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Urinary Neopterin Concentrations During Combination Therapy with Cetuximab in Previously Treated Patients with Metastatic Colorectal Carcinoma

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Abstract. *Background/Aim: Increased concentrations of neopterin, a biomarker of systemic immune response, have been reported after administration of cytokines, cytotoxic chemotherapy or external-beam radiation, but little is known about the effects of targeted-agents on neopterin. Patients and Methods: Urinary neopterin was studied in pre-treated patients with metastatic colorectal carcinoma during therapy with cetuximab, administered mostly in combination with irinotecan, 5-fluorouracil and leucovorin. Urinary neopterin was determined by high-performance liquid chromatography. Results: High initial urinary neopterin concentrations predicted poor prognosis. A significant correlation was observed between urinary neopterin and peripheral blood leukocyte count, hemoglobin and carcinoembryonic antigen concentrations. Urinary neopterin concentrations significantly increased during therapy only in patients with initially low neopterin concentrations. Conclusion: Urinary neopterin concentrations predict prognosis in patients with metastatic colorectal carcinoma treated with cetuximab. Rising neopterin concentrations indicate an activation of systemic immune response that could be responsible for the antitumor activity of cetuximab.*

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 types of cancer, such as breast carcinoma or colorectal carcinoma. Targeted-agents are, in general, less toxic and better-tolerated than conventional cytotoxic agents. Targeted-agents are thought to exert their antitumor activity through inhibition of a defined pathway(s) involved in cancer progression or metastasis (1). Most targeted-drugs may inhibit tumor growth through more than one mechanism acting on multiple molecular targets. Importantly, evidence is accumulating indicating that many targeted-agents may also activate the immune response.

Cetuximab is a chimeric antibody against epidermal growth factor receptor used in the therapy of advanced colorectal carcinoma. In prospective clinical trials, cetuximab has demonstrated significant clinical activity in patients with irinotecan-refractory metastatic colorectal carcinoma when administered as monotherapy or in combination with irinotecan (2, 3). Activity has also been demonstrated for cetuximab in combination with chemotherapy as first-line treatment of metastatic colorectal carcinoma in patients with tumors not harboring retrovirus-associated DNA sequences (*RAS*) mutations (4, 5). The precise mechanism of the antitumor activity of cetuximab is currently unknown. Among potential mechanisms, activation of the host immune response has also been implicated (6, 7).

Although biomarkers play an increasingly important role in the management of patients with cancer, the utilization of biomarkers associated with host response to neoplasia has so far been limited (8). The presence of systemic inflammatory or immune response may be studied by measuring circulating cytokine concentrations. A significant problem with this approach is posed by marked fluctuations of systemic cytokine levels. Neopterin is a pteridine produced from guanosine triphosphate (GTP) by activated macrophages in a reaction

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catalyzed by the enzyme GTP cyclohydrolase I. The activity of GTP cyclohydrolase I is induced by interferon-γ (IFN-γ) that is produced by T-lymphocytes and natural killer cells. Thus, the production of IFN-γ reflects systemic immune activation. Because the production of IFN-γ is enhanced by pro-inflammatory cytokines, such as interleukin-1 or interleukin-6, systemic concentrations of neopterin accompany both systemic immune and inflammatory responses (9). Neopterin in serum or in urine has been validated as a biomarker of systemic immune and inflammatory responses in disorders ranging from cancer to viral infections, transplant rejection, and atherosclerosis or its complications (9-14). The use of urine for neopterin measurements may help circumvent the need for repeated venipuncture. In addition, neopterin is stable in refrigerated samples for up to two weeks, and samples for repeat assessment may be collected and stored by the patient between regular office visits. Urinary neopterin concentrations are relatively stable in patients with cancer in the absence of complications (12). The presence of increased neopterin concentrations in serum or urine has been amply documented in patients with cancer (13, 14). Moreover, an increase of urinary neopterin concentrations has been demonstrated during administration of different anticancer therapies including cytokines (13), cytotoxic drugs (15) and external beam radiation (16), but little is known about systemic immune activation, reflected in urinary neopterin levels, during targeted therapy.

In the present study, we evaluated daily urinary neopterin concentrations in patients during therapy with cetuximab, administered to the majority of patients in combination with irinotecan-based chemotherapy.

Patients and Methods

Forty-five consecutive patients with metastatic colorectal carcinoma, 28 males and 17 females, aged (mean±standard deviation) 60±11 (range=32–78) years were included in the study. Forty-three patients were treated with the combination of cetuximab (loading dose 400 mg/m^2, subsequently 250 mg/m^2 weekly) followed by irinotecan (180 mg/m^2), leucovorin (200 mg/m^2), and 5-fluorouracil (400 mg/m^2 bolus and 1200 mg/m^2 for 46 hours) every two weeks (17) (including one patient who received a modification of this regimen). One patient with hyperbilirubinemia was treated with the above regimen omitting irinotecan, and one patient with cetuximab monotherapy. All patients had been previously treated with oxaliplatin, and all but one patient had been pre-treated with an irinotecan-containing regimen. The investigations were part of a project approved by the Institutional Ethical Committee (file number 200504 S14P), and the patients signed informed consent. For all patients, neopterin determination was performed in urine samples collected before therapy. Preliminary results on prognosis in the first 21 patients of this cohort have already been reported (18).

Urinary neopterin was determined as described elsewhere (19). Briefly, urine sample were collected and stored at –20˚C until analysis. After centrifugation (5 min, 1300 × *g*) and diluting 100 μl of urine specimens with 1.0 ml of mobile phase containing 2 g of disodium-EDTA per liter, samples were injected onto a column, and neopterin was determined using high-performance liquid chromatography system Prominence LC20 (Shimadzu, Kyoto, Japan). Neopterin was identified by its native fluorescence (353 nm excitation, 438 nm emission) and quantified by external standard method. Creatinine was determined by Jaffé reaction after dilution of the sample 1:50 on a Modular analyzer (Roche, Basel, Switzerland) using a commercial kit according the manufacturer's instructions, and neopterin concentrations were expressed as neopterin/creatinine ratio (μmol/mol creatinine).

Hemoglobin was measured by a photometric method using sodium lauryl sulfate, leukocytes and platelets were determined by impedance method using a Sysmex XE-2100 blood analyzer (Sysmex, Kobe, Japan). Serum carcinoembryonic antigen (CEA) was determined by radioimmunoassay using a commercial kit (Immunotech, Prague, Czech Republic), as described elsewhere (19).

Differences during therapy were evaluated using the Wilcoxon paired test. Correlations were examined using Spearman's rank correlation coefficient. Survival was analyzed using the Kaplan–Meier method, and differences were evaluated by 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).

Results

The mean (±standard deviation) of urinary neopterin at baseline was 272±225 μmol/mol creatinine. A significant correlation was observed between urinary neopterin and hemoglobin concentrations (r_s=–0.34; p<0.05; Figure 1A), peripheral blood leukocyte count (r_s=0.38; p<0.05; Figure 1B) and CEA concentrations (r_s=0.33; p<0.05; Figure 1C). Seventeen patients had urinary neopterin ≥214 μmol/mol creatinine (defined as upper limit of normal in an earlier study) (19). At the time of the analysis, 44 patients had died and one patient was alive after 74 months. Survival of patients with urinary neopterin concentration ≥214 μmol/mol creatinine was significantly inferior compared to patients with initial urinary neopterin <214 μmol/mol creatinine (median 10.1 *vs*. 17.7 months, p<0.05; Figure 2).

Daily neopterin measurements were obtained from 36 patients (Figure 3). The mean number of measurements obtained was 24±17 (range=1-63). Two fundamental patterns of urinary neopterin were evident based on initial neopterin concentrations. In patients with pre-treatment urinary neopterin ≥214 μmol/mol creatinine, a stable or decreasing pattern of urinary neopterin concentrations was usually observed. In contrast, urinary neopterin increased significantly in patients with initial neopterin <214 μmol/mol creatinine (Table I). In the patient treated with single-agent cetuximab, a rise of urinary neopterin concentrations was observed despite initially elevated neopterin concentrations (Figure 4).

Discussion

In addition to the prognostic significance of increased urinary neopterin concentrations, the present data demonstrate differential expression of neopterin during therapy with

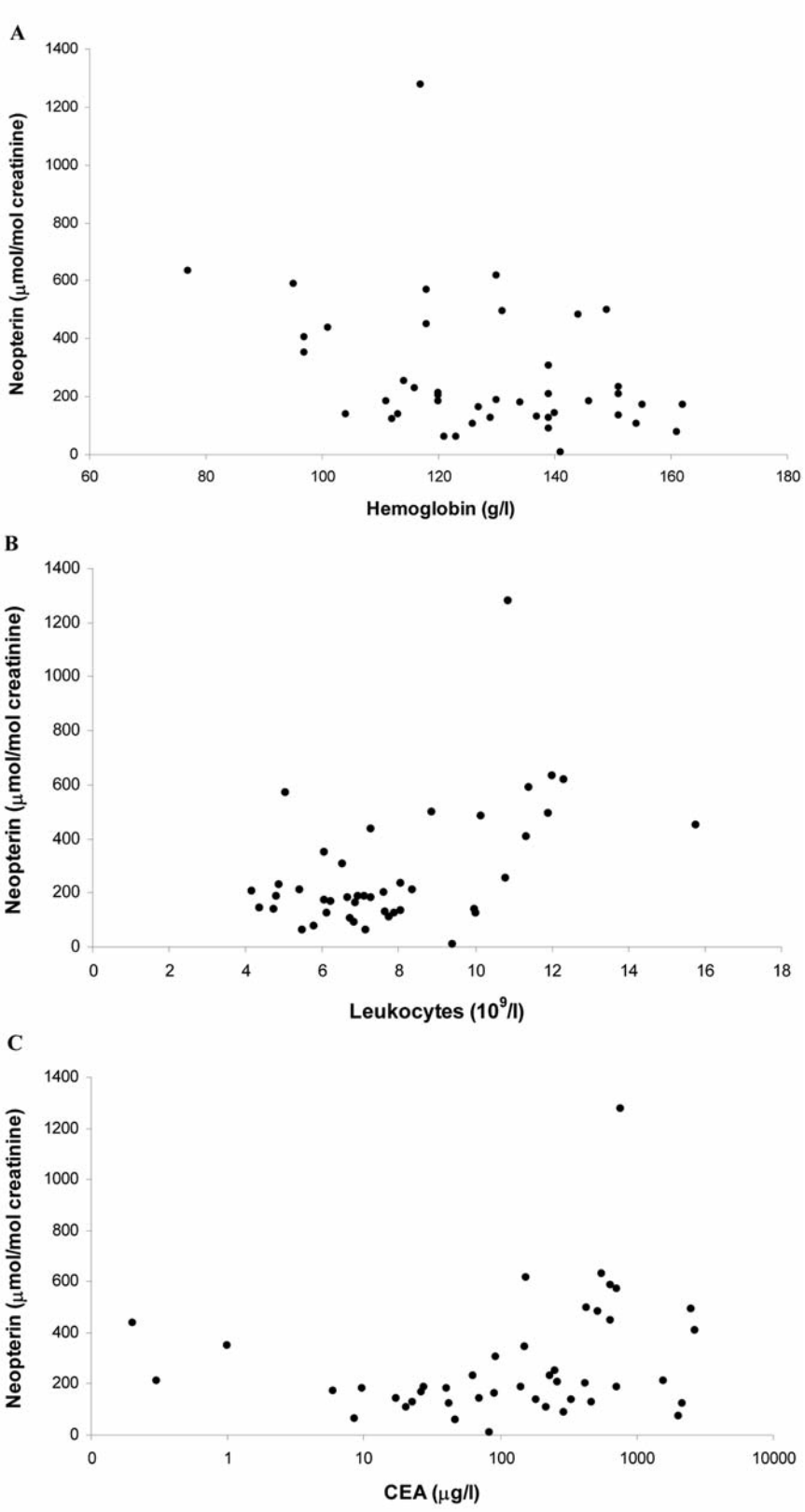

Figure 1. *Correlation between urinary neopterin concentrations and hemoglobin. (r_s=–0.34; p<0.05) (A), peripheral blood leukocyte count (r_s=0.38; p<0.05) (B) and serum carcinoembryonic antigen (CEA) (r_s=0.33; p<0.05) (C).*

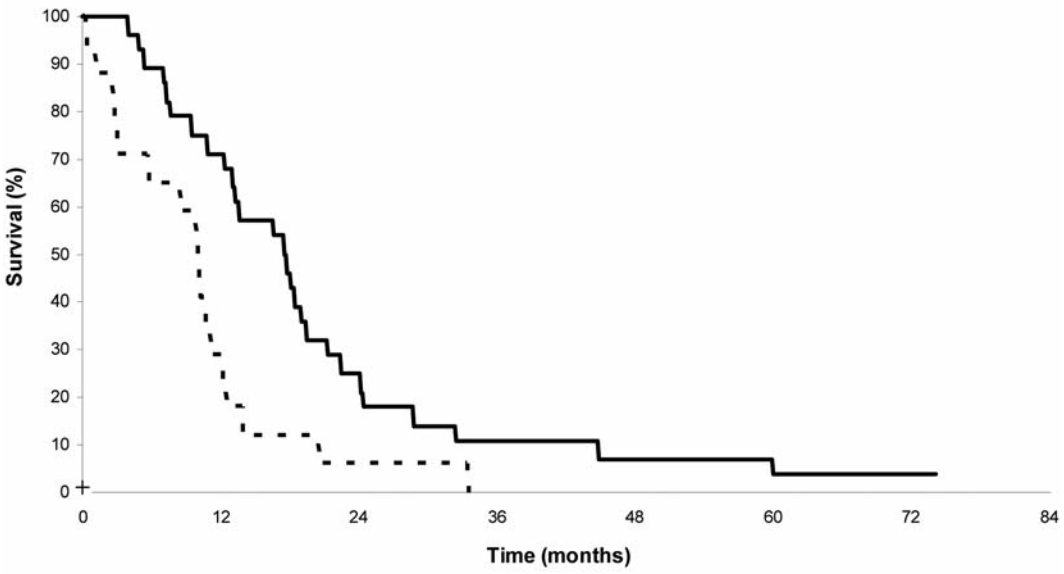

Figure 2. *Survival of patients based on initial urinary neopterin concentration. Kaplan–Meier survival curves of patients with initial urinary neopterin concentration ≥214 μmol/mol creatinine (dashed line) and <214 μmol/mol creatinine (bold line) are shown. Median survival was 10.1 versus 17.7 months for patients with urinary neopterin concentrations ≥214 μmol/mol creatinine and <214 μmol/mol creatinine, respectively (log-rank test, p<0.05).*

Table I. *Urinary neopterin concentrations during the course of therapy.*

	n	Urinary neopterin (μmol/mol creatinine)				
		Pre-treatment	Peak week 1	Before 2nd cetuximab dose	Peak week 2	Before 3rd cetuximab dose
Whole group	36	251±232	349±161[c]	263±186	345±209[b]	277±139
Pre-treatment neopterin						
<214 μmol/mol creatinine	24	138±53	299±115[d]	194±80[b]	280±124[c]	211±83[a]
≥214 μmol/mol creatinine	12	477±287	449±197	416±259	480±286	373±150

Data are means±standard deviation. Significantly different from the pre-treatment value at [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$, [d]$p<0.0001$.

cetuximab combined with chemotherapy. A significant increase was observed in patients with pre-treatment neopterin concentrations in the normal range, while a decreasing trend was evident in patients with initially high urinary neopterin. Thus, the present findings indicate the presence of systemic immune activation during therapy with cetuximab. Only one patient in the present cohort was treated with single-agent cetuximab, hence it is difficult to discern the effects of irinotecan-based chemotherapy and administration of cetuximab.

Although an increase of urinary neopterin concentrations in cancer patients has been well-documented and a therapy-induced rise has been described after the administration of chemotherapy or cytokines (13, 14, 20), so far there exist limited information about neopterin in patients treated with targeted agents, including monoclonal antibodies. Among patients with non-neoplastic disorders, daily neopterin measurements were reported in organ transplant recipients, and a rise in urinary neopterin was an early indicator of acute complications (9). Similarly, daily monitoring of urinary neopterin was performed in patients with cancer, and an increase in neopterin concentration preceded complications, while a decrease in urinary neopterin was associated with tumor control (12). An increase of neopterin production has been documented after systemic administration of different cytokines (13), and cytotoxic agents (15, 20).

In patients with tumors across the spectrum of primary locations, including colorectal carcinoma, increased serum or urinary neopterin concentrations were associated with poor prognosis (13, 14, 19, 21). The present study extends observation of the negative prognostic significance of increased

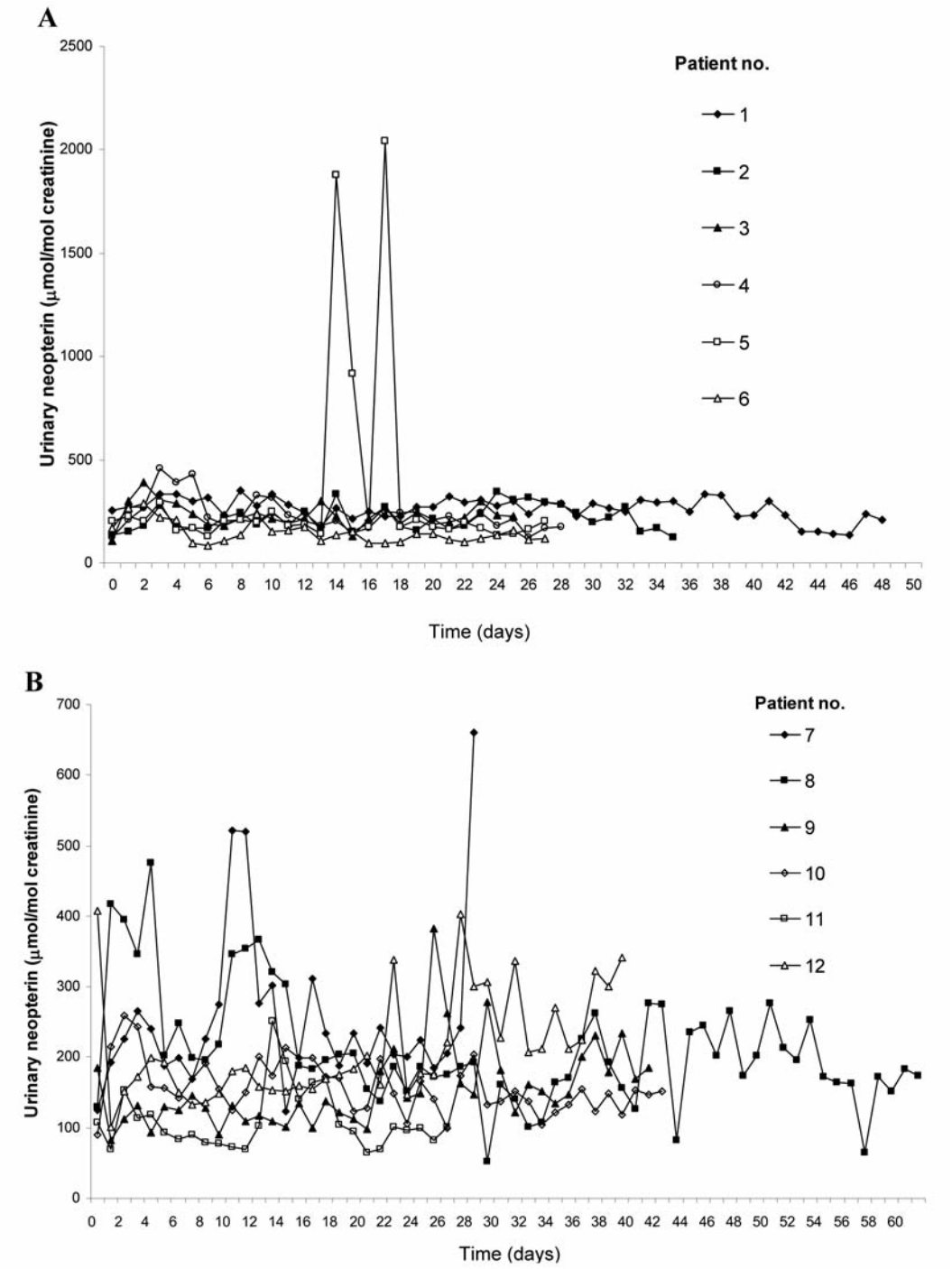

Figure 3. *Urinary neopterin concentrations during the course of therapy. A: Patients 1-6. The course of urinary neopterin concentrations during the course of therapy in patients 1-6 is shown. The marked peaks of urinary neopterin concentration in patient 5 occurred immediately after the start of the second cycle of the combination of cetuximab, irinotecan, 5-fluorouracil and leucovorin. B: Patients 7-12.*

urinary neopterin concentrations previously reported in patients with metastatic colorectal carcinoma (19) to patients treated in second or higher lines of therapy with the combination of chemotherapy and cetuximab. It remains to be tested whether the negative prognostic significance of high urinary neopterin concentrations observed in the present cohort is associated with the absence of systemic immune response reflected by a lack of an increase of urinary neopterin concentration.

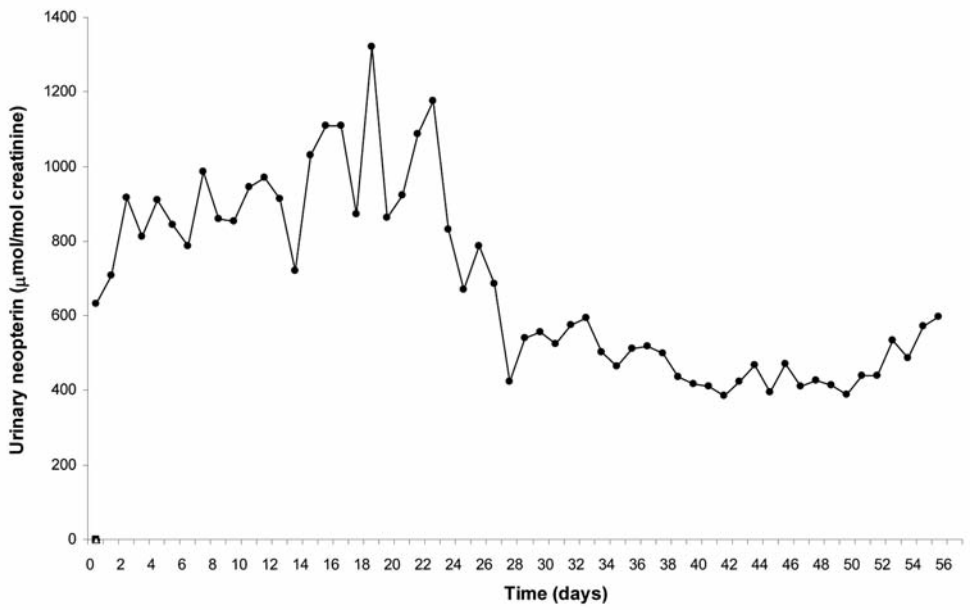

Figure 4. *Urinary neopterin concentrations during the course of therapy in a 78-year-old patient treated with single-agent cetuximab.*

A significant correlation was observed between urinary neopterin and peripheral blood leukocyte count, hemoglobin, and CEA concentration. These correlations may partly explain the association between high urinary neopterin concentrations and poor prognosis in the present cohort of patients. Similarly to prior reports, neopterin concentrations correlated with peripheral blood cell counts. In particular, the inverse correlation of hemoglobin with neopterin concentrations has been studied extensively (22-24). Correlation of urinary neopterin with peripheral blood leukocyte count and CEA concentrations has also been reported (19). In earlier studies, associations were also observed between lower numbers or impaired function of lymphocytes or dendritic cells and increased neopterin concentration (25-28). Thus, increased neopterin concentrations are thought to reflect immune dysregulation (13), similarly to other indicators of systemic immune or inflammatory activity, *e.g.* C-reactive protein.

The mechanism of action of many targeted-agents may involve, at least partly, the activation of the immune response. Cetuximab is an immunoglobulin G1 class antibody that could trigger antibody-dependent cell-mediated cytotoxicity (6), and there are data indicating that, indeed, the activation of host response may be one of the mechanisms responsible for antitumor activity of cetuximab (7, 29). The increase of urinary neopterin observed in the present study further supports the notion that the activation of host response may represent one of the mechanisms behind the antitumor activity of cetuximab alone or in combination with cytotoxic chemotherapy. An acute rise of the parameters of immune or inflammatory response may have different implications and reflect an association with effective host response resulting in tumor control. On the other hand, increased neopterin concentrations before the start of therapy may indicate the presence of a state refractory to further stimulation of the immune system.

In conclusion, urinary neopterin is a prognostic biomarker in patients treated with cetuximab in second or higher lines of treatment for metastatic disease. Urinary neopterin correlates with peripheral blood leukocyte count, hemoglobin and CEA concentrations. A marked increase of urinary neopterin observed during treatment may indicate an activation of the immune response.

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