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The Prognostic Value of Polymorphonuclear Leukocyte Elastase in Patients with Primary Breast Cancer[1]

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ABSTRACT

A variety of serine proteases, including urokinase-type plasminogen activator (uPA), plasmin, and polymorphonuclear leukocyte elastase (PMN-E), have been implicated in the processes of tumor cell invasion and metastasis. Besides degrading of matrix proteins, PMN-E has been shown to be able to cleave and inactivate plasminogen activator inhibitor-1 (PAI-1), the main inhibitor of uPA, and α_2-antiplasmin, the natural inhibitor of plasmin, thus enabling an uncontrolled matrix degradation by the fibrinolytic enzymes. Because only limited data are available on a relationship between the tumor level of PMN-E and prognosis in primary breast cancer patients, in the present study we have measured with an ELISA the levels of PMN-E (in complex with α_1-proteinase inhibitor) in cytosolic extracts of 1143 primary breast tumors. Levels of complexed PMN-E have been correlated with the lengths of metastasis-free survival (MFS), relapse-free survival, and overall survival, and a comparison was made with data previously obtained for uPA and PAI-1. Our results show that patients with a high PMN-E level in their primary tumor had a rapid relapse and an early death compared with patients with a low tumor level of PMN-E. This held true for node-negative and node-positive subgroups of patients as well. The relationship of PMN-E with a poor prognosis was especially obvious during short-term follow-up (0–60 months). In Cox multivariate regression analysis, corrected for the traditional prognostic factors, PMN-E was an independent prognostic factor, and high levels of PMN-E were associated with a poor MFS [hazard ratio (HR), 1.63; 95% confidence interval (CI), 1.23–2.16; $P < 0.001$], relapse-free survival (HR, 1.45; 95% CI, 1.10–1.89; $P = 0.01$), and overall survival (HR, 1.64; 95% CI, 1.20–2.23; $P = 0.003$). Furthermore, in all three multivariate models, PMN-E still added significantly to the model after the additional inclusion of the uPA. PMN-E was an independent prognostic factor for MFS even in the multivariate analysis including the traditional clinical prognostic factors and the strong established biochemical prognostic factors uPA and PAI-1. Our present study suggests that PMN-E is associated with breast cancer metastasis, and knowledge of the tumor PMN-E status might be helpful in selecting the appropriate individualized (adjuvant) treatment for patients with breast cancer.

INTRODUCTION

The serine protease uPA[3] and its main inhibitor, PAI-1, have been studied extensively for their prognostic value in patients with breast cancer, indicating that high tumor levels of both parameters are associated with a poor prognosis (1, 2). The finding of a relationship between high levels of the inhibitor PAI-1 and a poor prognosis can be attributed to its role as a direct enhancer of adhesion, migration, and tumor angiogenesis irrespective of its protease inhibitor potency (3, 4).

PAI-1 can be cleaved by the serine protease PMN-E, produced mainly in polymorphonuclear leukocytes, thereby loosing its inhibitory activity against uPA (5). Furthermore, PMN-E has been shown to inactivate α_2-antiplasmin, the natural inhibitor of the serine protease plasmin (6). Because PMN-E itself disintegrates matrix proteins such as elastin, collagen, and proteoglycans (7), a prominent role of PMN-E seems to be very likely in the process of tumor cell invasion and metastasis. Interestingly, PMN-E is produced not only by neutrophils but also by human breast cancer cells in cell culture (8). Although the main origin of PMN-E in tumor tissue extracts is not yet clear, the first pilot studies measuring PMN-E in tissue extracts of human breast cancer samples revealed a clear relation between elevated elastase levels and poor prognosis of the patients (9, 10).

Because PMN-E and the fibrinolysis parameters interact, we considered it of interest to evaluate the combined prognostic value of PMN-E, uPA, and PAI-1 in primary breast cancer patients ($n = 1143$). The findings were correlated with the patient and tumor characteristics as well as with traditional clinical prognostic factors, revealing PMN-E as an independent prognostic parameter for MFS when tested in the multivariate analysis.

MATERIALS AND METHODS

Patients and Tissue Samples. PMN-E levels were determined in cytosol preparations (as described below) from 1143 primary invasive breast tumors collected between 1978 and 1989. Our study design was approved by the medical ethical committee of the Erasmus University (Rotterdam, the Netherlands). Inoperable T_4 tumors and tissue specimens that were sampled after neoadjuvant treatment or obtained from a biopsy were excluded. Furthermore, patients admitted to our institute more than 100 days after primary surgery and patients with distant metastasis at the time of primary surgery [M_1 patients; staging according to the International Union Against Cancer tumor-node-metastasis (TNM) classification] were excluded.

Median age of the patients at the time of surgery (modified mastectomy = 615 patients, breast-conserving treatment = 528 patients) was 56 years (range, 27–90 years). The characteristics of the patients with respect to menopausal status, tumor size, nodal status, tumor histology and grade, and ER and PgR status are listed in Table 1. Radiotherapy was applied to 1006 patients (88%): on the breast/thoracic wall in 788 patients and/or on the axilla in 430 patients; and/or parasternal and/or supraclavicular lymph nodes in 516 patients. None of the node-negative patients received systemic adjuvant therapy. Adjuvant chemotherapy [mainly cyclophosphamide/methotrexate/5-fluorouracil (CMF)] was given to 213 node-positive patients (mainly premenopausal patients), whereas 58 patients received adjuvant hormonal therapy (mainly postmenopausal patients), either alone (44 patients) or in combination with chemotherapy (14 patients).

All patients were examined routinely every 3–6 months during the first 5 years of follow-up and once a year thereafter. The median follow-up period of patients alive ($n = 584$) was 124 months (range, 13–231 months). Patients with events after 120 months were censored at 120 months because after 10 years of observation, patients frequently are redirected to their general practitioner for checkups and mammography and cease to visit our outpatient breast cancer clinic. Of the 1143 patients included, 629 (55%) showed evidence of disease

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[3] The abbreviations used are: uPA, urokinase-type plasminogen activator; PMN-E, polymorphonuclear leukocyte elastase; ER, estrogen receptor; PgR, progesterone receptor; PAI-1, plasminogen activator inhibitor-1, HR, hazard ratio; CI, confidence interval; MFS, metastasis-free survival; RFS, relapse-free survival; OS, overall survival; IRA, isotonic regression analysis; d.f., degrees of freedom; α_1PI, α_1-proteinase inhibitor; EORTC, European Organization for Research and Treatment of Cancer.

Table 1 *Relationships of total PMN-E with patient and tumor characteristics*

Characteristic	Frequency[a]	Total PMN-E: Median value (quartiles)[b] (ng/mg protein)	*P*
All patients	1143	6.6 (3.6, 13.5)	
Age (yrs)			0.01[c]
≤40	161	7.8 (4.3, 19.8)	
41–55	382	6.6 (3.5, 13.4)	
56–70	406	6.2 (3.3, 12.3)	
>70	194	6.5 (3.7, 12.8)	
Menopausal status			0.03[d]
Premenopausal	457	6.8 (3.8, 16.3)	
Postmenopausal	686	6.4 (3.4, 12.6)	
T status			0.58[e]
T ≤ 2 cm	460	6.2 (3.5, 13.0)	
T > 2–5 cm	546	6.7 (3.8, 14.7)	
T > 5 cm	137	7.1 (3.3, 14.2)	
N status			0.45[e]
N_0	475	6.7 (3.7, 14.6)	
$N_{1–3}$	332	6.2 (3.4, 12.9)	
$N_{>3}$	336	6.7 (3.5, 12.9)	
Histology			
Intraductal	789	6.7 (3.6, 14.1)	<0.001[e]
Intralobular	48	4.4 (2.8, 7.5)	
Mucinous	23	5.2 (3.5, 7.9)	
Medullary	29	16.3 (8.0, 96.6)	
Others + unknown	254	6.6 (3.6, 13.5)	
Histological grade			0.07[e]
Poor	636	6.8 (3.9, 15.1)	
Moderate	206	6.3 (3.5, 11.6)	
Good	15	3.9 (2.4, 8.4)	
ER status[f]			<0.001[f]
Negative	244	14.9 (6.1, 41.7)	
Positive	896	5.8 (3.3, 10.3)	
PgR status[f]			<0.001[c]
Negative	321	9.1 (5.2, 30.3)	
Positive	796	6.0 (3.3, 10.7)	

[a] Due to missing values, numbers do not always add up to 1143.
[b] All values in ng/mg of protein (25th and 75th percentiles).
[c] *P* for Spearman rank correlation.
[d] *P* for Wilcoxon rank-sum test.
[e] *P* for Kruskal-Wallis test.
[f] Cut points used for ER and PgR: 10 fmol/mg of protein.

(including locoregional relapse) and count as failures in the analysis of RFS. In the analysis of MFS, 538 (47%) patients with distant metastasis were counted as failures. Sixty patients (5%) died without evidence of disease and were censored at last follow-up in the analysis of RFS and MFS. Four hundred and fifty-one (39%) patients died after a previous relapse. Thus, a total of 511 patients (45%) were failures in the analysis of OS.

Assays of ER, PgR, Total Protein, uPA, PAI-1, and PMN-E in Tumor Tissue Extracts. Processing of tumor tissues as recommended by the EORTC for cytosolic ER and PgR determinations by ligand binding assay or with enzyme immunoassay was as described previously (11). Total cytosolic protein was quantified with the Coomassie Brilliant Blue method (Bio-Rad) with human serum albumin as a standard. The cytosolic levels of uPA and PAI-1 were determined with ELISAs as described previously (12).

Taking into account that most of the PMN-E in body fluids or tissue cytosols is complexed with its main antagonist, α_1PI, we used a commercial two-site ELISA for quantification of the complex (Milenia-PMN Elastase; Milenia Biotec, Bad Nauheim, Germany). For measurement of the total amount of the enzyme, presumably free, proteolytically active elastase was complexed by adding a surplus of α_1PI (100 μg/ml) to an aliquot of each cytosol, and the sample was reassayed for an increase in the elastase-α_1PI complex. Free PMN-E was defined as the amount of PMN-E measured in the presence of additional α_1PI minus that measured in the original sample. Because total PMN-E and free PMN-E were significantly correlated with each other (Spearman rank correlation, $r_s = 0.54$; $P < 0.001$), we have restricted the data presentation to total PMN-E. Moreover, the PMN-E levels in cytosols significantly ($r_s = 0.85$; $P < 0.001$) correlated with those quantified in Triton X-100 extracts prepared according to Jänicke *et al.* (13). In the present study, we have used cytosol for total PMN-E determination. The EORTC receptor buffer used to prepare the cytosols did not interfere with the ELISA determination of PMN-E. This was checked by dilution of the elastase standard protein in EORTC buffer instead of calibrator diluent. The coefficients of variation were calculated from different samples in a total of 42 runs, demonstrating a mean intra- and interassay coefficient of variation CV of 2.6% and 6.4%, respectively.

Statistics. The strength of the associations of PMN-E with continuous variables was tested with Spearman rank correlation. The strength of the association of PMN-E with other variables (used as grouping variable) was tested with the nonparametric Wilcoxon rank-sum test or Kruskal-Wallis test, followed by a Wilcoxon-type test for trend across ordered groups where appropriate. Survival probabilities were calculated by the actuarial method of Kaplan and Meier. Both univariate and multivariate analyses were performed using the Cox proportional hazards model. The likelihood ratio test in the Cox

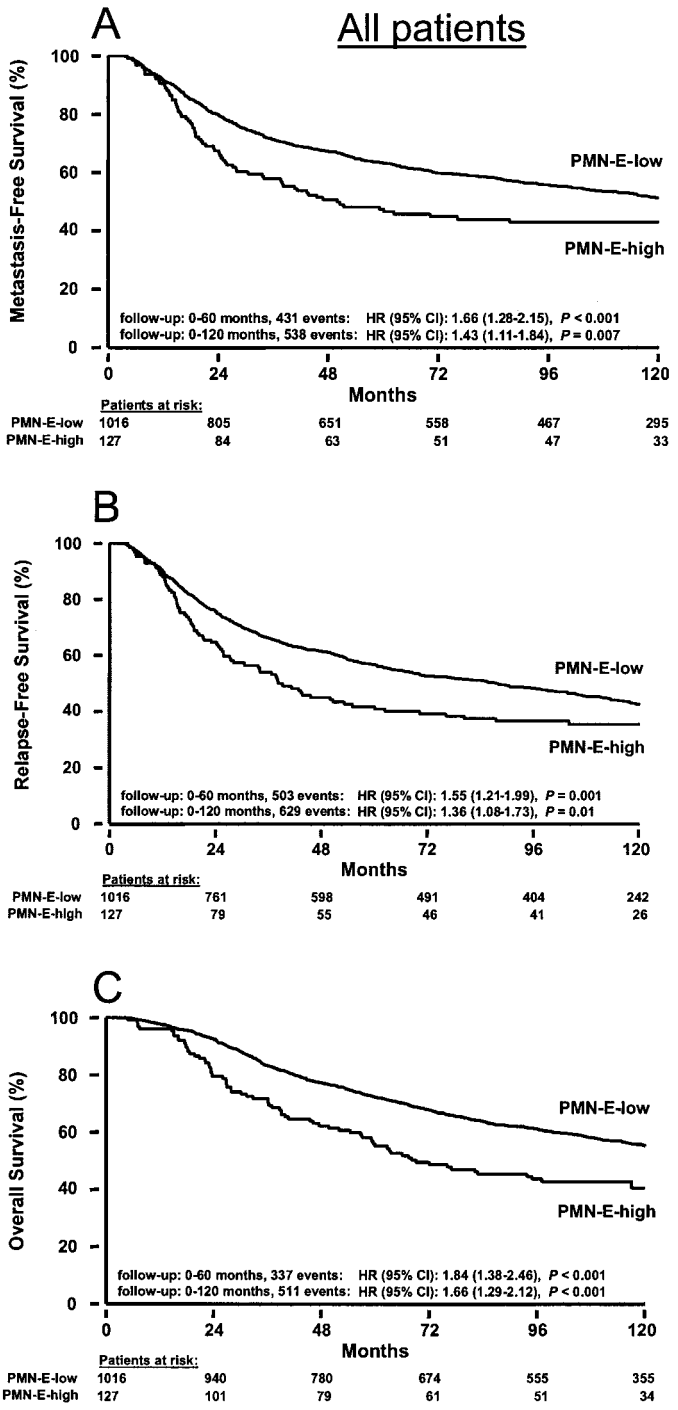

Fig. 1. MFS (*A*), RFS (*B*), and OS (*C*) as a function of total PMN-E status in 1143 primary breast cancer patients. Patients at risk are indicated. Cut point used, 36.4 ng PMN-E/mg protein.

regression models was used to test for differences and for interactions. In our search for a categorization of PMN-E, we used IRA (11, 14). With IRA, the hazard rate for failure is estimated as a function of the PMN-E value under the assumption of a monotone increasing failure rate with increasing PMN-E levels. The patients are ordered according to the PMN-E level and subsequently partitioned in ordered groups in such a way that average failure rates in the groups increase with increasing PMN-E level. The final partition is optimal in the sense that it is the maximum likelihood estimate for the exponential failure model. The cut point will be identified where the jump in the failure rates or the average time to failure is largest. The proportionality assumption was investigated using a test based on the Schoenfeld residuals (15). The residuals are retrieved, and a smooth function of time is fitted and then tested to determine whether there is a relationship. All computations were done with the STATA statistical package, release 7.0 (STATA Corp., College Station, TX). All *P*s are two-sided.

RESULTS

PMN-E Levels in Relation to Patient and Tumor Characteristics and uPA/PAI-1 Levels. The levels of PMN-E in 1143 tumor cytosols ranged from 0.4 to 1667 ng/mg protein (median, 6.6 ng/mg protein). Table 1 shows the median and the 25th and 75th percentile levels of PMN-E in subgroups of tumors and their relationship with patient and tumor characteristics. The tumor level of PMN-E was higher in premenopausal patients and was negatively related with age ($r_s = -0.07$), ER ($r_s = -0.28$), and PgR ($r_s = -0.23$). Compared with intraductal carcinomas, medullary tumors contained higher levels of PMN-E, and intralobular and mucinous tumors contained lower levels of PMN-E. There were no significant correlations with tumor size, lymph node status, or histological grade, whereas PMN-E levels were positively correlated with those of uPA ($r_s = 0.17$) and PAI-1 ($r_s = 0.30$).

MFS, RFS, and OS: Univariate Analysis. When studying PMN-E as a categorized variable divided in quartiles in Kaplan-Meier survival analyses, we noticed that the curves merged and occasionally crossed after 10 years of follow-up. This can be interpreted as a violation of the proportional hazards assumption during long-term prognosis and was confirmed when we checked the proportional hazards assumption for MFS ($\chi^2 = 8.9$, d.f. = 1, $P = 0.003$), RFS ($\chi^2 = 11.3$, d.f. = 1, $P < 0.001$), and OS ($\chi^2 = 14.7$, d.f. = 1, $P < 0.001$). MFS, RFS, and OS analyses as a function of continuous PMN-E levels showed a significant relationship with a poor prognosis ($P = 0.02$, $P = 0.01$, and $P < 0.001$, respectively) during short-term observation (0–60 months), whereas the proportional hazards assumption was no longer violated for MFS ($P = 0.14$), RFS ($P = 0.07$), or OS ($P = 0.22$). We therefore restricted our analysis to short-term prognosis.

The significant inverse relationship of continuous PMN-E levels with prognosis justified the search for a cut point to allow visualization with survival curves and analysis as a categorical variable in addition to analysis of PMN-E as a continuous variable. Using the results of the IRA with short-term MFS, RFS, and OS as end points, we chose 36.4 ng PMN-E/mg protein as a cut point to classify tumors as PMN-E-low and PMN-E-high. The Kaplan-Meier curves for all patients as a function of PMN-E status show that high levels of PMN-E were significantly associated with a poor MFS (Fig. 1*A*), RFS (Fig. 1*B*), and OS (Fig. 1*C*).

In separate analyses, we explored the prognostic value of PMN-E in

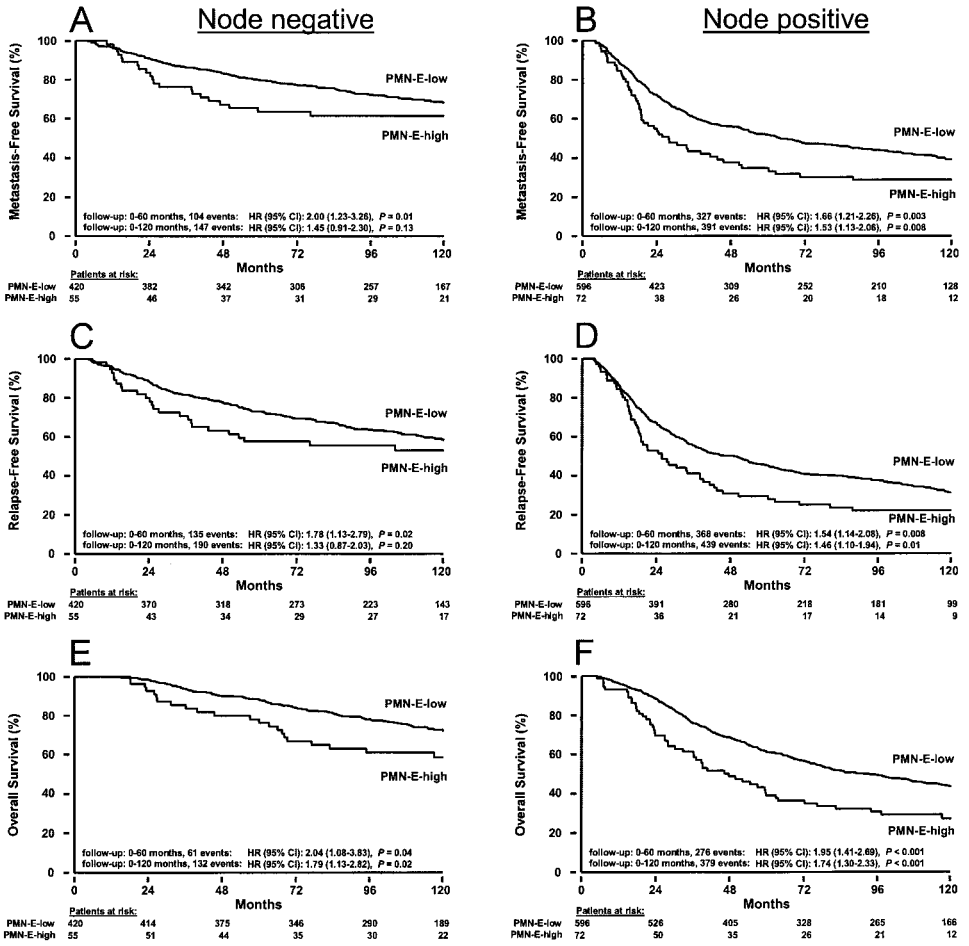

Fig. 2. MFS (*A* and *B*), RFS (*C* and *D*), and OS (*E* and *F*) as a function of total PMN-E status in subgroups of node-negative (*A*, *C*, and *E*) and node-positive patients (*B*, *D*, and *F*). Cut point used, 36.4 ng PMN-E/mg protein. Patients at risk are indicated.

Table 2 *Cox multivariate analysis of MFS, RFS, and OS*[a]

Factor	MFS		RFS		OS	
	HR[b]	*P*	HR[b]	*P*	HR[b]	*P*
Base model						
Age and menopausal status[c]		0.03		0.005		<0.001
Age premenopausal[d]	0.73 (0.57–0.92)		0.68 (0.55–0.85)		0.63 (0.47–0.85)	
Age postmenopausal[d]	0.91 (0.79–1.05)		0.96 (0.84–1.09)		1.08 (0.93–1.25)	
Post- *vs.* premenopausal	1.70 (1.15–2.52)		1.83 (1.27–2.64)		2.27 (1.38–3.73)	
Tumor size		<0.001		<0.001		<0.001
2–5 cm *vs.* ≤2 cm	1.71 (1.35–2.17)		1.69 (1.36–2.10)		1.69 (1.27–2.24)	
>5 cm *vs.* ≤2 cm	2.47 (1.81–3.36)		2.31 (1.73–3.10)		2.57 (1.82–3.63)	
Nodal status		<0.001		<0.001		<0.001
N_{1-3} *vs.* N_0	1.80 (1.37–2.35)		1.66 (1.30–2.11)		2.41 (1.73–3.35)	
$N_{>3}$ *vs.* N_0	3.23 (2.50–4.17)		2.75 (2.18–3.47)		4.00 (3.22–6.02)	
ER (positive *vs.* negative)[e]	0.94 (0.72–1.22)	0.62	0.87 (0.68–1.11)	0.25	0.72 (0.54–0.96)	0.02
PgR (positive *vs.* negative)[e]	0.67 (0.53–0.85)	<0.001	0.76 (0.61–0.95)	0.02	0.57 (0.44–0.74)	<0.001
Additions to base model[f]						
+PMN-E (high *vs.* low)[g,h]	1.63 (1.23–2.16)	0.001	1.45 (1.10–1.89)	0.01	1.64 (1.20–2.23)	0.003
+PMN-E (continuous)[g,i]	1.06 (0.98–1.15)	0.16	1.06 (0.98–1.14)	0.13	1.14 (1.05–1.25)	0.004
+PMN-E + uPA + PAI-1[j]						
PMN-E (high *vs.* low)[h]	1.36 (1.01–1.82)	0.048	1.22 (0.92–1.62)	0.17	1.32 (0.96–1.82)	0.10
uPA[k]		<0.001		<0.001		0.002
Intermediate *vs.* low	1.66 (1.20–2.30)		1.45 (1.09–1.94)		1.51 (1.04–2.19)	
High *vs.* low	2.29 (1.61–3.28)		1.99 (1.45–2.73)		2.02 (1.35–3.03)	
PAI-1[l]		0.03		0.02		0.003
Intermediate *vs.* low	1.23 (0.92–1.65)		1.28 (0.98–1.67)		1.16 (0.82–1.63)	
High *vs.* low	1.78 (1.17–2.69)		1.76 (1.19–2.60)		2.06 (1.30–3.26)	

[a] Final multivariate models included 1116 patients; follow-up truncated at 60 months.
[b] Numbers in parentheses, 95% CI.
[c] Age and menopausal status combined.
[d] Age in decades for pre- and postmenopausal patients.
[e] Positive, ≥10 fmol/mg protein; negative <10 fmol/mg protein.
[f] Factors added alone or together to the base model.
[g] PMN-E added alone to the base model.
[h] High, >36.4 ng/mg protein; low, ≤36.4 ng/mg protein.
[i] Log-transformed variable.
[j] PMN-E, uPA, and PAI-1 added together to the base model.
[k] Low, ≤0.19 (n = 244); intermediate, >0.19 and ≤1.21 (n = 550); high, >1.21 ng/mg of protein (n = 322) (for cut points, Ref. 12).
[l] Low, ≤9.33 (n = 294); intermediate, >9.33 and ≤45.28 (n = 729); high, >45.28 ng/mg of protein (n = 93) (for cut points, Ref. 12).

the clinically relevant subgroups of node-negative and node-positive patients. The results of the Kaplan-Meier analyses and log-rank tests again provided evidence that PMN-E was a stronger prognostic factor during short-term follow-up. This difference in HR between short-term and long-term follow-up was less pronounced for node-positive patients than for node-negative patients (Fig. 2).

MFS, RFS, and OS: Multivariate Analysis. The independent relationship of PMN-E with MFS, RFS, and OS was studied with Cox multivariate regression analysis during short-term follow-up. In the analysis of all patients, tumor size, nodal status, and young premenopausal age were associated with poor prognosis. In all three models, PgR positivity was associated with a favorable prognosis, whereas ER did not significantly add to the models of MFS and RFS. Tumor grade, which was a significant prognostic factor in both short-term and long-term univariate analyses of MFS, RFS, and OS (all $P < 0.05$), did not significantly contribute to any of the multivariate models. The multivariate model including age and menopausal status, tumor size, nodal status, ER, and PgR was defined as the base model (Table 2).

After inclusion of PMN-E as a dichotomized variable to the multivariable models, the increase in χ^2 ($\Delta\chi^2$) was 10.6 (d.f. = 1) in the analyses of MFS ($P = 0.001$), 6.7 (d.f. = 1) in the analysis of RFS ($P = 0.01$), and 8.9 (d.f. = 1) in the analyses of OS ($P = 0.003$). Analysis of PMN-E as a continuous variable only showed a significant contribution to the base model for OS ($\Delta\chi^2 = 8.4$, d.f. = 1, $P = 0.004$). When PMN-E was added as a dichotomized variable to the base models including the established strong prognostic factor uPA as well, PMN-E still significantly contributed to the multivariate models for MFS ($\Delta\chi^2 = 7.7$, d.f. = 1, $P = 0.005$), RFS ($\Delta\chi^2 = 4.5$, d.f. = 1, $P = 0.04$), and OS ($\Delta\chi^2 = 6.9$, d.f. = 1, $P = 0.009$). Additional inclusion of PAI-1 to these multivariate models resulted only in a better fit of PMN-E in the model for MFS ($\Delta\chi^2 = 3.9$, $P = 0.048$), whereas both uPA and PAI-1 were independent prognostic factors in all three models (Table 2).

Addition of adjuvant treatment as an indicator variable to the models did not change the coefficients of PMN-E. Furthermore, there were no statistically significant interactions between PMN-E and any of the other prognostic factors in any of the analyses. Moreover, after defining the final multivariate models for short-term prognosis, the proportional hazards assumption of PMN-E, either as a continuous or as a categorical variable, was not violated.

DISCUSSION

To identify the 60–70% of node-negative patients who do not need adjuvant treatment or to select node-positive patients who may be candidates for more aggressive and/or specific forms of treatment, the use of traditional prognostic factors is not sufficient. Therefore, the identification of prognostic factors that reflect the biology of breast cancer is important.

Proteases and their inhibitors have turned out to be essential for the invasive and metastatic capability of tumor cells. In this respect, the serine protease uPA and its inhibitor, PAI-1, have been studied extensively, especially in breast cancer, showing a clear relation with a poor prognosis. Recently, two level-of-evidence 1 type studies [according to proposed guidelines (16)] have unequivocally demonstrated the prognostic value of uPA and PAI-1 in node-negative (1, 2) and node-positive breast cancer (2).

PMN-E, a serine protease produced by polymorphonuclear leukocytes and breast cancer cells (8, 17), showed prognostic value in breast cancer (8–10). PMN-E may play a role in the urokinase system of plasminogen activation by affecting uPA-governed plasmin pro-

duction (18), by cleaving the inhibitor PAI-1 (5), or by inactivating α_2-antiplasmin, the main inhibitor of plasmin (6). Therefore, we studied the prognostic value of PMN-E combined with those of uPA and PAI-1. To our knowledge, thus far, no other reports have analyzed PMN-E, uPA, and PAI-1 levels together.

In our present study, tumor PMN-E levels were positively correlated with those of uPA and PAI-1, consistent with the known complex interactions between various proteases and inhibitors. Higher tumor levels of PMN-E were associated with a poor MFS, RFS, and OS in a large series of primary breast cancer patients. The proportional hazards assumption (that is, the effect of PMN-E over time) was violated when the total follow-up period of 120 months was used. Similar changes over time have been reported for several traditional prognostic factors in breast cancer (19). When used as a dichotomized variable in short-term MFS, RFS, and OS (60 months), during which period the proportional hazards assumption was not violated, the prognostic value of PMN-E was independent of that of the traditional prognostic factors age, menopausal status, tumor size, tumor grade, nodal status, and steroid hormone receptor status and even of the established strong prognostic factor uPA. Although uPA and PAI-1 appeared to be stronger prognostic factors than PMN-E, the observed prognostic value of PMN-E, independent of traditional prognostic factors, is in accordance with the data reported previously by Yamashita *et al.* (8–10) in studies in which up to 313 patients were included.

However, in contrast to Yamashita *et al.* (9), we could not demonstrate that only free-form elastase independently predicts recurrence in primary breast cancer. In our study, about 35% of all samples exhibited free elastase. Although the ratio of free-form to total immunoreactive elastase varied over a wide range between 5% and 95%, similar to the results of Yamashita *et al.* (9), the prognostic potency of active elastase was comparable with that of total elastase (data not shown). Taking into consideration that the pool of free-form elastase in cytosols of tumor tissues depends not only on the overall content of elastase in tumor cells and infiltrating polymorphonuclear leukocytes but even more on the amount of α_1PI in the stroma, it seems more rational to us to evaluate the prognostic potency of total elastase (after the addition of exogenous α_1PI) in tumor tissue samples than to focus on free-form elastase.

In the primary breast tumors, PMN-E levels were negatively correlated with those of ER and PgR. This was not reported in previous studies (8, 10), but direct comparison is not possible due to differences in assays and tumor extracts used, methods of reporting the statistical data, and the number of patients included.

Taking together our data and those of other authors, various complex proteolytic systems seem to play a role in tumor progression. There is a large redundancy among protease systems, suggesting that if one or more protease systems fail, other systems may be able to take over their (pathological) physiological role. Together with or separate from other protease systems, PMN-E seems to play a role in breast cancer metastasis in a relatively small subgroup of patients. Breast cancer is very heterogeneous disease, and individual risk assessment based on a specific tumor phenotype may be particularly helpful for small subgroups of patients to design individualized tailored treatment strategies. Therefore, knowledge of the PMN-E status of the primary tumor may be helpful in a detailed assessment of prognosis. Furthermore, the development of neutrophil elastase inhibitors (20) may eventually lead to novel targeted (combination) therapies for specific groups of primary and advanced breast cancer patients expressing a high level of PMN-E in their tumor.

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REFERENCES

1. Jänicke, F., Prechtl, A., Thomssen, C., Harbeck, N., Meisner, C., Untch, M., Sweep, C. G. J., Selbman, H-K., Graeff, H., and Schmitt, M. Randomized adjuvant chemotherapy trial in high-risk, lymph node-negative breast cancer patients identified by urokinase-type plasminogen activator and plasminogen activator inhibitor type 1. J. Natl. Cancer Inst. (Bethesda), *93:* 913–920, 2001.
2. Look, M. P., van Putten, W. L. J., Duffy, M. J., Harbeck, N., Christensen, I. J., Thomssen, C., Kates, R., Spyratos, F., Fernö, M., Eppenberger-Castori, S., Sweep, C. G. J., Ulm, K., Peyrat, J. P., Martin, P. M., Magdelenat, H., Brünner, N., Duggan, C., Lisboa, B. W., Bendahl, P. O., Quillien, V., Daver, A., Ricolleau, G., Meijer-van Gelder, M. E., Manders, P., Fiets, W. E., Blankenstein, M. A., Broët, P., Romain, S., Daxenbichler, G., Windbichler, G., Cufer, T., Borstnar, S., Kueng, W., Beex, L. V. A. M., Klijn, J. G. M., O'Higgins, N., Eppenberger, U., Jänicke, F., Schmitt, M., and Foekens, J. A. Pooled analysis of prognostic impact of urokinase-type plasminogen activators and its inhibitor PAI-1 in 8377 breast cancer patients. J. Natl. Cancer Inst. (Bethesda), *94:* 116–128, 2002.
3. Stefansson, S., and Lawrence, D. A. The serpin PAI-1 inhibits cell migration by blocking integrin $\alpha_v\beta_3$ binding to vitronectin? Nature (Lond.), *383:* 441–443, 1996.
4. Bajou, K., Noël, A., Gerard, R. D., Masson, V., Brünner, N., Holst-Hansen, C., Skobe, M., Füsenig, N. E., Carmeliet, P., Collen, D., and Foidart, J. M. Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. Nat. Med., *4:* 923–928, 1998.
5. Wu, K., Urano, T., Ihara, H., Takada, Y., Fujie, M., Shikimori, M., Hashimoto, K., and Takada, A. The cleavage and inactivation of plasminogen activator inhibitor type 1 by neutrophil elastase: the evaluation of its physiologic relevance in fibrinolysis. Blood, *86:* 1056–1061, 1995.
6. Gramse, M., Egbring, R., and Havemann, K. α2-plasmin inhibitor inactivation by granulocyte elastase. Hoppe-Seyler's Z. Physiol. Chem., *356:* 19–26, 1984.
7. Barrett, A. J. Leukocyte elastase. Methods Enzymol., *80:* 581–588, 1981.
8. Yamashita, J-I., Ogawa, M., Ikei, S., Omachi, H., Yamashita, S-I., Saishoji, T., Nomura, K., and Sato, H. Production of immunoreactive polymorphonuclear leukocyte elastase in human breast cancer cells: possible role of polymorphonuclear leukocyte in the progression of human breast cancer. Br. J. Cancer, *69:* 72–76, 1994.
9. Yamashita, J-I., Ogawa, M., and Shirakusa, T. Free-form neutrophil elastase is an independent marker predicting recurrence in primary breast cancer. J. Leukocyte Biol., *57:* 375–378, 1995.
10. Yamashita, J-I., Ogawa, M., and Sato, K. Prognostic significance of three novel biological factors in a clinical trial of adjuvant therapy for node-negative breast cancer. Surgery, *117:* 601–608, 1995.
11. Foekens, J. A., Portengen, H., van Putten, W. L. J., Peters, H. A., Krijnen, H. L. J. M., Alexieva-Figusch, J., and Klijn, J. G. M. Prognostic value of estrogen and progesterone receptors measured by enzyme immunoassays in human breast tumor cytosols. Cancer Res., *49:* 5823–5828, 1989.
12. Foekens, J. A., Peters, H. A., Look, M. P., Portengen, H., Schmitt, M., Kramer, M. D., Brünner, N., Jänicke, F., Meijer-van Gelder, M. E., Henzen-Logmans, S. C., van Putten, W. L. J., and Klijn, J. G. M. The urokinase system of plasminogen activation and prognosis in 2780 breast cancer patients. Cancer Res., *60:* 636–643, 2000.
13. Jänicke, F., Schmitt, M., Hafter, R., Hollreider, A., Babic, R., Ulm, K., Gössner, W., and Graeff, H. Urokinase-type plasminogen activator (uPA) antigen is a predictor of early relapse in breast cancer. Fibrinolysis, *4:* 69–78, 1990.
14. Barlow, R. E., Bartholomew, D. J., Bremner, J. M., and Brunck, H. D. Statistical Inference under Order Restrictions. New York: John Wiley & Sons, Inc., 1972.
15. Grambsch, P. M., and Therneau, T. M. Proportional hazards tests and diagnostics based on weighted residuals. Biometrika, *81:* 515–526, 1994.
16. Hayes, D. F., Bast, R. C., Desch, C. E., Fritsche, H., Kemeny, N. E., Jessup, J. M., Locker, G. Y., Macdonald, J. S., Mennel, R. G., Norton, L., Ravdin, P., Taube, S., and Winn, R. J. Tumor marker utility grading system: a framework to evaluate clinical utility of tumor markers. J. Natl. Cancer Inst. (Bethesda), *88:* 1456–1466, 1996.
17. Kao, R. T., and Stern, R. Elastases in human breast carcinoma cell lines. Cancer Res., *46:* 1355–1358, 1986.
18. Machovich, R., and Owen, W. G. An elastase-dependent pathway of plasminogen activation. Biochemistry, *28:* 4517–4522, 1989.
19. Hilsenbeck, S. G., Ravdin, P. M., de Moor, C. A., Chamness, G. C., Osborne, C. K., and Clark, G. M. Time-dependence of hazard ratios for prognostic factors in primary breast cancer. Breast Cancer Res. Treat., *52:* 227–237, 1998.
20. Inada, M., Yamashita, J-L, Nakano, S., and Ogawa, M. Complete inhibition of spontaneous pulmonary metastasis of human lung carcinoma all line EBC-1 by a neutrophil elastase inhibitor (ONO-5046·Na). Anticancer Res., *18:* 885–890, 1998.