

# Urokinase-Type Plasminogen Activator System in Breast Cancer: Association with Tamoxifen Therapy in Recurrent Disease

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## ABSTRACT

The prognostic value of components of the urokinase-type plasminogen activator (uPA) system, its receptor uPAR (CD87), and plasminogen activator inhibitors PAI-1 and PAI-2 is well established. We studied the predictive value of these proteolytic factors by evaluating the association of their tumor expression level and the efficacy of tamoxifen therapy in patients with recurrent breast cancer. The antigen levels of the four factors were determined by ELISA in cytosols prepared from estrogen receptor-positive primary breast tumors of 691 hormone-naïve breast cancer patients with recurrent disease and treated with tamoxifen as first-line systemic therapy. High tumor levels of uPA ( $P < 0.001$ ), uPAR ( $P < 0.01$ ), and PAI-1 ( $P = 0.01$ ) were associated with a lower efficacy of tamoxifen therapy. In the multivariable analysis, uPA ( $P < 0.001$ ) provided additional information independent of the traditional predictive factors to predict benefit from tamoxifen therapy. High levels of uPA, uPAR, and PAI-1 predicted a shorter progression-free survival (PFS) on tamoxifen in an analysis of the first 9 months of therapy. However in the analysis during the total follow-up period, high PAI-2 levels ( $P = 0.01$ ) showed a longer response to tamoxifen. In conclusion, uPA, uPAR, and PAI-1, components of the urokinase system, are predictive for the efficacy of tamoxifen therapy in patients treated for recurrent breast cancer. Knowledge of their tumor expression levels might be helpful for future individualized therapy protocols, including possible new-targeted therapies based on the interference in the urokinase system.

## INTRODUCTION

The urokinase-type plasminogen activator (uPA) system plays an important role in processes leading to cancer cell invasion and metastasis (1–3). The four major components of this system, the serine protease uPA, its cell surface receptor uPAR (CD87), and its inhibitors plasminogen activator inhibitor types 1 and 2 (PAI-1 and PAI-2, respectively), have been established as prognostic factors in primary breast cancer by various research groups (reviewed in Refs. 1, 4, and 5). In primary breast cancer, uPA and PAI-1 reached level-I evidence for their prognostic impact (6, 7), according to proposed guidelines (8). Less is known about their predictive value for clinical benefit to systemic therapy in patients treated for recurrent breast cancer. Recently, we showed that the uPA/PAI-1 tumor level in the primary breast tumor is predictive for response to adjuvant systemic chemotherapy (9, 10). The benefit of chemotherapy, but not of endocrine therapy, was strongly enhanced in patients with high uPA/PAI-1 levels compared with patients with low uPA/PAI-1 (9, 10). This finding supported the results of a prospective randomized multicenter trial (6), in which high-risk node-negative breast cancer patients, stratified by uPA and PAI-1 levels, benefited from adjuvant chemotherapy. However, clinical benefit of systemic therapy in the adjuvant and in the palliative setting is not necessarily identical.

In recurrent breast cancer, the steroid hormone-receptor status is one of the parameters often used for the choice of endocrine therapy. It is well established that hormone receptor-positive tumors commonly respond to a wide variety of hormonal therapies (11). Thus far, tamoxifen is the most extensively used therapeutic for hormonal treatment, although only about 50% of the treated patients will benefit (12–14).

Levels of all four parameters of the uPA system are higher in hormone receptor-negative tumors than in the more favorable, hormone receptor-positive tumors (15). In a pilot study, a series of 235 patients, we showed that uPA and PAI-1 can be useful in predicting the efficacy of tamoxifen therapy in recurrent breast cancer (16). In the present extended and updated study including 691 patients with estrogen receptor (ER)-positive primary tumors treated for recurrent breast cancer, we examined whether simultaneous knowledge of four major components of the uPA system provided information on the efficacy of first-line tamoxifen therapy additional to that of the traditional predictive factors.

## MATERIALS AND METHODS

**Patients and Treatment.** The Medical Ethical Committee of the Erasmus University Rotterdam, the Netherlands, approved our study design (MEC 02.953). This retrospective study included 691 patients with primary operable breast cancer, diagnosed between 1978 and 1994, who developed a recurrence—73 patients with local-regional relapse, 510 patients with distant metastasis, and 108 patients with local-regional relapse and distant metastasis—and were treated with first-line tamoxifen (40 mg daily). All patients had ER-positive tumors. None of the patients had received neoadjuvant therapy or were exposed to hormonal adjuvant treatment (hormone naïve). These patients were a subset from the series of 2780 primary breast cancer patients described before (15), extended with 60 patients who presented with distant metastasis at diagnosis or developed distant metastasis (including supraclavicular lymph nodes) within 1 month after primary surgery (M1 patients). Three patients were not tumor free after surgery because of an inadequate axillary dissection and were treated right after surgery with tamoxifen for measurable disease (M0 patients with local-regional relapse). Of all 691 patients, the median time between primary surgery and start of therapy was 28 months (range, 0–180 months). At the time of surgical removal of the primary tumor, the median age of the patients was 58 years (range, 26–90 years) and at start of tamoxifen therapy for recurrent disease, 61 years (range, 28–91 years). Response to tamoxifen therapy was defined by standard Union International Contre Cancer criteria (17). Objective response was observed in 118 patients [20 complete remission and 98 partial remission (PR)], and 228 patients had a tumor progression of 25% or more or showed new tumor lesions within 3 months. The 345 patients with no evident tumor reduction of 50% or more (PR) or a tumor progression were considered as patients with no change (NC). These patients with NC were divided in 287 patients who had a NC >6 months and 58 patients with a NC ≤6 months. The median progression-free survival (PFS) was for complete remission, 35 months; for PR, 15 months; for NC >6 months, 15 months; for NC ≤6 months, 5 months; and for progressive disease, 3 months. Because the patients with NC >6 months had a PFS similar to patients with PR, we classified these patients as responders to tamoxifen as described in the manual for clinical research and treatment in breast cancer of the European Organization for Research and Treatment of Cancer (18). Therefore, clinical benefit was defined in our study as objective response (complete remission + PR) and NC >6 months combined, as has been done before (12,

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19, 20). Only patients with measurable disease were evaluated, and for patients with NC, it was essential that they received tamoxifen at least for a period of 6 months.

The median follow-up of patients alive after surgery was 101 months (range, 10–196 months), and 43 months (range, 4–137 months) after start of tamoxifen therapy. At the end of the follow-up period, 683 (99%) patients had developed tumor progression and 575 (83%) patients had died.

**Assays.** Tumor cytosols were prepared and processed as recommended by the European Organization for Research and Treatment of Cancer (21), and quantitative ER and progesterone receptor levels were determined, as described previously (22). The cut point used to classify tumors as ER or progesterone receptor high and low was 10 fmol/mg cytosolic protein. uPA and PAI-1 levels were determined in breast tumor cytosols using ELISAs with reagents that are commercially available in assay kits (American Diagnostica Inc., Stamford, CT). The details of the assay procedures, including those of the specificity and performance of the uPAR and PAI-2 ELISAs, have been described elsewhere (23, 24–27).

**Statistics.** The strength of the associations between continuous variables was tested with Spearman rank correlation ( $r_s$ ). The relation with clinical benefit-to-therapy was examined with logistic regression analysis. Odds ratios (ORs) were calculated and presented with their 95% confidence interval. The likelihood ratio test in logistic regression models was used to test for differences and for interactions. Isotonic regression analysis (28) was applied to define cut points for the factors if in a test for trend using log-transformed continuous variables, the analysis for clinical benefit was statistically significant. Otherwise, median levels were used to categorize the variable in low and high. In isotonic regression analysis, the patients are ordered according to the level of the factor studied and subsequently partitioned in ordered groups in such a way that the average ORs in the groups increase with increasing or decreasing levels. The final partition is optimal in the sense that it is the maximum likelihood estimate for the exponential failure model. Previously, the results of the isotonic regression analysis were compared with the results of the maximum  $\chi^2$  value from the log rank test, and the agreement between both procedures was very high (22). Transformations of the variables were explored with fractional polynomials for the analysis of treatment benefit. The gain in  $\chi^2$  values was not substantial when using transformations other than log transformation. The Cox proportional hazards model was used to calculate the hazard ratios (HRs) and their 95% confidence interval in the analysis of PFS. PFS was the time that the patients were treated with tamoxifen as first-line systemic treatment for recurrent disease. The start of tamoxifen therapy was set at zero and the end point at the stop-date of tamoxifen therapy. The proportionality assumption was investigated using a test based on the Schoenfeld residuals (29). Survival curves were generated using the method of Kaplan and Meier (30), and the log-rank test was used to test for differences. All  $P$ s are two-sided and  $<0.05$  was considered statistically significant. For the test of interactions, a  $P < 0.01$  was used. Computations were done with the STATA statistical package, release 8.0 (STATA Corp., College Station, TX).

## RESULTS

The median levels of the factors of the urokinase system, measured in cytosols of primary breast tumors and reported in ng/mg protein, were for uPA ( $n = 691$ ), 0.96 (range, 0–16); for uPAR ( $n = 686$ ), 0.94 (range, 0–37); for PAI-1 ( $n = 691$ ), 18.08 (range, 0–489); and for PAI-2 ( $n = 686$ ), 2.03 (range, 0–260). The  $r_s$  between the various factors of the uPA system varied from  $r_s = 0.24$  (between uPA and PAI-2) to  $r_s = 0.57$  (between uPA and PAI-1). All factors were positively related to each other (all,  $P < 0.001$ ). A significant but weak correlation of the factors with the steroid hormone receptors was seen between uPAR and ER ( $r_s = -0.11$ ). For relationships of the factors of the uPA system with patient and tumor characteristics, we refer to Foekens *et al.* (15).

**Univariate Analysis.** When analyzing the relationship of log-transformed continuous tumor levels of the factors of the uPA system with benefit from tamoxifen treatment, increasing levels of uPA ( $P = 0.002$ ) and PAI-1 ( $P = 0.016$ ) were related with a poor treatment response. We determined cut points using isotonic regression analysis

for these two factors. For uPAR and PAI-2, of which the continuous variables were not significantly related with clinical benefit, we used the median level to classify tumors as low or high. The cut points chosen were 0.76, 0.94, and 2.03 ng/mg protein for uPA, uPAR, and PAI-2, respectively. For PAI-1, two cut-points, 7.66 ng/mg and 31.62 ng/mg protein, were defined to classify tumors as low, intermediate, and high.

The results of the univariate logistic regression analysis for treatment benefit are listed in Table 1. Of the clinical factors, only a short disease-free interval (DFI) showed less benefit from treatment ( $P < 0.001$ ). Age, menopausal status, adjuvant chemotherapy, dominant site of relapse, and, as might be expected in patients with recurrent disease, the traditional prognostic factors such as nodal status, size, and grade of the primary tumor were not significantly related with treatment outcome. Of the biological tumor factors, ER as log-transformed continuous variable showed a significant relation between increasing tumor levels and treatment benefit ( $P < 0.001$ ). High tumor levels of uPA (OR = 0.49,  $P < 0.001$ ) and uPAR (OR = 0.67,  $P < 0.01$ ) and intermediate and high levels of PAI-1 (OR = 0.61, and OR = 0.45, respectively,  $P = 0.01$ ) were significantly related with treatment failure. Such a significant relationship with treatment failure was not found for PAI-2 (OR = 1.08) in the univariate analysis.

In these ER-positive patients, the proportion of patients (Table 1) that showed benefit from tamoxifen treatment was 68% for the 296 patients with low uPA levels and 51% for the 395 patients with high uPA levels. Of the 350 patients with low uPAR levels, 63% benefited from treatment compared with 54% of the 336 patients with high uPAR levels. For the patients with low ( $n = 109$ ), intermediate ( $n = 432$ ), and high ( $n = 150$ ) tumor levels of PAI-1, these rates were 70%, 58%, and 51%, respectively. For both patients with low and high PAI-2 levels, these proportions were 58% versus 60%.

**Multivariable Analysis.** The statistically independent relationship of the levels of the factors of the uPA system and benefit of tamoxifen treatment was studied using multivariable logistic regression analysis (Table 1). A basic multivariable model was designed by the step-down procedure after including the traditional prognostic and predictive factors age and menopausal status at start of therapy, tumor size, grade, and ER/progesterone receptor status of the primary breast tumor nodal status, dominant site of relapse, DFI, and adjuvant chemotherapy. Factors with a  $P < 0.1$  were retained in the basic model. We used a basic model that included the factors menopausal status, dominant site of relapse, DFI, and ER tumor levels (log-transformed). Table 1 shows the results of the final multivariable analysis. High levels of uPA (OR = 0.48;  $P < 0.001$ ) provided significant additional predictive information over the traditional factors. The uPAR, PAI-1, and PAI-2 levels did not contribute to the model, however, uPAR and PAI-1 did contribute to the basic model when added separately ( $P = 0.03$  for both) in the absence of uPA. Using the levels of uPA as log-transformed continuous variable also showed a significant relation with treatment failure (OR = 0.63;  $P = 0.006$ ). The tests of interaction between ER status and the factors of the uPA system were not statistically significant. Therefore, interactions were not included in the multivariable model. Exclusion of the 63 patients with a DFI of 0 months did not affect the estimate of the regression coefficient of uPA (OR = 0.52;  $P < 0.001$ ) in the multivariable analysis for clinical benefit.

**PFS.** In Cox univariate regression analysis for PFS using the tumor levels of the four factors of the uPA system as log-transformed continuous variables, uPA ( $P = 0.05$ ), PAI-1 ( $P = 0.005$ ), and PAI-2 ( $P = 0.02$ ) were significantly associated with a rapid disease progression when all 638 failures during the total follow-up period were considered. However, in these analyses, the proportional hazards

Table 1 Cox univariate and multivariable analysis for clinical benefit of first-line tamoxifen and multivariable analysis for PFS

	Frequency <sup>b</sup>	Clinical benefit (%)	Univariate analysis (clinical benefit)			Multivariable analysis (clinical benefit) <sup>a</sup>			Multivariable analysis (PFS) <sup>a</sup>		
			P	OR	(95% CI)	P	OR	(95% CI)	P	HR	(95% CI)
All patients	691	59									
Age (years) <sup>c</sup>											
≤40	41	56		1							
41–55	214	54		0.92	(0.47–1.82)						
56–70	257	58		1.06	(0.55–2.07)						
>70	179	66	0.11	1.51	(0.76–3.02)						
Menopausal status <sup>c</sup>											
Premenopausal	152	53		1						1	
Postmenopausal	539	60	0.13	1.32	(0.92–1.90)	0.69	1.08	(0.73–1.61)	0.24	0.89	(0.73–1.08)
Adjuvant chemotherapy <sup>d</sup>											
No	472	59		1							
Yes	156	57	0.65	0.92	(0.64–1.33)						
Dominant site of relapse											
Local-regional relapse	81	63		1						1	
Bone	350	60		0.87	(0.53–1.44)		0.69	(0.39–1.14)		1.26	(0.98–1.63)
Viscera	260	56	0.43	0.74	(0.44–1.24)	0.11	0.56	(0.33–0.97)	<0.001	1.64	(1.26–2.13)
DFI											
≤1 year	164	45		1						1	
1–3 years	299	60		1.79	(1.22–2.63)		1.93	(1.29–2.89)		0.67	(0.55–0.81)
>3 years	228	67	<0.001	2.48	(1.64–3.75)	<0.001	2.59	(1.68–4.00)	<0.001	0.62	(0.51–0.76)
ER status											
As log-transformed Continuous variable	691	59	<0.001	1.37	(1.20–1.56)	<0.001	1.43	(1.24–1.64)	<0.001	0.83	(0.77–0.89)
PgR status <sup>e</sup>											
Negative	125	53		1							
Positive	553	60	0.13	1.35	(0.92–2.00)						
uPA levels <sup>f</sup>											
Low	296	68		1						1	
High	395	51	<0.001	0.49	(0.36–0.67)	<0.001	0.48	(0.35–0.67)	<0.001	1.31	(1.12–1.53)
uPAR levels <sup>f</sup>											
Low	350	63		1							
High	336	54	<0.01	0.67	(0.49–0.90)						
PAI-1 levels <sup>f</sup>											
Low	109	70		1							
Intermediate	432	58		0.61	(0.39–0.95)						
High	150	51	0.01	0.46	(0.27–0.77)						
PAI-2 levels <sup>f</sup>											
Low	345	58		1						1	
High	341	60	0.62	1.08	(0.80–1.46)				0.01	0.81	(0.69–0.95)

<sup>a</sup> Final model included 691 patients for clinical benefit and 686 patients for PFS. The same basic model as for response was used for PFS.

<sup>b</sup> Because of missing values, numbers do not always add to 691 patients.

<sup>c</sup> At time of start of first-line therapy for recurrent disease.

<sup>d</sup> Not included, 63 patients who were not disease-free within 1 month after surgery. One hundred thirteen patients received cyclophosphamide, methotrexate, and 5-fluorouracil, and 43 patients received adriamycin-containing chemotherapy.

<sup>e</sup> Cut points: for ER and PgR, 10 fmol/mg protein.

<sup>f</sup> Cut points: for uPA, 0.76 ng/mg protein; for uPAR, 0.94 ng/mg protein; for PAI-1, 7.66 and 31.62 ng/mg protein; for PAI-2, 2.03 ng/mg protein.

assumption for uPA ( $P = 0.03$ ) was violated. Before and after 8.5 months, an approximately equal number of patients showed disease progression. Therefore we explored the relationship of the four factors with PFS during the first 9 months of follow-up. In these short-term analyses (357 failures of a total of 683), the proportional hazards assumption was no longer violated for uPA ( $P = 0.86$ ). Increasing levels of log-transformed values of uPA ( $P = 0.006$ ) and of PAI-1 ( $P = 0.004$ ) were significantly related with a shorter PFS. There was no such relationship between the levels of uPAR ( $P = 0.42$ ) and PAI-2 ( $P = 0.52$ ) with PFS.

PFS as a function of the categorized tumor levels for the factors of the uPA system are shown in Fig. 1. Restricting the analyses to the first 9 months of follow-up resulted in HRs of 1.50 for tumors with high uPA levels ( $P < 0.001$ ; Fig. 1A), of 1.26 for those with high uPAR levels ( $P = 0.03$ ; Fig. 1B), and of 1.41 and 1.69 for those with intermediate and high PAI-1 levels, respectively ( $P = 0.014$ ; Fig. 1C). Also, when analyzed as a dichotomized variable, PAI-2 was not significantly associated with PFS ( $P = 0.39$ ; Fig. 1D) in the time-restricted analyses. In the analyses without time restriction, the strengths of the relationships were smaller for uPA (HR = 1.21,  $P = 0.015$ ), uPAR (HR = 1.05,  $P = 0.53$ ), and PAI-1 (HR = 1.13 and 1.31,  $P = 0.10$ ). However, the relationship between PFS and PAI-2 (HR, 0.82,  $P = 0.013$ ) was of statistical significance, suggesting that

a high PAI-2 level in the primary tumor predicts a longer benefit from tamoxifen therapy.

The Cox multivariable regression analysis for PFS showed that a high uPA tumor level (HR = 1.31;  $P < 0.001$ ) was an independent marker for an early progression of the disease (Table 1). In contrast to uPA, high PAI-2 tumor levels (HR = 0.81,  $P = 0.01$ ) were predictive for a longer response to tamoxifen. The proportional hazards assumption for uPA and PAI-2 were not violated in this final multivariable model ( $P > 0.05$ ).

## DISCUSSION

Tamoxifen is thus far the most extensively used hormonal agent for all stages of breast cancer (11, 31, 32). In the present retrospective study, we evaluated whether the four major factors of the uPA system can predict the outcome of first-line tamoxifen treatment in patients with recurrent breast cancer.

The ideal situation for determining predictive factors is a properly designed prospective study, because treatment policy today is different than in the time period of our study when therapy decisions were made on the basis of guidelines in force at that time. However, associations between biological factors and therapies can help us understand biological mechanisms playing a role in today's treatments

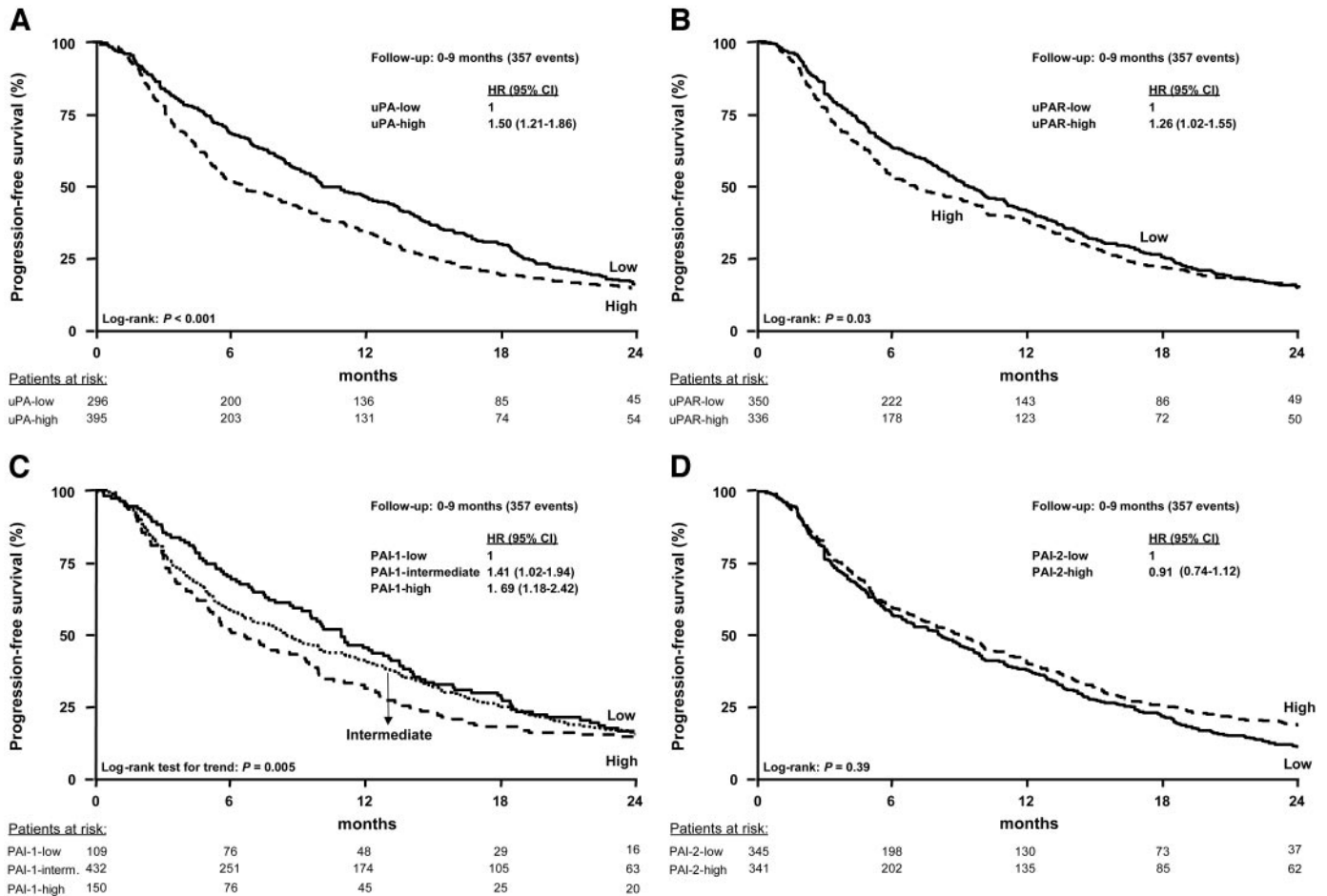


Fig. 1. PFS after start of first-line tamoxifen therapy as a function of uPA (A), uPAR (B), PAI-1 (C), and PAI-2 (D) status.

and point out targets for new therapeutics. Today, still a large group of patients are treated with tamoxifen (adjuvant, first-line, or second-line therapy) as first hormone therapy.

The primary end point of our study was clinical benefit from tamoxifen therapy. We defined the type of response strictly beforehand, and when there was any doubt, patients were not included in this study. The size of the metastases or the occurrence of new lesions is an objective measure of treatment effect. However, as a consequence of the retrospective nature of our study, the differentiation between PR and NC was difficult to assess, especially in patients with bone metastasis (51%). In our study, we showed that the PFS of patients with prolonged NC (> 6 months) was comparable with the PFS of patients with PR and therefore could be considered as responders. A prospective study reported as well that objective benefit was not always easy to assess, and prolonged stable disease was categorized as response (12). We selected hormone-naïve patients to exclude acquired therapy resistance to tamoxifen.

Both ER-positive and ER-negative breast cancer cells and breast tumor fibroblasts can express and release uPA, uPAR, and PAI-1 under *in vitro* conditions (33–39). In recent years, the significance of uPA and uPAR in invasion and metastases of hormone-dependent cancers (*e.g.*, breast and prostate) has been demonstrated (40, 41). uPA and PAI-1 expression have been shown to be regulated by estradiol and antiestrogens via an ER-mediated pathway (35–37), whereas uPAR expression was not affected (35). Because of the observed up- and down-regulation of the expression and secretion of the factors of the uPA system by various growth factors in breast fibroblasts *in vitro* (38, 39, 42, 43) and the capability of uPA and

uPAR to promote epithelial tumor cell proliferation directly (44) or indirectly through activation or release of growth factors, it is tempting to speculate that in the microenvironment of a tumor, the serine protease uPA may interfere with the efficacy of tamoxifen treatment in patients with (recurrent) breast cancer. Indeed, in our previous study, a high uPA tumor antigen level was found to be associated with a poor response to tamoxifen in patients with recurrent breast cancer (16). Furthermore, other serine proteases, *i.e.*, the human kallikreins 3 (45) and 10 (46), and polymorphonuclear leukocyte elastase (47) have been shown to be related with tamoxifen resistance in patients with advanced breast cancer.

Our study showed that increasing levels of uPA and PAI-1 were related with a higher probability of tamoxifen failure. In the multivariable model, high tumor levels of uPA provided additional predictive information, independent of the traditional predictive factors menopausal status, dominant site of relapse, DFI, and ER status. The analysis for PFS, the time during treatment with tamoxifen as first-line systemic therapy for recurrent breast cancer, showed that higher levels of uPA, uPAR, and PAI-1 were significantly related with a shorter PFS in the first 9 months of the analysis. Such a time-dependent relationship between clinical or cell biological factors and survival has been observed before (48–51). Although a high level of PAI-2 was not related with PFS in the time-dependent analysis, in the analysis without time restriction, it was significantly related with a prolonged PFS. The mechanism underlying a protection of PAI-2 against tamoxifen resistance may be attributed to its inhibition of uPA proteolytic activity (52). From earlier studies, we know that high levels of PAI-2 are associated with a favorable prognosis in patients with primary

breast cancer (25, 53, 54), particularly in those patients with a high tumor level of uPA (25).

In conclusion, knowledge of the expression levels of the components of the uPA system, which have been found to be of prognostic value in a large variety of solid cancers, might be valuable for individualized therapy decisions in patients with recurrent breast cancer as well. It should be emphasized that validation studies, including those of the defined cut points, are necessary. In our study, especially uPA for clinical benefit and PAI-2 for the length of response seemed to be strong predictive factors in patients treated with tamoxifen therapy. Whether patients with high tumor levels of uPA will be better off with other endocrine treatments or chemotherapy is hard to say in this stage. Maybe they will be candidates for novel promising agents targeting the uPA system alone or in combination with currently available treatment (40, 41, 55–57).

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