Gori, M. Colozza, L. Pistola, E. Rulli, I. Floriani, E. Pacifico, F.R. Tofanetti, A. Sidoni, C. Basurto, A. Rulli, L. Crinò, Evaluation of serum HER2 extracellular domain in early breast cancer patients: correlation with clinicopathological parameters and survival. Ann. Oncol. **19**, 883–890 (2008)
64. V. Rossi, I. Sarotto, F. Maggiorotto, P. Berchialla, F. Kubatzki, N. Tomasi, S. Redana, R. Martinello, G. Valabrega, M. Aglietta, R. Ponzone, F. Montemurro, Moderate immunohistochemical expression of HER-2 (2+) without HER- 2 gene amplification is a negative prognostic factor in early breast cancer. Oncologist **17**, 1418–1425 (2012)
65. R. Colomer, S. Montero, A. Lluch, B. Ojeda, A. Barnadas, A. Casado, B. Massutí, H. Cortés-Funes, B. Lloveras, Circulating HER2 extracellular domain and resistance to chemotherapy in advanced breast cancer. Clin. Cancer Res. **6**, 2356–2362 (2000)

66. D. Lüftner, P. Henschke, B. Flath, C. Akrivakis, S. Schnabel, B. Prinz, R. Geppert, K.D. Wernecke, K. Possinger, Serum HER2 /neu as a prediction and monitoring parameter in a phase II study with weekly paclitaxel in metastatic breast cancer. Anticancer Res. **24**, 895–906 (2004)
67. L.N. Harris, V. Liotcheva, G. Broadwater, M.J. Ramirez, P. Maimonis, S. Anderson, T. Everett, D. Harpole, M.B. Moore, D.A. Berry, D. Rizzeri, J.J. Vredenburgh, R.C. Bentley, Comparison of methods of measuring HER-2 in metastatic breast cancer patients treated with high-dose chemotherapy. J. Clin. Oncol. **19**, 1698–1706 (2001)
68. A. Lipton, S.M. Ali, K. Leitzel, L. Demers, V. Chinchilli, L. Engle, H.A. Harvey, C. Brady, C.M. Nalin, M. Dugan, W. Carney, J. Allard, Elevated serum Her-2/neu level predicts decreased response to hormone therapy in metastatic breast cancer. J. Clin. Oncol. **20**, 1467–1472 (2002)
69. G.B. Cook, I.E. Neaman, J.L. Goldblatt, D.R. Cambetas, M. Hussain, D. Lüftner, K.K. Yeung, D.W. Chan, M.K. Schwartz, W.J. Allard, Clinical utility of serum HER-2/neu testing on the bayer immuno 1 automated system in breast cancer. Anticancer Res. **21**, 1465–1470 (2001)
70. F.J. Esteva, V. Valero, D. Booser, L.T. Guerra, J.L. Murray, L. Pusztai, M. Cristofanilli, B. Arun, B. Esmaeli, H.A. Fritsche, N. Sneige, T.L. Smith, G.N. Hortobagyi, Phase II study of weekly docetaxel and trastuzumab for patients with HER-2-overexpressing metastatic breast cancer. J. Clin. Oncol. **20**, 1800–1808 (2002)
71. R.R. Mehta, J.H. McDermott, T.J. Hieken, K.C. Marler, M.K. Patel, L.D. Wild, T.K. Das Gupta, Plasma c-erbB-2 levels in breast cancer patients: prognostic significance in predicting response to chemotherapy. J. Clin. Oncol. **16**, 2409–2416 (1998)
72. I. Gruber, N. Landenbergen, A. Staebler, M. Hahn, D. Wallwiener, T. Fehm, Relationship between circulating tumor cells and peripheral T cells in patients with primary breast cancer. Anticancer Res. **33**, 2233–2338 (2013)
73. A. Lucci, C.S. Hall, A.K. Lodhi, A. Bhattacharyya, A.E. Anderson, L. Xiao, I. Bedrosian, H.M. Kuerer, S. Krishnamurthy, Circulating tumor cells in non-metastatic breast cancer: a prospective study. Lancet Oncol. **13**, 688–695 (2012)
74. V. Mikulova, M. Cabinakova, I. Janatkova, O. Mestek, T. Zima, P. Tesarova, Detection of circulating tumor cells during follow-up of patients with early breast cancer: clinical utility for monitoring of therapy efficacy. Scand. J. Clin. Lab. Invest. **19**, 1–11 (2013)
75. T. Fleitas, V. Martinez-Sales, V. Vila, E. Reganon, D. Mesado, M. Martín, J. Gómez-Codina, J. Montalar, G. Reynés, Circulating endothelial cells and microparticle as prognostic markers in advanced non-small cell lung cancer. PLoS ONE **7**, e47365 (2012)
76. P. Mancuso, F. Bertolini, Circulating endothelial cells as biomarkers in clinical oncology. Microvasc. Res. **79**, 224–228 (2010)
77. B. Dome, J. Timar, J. Dobos, L. Meszaros, E. Raso, S. Paku, I. Kenessey, G. Ostoros, M. Magyar, A. Ladanyi, K. Bogos, J. Tovari, Identification and clinical significance of circulating endothelial progenitors cells in human non-small cell lung cancer. Cancer Res. **66**, 7341–7347 (2006)
78. R.P. Naik, D. Jin, E. Chuang, E.G. Gold, E.A. Tousimis, A.L. Moore, P.J. Christos, T. de Dalmas, D. Donovan, S. Rafii, L.T. Vahdat, Circulating endothelial progenitors cells correlate to stage in patients with invasive breast cancer. Breast Cancer Res. Treat. **107**, 133–138 (2008)
79. P.K. Goon, G.Y. Lip, P.S. Stonelake, A.D. Blann, Circulating endothelial cells and circulating progenitors cells in breast cancer: relationship to endothelial damage/dysfunction/apoptosis, clinicopathologic factors, and the Nottingham Prognostic Index. Neoplasia **11**, 71–779 (2009)
80. D. Goodale, C. Phay, W. Brown, L. Gray-Statchuk, P. Furlong, M. Lock, I. Chin-Yee, M. Keeney, A.L. Allan, Flow cytometric assessment of monocyte activation markers and circulating endothelial cells in patients with localized or metastatic breast cancer. Cytometry B Clin. Cytom. **76**, 107–117 (2009)
81. H.K. Kim, K.S. Song, H.O. Kim, J.H. Chung, K.R. Lee, D.H. Lee, E.S. Lee, H.K. Kim, K.W. Ryu, J.M. Bae, Circulating numbers of endothelial progenitors cells in patients with gastric and breast cancer. Cancer Lett. **198**, 83–88 (2003)
82. C. Richter-Ehrenstaein, J. Rentzsch, S. Runkel, A. Schneider, G. Schonfelder, Endothelial progenitors cells in breast cancer patients. Breast Cancer Res. Treat. **106**, 343–349 (2007)
83. Y.H. Kuo, C.H. Lin, W.Y. Shau, T.J. Chen, S.H. Yang, S.M. Huang, C. Hsu, Y.S. Lu, A.L. Cheng, Dynamics of endothelial cells and progenitors cells in breast cancer patients receiving cytotoxic chemotherapy. BMC Cancer **12**, 620 (2012)
84. Y. Shaked, E. Henke, J.M. Roodhart, P. Mancuso, M.H. Langenberg, M. Colleoni, L.G. Daenen, S. Man, P. Xu, U. Emmenegger, T. Tang, Z. Zhu, L. Witte, R.M. Strieter, F. Bertolini, E.E. Voest, R. Benezra, R.S. Kerbel, Rapid chemotherapy-induced acute endothelial progenitor cell mobilization: implications for antiangiogenic drugs as chemosensitizing agents. Cancer Cell **14**, 263–267 (2008)
85. J.W. Ho, R.W. Pang, C. Lau, C.K. Sun, W.C. Yu, S.T. Fan, R.T. Poon, Significance of circulating endothelial progenitor cells in hepatocellular carcinoma. J. Hepatol. **44**, 836–843 (2006)