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Peripheral blood leukocyte populations and urinary neopterin during chemotherapy in patients with breast cancer

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Abstract: The aim of the present study was to investigate serial changes in urinary neopterin and the populations of peripheral blood lymphocytes in breast cancer patients treated with primary chemotherapy. Thirty patients with breast cancer, including 20 patients undergoing primary chemotherapy treated with the combination of doxorubicin and cyclophosphamide with sequential administration of paclitaxel-based therapy (cohort A), and 10 patients presenting for first-line therapy of metastatic disease and treated with the combination of bevacizumab, taxane and carboplatin (cohort M) were studied. Healthy female blood donors served as control group. Significant differences were observed in baseline relative and absolute counts of peripheral blood lymphocyte subpopulations between both patient cohorts and controls. Significant changes of peripheral blood lymphocyte subpopulations were also observed during the course of chemotherapy. However, no significant changes of urinary neopterin concentrations and no correlation between urinary neopterin and peripheral blood lymphocyte counts were observed. In conclusion, present data demonstrate the presence of significant differences in peripheral blood leukocyte phenotype in breast cancer patients even before the start of primary chemotherapy. Significant changes were observed during the course of chemotherapy.

Keywords: breast cancer; neopterin; peripheral blood lymphocytes; primary chemotherapy.

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Introduction

Breast cancer is the most common malignant tumor in women in the Western world [1]. Despite the advances in early diagnosis and multidisciplinary treatment, breast cancer remains one of the leading causes of cancer deaths in women. It is now widely accepted that even in patients with apparently localized tumors breast cancer represents a systemic disease. In fact, improved prognosis of patients with breast cancer during the last decades has been caused not only by timely diagnosis, but also by improved systemic therapy. Systemic therapy, including the administration of cytotoxic, hormonal or targeted drugs currently represents an essential integral part of the multidisciplinary management of cancer patients. In patients with breast cancer systemic therapy is commonly administered after surgery in the adjuvant setting. In patients with locally advanced breast cancer, the primary tumor is considered unsuitable for upfront surgery, and systemic treatment is administered as first therapeutic modality, followed by surgery. Based on the observation that this therapeutic approach converts inoperable tumors to tumors that can be surgically treated, primary systemic treatment is also being increasingly used in patients with operable tumors with the aim to allow less extensive surgery [2]. Primary (neoadjuvant) chemotherapy also represents a model system to directly examine the effect of systemic therapy on the tumor and the host-tumor interactions [3, 4].

The role of the immune response in the pathogenesis of neoplastic disorders is being increasingly recognized [5, 6]. In fact, evasion from the immune response has been defined as one of the hallmarks of cancer [5]. The immune response directed against the tumor cells can effectively control tumor growth and can even result in tumor eradication. However, in patients with advanced tumors, the immune response against the tumor is failing and advanced cancer is often associated with secondary immune deficiency [7, 8]. Moreover, the immune response plays a dual role in determining the outcome of the host-tumor interaction, and inflammatory response is one of the essential factors promoting tumor growth and progression [5].

The assessment of biomarkers currently represents an integral component of the multidisciplinary management of cancer patients. In patients with different primary tumors, including breast cancer, most of the biomarkers currently used reflect the properties of the tumor cells, and limited attention has been devoted to the biomarkers of the host response [6]. An association between the presence of immune cells in the tumor tissue or biological fluids of the tumor microenvironment and the host antitumor response has been demonstrated [9–11]. The presence of tumor infiltrating lymphocytes (TIL) has been associated with the prognosis of different primary tumors [4, 12–15]. Moreover, in patients with breast cancer, the numbers of TIL have been shown to predict the response to chemotherapy [3, 4, 15, 16]. However, repeated measurements are needed to assess the host response during the therapy. Thus, the utilization of TIL for the monitoring of the host response during the course of treatment is limited because of the need to perform repeated tumor biopsies. Changes in the peripheral blood leukocyte populations have been described in cancer patients. Using peripheral blood as the sample matrix for the assessment of the immune response has an obvious advantage of possibility to repeat the measurements during the course of treatment.

The aim of the present pilot study was to investigate serial changes in the populations of peripheral blood lymphocytes in breast cancer patients treated with chemotherapy.

Patients and methods

Thirty consecutive patients, all females, aged (mean ± standard deviation) 50 ± 11 (range 30–69) years with histologically verified breast cancer, including 20 patients (aged 49 ± 9, range 30–64 years) undergoing primary chemotherapy (cohort A) and 10 patients (aged 53 ± 15, range 37–69 years) presenting for first-line chemotherapy of metastatic disease (cohort M) were studied. Among patients with primary chemotherapy nine patients had stage II breast cancer, 10 patients had stage III breast cancer. One patient treated with primary chemotherapy had limited metastatic involvement of bone diagnosed shortly after the start of treatment (stage IV). Control examination 3 years after primary chemotherapy was performed in 10 patients aged (at the time of examination) 50 ± 10 (range 33–67) years. Twenty healthy female blood donors, aged 52 ± 8 (range 34–65) years served as controls. The present study was approved by institutional ethical committee and the subjects signed informed consent.

The primary chemotherapy regimen consisted of four cycles of the combination of doxorubicin and cyclophosphamide (AC) administered at the doses of 60 mg/m^2 and 600 mg/m^2 every 2 weeks, respectively. Filgrastim was administered to allow biweekly administration. The patients were sequentially treated with 12 weekly doses of paclitaxel (90 mg/m^2) that was combined with weekly trastuzumab (4 mg/kg loading dose and 2 mg/kg for subsequent doses) and weekly carboplatin (area under the concentration time curve 2) in eight patients with human epidermal growth factor receptor (HER)-2-positive tumors [2]. The cohort of patients with metastatic breast cancer treated with the combination of bevacizumab (10 mg/kg every 2 weeks), paclitaxel (90 mg/m^2 weekly) and carboplatin (area under the concentration time curve 2) has been described previously in a paper reporting changes of urinary neopterin and other biochemical parameters (in one patient with a history of infusion reactions after paclitaxel, weekly docetaxel at the dose of 40 mg/m^2 was used) [17].

Relative numbers of cluster of differentiation (CD)3$^+$, CD3$^+$CD8$^+$, CD3$^-$CD8$^+$, CD3$^+$CD69$^+$, CD8$^+$CD69$^+$, CD3$^-$CD8$^+$CD69$^+$, CD8$^+$CD28$^+$, CD8$^+$CD28$^-$ lymphocytes were analyzed by four-color flow cytometry. For surface staining, 100 µL of blood was added to tubes containing 10 µL of cocktail of fluorochrome-labeled mAbs including fluorescein isothiocyanate (FITC)-conjugated anti-CD28 (clone CD28.2), phycoerythrin (PE)-conjugated anti-CD69 (clone TP1.55.3), phycoerythrin-cyanin (PC)5-conjugated anti-CD8 (B9.11) and PC7-conjugated anti-CD3 (clone UCHT1), all supplied by Beckman Coulter (Miami, FL, USA). Class-matched isotype immunoglobulin FITC- and PE-conjugated negative control monoclonal antibodies were added simultaneously to separate tubes for all samples to detect nonspecific binding. Subsequently, 100 µL of heparinized peripheral blood was mixed with the cocktail monoclonal antibody solution and incubated for 15 min at room temperature. After the incubation, lysing solution (OptiLyse C, Beckman Coulter) was added, and the mixture was incubated for further 10 min. Flow cytometric analysis was performed using Cytomics FC 500 cytometer (Beckman Coulter) equipped with a 15-mW air-cooled 488-nm argon laser and a 625-nm neon diode laser, and the data were analyzed using the CXP Analysis Software (Beckman Coulter). Data on minimally 10,000 events was acquired for each staining and stored in list mode.

The events were gated based on scatter-gated fluorescence analysis. Subcellular debris and clumps were differentiated from single cells by size. A region was drawn around T lymphocytes based on side light scatter and CD3 staining. CD69 negativity was determined with regard to a negative isotype control. The percentage of CD69$^+$ cells from the gate of CD3$^+$ lymphocytes was determined. Similarly the gate of CD8$^+$ cells was drawn on the basis of light scatter and CD8 staining. The amount of CD69$^+$, CD28$^+$ cells and CD28$^-$ cells was defined in this gate with regard to a negative isotype control. CD3$^+$CD69$^+$ from all CD3$^+$, CD8$^+$CD69$^+$, CD8$^+$CD28$^+$ and CD8$^+$CD28$^-$ from all CD8$^+$ were analyzed using this approach. Natural killer (NK) CD69$^+$ cells were gated as CD3$^-$ lymphocytes.

For flow cytometry analysis of regulatory T-cell population (TREG), 100 µL of blood was added to tubes of monoclonal antibody cocktail containing FITC-conjugated anti-CD4 (clone 13B8.2), PE-conjugated anti-CD25 (clone B1.49.9), PC7-conjugated anti-CD3 (clone UCHT1) and PC5-conjugated anti-CD127 (clone R34.34; all manufactured by Beckman Coulter). The sample tubes were mixed and incubated for 20 min while shielding from the light. Lysing solution OptiLyse (Beckman Coulter) was added to the tubes and incubated for 10 min to promote lysis of residual erythrocytes.

The samples were analyzed within 1 h by a four-colour flow cytometry and the data were analysed using the CXP Analysis Software. Information on a minimum of 50,000 events was acquired for each staining and stored in list mode. The peripheral blood mononuclear cells were gated on $CD4^+$ lymphocytes (based on forward and side light scatter and CD4 staining) and a region was set to include 1% of isotype for CD25 PE, and was copied on the file labeled with CD25 to define the $CD4^+CD25^+$ population. CD127 negativity was determined with regard to a negative isotype control and the percentage of $CD127^-CD25^+$ cells within all $CD3^+CD4^+$ T cells was defined.

For the determination of dendritic cells (DC), the combination of anti-CD14, anti-CD16, anti-immunoglobulin-like transcript (ILT) 3 and anti-CD123 antibodies into one 3-color reagent provides a tool to analyze peripheral blood plasmacytoid dendritic cells (PDC), based on positive selection of all $CD123^+$ cells, $ILT3^+$ cells and exclusion of all $CD14^+$ or $CD16^+$ monocytes. The final cell population exhibits the following phenotype of $CD(14+16)^{dim/neg}/ILT3^+/CD123^+$ cells. The combination of antibodies to CD14, CD16, CD85k (ILT3) and CD33 into a single three-color reagent provides a straightforward tool to analyze peripheral blood myeloid dendritic cells (MDC) based on positive selection of all myeloid cells with CD33 positivity, $ILT3^+$ cells and exclusion of all monocytes with CD14 or CD16 positivity. The final cell population exhibits the following phenotype: $CD14CD16^{dim\ to\ neg}/ILT3^+/CD33^+$ cells.

To determine PDCs, whole blood was incubated with a cocktail of monoclonal antibodies that contained FITC-conjugated anti-CD14 and anti-CD16 (clones RMO52 and 3G8), PE-conjugated anti-CD85k (ILT3) (clone ZM3.8) and PC5-conjugated anti-CD123 (clone 107D2). To analyze MDCs PC5-conjugated anti-CD33 (clone D3HL60) in the cocktail was used instead of CD123 in the second tube. After the incubation, lysing solution (OptiLyse C, Beckman Coulter) was added, and the mixture was incubated for 10 min. The suspension was then washed in PBS buffer and centrifuged. Subsequently, the supernatant was discarded, and the sediment was resuspended in 350 µL of phosphate-buffered saline (PBS buffer). Flow cytometric analysis was performed using Cytomics FC 500 flow cytometer. The analysis was based on 50,000 gated events. The data files were analyzed with CXP Analysis software (Beckman Coulter, Miami, FL, USA).

The gating strategy consisted of excluding cell debris and dead cells from the analysis based on scatter signals. In the case of PDCs the events were gated based on forward and side light scatter and the cells that stained positively for CD123 were first identified in a plot of side light scatter versus CD123 staining and then displayed in a plot of ILT3 versus $CD14^+16^+$ to identify $CD14/CD16)^{dim\ to\ neg}/ILT3^+/CD123^+$ cells. Similarly, MDCs were gated based on forward and side light scatter, from this gate all cells with CD33 positivity were determined and subsequently displayed in a plot of ILT3 versus $CD14^+/CD16^+$ to identify $CD14/CD16^{dim\ to\ neg}/ILT3^+/CD33^+$ cells.

Urinary neopterin and creatinine were determined by high performance liquid chromatography as described, and the results were expressed as neopterin/creatinine ratio [18].

Statistical analysis was performed using IBM SPSS (Chicago, IL, USA) software by regression analysis. The aim was to analyze changes compared to baseline. To achieve this, two variables were created: variable diffDate representing the number of days from the baseline and variables diffX indicating the difference in the monitored parameters from baseline. Parameter B was calculated to characterize the changes of a given variable per unit of time (per day in this case). Descriptive data analysis was also performed. Subsequently, a regression analysis was performed that analyzed the dependence of the observed markers on the time. R^2 was calculated as a measure to quantify the proportion of changes explained on a given significance level by the linear regression model. Comparison of groups of patients and controls was performed using the Mann-Whitney U test. The comparison between baseline and 3 years after the treatment was performed using the Wilcoxon signed rank test. The correlation analysis was performed using Spearman's rank correlation coefficient. The decision on statistical significance was based on significance level ≤ 0.05.

Results

Significant differences were observed in baseline relative and absolute counts of peripheral blood lymphocyte subpopulations between the patients treated with primary chemotherapy and patients with metastatic relapse and between both patient cohorts and controls (Table 1). In particular, baseline relative and absolute lymphocyte counts, absolute $CD3^+$ lymphocyte, $CD3^+CD4^+$ lymphocyte, relative and absolute $CD8^+CD28^-$ and absolute $CD69^+$ counts were significantly lower, and relative $CD8^+CD28^+$ and regulatory T-cell counts were significantly higher in cohort A patients compared to the control group. Relative $CD3^+CD4^+$ and PDC counts and $CD4^+/CD8^+$ ratio were significantly higher, and relative $CD3^+CD8^+$, relative and absolute $CD8^+CD28^-$, relative and absolute $CD3^+CD69^+$, relative and absolute $CD8^+CD69^+$ counts were significantly lower in cohort A compared to cohort M patients. Compared to controls absolute lymphocyte counts, absolute $CD3^+$ lymphocytes, relative and absolute $CD3^+CD4^+$ lymphocyte counts and $CD4^+/CD8^+$ ratio were lower and relative $CD3^+CD8^+$, $CD3^+CD69^+$, $CD8^+CD69^+$ and $CD8^+CD28^-$ lymphocyte counts were higher in cohort M patients (Table 1). No statistically significant correlations were observed between baseline relative and absolute peripheral blood leukocyte counts and urinary neopterin concentrations (data not shown).

In the cohort A the changes of peripheral blood leukocyte phenotype were evaluated during four AC cycles as well as during sequential weekly paclitaxel-based chemotherapy. During the AC phase of primary chemotherapy, significant increase of relative $CD3^+$, $CD3^+CD8^+$, $CD8^+CD28^+$, $CD3^+CD69^+$ and $CD8^+CD69^+$ counts were observed (Table 2). During the sequential paclitaxel-based

Table 1: Baseline peripheral blood leukocyte phenotype.

Parameter	Cohort A (primary chemotherapy) median (Q1; Q3)	Cohort M (metastatic disease) median (Q1; Q3)	p Cohort A vs. cohort M	Controls median (Q1; Q3)	p Cohort A vs. controls	p Cohort M vs. controls
Lymphocytes, %	0.22 (0.18; 0.28)	0.21 (0.13; 0.34)	0.735	0.28 (0.24; 0.35)	**0.019**	0.109
Lymphocytes, 10^9/L	1.35 (1.04; 1.64)	1.08 (0.82; 1.82)	0.573	1.81 (0.53; 2.15)	**0.018**	**0.049**
$CD3^+$, %	74.25 (61.05; 79.50)	68.00 (63.05; 74.50)	0.448	73.75 (68.28; 77.55)	0.799	0.214
$CD3^+$, 10^9/L	0.97 (0.57; 1.13)	0.72 (0.54; 1.36)	0.542	1.35 (1.09; 1.62)	**0.005**	**0.035**
$CD3^+CD4^+$, %	46.75 (38.85; 51.78)	27.65 (23.23; 40.68)	**0.008**	48.10 (43.58; 53.90)	0.289	**<0.001**
$CD3^+CD4^+$, 10^9/L	0.59 (0.35; 0.76)	0.31 (0.24; 0.67)	0.138	0.89 (0.63; 1.18)	**0.013**	**0.003**
$CD3^+CD8^+$, %	23.95 (19.70; 27.38)	33.10 (23.70; 44.85)	**<0.001**	21.13 (16.77; 28.34)	0.640	**<0.001**
$CD3^+CD8^+$, 10^9/L	0.27 (0.20; 0.40)	0.37 (0.28; 0.64)	0.085	0.36 (0.32; 0.60)	0.113	0.914
$CD4^+/CD8^+$ ratio	1.76 (1.15; 2.11)	0.73 (0.43; 1.22)	**0.001**	1.81 (1.47; 2.19)	0.365	**<0.001**
$CD8^+CD28^+$, %	16.05 (12.83; 18.55)	10.40 (8.35; 14.80)	0.062	9.31 (6.24; 12.03)	**<0.001**	0.183
$CD8^+CD28^+$, 10^9/L	0.19 (0.13; 0.30)	0.14 (0.10; 0.23)	0.332	0.18 (0.12; 0.25)	0.444	0.627
$CD8^+CD28^-$, %	11.20 (7.90; 15.80)	27.20 (22.40; 36.85)	**<0.001**	16.66 (13.27; 22.50)	**0.004**	**0.002**
$CD8^+CD28^-$, 10^9/L	0.12 (0.10; 0.30)	0.32 (0.27; 0.50)	**0.005**	0.29 (0.21–0.46)	**0.002**	0.627
$CD3^+CD69^+$, %	1.85 (1.40; 2.73)	3.40 (2.28; 6.08)	**0.004**	1.95 (1.23; 2.68)	0.841	**0.005**
$CD3^+CD69^+$, 10^9/L	0.02 (0.02; 0.04)	0.04 (0.03; 0.06)	**0.021**	0.04 (0.02; 0.05)	0.101	0.559
$CD8^+CD69^+$, %	1.50 (1.05; 2.20)	3.15 (1.85; 5.20)	**0.003**	1.45 (01.10; 1.95)	0.779	**0.001**
$CD8^+CD69^+$, 10^9/L	0.02 (0.01; 0.04)	0.03 (0.03; 0.06)	**0.024**	0.03 (0.02; 0.04)	0.204	0.155
$CD69^+$, %	3.65 (3.10; 6.20)	6.00 (4.50; 9.70)	0.061	4.45 (3.95; 5.30)	0.265	0.067
$CD69^+$, 10^9/L	0.05 (0.03–0.09)	0.06 (0.04; 0.13)	0.247	0.09 (0.06; 0.10)	**0.014**	0.328
$NK69^+$, %	4.45 (2.25; 8.42)	5.45 (2.30; 7.78)	0.481	3.25 (2.32; 4.87)	0.361	0.145
$NK69^+$, 10^9/L	0.06 (0.04; 0.09)	0.05 (0.04; 0.10)	0.579	0.08 (0.03; 0.09)	0.558	0.619
TREG, %	2.80 (2.00; 4.00)	3.00 (2.05; 3.93)	0.775	2.15 (1.63; 2.50)	**0.026**	0.055
TREG, 10^9/L	0.04 (0.02; 0.07)	0.04 (0.02; 0.05))	0.605	0.04 (0.02; 0.06)	0.573	0.940
PDC, %	0.14 (0.09; 0.20)	0.08 (0.06; 0.12)	**0.040**	0.11 (0.06; 0.16)	0.186	0.267
PDC, 10^9/L	0.01 (0.01; 0.01)	0.00 (0.00; 0.01)	0.052	0.01 (0.00; 0.01)	0.158	0.055
MDC, %	0.14 (0.08–0.21)	0.11 (0.08; 0.20)	0.464	0.11 (0.08; 0.13)	0.072	0.650
MDC, 10^9/L	0.01 (0.00; 0.01)	0.01 (0.00; 0.01)	0.505	0.01 (0.00; 0.01)	0.110	0.681

MDC, Myeloid dendritic cells; PDC, plasmocytoid dendritic cells; Q1, first quartile; Q3, third quartile; TREG, regulatory T-cells. Significant p-values are highlighted by bold type.

therapy, a significant decrease of $CD4^+/CD8^+$ ratio, relative $CD3^+CD4^+$, TREG and MDC counts, and an increase of relative $CD3^+CD8^+$, relative and absolute $CD8^+CD28^-$, and absolute MDC counts were noted (Table 3). In cohort M patients, treatment with bevacizumab, taxanes and carboplatin resulted in significantly increased $CD4^+/CD8^+$ ratio, absolute $CD3^+CD4^+$, $CD3^+CD28^+$, $NK69^+$, relative and absolute PDC counts and relative regulatory T-cell counts, and decreased relative $NK69^+$ counts (Table 4). No significant changes were observed in urinary neopterin concentrations during the course of treatment.

In 10 cohort A patients, peripheral blood leukocyte phenotype before primary chemotherapy was compared with the measurements obtained 3 years after the treatment (Table 5). An increase of relative lymphocyte, relative $CD8^+CD28^-$, absolute $CD8^+CD28^-$ and $CD69^+$ counts, and decrease of relative $CD3^+$, $CD3^+CD4^+$, $CD8^+CD28^+$ counts and $CD4^+/CD8^+$ ratio compared to baseline were noted 3 years after the treatment. Compared to controls relative and absolute $CD3^+$, $CD3^+CD4^+$ counts and $CD4^+/CD8^+$ ratio were lower, and relative TREG, PDC and MDC counts were higher in patients compared to controls.

Discussion

Present data demonstrate that absolute peripheral blood lymphocyte counts are significantly decreased in breast cancer patients even before the start of any treatment. In addition, significant differences in multiple lymphocyte subsets were observed in peripheral blood leukocyte phenotype between patients and controls. Present data also indicate the immune response is activated during the administration of chemotherapy.

Breast cancer patients treated with primary chemotherapy as well as patients treated with first line therapy of metastatic disease were evaluated in the present study. Compared to controls both these patient groups

Table 2: Linear regression analysis of changes during the AC phase of primary chemotherapy (cohort A).

Parameter	B	Significance	R^2
Neopterin, μmol/mol creatinine	0.020	0.508	0.003
Lymphocytes, %	<0.001	0.500	0.003
Lymphocytes, 10^9/L	−0.003	0.272	0.009
$CD3^+$, %	**0.175**	**<0.001**	**0.091**
$CD3^+$, 10^9/L	<0.001	0.930	<0.001
$CD3^+CD4^+$, %	0.036	0.476	0.004
$CD3^+CD4^+$, 10^9/L	−0.001	0.634	0.002
$CD3^+CD8^+$, %	**0.112**	**0.002**	**0.071**
$CD3^+CD8^+$, 10^9/L	0.001	0.408	0.005
$CD4^+/CD8^+$ ratio	−0.002	0.456	0.004
$CD8^+CD28^+$, %	**0.082**	**0.006**	**0.055**
$CD8^+CD28^+$, 10^9/L	0.001	0.342	0.007
$CD8^+CD28^-$, %	−0.005	0.868	<0.001
$CD8^+CD28^-$, 10^9/L	<0.001	0.129	0.017
$CD3^+CD69^+$, %	**0.014**	**0.005**	**0.057**
$CD3^+CD69^+$, 10^9/L	<0.001	0.397	0.005
$CD8^+CD69^+$, %	**0.009**	**0.045**	**0.030**
$CD8^+CD69^+$, 10^9/L	<0.001	0.363	0.006
$CD69^+$, %	0.017	0.269	0.009
$CD69^+$, 10^9/L	<0.001	0.907	<0.001
$NK69^+$, %	0.020	0.280	0.009
$NK69^+$, 10^9/L	<0.001	0.595	0.002
TREG, %	0.008	0.314	0.008
TREG, 10^9/L	<0.001	0.913	<0.001
PDC, %	0.001	0.260	0.010
PDC, 10^9/L	<0.001	0.500	0.004
MDC, %	0.001	0.243	0.011
MDC, 10^9/L	<0.001	0.485	0.004

B values reflect changes of given parameter per unit of time (day in this case). Positive values correspond to an increase and negative values represent a decrease of the parameter during the course of treatment. Significance values <0.05 are considered statistically significant. R^2 values quantify changes explained by the linear model on a given significance level (when multiplied by 100 the percentage of observed value explained by the linear model is obtained at the given significance level). Significant changes are highlighted by bold type.

Table 3: Linear regression analysis of changes during the sequential weekly paclitaxel-based chemotherapy (cohort A).

Parameter	B	Significance	R^2
Neopterin, μmol/mol creatinine	−0.331	0.526	0.003
Lymphocytes, %	<0.001	0.995	0.015
Lymphocytes, 10^9/L	0.002	0.073	0.017
$CD3^+$, %	0.006	0.334	0.005
$CD3^+$, 10^9/L	0.001	0.280	0.007
$CD3^+CD4^+$, %	**−0.095**	**<0.001**	**0.087**
$CD3^+CD4^+$, 10^9/L	<0.001	0.912	<0.001
$CD3^+CD8^+$, %	**0.021**	**0.024**	**0.029**
$CD3^+CD8^+$, 10^9/L	<0.001	0.068	0.019
$CD4^+/CD8^+$ ratio	**−0.005**	**0.001**	**0.068**
$CD8^+CD28^+$, %	−0.019	0.261	0.007
$CD8^+CD28^+$, 10^9/L	<0.001	0.529	0.002
$CD8^+CD28^-$, %	**0.101**	**<0.001**	**0.160**
$CD8^+CD28^-$, 10^9/L	**0.002**	**<0.001**	**0.107**
$CD3^+CD69^+$, %	0.001	0.512	0.002
$CD3^+CD69^+$, 10^9/L	<0.001	0.263	0.007
$CD8^+CD69^+$, %	0.001	0.491	0.003
$CD8^+CD69^+$, 10^9/L	<0.001	0.306	0.006
$CD69^+$, %	<0.001	0.876	<0.001
$CD69^+$, 10^9/L	<0.001	0.387	0.004
$NK69^+$, %	0.001	0.883	<0.001
$NK69^+$, 10^9/L	<0.001	0.500	0.003
TREG, %	**−0.013**	**<0.001**	**0.104**
TREG, 10^9/L	<0.001	0.344	0.005
PDC, %	<0.001	0.949	<0.001
PDC, 10^9/L	<0.001	0.675	0.001
MDC, %	**−0.001**	**<0.001**	**0.091**
MDC, 10^9/L	**<0.001**	**0.028**	**0.027**

The meanings of B, significance and R^2 are explained in the footnote of Table 2. Significant changes are highlighted by bold type.

had markedly lower peripheral blood lymphocyte counts, and lower T-cell counts persisted in patients with early breast cancer 3 years after the primary diagnosis. Patients with metastatic disease (cohort M) had higher relative $CD3^+CD8^+$, lower relative $CD3^+CD4^+$ cell counts and $CD4^+/CD8^+$ ratio compared with both controls and patients with early disease (cohort A), indicating a shift from helper to cytotoxic T-cell phenotype. Within the $CD8^+$ T-cell population $CD8^+CD28^-$ cells reflect an activated phenotype, and, similarly to regulatory T cells, $CD8^+CD28^-$ lymphocytes have been reported to be increased in cancer patients and associated with poor prognosis [19].

Relative number of $CD8^+CD28^+$ T-cells were increased in the cohort A, while $CD8^+CD28^-$ lymphocytes were highest in cohort M patients. In fact, relative $CD8^+CD28^-$ T-cell counts in controls were significantly higher compared to cohort A and lower compared to cohort M. Present data are consistent with the hypothesis that the number of activated $CD8^+$ T-cells is low early in the course of disease, possibly even lower than in the control population, and increases during the subsequent course of disease, possibly in connection with the therapy. The data further indicate that the presence of $CD8^+CD28^-$ lymphocytes may be associated with the duration of the history of breast cancer rather than with the presence of the metastases. Breast cancer is currently regarded as a chronic disorder, and the duration of the disease seems to be associated with a switch from $CD8^+CD28^+$ to $CD8^+CD28^-$ phenotype. Moreover, a significant increase in $CD8^+CD28^-$ cells was observed during weekly paclitaxel-based chemotherapy in patients of cohort A. Higher proportion

Table 4: Linear regression analysis of changes in patients treated for metastatic disease (cohort M).

Parameter	B	Significance	R^2
Neopterin, μmol/mol creatinine	−0.034	0.702	0.002
Lymphocytes, %	<0.001	0.993	<0.001
Lymphocytes, 10^9/L	0.002	0.044	0.037
$CD3^+$, %	0.004	0.818	0.001
$CD3^+$, 10^9/L	0.002	0.016	0.054
$CD3^+CD4^+$, %	0.010	0.428	0.006
$CD3^+CD4^+$, 10^9/L	**0.001**	**0.001**	**0.098**
$CD3^+CD8^+$, %	−0.009	0.652	0.002
$CD3^+CD8^+$, 10^9/L	<0.001	0.567	0.004
$CD4^+/CD8^+$ ratio	**0.009**	**<0.001**	**0.132**
$CD8^+CD28^+$, %	−0.004	0.764	0.001
$CD8^+CD28^+$, 10^9/L	**<0.001**	**0.001**	**0.044**
$CD8^+CD28^-$, %	−0.010	0.582	0.003
$CD8^+CD28^-$, 10^9/L	<0.001	0.375	0.008
$CD3^+CD69^+$, %	−0.007	0.058	0.034
$CD3^+CD69^+$, 10^9/L	<0.001	0.164	0.019
$CD8^+CD69^+$, %	−0.005	0.162	0.018
$CD8^+CD69^+$, 10^9/L	<0.001	0.375	0.008
$CD69^+$, %	−0.008	0.148	0.020
$CD69^+$, 10^9/L	<0.001	0.347	0.009
$NK69^+$, %	**−0.018**	**0.006**	**0.069**
$NK69^+$, 10^9/L	**<0.001**	**0.022**	**0.051**
TREG, %	**0.040**	**0.010**	**0.062**
TREG, 10^9/L	<0.001	0.986	<0.001
PDC, %	**<0.001**	**0.028**	**0.047**
PDC, 10^9/L	**<0.001**	**0.025**	**0.050**
MDC, %	<0.001	0.682	0.002
MDC, 10^9/L	<0.001	0.955	<0.001

The meanings of B, significance and R^2 are explained in the footnote of Table 2. Significant changes are highlighted by bold type.

of T-cells in patients with metastatic breast cancer and after therapy also expressed the activation marker CD69 with an increase being observed during the therapy, and analogical hypothesis could be formulated with regard to changes during the course of disease for this marker of T-cell activation. Relative numbers of regulatory T-cell were increased in breast cancer patients compared to controls, but, probably because of low number of cohort M patients examined this difference was statistically significant only in cohort A patients. Relative numbers of PDCs were increased in cohort A compared to cohort M patients.

The concept of the tumor microenvironment is essential in understanding the host response against the neoplasia. However, the assessment of the immune response in the tumor microenvironment that is decisive for the outcome of the host-tumor interaction is difficult [9, 10]. The presence of lymphocytes infiltrating the tumor tissue termed tumor infiltrating lymphocytes (TIL) has been associated with the patient prognosis and outcome of treatment across a range of different primary tumors [14], including breast cancer [3, 4, 15, 16]. The changes of lymphocyte populations in the peripheral blood are more readily assessable.

Prior studies have reported the presence of differences in the peripheral blood lymphocyte phenotype in patients with breast cancer, even in patients with early disease. Low $CD4^+$ T-cell counts have been associated with poor prognosis in patients with metastatic breast cancer [20]. Using different assessment methods the relative number of regulatory T-cells has been reported to be increased in patients with breast cancer [21–23], similarly to the present study. Similarly to an earlier report, the relative number of regulatory T-cells was increased in the present investigation [21]. Interestingly, the percentage of regulatory T-cells decreased during weekly paclitaxel-based chemotherapy in cohort A (mostly patients with early breast cancer) while it increased during similar regimen in patients with metastatic disease.

Changes in peripheral blood lymphocyte counts associated with the administration of systemic chemotherapy have been previously reported in patients with breast cancer [24–27]. In an earlier study, an increase of peripheral blood T-lymphocytes was observed during primary chemotherapy [24]. In the present study, differential changes in peripheral blood cell phenotype have been observed in patients with metastatic disease and less advanced tumors, possibly reflecting also a difference in chemotherapy regimens. Moreover, differential changes have been observed in cohort A patients during the initial doxorubicin/cyclophosphamide therapy as compared to weekly paclitaxel-based regimen. As outlined above, these changes indicate an activated T-cell phenotype with a switch from $CD8^+CD28^+$ to $CD8^+CD28^-$, increased expression of CD69 and contrasting response of regulatory T-cells. However, the magnitude of these changes has been limited.

For a long time, cytotoxic therapy has been regarded as immunosuppressive. On the other hand, there has been recently an accumulating body of evidence indicating that the administration of some cytotoxic drugs may stimulate immune response. Based on experimental data the concept of immunogenic cell death has been formulated [28]. The changes observed in the present cohorts of patients are consistent with immune activation induced by the administration of systemic chemotherapy.

In addition, present data indicate that long-term changes of peripheral blood lymphocyte phenotype are evident in surviving patients when evaluated after 3 years. These results indicate that significant changes in the proportion of peripheral blood leukocytes persist

Table 5: Comparison of peripheral blood lymphocytes before and 3 years after treatment in cohort A.

Parameter	Before treatment median (Q1; Q3)	3 Years after treatment median (Q1; Q3)	Controls median (Q1; Q3)	p-Value pre-treatment compared to 3 years after the treatment	p-Value 3 years after the treatment compared to controls
Lymphocytes, %	0.22 (0.18; 0.28)	0.31 (0.30; 0.34)	0.28 (0.24; 0.35)	**<0.001**	0.198
Lymphocytes, 10^9/L	1.35 (1.04; 1.64)	1.69 (1.37; 1.93)	1.81 (0.53; 2.15)	0.104	0.328
$CD3^+$, %	74.25 (61.05; 79.50)	61.80 (51.05; 67.20)	73.75 (68.28; 77.55)	**0.018**	**<0.001**
$CD3^+$, 10^9/L	0.97 (0.57; 1.13)	0.91 (0.77; 1.24)	1.35 (1.09; 1.62)	0.982	**0.024**
$CD3^+CD4^+$, %	46.75 (38.85; 51.78)	33.33 (28.50; 39.15)	48.10 (43.58; 53.90)	**0.005**	**<0.001**
$CD3^+CD4^+$, 10^9/L	0.59 (0.35; 0.76)	0.58 (0.34–0.67)	0.89 (0.63; 1.18)	0.636	**0.007**
$CD3^+CD8^+$, %	23.95 (19.70; 27.38)	26.25 (19.19; 29.57)	21.13 (16.77; 28.34)	0.627	0.472
$CD3^+CD8^+$, 10^9/L	0.27 (0.20; 0.40)	0.43 (0.27; 0.54)	0.36 (0.32; 0.60)	0.085	0.880
$CD4^+/CD8^+$ ratio	1.76 (1.15; 2.11)	1.14 (0.83; 1.56)	1.81 (1.47; 2.19)	**0.016**	**0.004**
$CD8^+CD28^+$, %	16.05 (12.83; 18.55)	7.99 (6.01; 13.40)	9.31 (6.24; 12.03)	**0.002**	0.982
$CD8^+CD28^+$, 10^9/L	0.19 (0.13; 0.30)	0.14 (0.10; 0.21)	0.18 (0.12; 0.25)	0.129	0.365
$CD8^+CD28^-$, %	11.20 (7.90; 15.80)	22.81 (16.51; 25.44)	16.66 (13.27; 22.50)	**0.001**	0.127
$CD8^+CD28^-$, 10^9/L	0.12 (0.10; 0.30)	0.31 (0.25; 0.54)	0.29 (0.21–0.46)	**0.003**	0.390
$CD3^+CD69^+$, %	1.85 (1.40; 2.73)	1.60 (1.30; 1.85)	1.95 (1.23; 2.68)	0.274	0.501
$CD3^+CD69^+$, 10^9/L	0.02 (0.02; 0.04)	0.03 (0.02; 0.04)	0.04 (0.02; 0.05)	0.179	0.559
$CD8^+CD69^+$, %	1.50 (1.05; 2.20)	1.20 (1.05; 2.20)	1.45 (01.10; 1.95)	0.562	0.982
$CD8^+CD69^+$, 10^9/L	0.02 (0.01; 0.04)	0.02 (0.02; 0.04)	0.03 (0.02; 0.04)	0.151	0.983
$CD69^+$, %	3.65 (3.10; 6.20)	4.90 (3.80–5.65)	4.45 (3.95; 5.30)	0.317	0.908
$CD69^+$, 10^9/L	0.05 (0.03–0.09)	0.09 (0.07; 0.11)	0.09 (0.06; 0.10)	**0.035**	0.812
$NK69^+$, %	4.45 (2.25; 8.42)	4.5 (1.80; 6.4)	3.25 (2.32; 4.87)	0.869	0.409
$NK69^+$, 10^9/L	0.06 (0.04; 0.09)	0.09 (0.04; 0.11)	0.08 (0.03; 0.09)	0.353	0.650
TREG, %	2.80 (2.00; 4.00)	3.15 (2.38; 4.35)	2.15 (1.63; 2.50)	0.512	**0.010**
TREG, 10^9/L	0.04 (0.02; 0.07)	0.05 (0.03; 0.07)	0.04 (0.02; 0.06)	0.308	0.100
PDC, %	0.14 (0.09; 0.20)	0.15 (0.12; 0.21)	0.11 (0.06; 0.16)	0.382	**0.031**
PDC, 10^9/L	0.01 (0.01; 0.01)	0.01 (0.01; 0.01)	0.01 (0.00; 0.01)	0.980	0.067
MDC, %	0.14 (0.08–0.21)	0.15 (0.12; 0.17)	0.11 (0.08; 0.13)	0.869	**0.022**
MDC, 10^9/L	0.01 (0.00; 0.01)	0.01 (0.01; 0.01)	0.01 (0.00; 0.01)	0.286	0.880

Q1, First quartile; Q3, third quartile. Significant p-values are highlighted by bold type.

in survivors of early breast cancer, including lower $CD3^+$, $CD3^+CD4^+$ counts, $CD4^+/CD8^+$ ratio and higher proportion of regulatory T-cells, plasmocytoid and myeloid dendritic cells. These changes resemble the phenotype observed in patients with metastatic disease. However, the magnitude of these changes is relatively small, and biological significance of alterations of peripheral blood leukocyte phenotype during systemic therapy remains to be determined.

In contrast to some earlier reports [8, 18, 29], no correlation was observed between peripheral blood leukocyte counts or phenotype and urinary neopterin, a biomarker of systemic immune activation, and urinary neopterin did not increase during the administration of primary chemotherapy. The negative results regarding correlation between peripheral blood leukocyte phenotype and urinary neopterin or changes of urinary neopterin during chemotherapy may be explained by the relatively low number of patients examined in the present cohort. In addition, prior study reporting a negative correlation between urinary neopterin concentration and peripheral blood $CD3^+CD4^+$ counts was performed in patients with advanced cancer [8]. Neopterin that can be measured in the serum or urine represents a biomarker of systemic immune activation in a range of disorders [30–40], including cancer [33, 37–39, 41]. Moreover, increased neopterin production is associated with increased degradation of tryptophan to kynurenine [42, 43]. Although kynurenine and its metabolites have some cytotoxic activity against tumor cells [44] and tryptophan depletion may also inhibit tumor growth [45], the predominant effect of tryptophan metabolism to kynurenine *in vivo* is immune suppression [46]. It has been demonstrated that in patients with breast cancer increased urinary neopterin concentrations are associated with poor prognosis, but, in contrast to some other tumors increased neopterin concentrations are present only in a fraction of breast cancer patients [38, 39]. Consequently, in this cohort of limited size an effect of therapy on neopterin production could be missed. The fact that no significant change of urinary neopterin concentrations was observed in the present study during

chemotherapy could also reflect the relatively limited effect of the chemotherapy regimen administered on macrophage activation. Alternatively, initially increased urinary neopterin concentrations can decrease as a consequence of tumor control, and this decrease could balance an increase induced by chemotherapy administration in patients with initially low neopterin levels.

The present pilot study has obvious limitations. The cohorts of patients were of limited size and especially the cohort of patients treated with primary chemotherapy was rather heterogeneous. Because of the exploratory nature, the Bonferroni correction was not performed because this would reduce the statistical power. In general, an optimal method of analysis of serial data may be disputed. In the present study we used regression analysis. Inspection of the graphic presentation of the data did not reveal a trend that would approximate the data better than linear regression used in this study. However, it has to be kept in mind that this model explains only a small part of the variance. On the other hand, the fact that significant changes in peripheral blood leukocyte phenotype were evident even in this cohort of limited size with further alterations being observed during the treatment is in agreement with the hypothesis that immune response plays an important role in the pathogenesis of breast cancer and response to anticancer therapy. With the advent of antibodies targeting immune checkpoints these considerations are of increasing importance in the management of patients.

In conclusion, present study demonstrates the presence of significant differences in peripheral blood leukocyte phenotype in breast cancer patients even before the start of primary chemotherapy. Changes were observed during the course of chemotherapy indicating the activation of the immune response during the chemotherapy.

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