Neopterin Is an Independent Prognostic Variable in Females with Breast Cancer

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Background: Neopterin, produced by human monocytes/macrophages upon stimulation by interferon-γ, is a sensitive marker for monitoring Th1-cell immune response in humans. In malignant diseases, the frequency of increases in neopterin in the serum and urine of patients depends on tumor stage and type.

Methods: In a retrospective study comprising 129 females with breast cancer, urinary neopterin/creatinine ratios were measured at the time of diagnosis. Tumor characteristics were determined concomitantly.

Results: Urinary neopterin was increased in 18% of the patients. It did not correlate with tumor size or lymph node status, but it was influenced by the presence of distant metastases ($P < 0.05$) and by tumor differentiation ($P = 0.01$). When product-limit estimates were calculated after follow-up for up to 13 years (median follow-up, 56 months), the presence of distant metastases ($P < 0.001$), neopterin ($P < 0.001$), tumor size ($P = 0.001$), and lymph node status ($P < 0.01$) were significant predictors of survival. By multivariate analysis, a combination of the variables presence of distant metastases ($P < 0.001$), neopterin ($P < 0.01$), and lymph node status ($P < 0.05$) was found to jointly predict survival. In lymph node-negative patients without distant metastases, the relative risk of death associated with increased neopterin concentrations was 2.5 compared with patients with neopterin concentrations within the reference interval.

Conclusion: Urinary neopterin provides additional prognostic information in patients with breast cancer.

Breast cancer is the leading cause of cancer death in Austrian women today (1). Women have a 1 in 10 risk of developing this cancer during their lifetimes (2). The risk of death in breast cancer patients is related to tumor size, the axillary lymph node situation, and the progression of metastatic disease (3, 4). Conventional clinical and histopathological tumor characteristics provide some guide to estimate the risk of relapse after surgical therapy, but doubtless, other prognostic markers are necessary to create a medical regimen of most benefit to an individual (5). In addition to biomarkers such as the presence of estrogen or progesterone receptors in the tumor tissue, immune system markers such as neopterin might also be important: Upon stimulation by interferon-γ, human monocytes/macrophages produce and release large amounts of neopterin, 6-p-erythro-1,2,3-trihydroxypropyl-pterin, which is synthesized from GTP by GTP cyclohydrolase I (EC 3.5.4.16) (6). Increases in neopterin concentrations in serum and urine have been found in viral infections, including HIV-1 infection (7, 8), autoimmune diseases such as rheumatoid arthritis (9) and systemic lupus erythematosus (10), and during allograft rejection episodes (11). These in vivo studies have shown that neopterin is a useful marker for monitoring the activation of cellular immunity in patients. In various malignant disorders, such as multiple myeloma, hematological neoplasia, or gynecological cancer [for a review, see Ref. (12)], higher neopterin concentrations in serum or urine were significantly associated with rapid disease progression and death, usually being jointly predictive with the stage of the tumor. In a recent study of squamous cell carcinoma of the oral cavity, urinary neopterin concentrations were a significant predictor for patients’ outcomes (13), although the frequency of increased urinary neopterin concentrations was only 43%, which was rather low compared with the 50–95% observed in the malignancies mentioned above. In females with breast cancer, the frequency of increased urinary neopterin concentrations was ~20% (14). In this study, the ability of urinary neopterin measured at the time of diagnosis to predict the survival probability was assessed in a retrospective analysis of women with breast cancer.
Materials and Methods

PATIENTS
The study was based on 129 female patients—39 premenopausal and 90 postmenopausal women—with breast cancer who were diagnosed and treated at the Department of Gynecology and Obstetrics of the University Hospital of Innsbruck between August 1984 and June 1994. All investigations reported here were performed at the time of diagnosis just before initiation of any specific tumor therapy. As seen from their histories, all patients were clinically free of infections at this moment. Patient ages at diagnosis ranged from 30 to 93 years, with a median age of 61 years. After diagnosis, patients were followed up until May 1998 (median follow-up time, 56 months; range, 0.2–156 months). Fifty-six of these 129 patients died as a result of the malignant process, 1 patient died from pulmonary embolism, and 1 from liver cirrhosis. Together with other patients, still alive or out of observation, these three were regarded as “censored” for statistical analyses.

TUMOR COLLECTION, STAGING, AND THERAPY
Invasive ductal carcinoma, classified according to criteria of the Armed Forces Institute of Pathology (15), was found in 96 patients; 3 had ductal carcinoma in situ, 1 had lobular carcinoma in situ, 17 had invasive lobular carcinoma, 3 had medullary carcinoma, 4 had mucinous lobular carcinoma in situ, 17 had invasive lobular carcinoma; T 1 in 52 patients, T 2 in 51 patients, T 3 in 8 patients, and T 4 in 14 patients. Lymph node status N 0 was seen in 51 patients, 58 had N 1, 7 had N 2, and 13 had N x. In 32 patients, the tumor was highly differentiated; 61 patients had moderately differentiated, and 26 had poorly differentiated tumors. In 10 patients, tumor grading was not performed. Distant metastases were present in 11 patients at the time of diagnosis; 118 patients were obviously without metastases.

Because of disease progression, in 21 cases only lumpectomies and in 41 cases only quadrantectomies were performed, each with or without axillary dissection. Simple mastectomies were performed in 4 patients; in 63 patients, radical mastectomies according to Patey were performed. The tumor and lymph node tissues were processed for routine histopathology (16); 76 tumors presented estrogen receptors (>10 fmol/mg protein), 71 presented progesterone receptors (>25 fmol/mg protein), and in 19 cases, receptor status was not determined.

In 14 patients, therapy consisted of surgery only. Additional radiotherapy only was administered to 33 patients, additional combined chemotherapy only to 5, and additional therapy with tamoxifen only to 2 patients. Twenty-one patients were treated by surgery and radiotherapy combined with chemotherapy, 25 by surgery and radiotherapy combined with tamoxifen, and 2 by surgery combined with chemotherapy and tamoxifen. Twenty-seven patients received surgery combined with radiotherapy, chemotherapy, and tamoxifen. Therapy was not included when patients were categorized in the analysis of survival because of the small numbers of patients in different therapeutic regimens. In addition, it was considered that therapy was influenced by tumor staging and was, therefore, not an independent variable.

LABORATORY EXAMINATIONS
Neopterin determinations were performed in first-morning urine specimens. Analysis was performed immediately, or specimens were stored at −20 °C until analysis. Urinary neopterin and creatinine were determined by an optimized and fully automated HPLC technique on a Vista 5000 (Varian) as described previously (17). In short, 100 μL of urine was diluted with Sørensen potassium phosphate buffer (0.015 mol/L, pH 6.4), and 20 μL of the diluted sample was injected onto a reversed-phase C 18 column (LiChrosorb, 7 μm, 155 × 4 mm; Merck) and chromatographed with a Sørensen potassium phosphate buffer. Creatinine was monitored by its ultraviolet absorbance at 235 nm, neopterin by fluorescence detection (emission at 438 nm with excitation at 353 nm). Neopterin concentrations were related to urinary creatinine concentrations to account for physiologic variations in urine volumes. Within-run imprecision (CV) was 4.7% and day-to-day imprecision was 5.8% for the neopterin/creatinine ratio. A mean recovery of 99.3% was obtained for this ratio. The neopterin/creatinine ratios of adults are slightly age and sex dependent (17), with upper limits of normal (97.5 percentiles) between 208 μmol neopterin/mol creatinine (for women 18–25 years) and 251 μmol neopterin/mol creatinine (for women >65 years).

STATISTICS
Differences of distributions of urinary neopterin/creatinine ratios among patient groups differing by clinical variables (such as tumor size) were tested for significance by a nonparametric analysis of variance (Kruskal–Wallis test).

Univariate analyses of survival were performed by the product-limit method (18); differences between survival curves were assessed for significance by the generalized Savage test (Mantel–Cox test statistic). Categorization of patients according to the continuously coded variable neopterin was based on the reference ranges. Urinary neopterin values were dichotomized by the upper limit of normal because higher values of this marker are generally associated with disease progression.

Multivariate analysis of survival was performed by a stepwise version of Cox’s proportional hazards model (19), as implemented in the program BMDP2L (BMDP Statistical Software, 1990 edition; University of California Press). This technique identifies the subset of variables that discriminate best between patients at high, medium, or low risk for death. BMDP2L uses forward stepping of variables to define the stepwise process of variable selec-
Results

At the time of diagnosis, only 18% of the patients with breast cancer had increased urinary neopterin, with values ranging between 57 and 633 μmol/mol creatinine (median, 173 μmol/mol creatinine). Notably, by the Kruskal–Wallis test, tumor size and lymph node status showed no significant effect on urinary neopterin values. There was only a weak effect of the presence of distant metastases (n = 129; H = 5.74; P < 0.05) and a stronger effect of tumor grading on neopterin concentrations (n = 119; H = 8.82, P = 0.01).

Univariate analysis of survival

The product-limit estimates of cumulative survival probabilities for patients, grouped according to clinical variables and the variable urinary neopterin, are shown in Table 1. For these estimates, categorical variables were grouped according to the categories; calculations for the variables tumor size, lymph node status, and morphology according to dichotomized categories are also shown. Urinary neopterin was dichotomized according to the individual upper limits of normal. From all of the variables, only the presence of distant metastases, tumor size, lymph node status, and urinary neopterin were highly significant predictors of survival, whereas all of the others showed no correlations at all with survival expectations. Fig. 1 shows the computed cumulative survival expectations for patients, grouped by the three statistically significant clinical variables—tumor size, lymph node status, and presence of distant metastases—and by urinary neopterin concentrations in urine. Notably, no patient with tumor T4 died during the observation period, and no cancer-related deaths occurred later than 106 months after diagnosis (maximum observation period, 156 months).

Multivariate analysis of survival

In multivariate analysis of survival probability using the Cox proportional hazard model (19), continuously coded variables were dichotomized in the same way as in univariate survival analysis. When the model was tested, univariate analysis with the proportional hazard model produced essentially the same results as product-limit estimates (not shown). Because not all data sets in our study were complete, only the univariately statistically significant variables, rather than all candidate predictors, were included in the stepwise regression process to prevent a loss of information by the omission of too many partially incomplete data sets. When a stepwise regression was performed, the variables neopterin, presence of distant metastases, and lymph node status were found to jointly predict survival (Table 2). As can be estimated from the regression coefficients of this model, the relative risk of death associated with patients with the presence of distant metastases is exp (1.66) = 5.3, the relative risk of death associated with an urinary neopterin value higher than the individual upper limits of the normal is exp (0.90) = 2.5, the relative risk of death associated with patients with lymph node status N1 and N2 is exp (0.73) = 2.1, and the relative risk associated with all three unfavorable indicators is exp (1.66 + 0.90 + 0.73) = 26.8 times higher than that of patients with no distant metastases, normal urinary neopterin values, and lymph node status N0.

The predictive power of urinary neopterin values in 50 patients with negative axillary lymph node status (N0)
and absence of distant metastases (M0) is demonstrated in Fig. 2. Those with increased neopterin (n = 5) have a dramatically worse outcome than those with normal (n = 45) neopterin (Mantel–Cox test statistic = 21.910;  \( P < 0.0001 \)); in fact, four of five patients with increased neopterin died within 32 months after diagnosis.

Discussion
In this study, the presence of distant metastases, lymph node status, tumor size, and urinary neopterin were the only variables that significantly predicted fatal outcome of women with breast cancer. The predictive power for a patient’s outcome of the clinical variables presence of

Table 2. Multivariate proportional hazards model of prognosis in patients with breast cancer (n = 116).\(^{a}\)

<table>
<thead>
<tr>
<th>Variable(^{b})</th>
<th>Value</th>
<th>( S_{\text{vz}} )</th>
<th>( \text{Exp (coeff.)} )</th>
<th>( P )</th>
<th>Log L(^{d})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distant metastases</td>
<td>1.66</td>
<td>0.527</td>
<td>5.3</td>
<td>&lt;0.0001</td>
<td>-188.70</td>
</tr>
<tr>
<td>Neopterin</td>
<td>0.90</td>
<td>0.378</td>
<td>2.5</td>
<td>0.0069</td>
<td></td>
</tr>
<tr>
<td>Lymph node status</td>
<td>0.73</td>
<td>0.341</td>
<td>2.1</td>
<td>0.0260</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
<td>Not included(^{e})</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) n, denotes the number of data sets included in the model.

\(^{b}\) The variable, distant metastases, was used without transformation; lymph node status was dichotomized into  \( N_0 \) and  \( N_1 + N_2 \), and tumor size into  \( T_{1s} + T_1 \) and  \( T_2 + T_3 + T_4 \). Urinary neopterin was dichotomized by the upper limit of normal.

\(^{c}\) The exponential function of the regression coefficient \( \text{Exp (coeff.)} \) denotes the relative risk associated with a variable; \( S_{\text{vz}} \), standard estimation error of regression coefficient;  \( P \), statistical significance estimated from a \( \chi^2 \)-to-remove statistic.

\(^{d}\) Logarithm of the likelihood associated with a model.

\(^{e}\) Not included in the model because of the lack of joint significance.
distant metastases, lymph node status, and tumor size is in good agreement with previous experiences of breast cancer treatment (3, 4). Most importantly, tumor grading, tumor morphology, and estrogen or progesterone receptor status did not contribute to survival expectations. This is in agreement with some previous studies (16, 20, 21), whereas other studies showed a predictive impact of tumor grading or receptor status (22, 23). Our study supplies no definite explanation for these contrary results, but possibly different methodologies of receptor status determination or different study populations might be responsible.

Urinary neopterin was increased in 18% of the patients. Because of this low diagnostic sensitivity, it is certainly an insufficient tool to support the diagnosis of breast cancer. Although this was the lowest frequency of neopterin investigated to date (12), urinary neopterin was the strongest of all investigated variables with the exception of the presence of distant metastases to predict survival in this study. This was true in univariate and multivariate analyses of survival. The study suffered from the relatively low number of investigated patients and the fact that some data sets were partially incomplete. Nevertheless, it clearly demonstrates the predictive power of increased urinary neopterin concentrations compared with other variables concerning survival expectations. To date, the number of data points are too few to draw definite conclusions for clinical practice, but the study encourages further clinical studies concerning neopterin measurements in female breast cancer: as demonstrated in patients with negative axillary lymph node status and absence of distant metastases (Fig. 2), neopterin measurements in breast cancer might be useful in addition to TNM-staging for a better estimation of survival expectations.

This predictive value of neopterin is further confirmed when the study population is restricted to an “intermediate risk” group: tumor size T1 or T2, lymph node status N0 or N1, and absence of distant metastases (M0, not shown).

Similar observations were made in other malignancies as well, but all of them showed higher frequencies (43–92%) of increased neopterin: neopterin was found to be a significant and independent predictor of survival in, e.g., carcinoma of the uterine cervix or the ovaries, in various hematological neoplasms [for a review, see Ref. (12)], in colon carcinoma (24), in lung cancer (25), and in squamous cell carcinoma of the oral cavity (13).

For clinical practice, it is important to stress that increased neopterin production, which is a sign of an activated cellular immune system, is not specific for a tumor disease (6). When neopterin is used as a risk factor in patients with cancer, it appears sufficient to exclude, by clinical anamnesis and by basic laboratory tests, other diseases, e.g., acute virus infections, as was done in our study. An improvement of risk estimation might be achieved by combining the predictive immunological marker neopterin with a predictive tumor marker. This was shown earlier in malignancies of the ovaries by a study combining neopterin measurements with measurements of the tumor marker CA 125 (26). Recently, the prognostic impact of increased serum concentrations of the tumor marker CA 15-3 in breast cancer was reported (27). CA 15-3 serum concentrations were not available in our retrospective study, but we think that a combination of a prognostic tumor marker such as CA 15-3 with a prognostic immunological marker such as neopterin could be useful and should be investigated.

The impact of increased urinary neopterin to predict poor survival in patients with malignancies might be explained by studying the role of neopterin in the immune system: neopterin is not a tumor marker in the usual sense of the word, there is no indication that cancer cells themselves are excreting relevant amounts of neopterin (6). In fact, to date only the myelocytoma cell line THP-1 is known to produce substantial neopterin on stimulation with interferon-γ. Rather, neopterin concentrations during malignant disease indicate a chronic cellular immune response in patients and appear to be exclusively attributable to production of cytokines such as interferon-γ by activated T cells, which in turn induce macrophages for neopterin release (6, 7). Thus, increased urinary neopterin concentrations in a subgroup of patients with cancer might be attributable to chronic immune stimulation that indicates a poor prognosis because the immune system is unable to eliminate the stimulating agent. This corresponds well to the fact that functional deficiency of cellular immunity develops preferentially in patients with signs of an activated immune response (28). From this point of view, higher neopterin concentrations may indicate a higher risk for developing metastases but not already existing metastatic deposits.

Recent observations have implied a more direct association between neopterin production and malignant
growth because, in addition to the close relationship between the formation of reactive oxygen species and neopterin by the activated monocytes/macrophages, the effects of various reactive compounds are also modulated by neopterin derivatives (29,30). Thus, increased neopterin concentrations seem to be an indicator of increased oxidative stress in humans (31). Reactive oxygen species have been implicated in the initiation and promotion of carcinogenesis, and direct effects on growth factors and other signaling pathways of both antioxidants and oxidants have been demonstrated. Recently, neopterin derivatives have been shown to significantly enhance c-fos oncogene expression in rat NIH3T3 fibroblasts in vitro (32). Therefore, the prognostic value of higher neopterin concentrations to predict disease progression and death in malignant diseases could be related to the capacity of neopterin derivatives to induce oncogene expression. Additional studies will be needed to eventually demonstrate a potential role of neopterin derivatives in malignant transformation in humans.

In conclusion, although the diagnostic sensitivity and specificity of increased neopterin concentrations for patients with breast cancer was low, a significant predictive value for urinary neopterin concentrations concerning patient outcome could be demonstrated.

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