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ORIGINAL RESEARCH

Role of urokinase plasminogen activator and plasminogen activator inhibitor mRNA expression as prognostic factors in molecular subtypes of breast cancer

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Background: Protein levels of urokinase plasminogen activator (uPA) and its inhibitor (PAI-1) determined by enzyme-linked immunosorbent assay from fresh-frozen tumor tissue have been evaluated as prognostic factors in prospectively randomized trials in breast cancer. However, the role of uPA and PAI-1 in the context of breast cancer subtypes and for mRNA expression of these factors is less clear.

Methods: We evaluated uPA and PAI-1 mRNA expression using the Affymetrix HG-U 133A array within molecular subgroups of breast cancer in cohorts of patients with systemic treatment (cohort A, n=362) and without systemic treatment (cohort B, n=200). We validated mRNA expression in a cohort of HER2-positive breast cancer patients (cohort C, n=290). Luminal, triple-negative, and HER2-positive subcohorts were defined by ESR1 and ERBB2 mRNA expression using predefined cutoffs.

Results: In the entire cohort A, elevated PAI-1 but not uPA mRNA expression was associated with shorter disease-free survival (P=0.007 for PAI and 0.069 for uPA). Regarding different molecular subgroups, 67% (n=244) of tumors were luminal, 14% (n=49) were HER2-positive, and 19% (n=69) were triple-negative. Elevated PAI-1 mRNA expression was associated with shorter disease-free survival only in the HER2-positive subgroup (P=0.031). The same disease-free survival results were found for uPA in HER2-positive patients (P=0.011). In contrast, no association between either marker and survival was observed in the luminal or triple-negative subgroups. In the HER2-positive validation cohort C, elevated uPA and PAI-1 mRNA expression also showed strong associations with shorter disease-free survival (P=0.014 for PAI-1, $P<0.001$ for uPA).

Conclusion: In this study, the prognostic impact of uPA and PAI-1 expression was mainly observed in patients with HER2-positive tumors.

Keywords: urokinase plasminogen activator, urokinase plasminogen activator inhibitor-1, HER2, breast cancer, prognosis

Introduction

Protein and nucleic acid based markers have been introduced as prognostic and predictive factors in breast cancer therapy. As established clinicopathological markers are not sufficient to guide the decision whether a patient needs adjuvant chemotherapy or not, these factors mainly focus on information about the individual patient's benefit of adjuvant chemotherapy. For this purpose, determination of antigen levels of urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) in fresh-frozen tumor tissue from primary breast cancers by a commercially available

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enzyme-linked immunosorbent assay (ELISA, Femtelle®; Sekisui Diagnostics, Lexington, MA, USA) has already entered clinical practice in the context of large studies for risk-adapted, individual therapy decisions, particularly in patients with node-negative breast cancer. Measurement by ELISA is the standard method in uPA and PAI-1 protein determination and has been validated in several studies demonstrating their clinical relevance as prognostic factors. Patients with both factors being low (uPA <3 ng/mg protein; PAI-1 <14 ng/mg protein) have a significantly better prognosis than patients with either or both factors being high.[1] uPA/PAI-1 has been validated at level I of evidence by a European Organization for Research and Treatment of Cancer pooled analysis (n=8,377)[2] and a prospective clinical therapy trial, Chemo N0.[1] Ten-year-follow-up analysis of the Chemo-N0 trial confirmed the prognostic and predictive impact of both factors.[3] The prospective trials NNBC-3 and the WSG-PlanB are also looking at the prognostic and predictive impact of uPA and PAI-1.[4]

Since the ground-breaking publications of Perou et al based on gene expression analysis, it is widely accepted that mammary carcinomas can be divided into at least four molecular subtypes that differ in biology and prognosis, namely, HER2-positive tumors, basal-like carcinomas (mostly identical to triple-negative tumors), and two groups of estrogen receptor-positive tumors, ie, luminal A and luminal B.[5] Yet, in older validation cohorts using uPA and PAI-1 ELISA tests, HER2 status was not routinely determined. In addition, uPA/PAI-1 ELISA tests are difficult and time-consuming, and in the light of technical progress in RNA-based methods, the value of uPA and PAI-1 mRNA expression as a prognostic or predictive marker should be further analyzed.

The aim of the present study was to investigate the potential role of uPA and PAI-1 mRNA levels and whether expression levels of both factors have different prognostic values in molecular subtypes of breast cancer.

We evaluated the role of uPA/PAI-1 expression in a cohort of untreated breast cancer patients and compared the results with a cohort of patients who received adjuvant chemotherapy or endocrine treatment. In order to verify the findings obtained in both cohorts, we determined the effect of uPA/PAI-1 in a third group of breast cancer patients with HER2-positive tumors.

Materials and methods

All analyses were performed according to REMARK (REporting recommendations for tumor MARKer prognostic studies).[6]

Finding cohort A

Tissue samples of 362 patients (n=186 in Hamburg, n=171 in Frankfurt) with primary breast cancer were collected during surgery, snap-frozen, and stored in liquid nitrogen. All patients were treated for breast cancer either at the University Medical Center Hamburg Eppendorf or Frankfurt, Germany, between 1992 and 2003. Patient selection was based upon availability of tumor tissue. Patients gave written informed consent to access their tissue and review their medical records according to ethics committee guidelines of Hamburg and Hessen, Germany.

The median age of the patients at surgery was 56 (range 28–93) years. The median duration of follow-up was 80 months. Sixty-five percent of patients (n=235) had received taxane-free chemotherapy in the adjuvant setting, and 35% had received only endocrine treatment (n=126). No radiotherapy or neoadjuvant chemotherapy had been performed prior to surgery. None of the patients had received trastuzumab.

Finding cohort B

The 200 patients in this cohort consisted of lymph node-negative breast cancer patients who did not receive any systemic therapy in the adjuvant setting according to former treatment standards. This cohort was treated at the Department of Obstetrics and Gynaecology of the Johannes Gutenberg University Mainz between 1988 and 1998.[7] The median age of the patients at surgery was 60 (range 34–89) years. The median duration of follow-up was 92 months. Patients were treated with either modified radical mastectomy (n=75) or breast-conserving surgery followed by irradiation (n=125) and did not show evidence of regional lymph node or distant metastases at the time of surgery. In these patients, uPA and PAI-1 protein levels measured by ELISA were also available.

Validation cohort C

We included HER2-positive samples from our previously published compilation of gene expression data (U133A or U133Plus2.0 arrays from Affymetrix, Santa Clara, CA, USA) for 4,467 breast cancer patients from 40 publicly available datsets.[8] We identified 589 HER2-positive samples by applying our previously defined cutoff for HER2 from Affymetrix array.[9] We then excluded all patients without sufficient follow-up information as well as samples already included in cohort A, leading to a final cohort of 290 HER2-positive tumors with follow-up. These 290 samples are listed in a Supplementary Table which also contains hyperlinks to the respective source files.

Detailed patient characteristics for all cohorts are listed in Table 1.

RNA isolation (cohorts A and B)

Approximately 50 mg of frozen breast tumor tissue was pulverized in liquid nitrogen. RLT-Buffer (Qiagen, Hilden, Germany) was added and the homogenate was centrifuged through a QIAshredder column (Qiagen). From the eluate, total RNA was isolated by the RNeasy Kit (Qiagen) according to the manufacturer's instructions. RNA yield was determined by ultraviolet absorbance and RNA quality was assessed by analysis of ribosomal RNA band integrity on an Agilent 2100 Bioanalyzer RNA 6000 LabChip kit (Agilent Technologies, Palo Alto, CA, USA).

Microarray analysis

The HG-U133A array and GeneChip System™ (Affymetrix) was used to quantify the relative transcript abundance in breast cancer tissues. Starting from 5 µg total RNA, labeled cRNA was prepared using the Microarray cDNA Synthesis, Microarray RNA Target Synthesis (T7), and Microarray Target Purification Kit (Roche, Penzberg, Germany), according to the manufacturer's instructions. Arrays were analyzed using the MAS5 algorithm (Affymetrix Microarray Suite 5.0 software) with global scaling of each array to a mean target intensity of 500. Samples with suboptimal average signal intensities (ie, scaling factors >25) or glyceraldehyde 3-phosphate dehydrogenase 3′/5′ ratios >5 were relabeled and rehybridized on new arrays.

Statistical analysis

Statistical analyses were calculated using IBM SPSS statistics version 21 software (IBM Corp, Armonk, NY, USA). For survival analyses, uPA and PAI-1 mRNA values below and above the median were compared. Based on correlations with biochemical methods for estrogen receptor and HER2 detection

Table 1 Clinical and histopathological characteristics in all cohorts

	Cohort A (treated, n=362)		Cohort B (untreated, n=200)		Cohort C (HER2-positive, treated, n=290)	
	Cases (n)	Total %	Cases (n)	Total %	Cases (n)	Total %
Age, years						
≤50	125	34.5	51	25.5	120	41.4
>50	237	65.5	149	74.5	138	47.6
Unknown					32	11.0
Event*						
No	237	65.5	154	77	186	64.1
Yes	111	30.7	46	23	104	35.9
Unknown	14	3.9				
Grade						
1 and 2	207	57.2	165	82.5	94	32.4
3	153	42.3	35	17.5	161	55.5
Unknown	2	0.6			35	12.1
Nodal status						
Node negative	227	62.7	200	100	172	59.3
Node positive	132	36.5			96	33.1
Unknown	3	0.8			22	7.6
Estrogen receptor						
Negative	106	29.3	37	18.5	176	60.7
Positive	256	70.7	163	81.5	114	39.3
HER2 status						
Negative	313	86.5	180	90.0		
Positive	49	13.5	20	10.0	290	100
Chemotherapy						
No	126	34.9	200	100	174	60.0
Yes	235	65.1			32	11.0
Unknown					84	29.0
Molecular subgroups						
Luminal	244	67.4	154	77.0		
Triple-negative	69	19.1	26	13.0		
HER2-positive	49	13.5	20	10.0	290	100

Note: *Defined as relapse or metastasis.

and the bimodal distribution of expression values for these genes, the following probe sets and cutoff values after magnitude normalization had been chosen for subtype definition in a large meta-analysis[9] and were also used in the present study:

Luminal: Affymetrix #205225_at (ESR1) >0.0075, #216836_s_at (HER2) <0.0135
HER2-positive: Affymetrix #216836_s_at (HER2) >0.0135
Triple-negative_ Affymetrix #205225_at (ESR1) <0.0075, #216836_s_at (HER2) <0.0135.

Based on these cutoffs, all samples were classified into one of these groups (Table 1).

uPA and PAI-1 protein levels were compared according to published cutoffs (uPA, 3 ng/mg protein; PAI-1, 14 ng/mg protein). For survival analysis, patient cohorts A and B were stratified into medians according to uPA and PAI-1 mRNA expression. In cohort C, with 290 HER2-positive patients, four groups of equal size (quartiles Q1–Q4) were generated according to uPA or PAI-1 mRNA levels, and different cutoffs were compared. Disease-free survival was computed from the date of surgery to the date of first metastasis or recurrence, and overall survival from the date of surgery to the date of death. Survival curves were compared with the log-rank test. Univariate as well as multivariate *P*-values for the respective risk factors in the survival model were obtained by a Cox proportional hazards model. All tests were performed at a significance level of $P=0.05$ (two-sided).

Results

uPA and PAI-1 mRNA expression

Two different probe sets for measuring uPA or PAI-1 expression are present on the Affymetrix U133A microarray (probe set 211668_s_at and 205479_s_at for uPA; 202627_s_at and 202628_s_at for PAI-1). We found a strong correlation between both probe sets ($r=0.8$, $P<0.001$ for uPA and $r=0.89$, $P<0.001$ for PAI-1) and therefore selected probe sets 211668_s_at and 202627_s_at for all subsequent analyses. uPA and PAI-1 mRNA expression levels varied slightly between the cohorts (Figure S1). For survival analysis, the median was used for each cohort, and the results were similar when calculated with the alternative probe set (data not shown).

uPA and PAI-1 mRNA expression in treated cohort A

In cohort A with systemic treatment, 67% (n=244) of the tumors had a luminal, 14% (n=49) a HER2-positive, and 19% (n=69) a triple-negative subtype (Table 1). In this cohort, PAI-1 mRNA expression above the median was associated with shorter disease-free survival (60% versus 67% at 10 years, $P=0.007$, Table 2 and Figure 1D) and overall survival (62% versus 77% at 10 years, $P=0.033$, data not shown). This was not the case for uPA.

In order to evaluate the prognostic relevance of uPA and PAI-1 mRNA expression in molecular subgroups, a stratified Kaplan–Meier analysis was performed. In the HER2-positive subgroup, elevated PAI-1 and uPA mRNA expression was associated with significantly shorter disease-free survival (37% versus 63% at 10 years for PAI-1, $P=0.031$, Figure 1B; and 28% versus 76% at 10 years for uPA, $P=0.011$, Table 2) and shorter overall survival (31% versus 71% at 10 years for PAI-1, $P=0.021$; and 35% versus 68% at 10 years for uPA, $P=0.036$, data not shown). In contrast, no association between either marker and disease-free survival or overall survival was seen in the luminal or triple-negative subgroups (Table 2, Figure 1A and C).

uPA and PAI-1 mRNA expression in untreated cohort B

For comparison, a second cohort of breast cancer patients without systemic treatment (n=200) was analyzed. In this group, 77% of the tumors (n=154) had a luminal, 10% (n=20) had a HER2-positive, and 13% (n=26) had a triple-negative subtype (Table 1). No significant association between uPA or PAI-1 mRNA expression and survival was observed for the entire cohort and for subtypes (Table 2). Yet, regarding the 10-year disease-free survival in molecular subgroups, there was a strong difference in HER2-positive tumors with high versus low uPA expression (39% versus 75%) that was not observed in luminal and triple-negative tumors (Table 2).

uPA and PAI-1 mRNA expression in HER2-positive validation cohort C

Based on the results obtained with two cohorts, we decided to study the prognostic impact of uPA and PAI-1 in an additional cohort with exclusively HER2-positive tumors. For statistical analysis, this HER2-positive cohort C (n=290) was first separated into four quartiles (Q1–Q4) with low, moderate, strong, or very strong uPA or PAI-1 mRNA expression in order to identify the most suitable cutoff for survival analysis. For PAI-1, the median was selected as the cutoff for further analysis, whereas for uPA, a cutoff of Q3 (lower 75% versus higher 25% of the cases) gave the best results. Based on these cutoffs, elevated uPA and PAI-1 mRNA expression showed a strong association with shorter

Table 2 Comparison of survival data in three different cohorts according to uPA and PAI-1 levels (univariate analysis)

	Survival					
	Cohort A (treated, n=362)		Cohort B (untreated, n=200)		Cohort C (HER2-pos, treated, n=290)	
	10-year DFS# (%)	*P*-value	10-year DFS# (%)	*P*-value	10-year DFS# (%)	*P*-value
PAI-1 mRNA						
All	60 versus 67	0.007*	72 versus 76	0.278		
HER2-positive	37 versus 63	0.031*	52 versus 66	0.557	48 versus 66	0.014*
Luminal	62 versus 67	0.143	82 versus 77	0.829		
TNT	56 versus 59	0.583	52 versus 75	0.342		
uPA mRNA						
All	61 versus 65	0.069	71 versus 76	0.952		
HER2-positive	28 versus 76	0.011*	39 versus 75	0.165	36 versus 67	<0.001*
Luminal	59 versus 69	0.228	78 versus 80	0.688		
TNT	52 versus 67	0.169	63 versus 57	0.583		
			10-year OS (%)	*P*-value		
PAI-1 protein						
All			68 versus 82	0.010*		
HER2-positive			31 versus 63	0.031*		
Luminal			77 versus 85	0.24		
TNT			71 versus 72	0.76		
uPA protein						
All			72 versus 77	0.077		
HER2-positive			44 versus 57	0.26		
Luminal			76 versus 79	0.89		
TNT			67 versus 82	0.066		

Notes: #Groups with high versus low uPA and PAI-1 expression, respectively, are shown; *statistically significant.
Abbreviations: TNT, triple-negative tumors; DFS, disease-free survival; OS, overall survival; uPA, urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor.

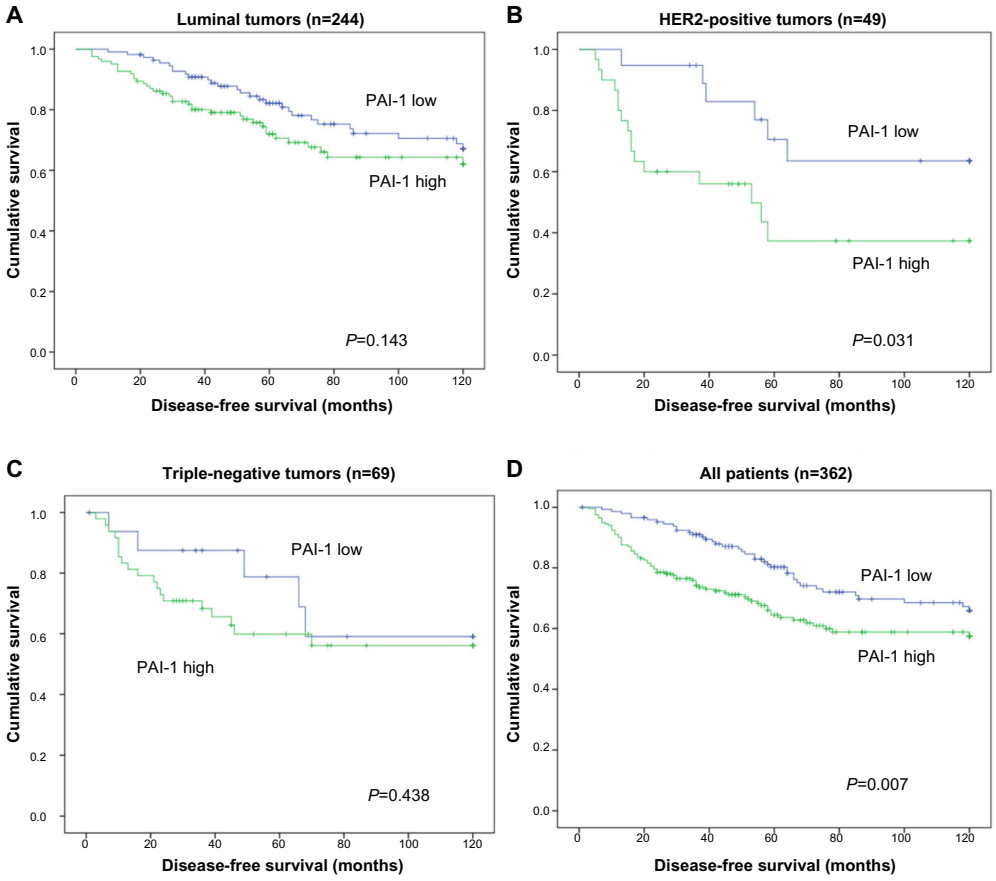

Figure 1 Disease-free survival according to PAI-1 mRNA expressions in the entire cohort A (**D**) and in molecular subtypes (**A**–**C**).

disease-free survival in univariate analysis (48% versus 66% at 10 years for PAI-1, P=0.014, Figure 2A; 36% versus 67% at 10 years for uPA, P<0.001, Figure 2B and Table 2). Notably, high mRNA expression of both markers retained prognostic significance in multivariate analysis adjusted for tumor size, nodal status, grading, and estrogen receptor status (hazard ratio 1.85, 95% confidence interval 1.01–3.42, P=0.048 for PAI-1; and hazard ratio 2.86, 95% confidence interval 1.36–6.01, P=0.006 for uPA, Table 3).

uPA and PAI-1 protein levels in untreated cohort B patients

In addition to mRNA expression levels, uPA and PAI-1 protein levels determined by ELISA tests were available in the untreated group of patients (cohort B). For all patients, high PAI-1 protein levels were significantly associated with shorter overall survival (P=0.010, Table 2 and Figure S2A). This was not the case for uPA protein levels (P=0.077, Table 2 and Figure S2B). Regarding the molecular subgroups, PAI-1 protein levels remained a prognostic factor only in HER2-positive but not in luminal or triple-negative carcinomas (Table 2). We found no significant associations between either marker and disease-free survival (data not shown).

Discussion

Our data demonstrate a prognostic impact of uPA and PAI-1 mRNA levels in breast cancer that is driven by the subgroup of patients with HER2-positive tumors. To our knowledge, this is the first investigation of the prognostic or predictive value of uPA/PAI-1 in molecular subtypes of breast cancer. In our analysis of patients who received systemic treatment, high PAI-1 RNA levels correlated with shorter disease-free survival in the whole cohort, but both parameters (uPA and PAI-1) were significantly associated with disease-free survival in HER2-positive patients. In cases with luminal or triple-negative tumors that make up the majority of these patients, uPA and PAI-1 had no prognostic impact in our cohorts. In the untreated cohort B, neither uPA nor PAI-1 mRNA was associated with shorter disease-free survival in the whole cohort or in the HER2-positive subgroup, which might be due to the small number of only 20 HER2-positive tumors. Yet, high PAI-1 protein levels correlated with shorter survival in the whole cohort and again in the subgroup of HER2-positive patients. The strong prognostic impact of uPA and PAI-1 in the HER2-positive molecular subgroup could be further validated in a large combined group of patients with high HER2 mRNA expression (n=290).

Most of the commercially available prognostic tests have been validated in retrospective clinical cohorts. Therefore, most patients in those cohorts were not characterized with respect to molecular subtype and were treated according to former treatment standards. Most of the validated tests do not give information about the molecular subtypes of the patients in their validation cohorts, although this might have had a strong influence on the results. For example, the 21-gene recurrence score assay was calculated in a mixed population of patients including tumors of all molecular subtypes. HER2 is part of the algorithm to calculate the score although the test is currently only recommended in HER2-negative patients, indicating that HER2 positivity might have influenced the prognostic impact of test results in the validation cohort.[10] In the National Surgical Adjuvant Breast and Bowel Project (NSABP)-B14 trial, the

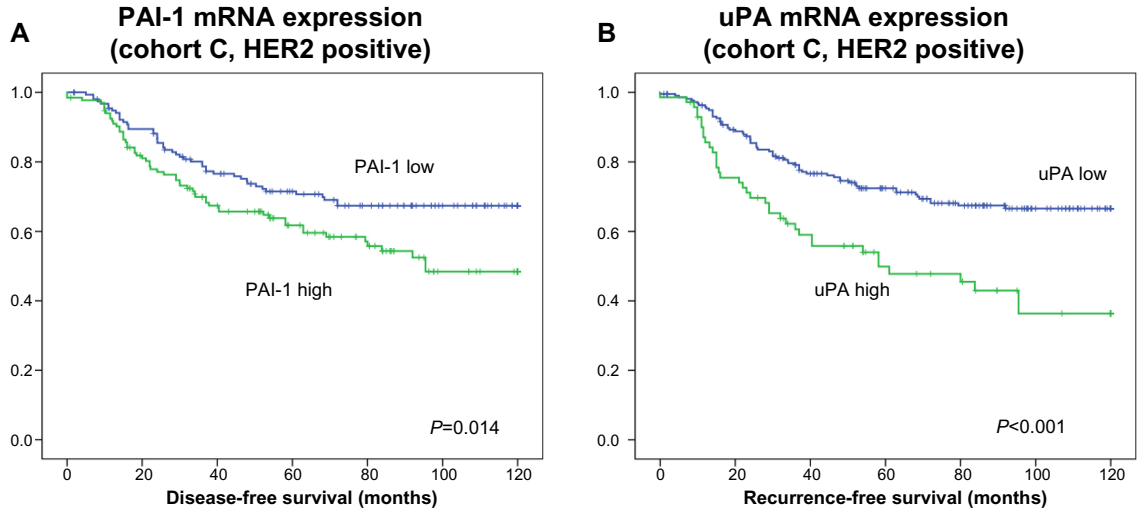

Figure 2 Disease-free survival according to PAI-1 mRNA expression (**A**) and uPA mRNA expressions (**B**) in the HER2-positive cohort C.

Table 3 Disease-free survival in validation cohort C (HER2-positive patients, n=290) in the multivariate Cox regression analysis

Parameter	Cohort C (n=290)		
	Hazard ratio	95% CI	*P*-value
uPA mRNA (Q4 versus Q1–3)	2.86	1.36–6.01	0.006*
PAI-1 mRNA (>median)	1.85	1.01–3.42	0.048*
Tumor size (<2 cm)	0.43	0.23–0.78	0.006*
Grade (G3)	1.10	1.04–1.18	0.003*
Nodal status (positive)	1.16	0.61–2.23	0.652
ER status (positive)	0.55	0.29–1.06	0.072

Note: *Statistically significant.
Abbreviations: CI, confidence interval; ER, estrogen receptor; uPA, urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor.

21-gene recurrence score identified 22% of patients in the intermediate-risk group,[11] and 20.6% in the NSABP-20 trial.[12] Today, as the 21-recurrence score is only recommended for luminal tumors, 40%–66% of patients are classified into the intermediate-risk group.[13] In the Chemo-N0 trial, elevated uPA and PAI-1 protein levels were associated with shorter disease-free survival, and patients with elevated levels had a benefit from chemotherapy (6× cyclophosphamide, methotrexate and fluorouracil, n=117).[3] However, in this trial (recruitment 1993–1998), HER2 testing was not performed and HER2-directed therapies were not yet available. It should be noted that the patients in our study also did not receive HER2-directed therapy. Therefore, our data only suggest a prognostic impact in untreated patients and a predictive role for response to systemic chemotherapy and endocrine therapy, but do not give any information about the predictive value of uPA and PAI-1 with regard to treatment with trastuzumab. Results of the NNBC-3 and Plan B trials that examined the role of proteases in the context of modern therapy are not yet available.[14,15] Regarding our results, an investigation of the prognostic or predictive impact of uPA and PAI-1 mRNA and/or protein expression in trastuzumab-treated patients would be highly desirable.

The prognostic impact of HER2 in addition to uPA/PAI-1 protein levels has been evaluated in 112 patients with node-negative breast cancer. In this study, combining the HER2 gene status measured by fluorescence in situ hybridization with levels of tumor invasion markers uPA and PAI-1 improved clinically relevant risk group assessment.[16] Another study of 587 patients with node-negative or node-positive breast cancer analyzed the effects of HER2 and the uPA/PAI-1 axis on prognosis in primary breast cancer, and demonstrated that overexpression of HER2 indicates a poor prognosis among uPA-positive and PAI-1-positive patients.[17] In that study, uPA gave additional prognostic information in HER2-positive patients, whereas the influence of PAI-1 on survival was lower. The authors concluded that the association between uPA and HER-2 was more likely to be based on indirect interactions than on direct regulation. In another investigation of 117 lymph node-negative breast tumors, the effect of uPA and PAI-1 was independent of HER2 status.[18] Importantly, HER2 status in these studies was determined by a protein (ELISA, immunohistochemistry) or DNA level (fluorescence in situ hybridization), whereas we used mRNA expression levels for identification of molecular subgroups. In addition, these studies aimed at the role of HER2 in subgroups of patients with low or high proteases, whereas our study analyzed the impact of uPA and PAI-1 in different molecular subtypes of breast cancer.

A potential drawback of our study is its retrospective nature and its assessment of a biomarker in a patient cohort that was not predefined. Moreover, in our cohorts, uPA/PAI-1 could be determined mainly on an mRNA level, and protein determination by ELISA was only performed in a subset of patients.

In those patients with protein determination of uPA and PAI-1 (cohort B), only PAI-1 protein levels showed a significant prognostic impact on survival. A lack of correlation between PAI-1 measured on mRNA and protein level was previously described.[19,20] In a prior study, we showed that PAI-1 mRNA expression adds prognostic information in breast cancer patients in addition to protein levels and might also have a predictive effect.[21] We could confirm this strong association of PAI-1 mRNA levels with disease-free survival in the treated cohort A and also in the larger HER2-positive cohort C. This is in accordance with several previous studies showing a stronger prognostic impact for PAI-1 mRNA on survival than uPA mRNA also in patients with lymph node-positive breast cancer receiving adjuvant treatment.[22,23]

In conclusion, we provide evidence that there is an important difference in the prognostic and predictive role of uPA and PAI-1 mRNA levels in molecular subtypes of mammary carcinomas. This could be of clinical relevance also for interpretation of the results of other tests developed in breast cancer. In the subgroup of HER2-positive carcinomas, uPA and PAI-1 might be interesting prognostic or predictive factors for treatment decisions.

Disclosure

RW is the founder and chief executive officer of STRATIFYER Molecular Pathology GmbH. The other authors report no conflicts of interest in this work.

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Supplementary materials

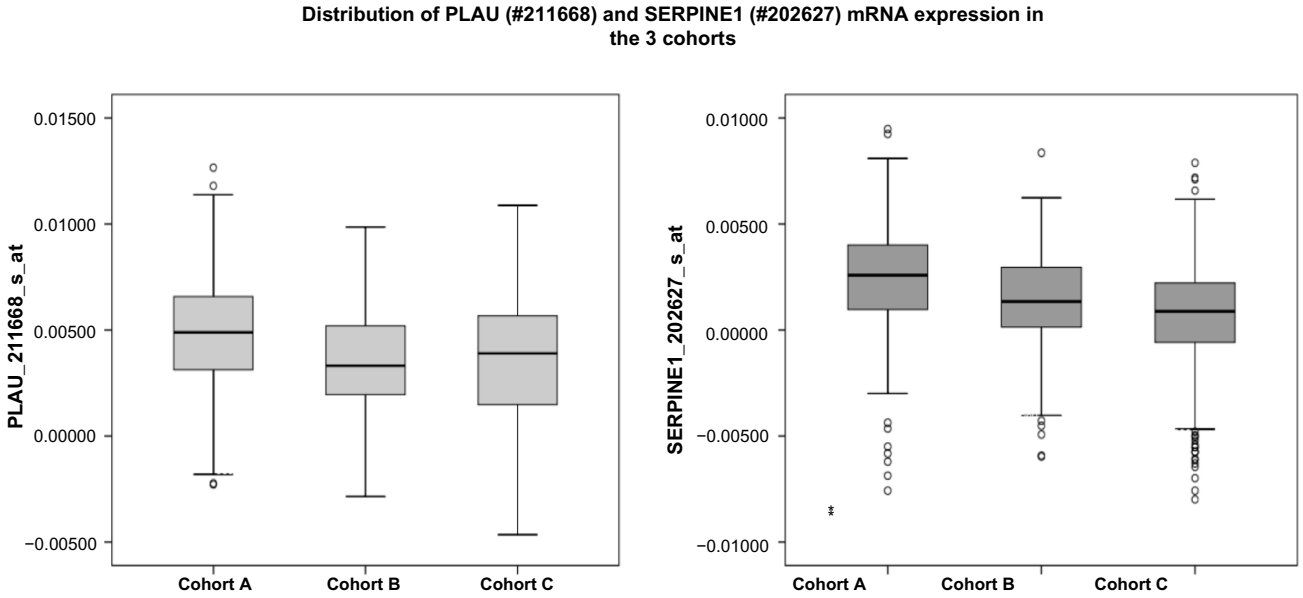

Figure S1 Distribution of urokinase plasminogen activator and plasminogen activator inhibitor mRNA expression in all three cohorts.

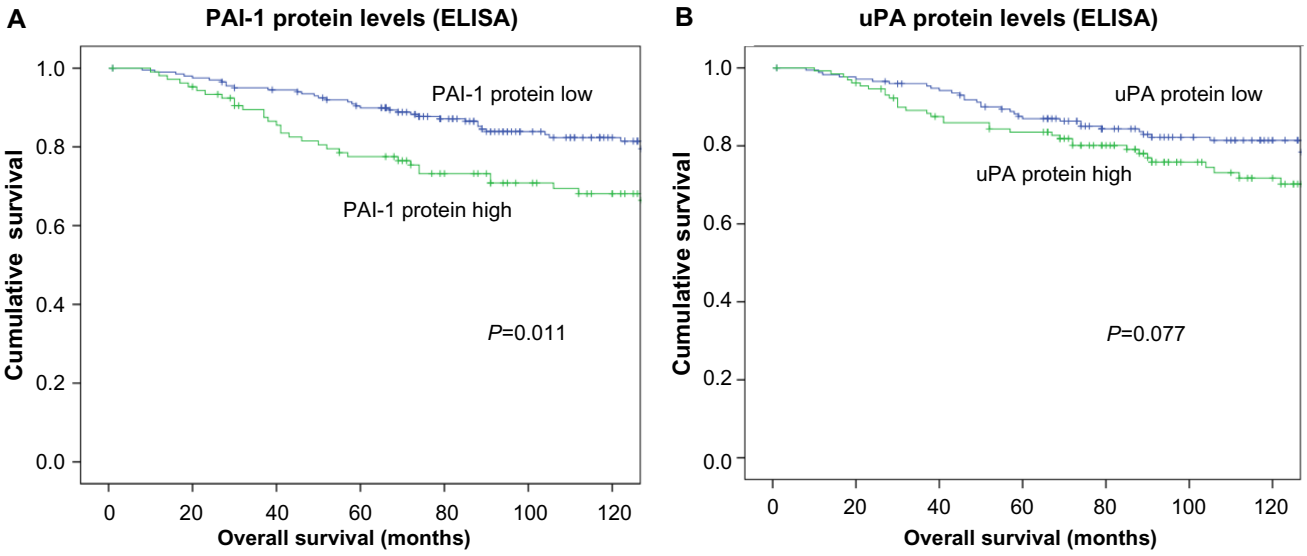

Figure S2 Overall survival in the untreated group of patients (cohort B) according to PAI-1 protein levels (**A**) and uPA protein levels (**B**).
Abbreviations: ELISA, enzyme-linked immunosorbent assay; uPA, urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor.