Independent validation of stromal uPA in ABCSG-08: Level 1b evidence for the prognostic value of uPA immunohistochemistry

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ABSTRACT

Purpose: To validate the prognostic role of urokinase-type plasminogen-activator (uPA) and plasminogen activator inhibitor type-1 (PAI-1) protein expression in FFPE archived tumor samples when assessed by immunohistochemistry.
Patients and methods: Fresh-frozen, paraffin-embedded (FFPE) samples from 303 postmenopausal women with hormone receptor-positive, early breast cancer were investigated. The patients had received 5 years of endocrine therapy in the prospectively randomized ABCSG-8 trial. Immunohistochemistry for stromal uPA and PAI-1 protein expression was correlated with distant recurrence-free survival (DRFS) and overall survival (OS).
Results: We detected stromal uPA in 132 of 297 tumors (44.4%) and stromal PAI-1 expression in 74 out of 299 samples (24.7%). Co-expression of uPA and PAI-1 was present in 48 of 294 (16.3%) cases. Neither uPA nor PAI-1 expression was associated with tumor size, age, nodal status, grading, or quantitative receptor status. Patients whose tumor stroma expressed uPA protein had a significantly shorter DRFS (adjusted HR for relapse: 2.78; 95% CI 1.31–5.93; p = 0.008 Cox regression analysis) than women without uPA expression. No such association was seen for PAI-1 and the uPA/PAI1 ratio. After a median follow-up of 5.6 years, women with uPA-positive tumors demonstrated significantly shorter DRFS (93.3% vs. 84.8%; p < 0.013 log-rank test), and tended to have a worse OS (83.0% vs. 77.3%; p = 0.106) compared to women with uPA negative tumors.
Conclusion: This independent validation in archived tumor samples from a large prospective randomized trial confirms the clinical utility of stromal uPA evaluation by immunohistochemistry. This provides level 1b evidence for the prognostic role of stromal uPA in women with endocrine-responsive early breast cancer.

1. Introduction

Urokinase-type plasminogen activator (uPA) is an extracellular matrix-degrading protease that mediates pericellular proteolysis. Together with its physiological inhibitor, the plasminogen activator inhibitor type 1 (PAI-1), it interacts with several extracellular matrix proteins and transmembrane receptors and modulates cell migration, cell-matrix interactions, and signaling pathways [1,2]. A large body of experimental evidence from *in-vitro* and *in-vivo* evidence, as well as from clinical trials, suggests that uPA and PAI-1 also have a crucial role in local tumor invasion and metastatic behavior in breast cancer [3–6]. When measured by enzyme-linked immunosorbent assay (ELISA) in

fresh tissue [7], patients with high levels of intra-tumoral uPA and/or PAI-1 protein experience a significantly shorter disease-free (DFS) and overall survival (OS) compared to women with low uPA/PAI-1 expression. The prospectively designed randomized phase III Chemo-N0 study has previously demonstrated a 10-year recurrence rate of 23% in tumors with high intra-tumoral uPA/PAI-1, compared to 13% in tumors with low uPA/PAI-1 expression. Patients who received cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) chemotherapy and exhibited high intratumoral uPA/PAI-1 expression by ELISA had a 26% lower recurrence rate and a significantly longer DFS than women with low uPA/PAI1 expressing tumors. Therefore low intra-tumoral uPA/PAI-1 expression suggests that patients can safely forgo adjuvant systemic chemotherapy, while patients with high-uPA/PAI-1 expressing tumors derive a significant benefit from the addition of adjuvant CMF [8].

In addition, two randomized prospective trials investigate the predictive utility of uPA/PAI-1 for anthracycline and taxane-based regimen. One of these, the NNBC-3 trial, has enrolled 4147 patients, and comparing fluorouracil (5-FU), epirubicin, and cyclophosphamide followed by docetaxel (3xFEC-3xDoc; FEC-D) with 5-FU, epirubicin, and cyclophosphamide (6xFE100C; FEC) as adjuvant chemotherapy for high-risk lymph node-negative patients [9].

WSG Plan B is another trial which compares an anthracycline- and taxane-based adjuvant chemotherapy combination with an anthracycline-free taxane-based regimen in patients with HER-2-negative breast cancer. Both trials have finished recruitment are aimed at establishing the prognostic and predictive potential of uPA/PAI-1 with current chemotherapy standards [10].

Based mainly on the results from the chemo-N0 study, ELISA-based intra-tumoral uPA/PAI-1 protein expression analysis is now endorsed for risk assessment by several national and international societies and is included in the American Society of Clinical Oncology 2007 Update of Recommendations for the Use of Tumor Markers in Breast Cancer [11]. Nevertheless, despite level-1 evidence on the validity of uPA/PAI-1 prognostication, the clinical utility of ELISA-based uPA/PAI1 analysis is limited by the requirement of relatively large amounts of fresh tumor tissue extracts, and by the availability of gene expression profiles [12–14].

In order to overcome these assay-inherent limitations, FFPE-based uPA/PAI-1 ELISA have been developed [15]. However, a thorough head-to head comparison between both techniques, which would allow for a comparison of FFPE and fresh tissue-based ELISA in a sufficiently large prospective trial has never been reported.

Another strategy uses immunohistochemical analysis of intra-tumoral uPA and PAI-1 protein expression, which has also been suggested for the identification of women with poor prognosis [16,17]. We have previously investigated stromal uPA and PAI-1 expression by immunohistochemistry in FFPE-based tumor samples from the prospectively designed ABCSG 6 study. In that phase III study, we demonstrated that postmenopausal women with HR + early breast cancer who had received 5 years of adjuvant tamoxifen with or without aminoglutethimide for the first 2 years of treatment showed a significantly worse DRFS and OS if their tumors exhibited stromal co-expression of uPA and PAI-1 assessed by immunohistochemistry (IHC) [18].

We hereby report the results of an independent uPA and PAI-1 biomarker validation in another cohort of patients with HR + positive early breast cancer who received adjuvant endocrine therapy in the multicentric phase III ABCSG-8 trial, in which postmenopausal women with HR + tumors with good to moderate differentiation received endocrine therapy for 5 years. All patients initially received tamoxifen for 2 years, which was either continued for another 3 years or switched to anastrozole for 3 years in a prospectively randomized manner [19].

2. Patients and methods

The current investigation is part of the ABCSG translational research program (abcsg. research). Women included in the ABCSG-8 trial were recruited between 1996 and 2004. Participants were postmenopausal, below 80 years of age, with primary, operable, histologically verified, estrogen receptor (ER)+ and/or progesterone receptor (PR)+, grade 1 or 2 ductal, and Gx lobular invasive breast cancer. Patients were randomized immediately after surgery, initially treated with TAM for 2 years, and received either tamoxifen (TAM) or anastrozole (ANA) for the subsequent 3 years. None of the patients had received adjuvant chemotherapy or HER2-directed therapy. Additional information concerning the definition of menopausal status, endocrine receptor assessment, surgery, radiotherapy, random assignment, stratification, study treatment, and patient follow-up has been published previously [20]. FFPE tumor blocks were collected from participating centers at the time of surgery and were stored at room temperature. Approval was obtained from Institutional Review Boards. A REMARK diagram detailing the study cohort is shown in Fig. 1.

2.1. uPA and PAI1 immunohistochemistry

Immunostaining for uPA and PAI-1 has been previously described [18]. In brief, consecutive FFPE tissue sections (3–5 μm) were deparaffined with EZPrep (Ventana Inc), endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide, and unspecific antibody binding was blocked with 10% goat serum. We used the anti-uPA monoclonal antibody (Sekisui Diagnostics, clone #3689) at a dilution of 1:300 and incubated for 30 min at 37 °C. The anti-PAI1 monoclonal antibody (Sekisui Diagnostics, clone #ADG3786) was used at a dilution of 1:35, and slides were incubated for 30 min at 37 °C. Samples were then subjected to biotinylated goat anti-mouse immunoglobulin for 30 min and incubated with streptavidin-HRP complex before DAB substrate was added (Dako Inc). Sections were washed using Ultra Wash (Ventana Inc.), and counterstained with hematoxylin, dehydrated, and mounted with Aquatex (Merck, Germany). Tumors were evaluated for the presence of uPA and PAI1 reactivity of the tumor stroma by a board-certified pathologist (S.J.). Immunohistochemical uPA and PAI1 expression in more than 10% of the tumor stroma was (i.e. stromal cells) scored as "positive" for the respective marker. In accordance with our previous study a cut-off of 10% positively stained tumor stroma was chosen to stratify tumors into uPA/PAI1 negative cases (=<10% positively stained tumor stroma) and uPA/PAI1 positive cases (>10% stromal positivity) [18]. For reasons of readability, uPA/PAI1 negative tumors, as defined above, are also referred to as "without detectable uPA/PAI1 expression" in the manuscript. Immunohistochemical staining of tumor stroma was assessed at 4x and 10x magnification.

2.2. Statistical analysis

The primary endpoints of the statistical analyses were distant recurrence-free survival (DRFS) and overall survival (OS). DRFS was defined as the interval between the date of surgery and the first evidence of relapse at any distant site. Since 62% of patients were older than 60 years at trial initiation, and because of the long-term follow up, patients were censored if they were recurrence-free and had died from reasons unrelated to their malignancy. Baseline data were compared in univariate analyses using the χ^2 and in a multiple logistic model. Survival rates were estimated using the Kaplan-Meier method. The prognostic value of stromal uPA and/or PAI-1 expression was evaluated using univariate and multiple Cox models. All p values were two-sided, with $p \leq 0.05$ considered statistically significant. All statistical analyses were performed using SPSS software version 15.0 (SPSS, Inc.)

3. Results

Of the 3714 women who had entered the ABCSG 8 trial, evaluable FFPE tumor samples and clinical data were analyzed in a subset of 303 patients. This subset was representative of the overall study population (data not shown). Patient characteristics are shown in Table 1.
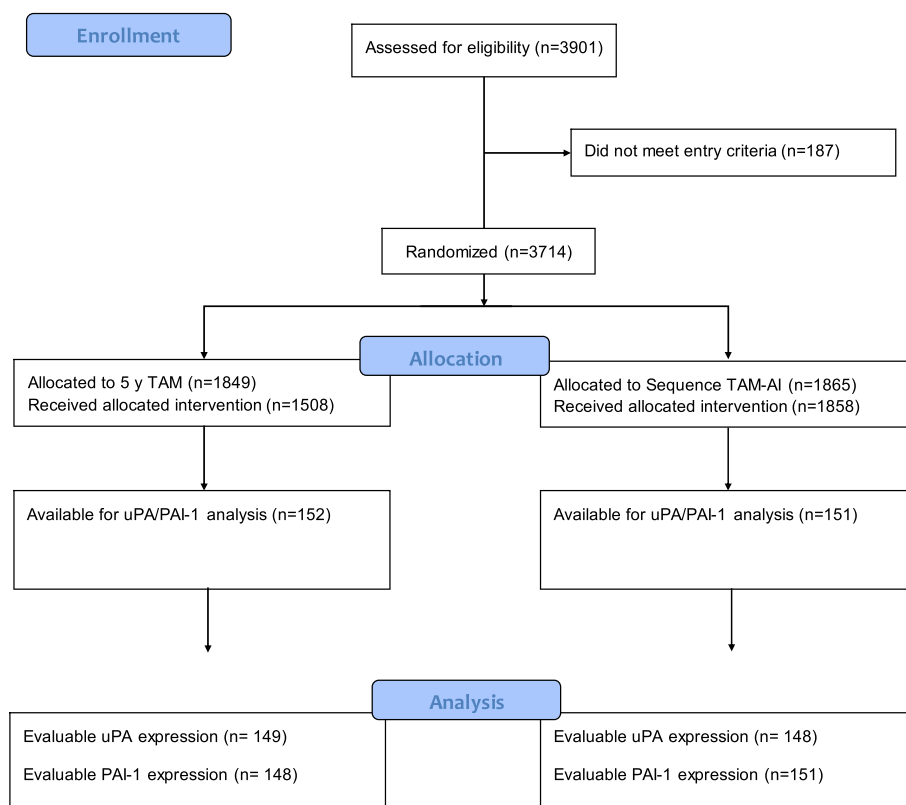
Fig. 1. REMARK diagram describing the study cohort.

Table 1

Patient characteristics.

	uPA $n=132/297$ (44.4%)	PAI1 $n=74/299$ (24.7%)	UPA + PAI1 $n=48/294$ (15.3%)
Age			
≤60	46 (41.1%)	33 (29.2%)	19 (17.3%)
>60	86 (46.5%)	41 (22.0%)	29 (15.8%)
Tumor size			
T1	79 (41.8%)	42 (22.1%)	26 (13.8%)
T2	50 (50.0%)	30 (29.7%)	20 (20.4%)
T3	3 (37.5%)	2 (25.0%)	2 (25.0%)
Nodal status			
N0	94 (46.1%)	52 (25.4%)	34 (16.7%)
1–3 positive nodes	35 (43.2%)	19 (23.2%)	13 (16.5%)
4–10 positive nodes	3 (27.3%)	2 (18.2%)	1 (9.1%)
>10 positive nodes	0 (0%)	1 (100.%)	0 (0%)
Tumor grade			
G1	25 (49.0%)	14 (28.0%)	9 (18.4%)
G2	99 (44.2%)	55 (24.2%)	36 (16.1%)
GX	8 (36.4%)	5 (22.7%)	3 (13.6%)
Estrogen Receptor			
0	1 (33.3%)	1 (33.3%)	1 (33.3%)
+	13 (44.8%)	7 (24.1%)	3 (10.3%)
++	37 (42.0%)	24 (27.0%)	17 (19.8%)
+++	81 (45.8%)	32 (23.6%)	27 (15.3%)
Progesterone Receptor			
0	35 (46.7%)	21 (28.0%)	35 (64.7%)
+	20 (38.5%)	11 (20.0%)	20 (38.5%)
++	46 (43.4%)	27 (26.2%)	46 (43.4%)
+++	31 (48.4%)	15 (22.7%)	31 (48.4%)
Treatment Arm			
Tam > Tam	68 (45.6%)	36 (24.3%)	23 (15.8%)
Tam > AI	64 (43.2%)	38 (25.2%)	25 (16.9%)

Intra-tumoral stromal uPA expression was evaluable in 297 of 303 cases (98.0%), PAI-1 expression in 299 of 303 (98.7%) cases and both uPA and PAI-1 in 294 cases (97.0%). We detected the expression of uPA

in 132 of 297 tumors (44.4%), and of PAI1 in 74 out of 299 samples (24.7%). Concomitant uPA and PAI-1 expression were seen in 48 of 294 (16.3%) of cases. Heterogeneous staining was frequent with a propensity of positive staining for both antibodies in centrally located fibrotic areas as well as in tumor areas rich in fibroblasts. In tumors with heterogenous expression uPA and PAI-1 expression was considered positive. The expression of uPA, PAI-1, as well as the co-expression of uPA and PAI-1 was not significantly associated with age or with any of the classical prognostic parameters such as grading, size, nodal status, ER or PR expression (data not shown).

At a median follow-up of 5.6 years, 31 of the 303 patients (10.2%) evaluated had experienced distant relapses: There were 20 out of 132 (15.2%) women with DDFS events in uPA positive tumors vs. 11 out of 165 (6.67%) women with DDFS events in uPA negative tumors. 8 out of 74 (10.8%) women with DDFS events were seen in PAI1 positive tumors, vs. 23 out of 225 (10.2%) such events in PAI1 negative tumors. 58 of the 297 patients (19.5) evaluated had died: 30 out of 132 women (22.7%) with uPA positive tumors, and 28 out of 165 women (17.0%) with uPA negative tumors. We observed OS events in 15 out of 74 (20.3%) women with PAI1 positive tumors, vs. 43 out of 225 (19.1%) such events in PAI1 negative tumors.

Expression of uPA, was associated with a significantly worse DRFS in univariate analysis (HR for relapse, 2.48; 95% C.I. 1.19–5.18, *p=0.013*), but this was not the case for PAI-1 expression (HR 1.14; 95% C.I. 0.51–2.54, *p=0.759*), or the co-expression of uPA and PAI-1 (HR 1.04; 95% C.I. 0.40–2.72, *p=0.993*). We also observed a trend towards a decreased OS in women with uPA expressing tumors (HR 1.52; 95% C.I. 0.91–2.55, *p=0.110*), while neither PAI-1, nor uPA and PAI-1 co-expression were predictive for overall survival in univariate analysis (HR 1.12; 95% C.I. 1.62–2.02, *p=0.702*; and HR 0.87; 95% C.I. 0.41–1.83, *p=0.706*, respectively; Table 2).

The Kaplan Meier curves for breast cancer patients with and without intratumoral uPA expression describe a significantly different DRFS (log-rank Mantel-Cox; *p=0.013*) and are shown in Fig. 2a. The Kaplan

Table 2
Cox proportional hazard models for DRFS and OS.

| Variable	HR for distant recurrence	95% CI	P	HR for death	95% CI	P
Univariate						
uPA	2.48	1.19–5.18	0.013	1.52	0.91–2.55	0.110
PAI1	1.14	0.51–2.54	0.759	1.12	0.62–2.02	0.702
uPA + PAI1	1.04	0.40–2.72	0.993	0.87	0.41–1.83	0.706
Multivariate						
Age	1.72	0.75–3.95	0.20	4.39	1.92–9.87	<0.0001
Tumor size	1.46	0.78–2.75	0.241	2.28	1.44–3.61	<0.0001
Nodal status	2.05	1.12–3.74	0.019	1.41	0.90–2.21	0.133
Tumor grade	0.98	0.61–1.60	0.95	0.72	0.48–1.08	0.116
ER	0.89	0.53–1.49	0.659	0.84	0.57–1.22	0.352
PR	0.87	0.63–1.21	0.397	1.01	0.80–1.29	0.917
Therapy arm	1.31	0.63–2.72	0.472	1.06	0.62–1.82	0.917
uPA	2.78	1.31–5.93	0.008	1.47	0.86–2.50	0.161
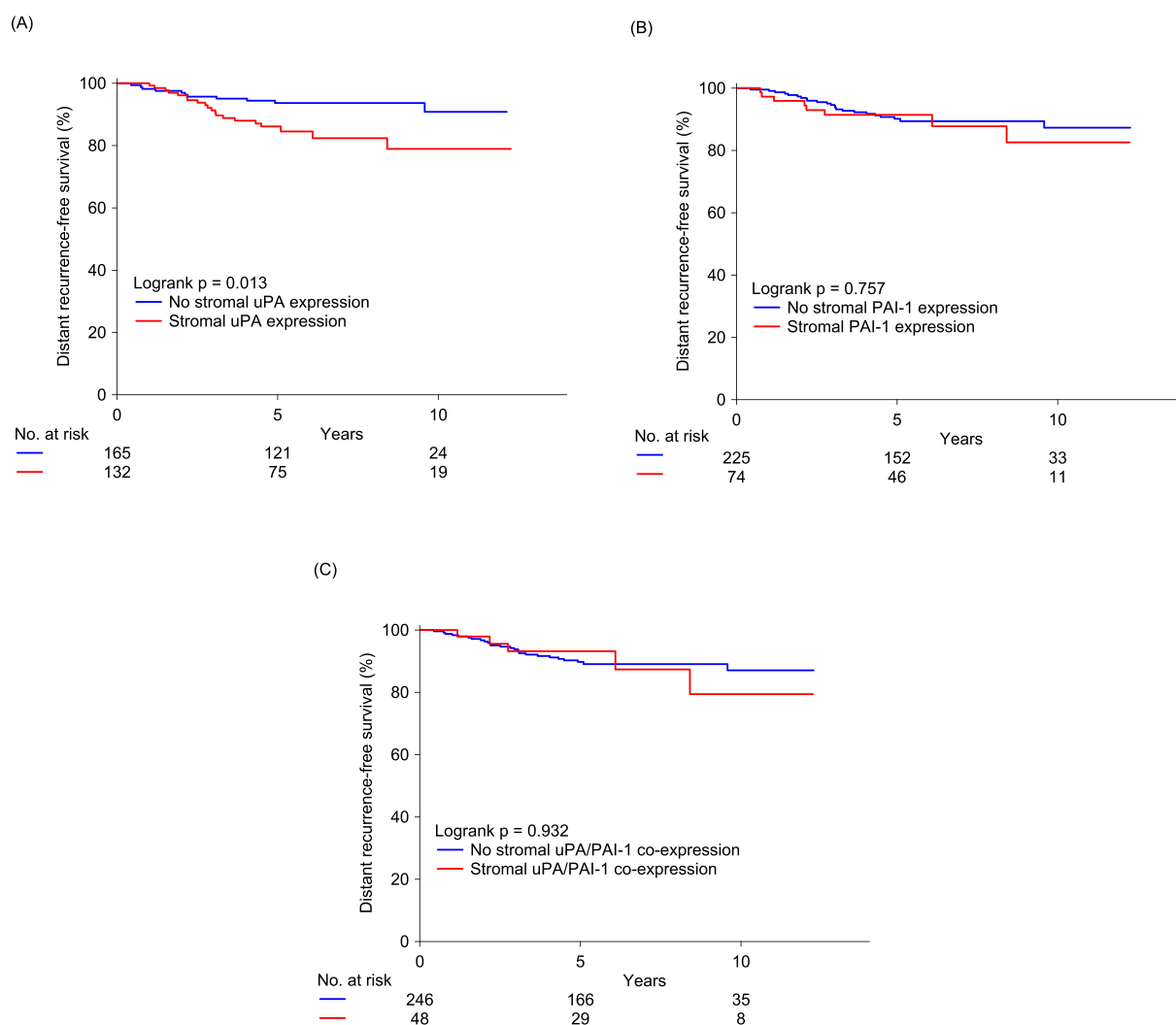
Fig. 2. DRFS in postmenopausal breast cancer patients with endocrine-responsive tumors according to uPA protein expression (A), PAI1 protein expression (B), and co-expression of uPA and PAI1 (C).

Meier curves for PAI1 (log-rank *p=0.757*), and for the co-expression of uPA and PAI1 (log-rank *p=0.932*) are shown in Fig. 2b and c, respectively.

A similar, albeit non-significant, trend towards a decreased OS was observed for women with intra-tumoral uPA expression (log-rank *p=0,106*; Fig. 3a). Neither PAI-1 expression (Fig. 3b) nor the co-expression of uPA and PAI-1 (Fig. 3b) was prognostic for OS in our study (log-rank *p=0.699* and log-rank *p=0.710*, respectively).

The independent effect of stromal uPA and PAI-1 expression on DRFS and OS was assessed by multivariate Cox proportional hazard models adjusted for age, treatment, tumor size, nodal status, tumor grade, as well as ER and PR expression. In multivariate analyses, the expression of stromal uPA remained significantly associated with prolonged DRFS (adjusted HR for distant relapse 2.78; 95% CI 1.31–5.93; *p=0.008* Cox regression analysis) and again showed a trend towards an improved OS (adjusted HR for death 1.47; 95% CI 0.86–2.50; *p=0.161*) when

(A)

(B)

(C)
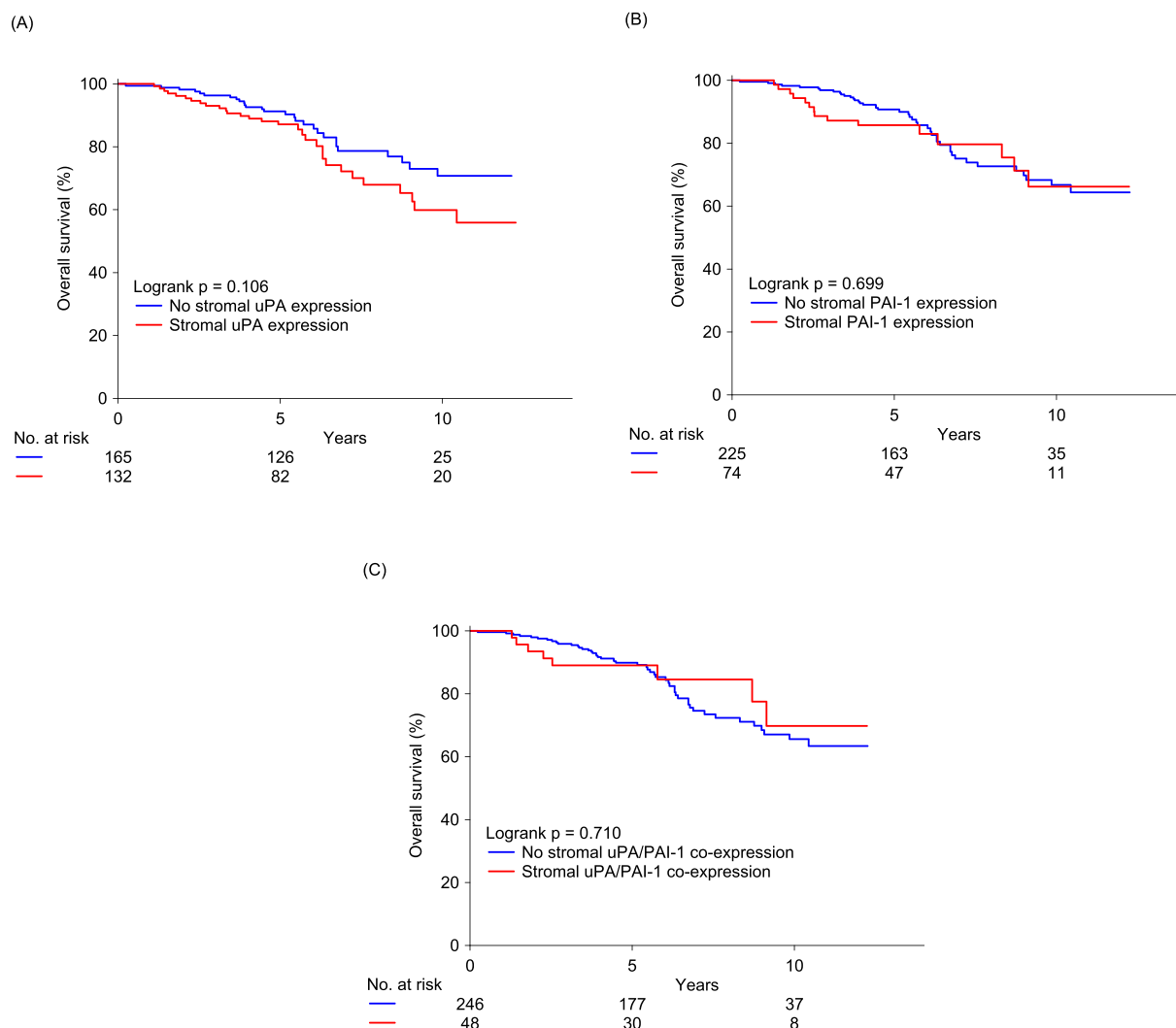
Fig. 3. OS in postmenopausal breast cancer patients with endocrine-responsive tumors according to uPA protein expression (A), PAI1 protein expression (B), and co-expression of uPA and PAI1 (C).

compared to women whose tumors did not express uPA (Table 2). In contrast, we did not observe an association of DRFS or OS with PAI-1 or uPA/PAI1 co-expression in this model (data not shown).

4. Conclusions

Urokinase-type plasminogen activator (uPA) is a protease, which has a key role in tumor invasion and metastatic behavior in several cancer entities [1]. it is therefore not surprising that uPA and its physiological inhibitor PAI-1 are established biomarkers which predict for long-term outcome in breast cancer patients [21]. The clinical value of uPA/PAI-1 levels as prognostic biomarkers in lymph node-negative breast cancer has been established in the randomized prospective Chemo N0 trial, and was confirmed in a pooled analysis of individual data sets of more than 8000 individual patients from retrospective and prospective studies [8,22]. Independent level 1a evidence has led to several guideline recommendations including from ASCO to endorse uPA/PAI-1 expression measured by ELISA as biomarkers for risk assessment in node-negative early breast cancer [22,23]. Routine application of ELISA based risk-prediction, however, is hampered by the availability of adequate amounts of fresh tumor tissue required for analysis, and hence clinical implementation has remained limited.

To overcome this limitation, we have previously demonstrated the technical feasibility and the prognostic value of stromal uPA and PAI-1

expression in archived FFPE tumor samples in a subset of the 606 postmenopausal endocrine-treated women with early breast cancer who were included in the phase III ABCSG 6 trial. In this study we detected stromal uPA 54.3% and stromal PAI-1 in 53.3% of cases, while co-expression of both proteins occurred in 37.3% of samples. We validated the cut-off of 10% ER positivity, which we have already used in a previous assessment of the prognostic role of uPA/PAI-1 the ABCSG 6 patient cohort [24]. While we have no evidence that a cut-off of 10% is clinically relevant for uPA and PAI-1 as well, we found it difficult to distinguish tumors that do not express either of the two proteins at all from those which did express the respective biomarkers in very low levels. The 10% cut-off was found to be much more straight-forward – and had also been used in our previous publication.

Despite some differences in trial design, patient characteristics, treatment and follow-up durations in ABCSG 6 and ABCSG 8, the results of the present study confirm the findings of our previous study regarding the prognostic value of uPA and PAI1 as single markers: uPA expression was significantly associated with DDFS in both ABCSG 6 (univariate adjusted HR for relapse: 1.86; 95% CI 1.20–2.88; *p=0.005*; multivariate adjusted HR for relapse: 1.64; 95% CI 1.04–2.57; *p=0.032*) and ABCSG 8 (univariate adjusted HR for relapse: 2.48; 95% CI 1.19–5.18; *p=0.013*; multivariate adjusted HR for relapse: 2.78; 95% CI 1.31–5.93; *p=0.008*) [18]. PAI-1, by contrast, only showed a trend for DDFS in ABCSG 6 in univariate analysis (adjusted HR for relapse: 1.49; 95% CI 0.99–2.26;

$p=0.057$), and was not associated with outcome in multivariate analysis (multivariate adjusted HR for distant relapse: 1.28; 95% CI 0.83–1.98; $p=0.264$). Similarly, in ABCSG-8, PAI1 was not significantly associated with DDFS in univariate analysis (adjusted HR for relapse: 1.14; 95% CI 0.51–2.54; $p=0.759$), and was thus not included in the multivariate model.

In contrast to results for uPA and PAI-1 as single markers, we could not confirm the prognostic utility of uPA/PAI-1 co-expression in ABCSG 8. The reason for this discrepancy is currently unclear. Differences might be related to a lower rate of PAI-1 positive tumors in the current study compared to ABCSG-6. In the latter cohort, 54.3% of tumors were uPA positive, and 53.3% were PAI-1 positive, while in ABCSG 8 44.4% of tumors were uPA positive, but only 24.7% were PAI-1 positive. Consequently, rates of uPA/PAI1 co-expression also differed between both studies with 37.3% in ABCSG 6 vs. 16.3% in ABCSG 8. The lower rates of uPA/PAI-1 co-expression in ABCSG 8 might have impaired the power to detect a prognostic value of uPA/PAI-1 co-expression.

An indirect comparison with the gene expression profile results, which are available for PAM50 in the very same ABCSG 8 study population [25], demonstrate that patients with a low and intermediate PAM50 Risk-of-Recurrence ('ROR') score exhibit a 10-year DRFS of 96.7% and 91.3%, respectively, which is comparable to the DRFS of 93.3%, which we observed in women with non-uPA expressing tumors. By contrast, patients with a high ROR score had median distant disease-free survival of 79.9%, which was again comparable to women, whose tumors expressed uPA (84.8%). uPA expression analysis is, however, superior to PAM50 by classifying fewer patients into the high risk group than PAM 50 (44.4% vs 33.7%).

It should also be noted that ABCSG 6 and ABCSG 8 trials differed from comparable trials in the sense that recruitment was restricted to postmenopausal women, whereas both pre- and postmenopausal women were recruited into the Chemo-N0 trial. In addition, while the Chemo-N0 study was confined to nodal-negative tumors per definition, the ABCSG6 and ABCSG 8 populations comprised almost 40% and 25% nodal-positive patients, respectively, thus permitting to evaluate the prognostic effect of uPA/PAI1 in a patient population with a particularly aggressive tumor subtype.

Due to the retrospective nature of our analysis, our study has several limitations: ABCSG 8 patients were recruited between 1996 and 2004, at a time when uPA/PAI-1 ELISA assays were not yet available, and thus no direct comparison between ELISA and our IHC results is possible. In addition, Ki67 was not routinely measured at that time. Individual Ki67 values were therefore not available for uni- and multivariate analysis. This is important in the light of two recent publications which demonstrated that Ki67 expression was lower in uPA/PAI-1-negative than in uPA/PAI-1-positive tumors [26,27]. Furthermore, the subset of patients with available and thus analyzable tumor tissue was only a relatively small fraction of the overall trial population of 3714 women who had been randomized into the trial, thus potentially obscuring a potential prognostic effect of the uPA/PAI1 ratio, simply because not enough DDFS events had occurred.

The extended follow-up of our study and the resulting FFPE storage periods, as well as variations in tissue fixation conditions between the contributing centers, might have affected the uPA and PAI-1 epitope preservation, although this would also have been the case in ABCSG 6, in which recruitment was from 1990 to 1995.

In summary, we have analyzed the stromal expression of uPA and PAI-1 in endocrine-responsive early-stage breast cancers from postmenopausal women who had been enrolled in the prospectively-designed ABCSG-8 phase III study. Together with our previously published data from ABCSG 6, we now provide independent level 1b evidence for the prognostic value of immunohistochemically determined uPA protein expression in endocrine-treated postmenopausal breast cancer patients [28].

Declaration of competing interest

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