Urinary Neopterin, Serum Retinol, α-tocopherol and Homocysteine in Breast Cancer Patients During Treatment with Bevacizumab and Chemotherapy

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Abstract

Bevacizumab, monoclonal antibody targeting vascular endothelial growth factor, is effective in different tumors, including colorectal carcinoma, non-small cell lung cancer, renal cell carcinoma and breast cancer. Increased serum or urinary concentrations of neopterin, an indicator of systemic immune response, have been described in patients with tumors of different primary locations, and further increase has been observed during anticancer therapy. An increase of urinary neopterin has been described after administration of cytokines, cytotoxic chemotherapy, or external beam radiation, but less is known about the effects of targeted agents on systemic immune response. We have studied serum homocysteine, C-reactive protein, α-tocopherol and retinol, and urinary neopterin in patients with metastatic breast cancer treated with bevacizumab, taxane and carboplatin. Homocysteine and C-reactive protein were determined immunochemically. α-tocopherol, retinol and urinary neopterin were determined by high performance liquid chromatography. Homocysteine, C-reactive protein and urinary neopterin decreased, while retinol and α-tocopherol increased during the therapy. In conclusion, the treatment of patients with metastatic breast cancer with bevacizumab, taxane and carboplatin resulted in the suppression of systemic inflammatory and immune response. The suppression of systemic inflammatory and immune response was associated with an increase in serum vitamin concentrations.

Key words: α-tocopherol; bevacizumab; C-reactive protein; homocysteine; neopterin; retinol

Introduction

The advent of targeted agents has changed the landscape of medical oncology, resulting in significant improvement of survival of patients with a wide range of malignant disorders, including the most common cancers, e.g. breast carcinoma. Targeted agents exert antitumor activity through the inhibition of a defined pathway(s) involved in cancer progression or metastasis, and most agents inhibit tumor growth through more than one mechanism, acting on multiple molecular targets. In general, targeted agents are less toxic and better tolerated than conventional cytotoxic agents. On the other hand, a new spectrum of side effects has emerged. Many of these side effects have only limited immediate impact on the patients, but may lead to significant chronic toxicity. Prominent among these side effects are changes in parameters determining cardiovascular risk, including hypertension, hyperlipidemia, impaired glucose tolerance and hypercholesterolemia. Administration of anticancer agents is associated with oxidative stress. Oxidative stress plays an important role in atherosclerosis as atherogenic potential of serum lipids is dependent on oxidation (1) and is affected by liposoluble antioxidants (2). Moreover, antioxidant administration may retard atherosclerosis (3). With the remarkable improvement of long-term prognosis of patients with metastatic breast carcinoma, chronic effects of treatment on cardiovascular system may become an important consideration. Risk factors
of atherosclerosis are complex, and along with hypertension or hypercholesterolemia include high serum concentrations of homocysteine, C-reactive protein (CRP), neopterin or parameters of oxidative stress (4). The effect of anticancer therapy on laboratory parameters associated with the risk of atherosclerosis, e.g. serum lipids, has been best defined for hormonal agents (5, 6), and less is known about the effect of targeted agents or chemotherapy on serum cholesterol or other parameters of cardiovascular risk.

Vascular endothelial growth factor (VEGF) is a cytokine that plays fundamental role in angiogenesis and tumor progression. VEGF also represents an important therapeutic target, and the monoclonal antibody against VEGF, bevacizumab, is currently component of standard combination therapy in different tumors, including metastatic colorectal carcinoma, non-small cell lung carcinoma, renal cell carcinoma and breast carcinoma (7-9). With regard to risk factors of atherosclerosis, VEGF inhibition causes hypertension (10) and proteinuria (11). Less is known about the effects of VEGF inhibition or combination therapy on serum parameters indicative of the risk of atherosclerosis. Recently, we have described a decrease of serum cholesterol, homocysteine and CRP concentrations and increased serum retinol in patients with metastatic colorectal carcinoma treated with bevacizumab combined with oxaliplatin, folinic acid and 5-fluorouracil (12). In patients treated with combination therapy, it is difficult to discern the effect of targeted agent and cytotoxic chemotherapy. Evaluation of the combination of targeted agent with different cytotoxic drugs could be helpful to understand the effects of both therapeutic components.

In the present study, we have evaluated urinary neopterin, serum CRP, α-tocopherol, retinol and homocysteine during therapy with bevacizumab combined with taxanes and carboplatin in patients with metastatic breast carcinoma.

### Material and Methods

Ten patients with metastatic breast carcinoma, aged 48 ± 14 (range 30 - 72) years, were included in the study. The patients were treated with combination of bevacizumab (10 mg/kg) every two weeks, and paclitaxel (90 mg/m²) and carboplatin (area under the curve 2) every week. In one patient allergic to paclitaxel, weekly docetaxel (40 mg/m²) was used. In all patients, neopterin determination was performed in urine samples after the patient consent before and during therapy.

Blood samples were taken before the start of therapy and on weekly visits during the treatment. Hemoglobin was measured by a photometric method with sodium lauryl sulfate using a SysmexXE-2100 blood analyzer (Sysmex, Kobe, Japan). Homocysteine concentration was determined immunochromatically (Immulite 2000, Siemens Healthcare Diagnostics, Deerfield, IL, USA). CRP and uric acid were determined using particle-enhanced immunoturbidimetric assay and uricase method, respectively, as commercially available on MODULAR analyzer (Hoffmann-La Rocha, Basel, Switzerland).

Serum α-tocopherol and retinol were determined before and during the therapy using high performance liquid chromatography as described (13). Blood samples were drawn from a peripheral vein after an overnight fast. The samples were transferred immediately to the laboratory, centrifuged (2000 x g, 10 minutes, 4°C), the serum was separated and frozen at -20°C until analysis. In the liquid-liquid extraction procedure, 500 µL of serum was de-proteinized by cool ethanol denatured with 5 % methanol (500 µL, 5 minutes, 4°C). Subsequently, 2 500 µL of n-hexane was added to this mixture and extracted for 5 minutes by a vortex apparatus. After centrifugation (2000 x g, 10 minutes, 0°C), the aliquot (2 000 µL) of the clean extract was separated and evaporated in an AD 5301 concentrator (Eppendorf, Hamburg, Germany; 45°C). The residue was dissolved in 400 µL methanol and analyzed by reversed-phase high performance liquid chromatography using the external standard calibration. The analyses were performed using the Perkin Elmer high performance liquid chromatography set (Norwalk, CT, USA) comprising a LC 200 pump, a LC 200 autosampler, LC Column Oven 101 thermostat and LC 235C Diode Array Detector attached to the Perkin Elmer Turbochrom Chromatography Workstation version 4.1. Separation of α-tocopherol and retinol was performed using the Chromolith Performance RP-18e, 100 x 4.6 mm monolithic columns (Merck, Darmstadt, Germany). As the mobile phase 100 % methanol was used at the flow rate of 2.5 mL.min⁻¹ and column pressure 3.3 MPa. The block heater LC Oven 101 (Perkin Elmer) was utilized to keep the analytical column temperature at 25°C. The injection volume was 50 µL. The detection of α-tocopherol and retinol was carried out at 295 nm and at 325 nm, respectively.

Early urine samples were collected and stored at -20°C until analysis. Urinary neopterin was determined using modification of the method described earlier (14). After centrifugation (45 seconds, 12000 × g) and diluting 100 µL of urine specimens with 1.0 ml of mobile phase containing 2 g of disodium-EDTA per liter, the samples were filtered using Microfilter, AcroPrep 96 Filter Plate 0.2 µm/ 350 µL, Pall Life Science (Ann Arbor, MI, USA) and Vacuum manifold.
Results

At baseline, mean CRP and neopterin concentrations were above, hemoglobin, retinol and α-tocopherol were below, and homocysteine and uric acid were within (Table 1) the normal range observed in healthy subjects or patients with early breast carcinoma (15).

Compared to pre-treatment (baseline) concentrations, homocysteine, CRP and urinary neopterin decreased, while retinol and α-tocopherol increased during the therapy. A gradual decrease of hemoglobin concentrations was also observed. Serum uric acid concentrations remained constant. Statistically significant (p < 0.05) decrease was observed for serum homocysteine at visits 7 and 10, for CRP at visits 3, 4, 8, 9 and 11, and for urinary neopterin concentrations at visits 2 and 4 (Table 1). Retinol concentrations were significantly increased at visits 2 through 4 and α-tocopherol was significantly increased at visit 9. After 4 months, only 5 patients were still on treatment and evaluable.

Significant correlations between the parameters investigated were observed. At visit 1, a significant negative correlation was observed between CRP and hemoglobin (r = -0.72; p < 0.05; Figure 1), at visit 2 significant negative correlation were observed between CRP and retinol (r = -0.76; p < 0.05), CRP and α-tocopherol (r = -0.67; p < 0.05), and a significant correlation was observed between retinol and α-tocopherol (r = 0.72; p < 0.05). A significant negative correlation between retinol and CRP (r = -0.75; p < 0.05) and a positive correlation between α-tocopherol and hemoglobin (r = 0.81; p < 0.05) were also observed at visit 3. No other significant correlations were observed.

Table 1.: Homocysteine, C-reactive protein, uric acid, retinol, alpha-tocopherol and urinary neopterin during the therapy

<table>
<thead>
<tr>
<th>Visit</th>
<th>Time (days)</th>
<th>n</th>
<th>Hemoglobin (g/L)</th>
<th>Homocysteine (µmol/L)</th>
<th>Uric acid (µmol/L)</th>
<th>Retinol (µmol/L)</th>
<th>Alpha-tocopherol (µmol/L)</th>
<th>Neopterin (µmol/mol creatinine)</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0 ± 0</td>
<td>10</td>
<td>127 ± 17</td>
<td>12.7 ± 4.1</td>
<td>38 ± 56</td>
<td>234 ± 80</td>
<td>1.05 ± 0.45</td>
<td>20.7 ± 5.2</td>
</tr>
<tr>
<td>2</td>
<td>7 ± 0</td>
<td>10</td>
<td>125 ± 19</td>
<td>10.8 ± 2.5</td>
<td>27 ± 47</td>
<td>218 ± 74</td>
<td>1.30 ± 0.42</td>
<td>22.6 ± 4.8</td>
</tr>
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<td>3</td>
<td>14 ± 1</td>
<td>10</td>
<td>123 ± 17*</td>
<td>10.6 ± 2.7</td>
<td>10 ± 13*</td>
<td>204 ± 48</td>
<td>1.44 ± 0.39</td>
<td>21.8 ± 4.9</td>
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<td>4</td>
<td>23 ± 3</td>
<td>8</td>
<td>120 ± 22</td>
<td>10.9 ± 3.0</td>
<td>5 ± 4*</td>
<td>240 ± 56</td>
<td>1.58 ± 0.42</td>
<td>25.6 ± 2.3</td>
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<tr>
<td>5</td>
<td>31 ± 4</td>
<td>8</td>
<td>117 ± 13*</td>
<td>12.2 ± 3.2</td>
<td>9 ± 9</td>
<td>219 ± 36</td>
<td>1.34 ± 0.46</td>
<td>23.8 ± 4.4</td>
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<td>6</td>
<td>38 ± 4</td>
<td>6</td>
<td>114 ± 14*</td>
<td>10.2 ± 1.9</td>
<td>8 ± 10</td>
<td>199 ± 37</td>
<td>1.16 ± 0.27</td>
<td>19.8 ± 3.8</td>
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<tr>
<td>7</td>
<td>47 ± 4</td>
<td>8</td>
<td>114 ± 15</td>
<td>9.3 ± 2.5*</td>
<td>11 ± 17</td>
<td>208 ± 39</td>
<td>1.24 ± 0.33</td>
<td>23.1 ± 3.1</td>
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<tr>
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<td>54 ± 3</td>
<td>7</td>
<td>110 ± 12*</td>
<td>10.2 ± 3.5</td>
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<td>245 ± 53</td>
<td>1.20 ± 0.50</td>
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<td>112 ± 14</td>
<td>9.8 ± 3.7</td>
<td>8 ± 6*</td>
<td>204 ± 30</td>
<td>1.35 ± 0.55</td>
<td>23.4 ± 4.7</td>
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<td>112 ± 15*</td>
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<td>5 ± 5</td>
<td>220 ± 70</td>
<td>1.36 ± 0.51</td>
<td>22.0 ± 3.5</td>
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<td>8</td>
<td>112 ± 10</td>
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<td>1.51 ± 0.47</td>
<td>24.5 ± 2.9</td>
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<td>102 ± 16</td>
<td>6</td>
<td>110 ± 9*</td>
<td>9.7 ± 2.8</td>
<td>6 ± 5</td>
<td>258 ± 70</td>
<td>1.30 ± 0.50</td>
<td>23.0 ± 4.0</td>
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<tr>
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<td>8</td>
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<td>9.5 ± 3.9</td>
<td>12 ± 18</td>
<td>241 ± 57</td>
<td>1.22 ± 0.30</td>
<td>22.8 ± 2.9</td>
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<tr>
<td>15</td>
<td>116 ± 18</td>
<td>5</td>
<td>118 ± 7</td>
<td>9.3 ± 3.0</td>
<td>7 ± 6</td>
<td>212 ± 33</td>
<td>1.31 ± 0.33</td>
<td>24.2 ± 4.8</td>
</tr>
</tbody>
</table>

Shown are the mean ± standard deviation. * p < 0.05.

Pumps LC-20 AB, Auto sampler SIL-20 AC, Column Oven CTO-20AC Thermostat, Fluorescence detector RF-10AXL, Diode array detector SPD-M20A and communications bus module CBM-20A. Phosphate buffer 15 mmol/L, pH 6.4, with flow rate 0.8 ml/min was used as mobile phase. Separation was performed using hybrid analytical column Gemini Twin 5µ, C18, 150 × 3 mm (Phenomenex, Torrance, CA USA) at 25°C, injection volume was 1µL. Neopterin was identified by its native fluorescence (353 nm excitation, 438 nm emission wavelengths). Creatinine was monitored simultaneously in the same urine specimen with diode array detector at 235 nm. Time of analysis for urine neopterin and creatinine was 10 minutes and the analytes were quantified by external standard calibration. The neopterin concentrations were expressed as neopterin to creatinine ratio (µmol/mol creatinine).

Differences during therapy were evaluated using the Wilcoxon paired test. Correlations were examined using Spearman’s rank correlation coefficient. The survival of patients was analyzed by the Kaplan-Meier method, and survival of patients with laboratory parameters below or above median was compared by the log-rank test. The decision on statistical significance was based on p = 0.05 level. The analyses were performed using NCSS software (Number Cruncher Statistical Systems, Kaysville, UT, USA).
between the parameters investigated were observed. Two patients were alive at the time of the analysis, more than two years after the start of treatment. The survival of these patients was censored at 930 days. Days of survival calculated from the sample date significantly correlated at visit 1 with hemoglobin ($r_s = 0.74; p < 0.05$), $\alpha$-tocopherol ($r_s = 0.84; p < 0.005$) and inversely with CRP ($r_s = -0.73; p < 0.05$). Survival calculated from visit 2 correlated with hemoglobin ($r_s = 0.67; p < 0.05$), $\alpha$-tocopherol ($r_s = 0.75; p < 0.05$) and negatively with CRP ($r_s = -0.88; p < 0.001$). The survival (calculated from the sample date) of patients with serum $\alpha$-tocopherol above median at visit 1 (19.4 $\mu$mol/L) was significantly longer compared to patients with serum $\alpha$-tocopherol below the median (median 910 vs. 293 days, $p < 0.05$). The survival of patients with serum $\alpha$-tocopherol above median at visit 2 (23 mol/L) was also longer (median 903 vs. 286 days, $p < 0.05$) as was the survival of patients with CRP below median (5 mg/L) at this visit (median 903 vs. 309 days, $p < 0.05$).

**Discussion**

As expected, patients with metastatic breast cancer had CRP and neopterin concentrations above as well as hemoglobin, retinol and $\alpha$-tocopherol concentrations below the values observed in healthy subjects or patients with early breast carcinoma (15). The finding of higher urinary neopterin concentrations is in line with earlier studies reporting increased urinary neopterin in patients with metastatic breast cancer (16). Increased urinary neopterin concentrations were also reported to represent an independent prognostic indicator for overall survival in patients with breast cancer (16).

In the present study, we have observed a decrease of serum homocysteine, CRP and urinary neopterin as well as an increase of serum retinol and $\alpha$-tocopherol in patients with metastatic breast carcinoma treated with the combination of bevacizumab, taxane and platinum. The number of patients was limited and so was the power to detect significant differences during the treatment. Nevertheless, statistically significant differences demonstrating a decrease in systemic inflammatory and immune response and serum homocysteine were evident even in a cohort of limited size. From a point of view of risk factors of atherosclerosis, the present analysis indicates that besides unfavorable effects on hypertension or proteinuria, combined treatment with bevacizumab also has favorable effects on laboratory parameters associated with the risk of atherosclerosis. However, because of limited number of patients included in the present investigation the results have to be regarded as only exploratory. In future studies, the effect of bevacizumab in combination with chemotherapy on systemic immune activation should be assessed on a larger patient cohort.

Spearman's rank correlation is usually not used to study association between laboratory parameters and survival, and the present analysis with 2 patients being still alive at the time of analysis must also be regarded as exploratory. Prognostic significance of $\alpha$-tocopherol was also indicated by the results of the standard log-rank test. These results indicate possible prognostic significance of hemoglobin, CRP and $\alpha$-tocopherol in this group of patients that should be confirmed using standard statistical methods in a larger cohort.

Only a slight decrease of serum homocysteine was observed in the present cohort. A decrease of serum homocysteine and CRP has been recently reported in patients with metastatic colorectal carcinoma treated with the combination of bevacizumab, oxaliplatin, 5-fluorouracil and folinic acid (12). Homocysteine is an intermediate in the metabolism of methionine. Increased serum concentration of homocysteine may be caused by a deficiency of folate or vitamin B12. Increased homocysteine concentrations are a well established risk factor of atherosclerosis and thrombosis (17, 18). Hyperhomocysteinemia has also been documented in cancer patients (19-21). Homocysteine may be produced by both tumor cells as well as activated leukocytes (22). In cancer patients, serum homo-

![Figure 1: Correlation between baseline C-reactive protein (CRP) and hemoglobin concentrations (rs = -0.72; p < 0.05; note log-scale of CRP concentrations!)](image)
cysteine may behave as a tumor marker, and a decrease in homocysteine concentrations observed in cancer patients may reflect tumor control by the therapy.

Present data demonstrating a decrease of CRP and neopterin indicate suppression of systemic inflammatory and immune response during therapy with bevacizumab and cytotoxic agents. Patients with advanced cancer have laboratory evidence of activation of acute phase response. CRP is the parameter most widely used to assess acute phase response. CRP concentrations are also increased in the serum of patients with atherosclerosis, and elevated serum CRP levels predict the risk of future cardiovascular events (23-25). Increased urinary neopterin concentrations in cancer patients are also well documented (26, 27). An increase of serum or urinary neopterin has been described after systemic administration of different cytokines (26) or after chemotherapy (28, 29), but there is so far only limited information about neopterin in patients treated with targeted agents. Similarly to CRP, urinary neopterin is increased in atherosclerosis and predicts the risk of cardiac events (30). In patients with tumors of different primary locations, increased serum or urinary neopterin concentrations were associated with poor prognosis (16, 26, 27). In earlier studies, correlations were observed between lower numbers or impaired function of lymphocytes or dendritic cells and neopterin concentrations (31-34). Thus, increased neopterin concentrations are thought to reflect immune dysregulation (26), similar to CRP, and both these laboratory parameters reflect disease activity. As in the case of homocysteine, decreased concentrations of neopterin or CRP may be the result of tumor control and, in addition, could reflect an environment more favorable for host immune response against the tumor.

The decrease of inflammatory response reflected in lower CRP and neopterin concentrations was in the present cohort of patients associated with increased concentrations of retinol and α-tocopherol. Vitamin E represents major antioxidant in the serum (35). The term vitamin E denotes several naturally occurring tocopherols and tocotrienols, but α-tocopherol is responsible for most of vitamin E activity in animal tissues. Disorders of antioxidant balance involving vitamin E may also be thought to be involved in the toxicity associated with radiotherapy (36), or chemotherapy (37). In general, a decrease in serum α-tocopherol has been reported during systemic chemotherapy (38-41). Retinol is a major circulating form of vitamin A that has also antioxidant activity and plays an essential role in many physiological functions, including vision, growth, development and differentiation, and immune response (35). Serum concentrations of α-tocopherol and retinol are significantly decreased in patients with advanced cancer (42, 43), and it has been demonstrated earlier that this decrease correlates with the systemic inflammatory response (42, 44). It has been demonstrated earlier that an effective anti-tumor therapy could result in increased serum retinol and α-tocopherol concentrations (12, 45), and the suppression of inflammatory response has been invoked as a possible explanation of this phenomenon. Significant negative correlation was observed in the present cohort between CRP and retinol concentrations. In an earlier study, negative correlation was also observed between neopterin and α-tocopherol (46). The absence of such correlation in the present cohort may be explained by the low number of patients examined. An increase of dietary vitamin intake could also result in higher retinol or α-tocopherol concentrations. However, the rise of serum retinol was observed already one week after the start of treatment that is emetogenic and would acutely results in a decrease of food intake. Because of the timing of the increase and the consistent presence of negative correlation between CRP and retinol, the suppression of systemic inflammatory response resulting from tumor control appears as a likely cause of the increased serum retinol concentrations observed in the present study.

In conclusion, serum homocysteine, CRP and urinary neopterin decreased while retinol and α-tocopherol increased in metastatic breast cancer patients during treatment with bevacizumab and chemotherapy. Present data demonstrate that the treatment may have also favorable effects on the risk of atherosclerosis.

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References


