

# Prognostic Significance of Soluble Urokinase Plasminogen Activator Receptor in Serum and Cytosol of Tumor Tissue from Patients with Primary Breast Cancer<sup>1</sup>

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## ABSTRACT

**Purpose:** The aim of the study was to evaluate the prognostic value of soluble urokinase plasminogen activator receptor (suPAR) in preoperatively obtained sera samples (s-suPAR) from breast cancer patients.

**Experimental Design:** suPAR levels were determined by the use of a kinetic ELISA in sera from 274 breast cancer patients and in tumor cytosols (c-suPAR) from 188 of these patients. In addition, s-suPAR levels were analyzed in 174 female blood donors.

**Results:** The mean s-suPAR level was 3.8 ng/ml (range, 1.6–9.2 ng/ml) in the patients and 3 ng/ml (range, 1.3–6.4 ng/ml) in the donors. The mean c-suPAR level was 0.55 ng/mg protein (range, 0.07–2.83 ng/mg protein). A weak but significant linear association was found between s-suPAR and age in the donors; thus, all of the s-suPAR levels were adjusted for this age dependency (aa-s-suPAR). The aa-s-suPAR levels were significantly increased in the patients as compared with the donors ( $P < 0.0001$ ). No difference was found in aa-s-suPAR levels between the lymph node-positive and -negative patients ( $P = 0.27$ ), and no correlation was seen between aa-s-suPAR and c-suPAR ( $\sigma = 0.08$ ;  $P = 0.71$ ). During the follow-up period (5.9 years) 77 patients experienced a relapse and 69 died. aa-s-suPAR as a continuous variable was significantly associated with relapse-free

survival [hazard ratio (HR), 1.4; 95% confidence interval (CI), 1.1–1.8;  $P = 0.003$ ] and overall survival (HR, 1.6; 95% CI, 1.2–2.0;  $P < 0.0001$ ). In multivariate Cox analysis including the classical prognostic parameters in breast cancer, continuous aa-s-suPAR was significantly associated with both relapse-free survival (HR, 1.4; 95% CI, 1.1–1.7;  $P = 0.001$ ) and overall survival (HR, 1.4; 95% CI, 1.1–1.8;  $P = 0.002$ ). In these analyses positive lymph nodes, tumor size  $>2$  cm, and negative estrogen receptor content were also significantly associated with patient outcome.

**Conclusion:** This study shows that high preoperative aa-s-suPAR levels are significantly associated with poor outcome for breast cancer patients independent of lymph node status, tumor size, and estrogen receptor status.

## INTRODUCTION

To select patients for adjuvant therapy there is a critical need to distinguish between breast cancer patients at high versus low risk of disease recurrence. At present, such an evaluation is mainly based on determination of cancer cell dissemination to ipsilateral axillary lymph nodes. Over the last years, there have been several reports on the tumor tissue concentrations of molecules involved in the aggressive malignant spread of cancer cells, *e.g.*, proteolytic enzymes secreted by either the cancer cells or other cells in the surrounding tumor stroma, and the possibility that elevated levels of these proteins reflect the metastatic potential of the tumor (1, 2). Among the proteases being investigated for their prognostic significance in breast cancer are the aspartic protease cathepsin D (3–5), the serine protease urokinase plasminogen activator (uPA; Refs. 6, 7),<sup>3</sup> the matrix metalloproteases such as collagenases and stromelysins (8, 9), and the cysteine proteases such as cathepsin B and L (10–12).

uPA is secreted as a virtually inactive single-chain proenzyme (pro-uPA), which by limited proteolysis can be converted into active uPA (13–15). uPA binds to its receptor, uPAR, which is a three-domain, glycolipid-anchored cell surface protein (16, 17). Binding of uPA to uPAR strongly en-

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<sup>3</sup> The abbreviations used are: uPA, urokinase plasminogen activator; aa-s-suPAR, age-adjusted serum soluble urokinase plasminogen activator receptor; CV, coefficient of variation; CI, confidence interval; c-suPAR, cytosol soluble urokinase plasminogen activator receptor; HR, hazard ratio; OS, overall survival; PAI-1, plasminogen activator inhibitor type 1; PAI-2, plasminogen activator inhibitor type 2; RFS, relapse-free survival; s-suPAR, serum soluble urokinase plasminogen activator receptor; suPAR(2+3), cleaved soluble urokinase plasminogen activator receptor containing domain 2 + 3; suPAR, soluble urokinase plasminogen activator receptor; uPAR, urokinase plasminogen activator receptor.

hances and localizes the activation of surface-bound plasminogen into plasmin (17, 18), which, with broad specificity, degrades most components of the extracellular matrix and basement membranes (19). The specific inhibitors PAI-1 and PAI-2 can inhibit the activity of uPA. Binding of the uPA:PAI complex to uPAR results in internalization of this tertiary complex with subsequent degradation of uPA:PAI and recycling of uPAR (20). suPAR is present in the circulation under normal physiological conditions (21–23). The mechanism(s) by which soluble forms of uPAR may be released from cell surfaces include the action of phospholipase D (24) and the activity of several proteases, which can cleave uPAR between domain 1 and 2 (25–27). It has been shown that uPAR and suPAR are detectable in extracts of tumor tissue prepared with and without detergent, respectively (25, 28), and in bodily fluids such as blood (21), urine (29), and cystic fluid (30). However, even though three suPAR forms have been identified, namely full-length, three-domain suPAR, suPAR(2 + 3), and liberated domain 1, all of the published data indicate that suPAR in blood, both from healthy blood donors and from cancer patients, consists of the full-length, three-domain protein (21).

We have reported recently on a highly significant association between the suPAR levels in preoperatively obtained citrate plasma and survival in colon and rectal cancer patients; high suPAR levels were associated with shorter survival in a Dukes' stage independent manner (31, 32). Furthermore, high suPAR levels in serum and citrate plasma were significantly associated with poor survival in ovarian cancer patients (33, 34). Finally, two independent studies found high tumor tissue suPAR levels to be associated with shorter survival of breast cancer patients (7, 28).

In the present study, we measured suPAR levels in preoperatively obtained sera from 274 patients with primary breast cancer and in sera from 174 healthy female blood donors. In addition, suPAR was measured in breast tumor cytosols from 188 of the patients. We found a highly significant association between preoperative aa-s-suPAR levels and breast cancer patient survival; the association being independent of clinical parameters including nodal status. In addition, this study confirmed our previous finding of an association between c-suPAR levels and OS.

## MATERIALS AND METHODS

**Female Blood Donors.** Serum samples were drawn from 113 volunteer female blood donors (median age, 40; range, 17–58 years) attending the blood bank at the John Radcliffe Hospital, Oxford, England. In addition, a set of sera obtained from 61 healthy Danish volunteer donors (median age, 65; range, 51–79 years) was available for this study.

**Breast Cancer Patients.** Included in this study were 274 patients who underwent surgery for histologically verified primary breast cancer at John Radcliffe Hospital, Oxford, England, between October 1990 and December 1994. Patients were entered into the study consecutively provided that a preoperative serum sample was available. None of the patients had evident distant metastases. For 188 of the patients a tumor tissue cytosol prepared from the primary breast tumor was available for suPAR measurements. Sixty-six of the patients (24%) underwent

modified radical mastectomy and 208 (76%) had lumpectomy of whom 189 (91%) received breast irradiation. The remaining 19 patients did not receive irradiation because of the following: (a) radiation therapy planned but the patient progressed while receiving chemotherapy; (b) good prognostic features of the tumor, thus Tamoxifen was given alone with radiation therapy in reserve; or (c) the patient decided not to receive irradiation. All of the patients had axilla dissection, and axillary lymph nodes were histologically examined for the presence of cancer cells. If lymph node involvement was found then adjuvant radiotherapy was delivered to the axilla. Adjuvant systemic treatment consisted of 20 mg of Tamoxifen daily for 5 years for postmenopausal women regardless of hormonal receptor status, and six cycles of i.v. cyclophosphamide, methotrexate, and 5-fluorouracil delivered every 3 weeks for premenopausal women with positive lymph nodes. Information on adjuvant treatment was available for all but 1 patient (42 years old, node negative, tumor size  $\leq 2$  cm, ductal carcinoma grade II, estrogen receptor-positive, alive at follow-up) whose hospital record notes were missing. Two patients had a favorable prognosis on the basis of the histological examination of their tumors and were not offered any adjuvant treatment, 2 patients decided not to receive any adjuvant treatment, and 1 patient had a too-poor performance status (80-year-old, died 1 year after the primary operation). The 4 other patients who did not receive any adjuvant treatment were all alive at the time of last follow-up. All of the patients were followed every 3 months for the first 18 months after the operation and every 6 months thereafter. Treatment for confirmed recurrent disease was delivered by endocrine manipulation of soft tissue or skeletal metastases, or by chemotherapy for visceral disease and failed endocrine therapy. Clinical and pathological data were registered for all of the patients (Table 1). The median time of observation from date of operation to the date of data analysis was 5.9 years. During the observation period 77 (28%) patients experienced a relapse and 69 (25%) died. The actual cause of death was not available for all of the patients; thus, recording of survival was based on death from all causes.

**Blood Samples.** All of the blood samples were drawn with informed consent in accordance with the 1996 Helsinki Declaration and with approval from the local ethical committees.

Blood samples from the English women (donors and preoperative from breast cancer patients) were drawn into dry tubes, allowed to clot at 4°C for maximal 2 h, and the sera separated at 1600  $\times g$  at 4°C for 10 min. The blood samples from the Danish women were drawn into dry tubes, allowed to clot at 20°C for maximal 3 h, and the sera separated at 1500  $\times g$  at 20°C for 10 min. All of the sera were stored at  $-80^{\circ}\text{C}$  until analysis.

**Tumor Cytosols.** The tumors were collected into polythene bags, immersed in ice for transport to the pathology laboratory, and then cut up by a pathologist, selecting tumor areas for snap freezing in liquid nitrogen. The frozen tumor tissue was pulverized in pestles cooled by liquid nitrogen, then extracted by addition of 20 ml of ice-cold homogenizing buffer [20 mM HEPES, 1.5 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 0.5 mM benzamidine, and 10  $\mu\text{g/ml}$  trypsin inhibitor (pH 7.4; all reagents from Sigma Chemical Co., St. Louis, MO)] to 1 g cancer tissue using a Ultra Turrax T24 (Janke and Kunkel

Table 1 Patient and tumor characteristics<sup>a</sup>

	n	(%)
Age		
<41	19	(7)
41–50	69	(25)
>50	186	(68)
Tumor size		
≤2 cm	144	(53)
>2 cm	130	(47)
Positive lymph nodes		
no	164	(58)
yes	110	(42)
Estrogen receptor		
Positive <sup>b</sup>	178	(65)
Negative	96	(35)
Menopausal status		
Pre <sup>c</sup>	88	(32)
Post	186	(68)
Histology		
Ductal	237	(86)
Nonductal	37	(14)
Tumor grade <sup>d</sup>		
I	37	(16)
II	94	(40)
III	61	(26)
Unknown	45	(19)
Tumor tissue cytosol	188	(69)
Relapses	77	(28)
Deaths	69	(25)

<sup>a</sup> Median age (range), 58 (26–83).

<sup>b</sup> ≥fmol/mg protein.

<sup>c</sup> Age ≤50 years.

<sup>d</sup> Ductal carcinomas.

GmbH; IKA Labortechnik, Germany) for 5 min, followed by centrifugation at  $100,000 \times g$  at 4°C for 45 min. The resulting supernatant (cytosol) was separated from the pellet and stored at –80°C. This extraction without detergent produces a supernatant containing only suPAR and not the glycolipid-anchored uPAR present on cell membranes.

**suPAR ELISA.** The suPAR analyses were performed without knowledge of the clinical and pathological data including patient outcome.

s-suPAR were measured with the kinetic ELISA described by Riisbro *et al.* (35), which is a modified version of the suPAR ELISA described previously (31). The modifications were applied because of the finding of a variable and often high non-specific signal in noncoated blocked wells when analyzing serum, whereas a constant low nonspecific signal was obtained with citrate and EDTA plasma. The nonspecific signal could be totally removed in assays of plasma samples and significantly lowered in assays of serum samples by addition of 50 units/ml of unfractionated heparin (Sigma) to the assay buffer [0.81 M Na<sub>2</sub>HPO<sub>4</sub> (Merck, Darmstadt, Germany), 0.19 M NaH<sub>2</sub>PO<sub>4</sub> (Merck), 0.1 M NaCl (Merck), 10 g/liter BSA (Fraction V; Boehringer-Mannheim, Penzberg, Germany), 1 g/liter Tween 20 (Merck; pH 7.2)]. In addition, coating was performed with 3 μg/ml of the mouse monoclonal anti-uPAR antibody R2 overnight at 4°C. Samples and standards were incubated for 2 h at 30°C, and detection was performed with 1 μg/ml of rabbit polyclonal anti-suPAR antibody (immunoabsorbed against mouse IgG) overnight at 4°C. The remaining part of the assay

was performed as described previously (31). All of the incubations were performed using a volume of 100 μl/well. This suPAR assay was rigorously tested with dilution and recovery experiments, and immunoabsorption as described previously (31). The analytical sensitivity of the assay used in this study was 2.5 pg/ml (calculated from the blank measurements: mean rate +3 SD). A 10% dilution of the serum pool in assay buffer was included in each analysis as an internal control [interassay variation 8.6% ( $n = 13$ ); intra-assay variation 8.3% ( $n = 13$ )]. The serum pool had a suPAR concentration of 2.7 ng/ml, equivalent to the 34<sup>th</sup> centile of the donor sera. All of the serum samples were analyzed in duplicate diluted 1:10 in sample buffer (CV <15%).

c-suPAR levels were determined after validation of the assay using a cytosol pool made from equal volumes of 36 randomly chosen breast cancer cytosols. Good linearity was obtained in the range of 5–80% cytosol pool, and the suPAR recovery in a 20% cytosol pool was 105%. A 20% dilution of the cytosol pool in sample buffer was included in each analysis as an internal control [interassay variation 9.6% ( $n = 10$ ); intra-assay variation 5.5% ( $n = 6$ )]. All of the cytosol samples were analyzed in duplicate diluted 1:5 in sample buffer (CV <15%).

**Protein Assay.** The total protein concentration in the cytosols (diluted 1:10 in PBS) was analyzed by the BCA Protein Assay (Pierce Chemical Co., Rockford, IL) at the time of suPAR determination. The cytosol pool was included in each analysis as an internal control [intra-assay variation 2.4% ( $n = 12$ ); inter-assay variation 2.0% ( $n = 6$ )]. All of the samples were analyzed in duplicate (CV <15%).

**Statistical Methods.** The SAS software package (version 6.12; SAS Institute, Cary, NC) was used to manage the patient data and to perform all of the statistical analyses. Pearson moment correlations were used to calculate correlation coefficients, and ANOVA was used where applicable. Tests of independence were performed using the  $\chi^2$  test. Linear regression was used to estimate the age dependence of s-suPAR in the blood donors. All of the s-suPAR levels were adjusted for age dependency, resulting in an aa-s-suPAR level for each individual, that is the aa-s-suPAR level was defined as the observed s-suPAR level minus the expected s-suPAR level for the given age as estimated by linear regression of the donors (36). The Kaplan-Meier method was used to estimate survival probabilities and the log rank test to test for equality of strata. The Cox proportional hazards model was used for analysis of continuous covariates as well as for multivariate analysis. The assumption of proportional hazards was verified graphically. To illustrate the continuous association between aa-s-suPAR level and patient outcome, the patients were divided into four groups according to increasing aa-s-suPAR level. The following cut points were defined as: the mean aa-s-suPAR value minus 1 SD, the mean aa-s-suPAR value, and the mean aa-s-suPAR value plus 1 SD. Survival curves for patients grouped by dissemination to ipsilateral axillary lymph nodes were dichotomized by the mean of the aa-s-suPAR values. c-suPAR was scored as the actual value (log transformed). The patients were dichotomized by the median c-suPAR level for comparison with earlier published results on suPAR in breast tumor cytosols (28). The significance level was set to 5%.

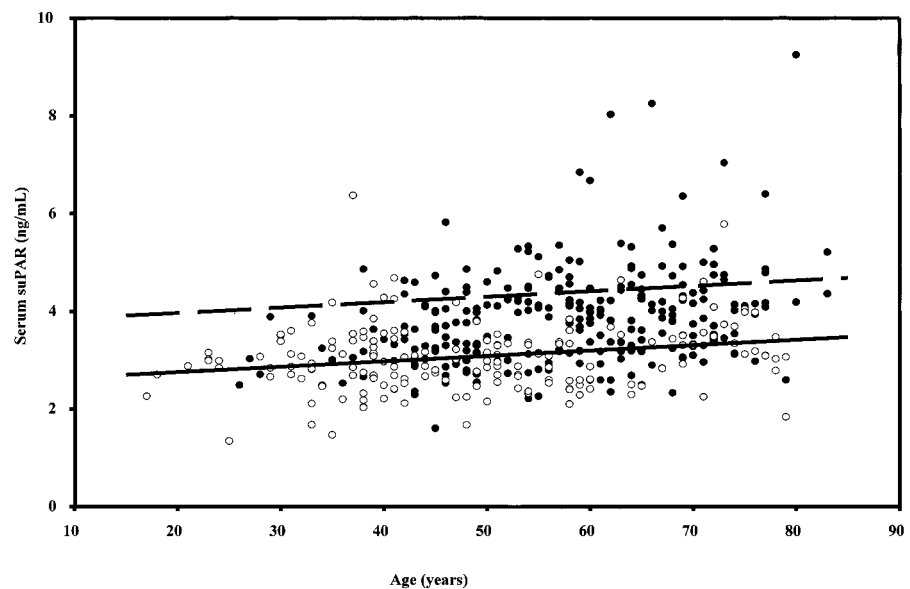


Fig. 1 Scatter plot of age and s-suPAR for the donors ( $\circ$ ,  $n = 174$ ) and the patients with primary breast cancer ( $\bullet$ ,  $n = 274$ ). The solid line shows the linear regression line between donor age and serum suPAR level; the broken line indicates the upper 95% CI; 24% of the patients had s-suPAR levels above the upper 95% CI.

## RESULTS

**suPAR in Sera from Female Blood Donors.** The English female blood donors ( $n = 113$ ; median age, 40 years) had a mean s-suPAR level of 3 ng/ml (median, 2.9; range, 1.3–5.8 ng/ml), and the Danish female blood donors ( $n = 61$ ; median age, 65 years) had a mean s-suPAR level of 3.2 ng/ml (median, 3.1; range, 1.7–6.4 ng/ml). The s-suPAR levels approached a normal distribution in both groups of blood donors.

Linear regression analysis of s-suPAR on age was performed for the pooled female blood donors corrected for sex. This correction was not significant, and it was therefore found justified to pool the two sets of samples from blood donors. A significant trend was found between donor age and s-suPAR level (s-suPAR (ng/ml) = 0.011 (ng/ml  $\times$  year)  $\times$  age (year) + 2.54 (ng/ml); SD = 0.70;  $P = 0.003$ ). Fig. 1 shows the trend between age and s-suPAR for the donors; the solid line indicates the estimated regression line, and the broken line shows its upper 95% CI.

**suPAR in Sera from Breast Cancer Patients.** The mean s-suPAR level of the patients was 3.8 ng/ml (median, 3.8; range, 1.6–9.2 ng/ml). The association between patient age and s-suPAR is also illustrated in Fig. 1 showing that 65 of the 274 patients (24%) have s-suPAR levels above the upper 95% CI of the donor s-suPAR level. Table 2 shows the associations between s-suPAR and axillary lymph node status, tumor size, estrogen receptor status, menopausal status, tumor histology, and tumor grade. The s-suPAR levels were significantly higher in postmenopausal patients as compared with premenopausal patients ( $P < 0.0001$ ). No significant associations were found with any of the other parameters including nodal status ( $P = 0.35$ ).

**Age-adjusted s-suPAR Levels.** Using the equation calculated above, the s-suPAR level in each patient was adjusted for the age dependency. The  $P$ s for tests of association between aa-s-suPAR and axillary lymph node status, tumor size, estrogen receptor status, menopausal status, tumor histology, and tumor

Table 2 Relationship between serum (s-suPAR) and cytosol suPAR (c-suPAR) levels, and patient and tumor characteristics

	s-suPAR ( $n = 274$ )		c-suPAR ( $n = 188$ )	
	mean, 95% CL (ng/mL)	$P$	g-mean, <sup>a</sup> 95% CI (ng/mg protein)	$P$
Positive lymph nodes				
No	3.8 (3.7–3.9)	0.35	0.41 (0.37–0.47)	0.19
Yes	3.9 (3.7–4.1)		0.47 (0.40–0.56)	
Tumor size				
$\leq 2$ cm	3.7 (3.6–3.9)	0.06	0.42 (0.36–0.49)	0.51
$> 2$ cm	4.0 (3.8–4.1)		0.45 (0.39–0.52)	
Estrogen receptor				
Positive <sup>b</sup>	3.8 (3.6–4.0)	0.86	0.40 (0.36–0.45)	0.01
Negative	3.8 (3.7–4.0)		0.52 (0.43–0.62)	
Menopausal status				
Pre <sup>c</sup>	3.4 (3.3–3.6)	$< 0.0001$	0.50 (0.43–0.59)	0.05
Post	4.0 (3.9–4.2)		0.41 (0.34–0.46)	
Histology				
Ductal	3.8 (3.7–4.0)	0.68	0.45 (0.41–0.50)	0.04
Nonductal	3.9 (3.6–4.2)		0.34 (0.15–0.45)	
Tumor grade <sup>d</sup>				
I	3.5 (3.3–3.7)	0.14	0.45 (0.33–0.60)	0.27
II	3.8 (3.6–4.0)		0.41 (0.35–0.49)	
III	3.8 (3.6–4.1)		0.51 (0.43–0.61)	

<sup>a</sup> Geometric mean.

<sup>b</sup>  $\geq 10$  fmol/mg protein.

<sup>c</sup>  $\leq 50$  years.

<sup>d</sup> Only ductal carcinomas.

grade were 0.27, 0.06, 0.99, 0.09, 0.34, and 0.11, respectively. The significant difference found in s-suPAR levels between pre- and postmenopausal patients disappeared when the s-suPAR levels were age adjusted.

**suPAR in Breast Tumor Cytosols.** suPAR was measured in corresponding tumor cytosols from 188 (69%) of the patients. The mean c-suPAR level was 0.55 ng/mg protein (median, 0.44; range, 0.07–2.83 ng/mg protein). The associations between c-suPAR and parameters with known prognostic

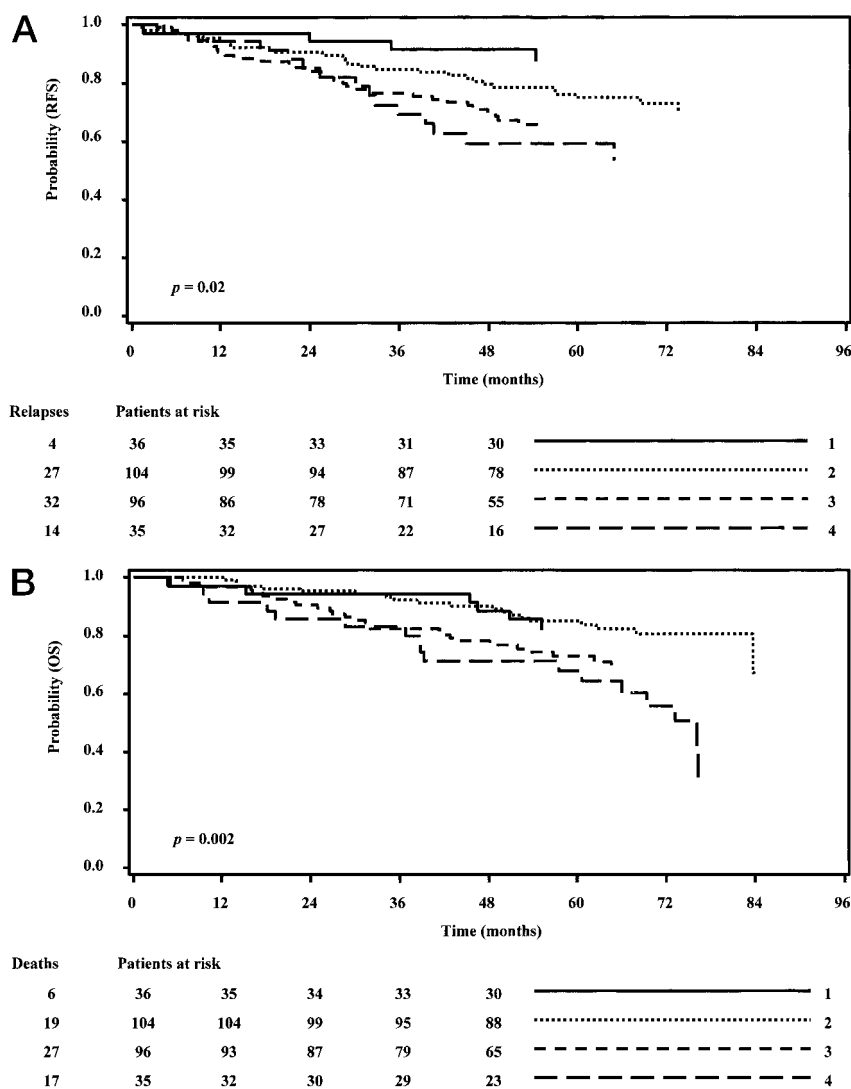
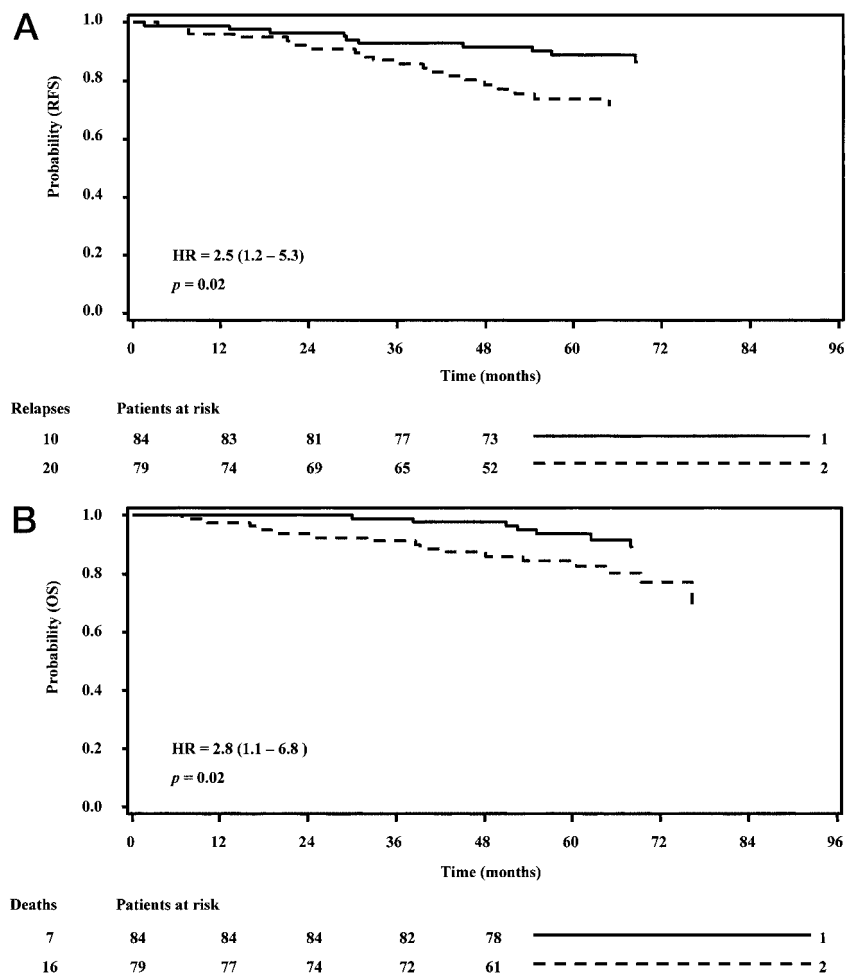


Fig. 2 Survival curves showing the association between aa-s-suPAR and RFS (a), and aa-s-suPAR and OS (b) for all patients. The patients were stratified into four groups with increasing aa-s-suPAR levels. Group 1 was defined as: aa-s-suPAR ≤ mean aa-s-suPAR value - SD; group 2 as: mean aa-s-suPAR value - SD < aa-s-suPAR < mean aa-s-suPAR; group 3 as: mean aa-s-suPAR < aa-s-suPAR ≤ mean aa-s-suPAR + SD; and group 4 as: aa-s-suPAR > mean aa-s-suPAR + SD. The numbers of events during the follow-up period and the numbers of patients at risk after each 12-month interval in each group are indicated below each figure. The log-rank test was used to test for differences between strata.

value in breast cancer are shown in Table 2. c-suPAR showed a significant positive association with both menopausal status ( $P = 0.05$ ) and histological subtype ( $P = 0.04$ ), whereas a significant negative association was found with estrogen receptor status ( $P = 0.01$ ). However, c-suPAR did not correlate with either s-suPAR ( $\sigma = 0.05$ ;  $P = 0.47$ ) or aa-s-suPAR ( $\sigma = 0.08$ ;  $P = 0.28$ ).

**Association of aa-s-suPAR with Survival.** Survival analyses were performed for 271 (99%) patients for whom there was complete information on RFS and OS. During the follow-up period 77 (28%) of these patients experienced a relapse and 69 (25%) died. The 2 patients who decided not to receive adjuvant treatment and the patient who had too-poor performance status to receive any adjuvant treatment were excluded from the analyses. Lymph nodal status was significantly associated with RFS ( $P < 0.0001$ ) and OS ( $P < 0.0001$ ), as were tumor size ( $P < 0.0001$  for RFS and  $P < 0.0001$  for OS) and estrogen receptor status ( $P = 0.005$  for RFS and  $P = 0.002$  for OS). Menopausal status and patient age were not significantly associated with

survival, but the group of premenopausal patients ≤40 years of age had a significantly shorter RFS ( $P = 0.03$ ) but not OS ( $P = 0.67$ ) as compared with premenopausal patients >40 years of age. aa-s-suPAR level treated as a continuous variable was statistically significantly associated with RFS (HR, 1.4; 95% CI, 1.2–1.9;  $P = 0.003$ ) and OS (HR, 1.6; 95% CI, 1.3–2.0;  $P < 0.0001$ ). The Kaplan-Meier survival curves for RFS and OS in all of the patients stratified into four groups (see “Materials and Methods”) with increasing aa-s-suPAR levels are shown in Fig. 2. A significant trend existed between aa-s-suPAR levels and patient survival; shorter survival was found with increasing aa-s-suPAR levels ( $P = 0.02$  for RFS and  $P = 0.002$  for OS). Analyses of RFS and OS were performed individually for the group of lymph node-positive and lymph node-negative patients. The patients in each subgroup were divided into two groups according to the mean aa-s-suPAR level. In the node-negative patients, a significant association was found between aa-s-suPAR and RFS (HR, 2.5; 95% CI, 1.2–5.3;  $P = 0.02$ ) and OS (HR, 2.8; 95% CI, 1.1–6.8;  $P = 0.02$ ); aa-s-suPAR levels



**Fig. 3** Survival curves showing the association between aa-s-suPAR and RFS (a), and aa-s-suPAR and OS (b) for the lymph node-negative patients. The patients have been divided into two groups by the mean aa-s-suPAR level. The numbers of events during the follow-up period and the numbers of patients at risk after each 12-month interval are indicated below each figure. The log-rank test was used to test for differences between strata. HR and 95% CI were calculated by Cox regression.

above the mean were associated with shorter survival (Fig. 3). In the patients with positive axillary lymph nodes, a trend between high aa-s-suPAR and shorter RFS was found (HR, 1.7; 95% CI, 0.9–3.0;  $P = 0.08$ ), whereas aa-s-suPAR was significantly associated with OS (HR, 2.0; 95% CI, 1.1–3.6;  $P = 0.02$ ). The Kaplan-Meier survival curves are shown in Fig. 4. Similar results were obtained when aa-s-suPAR was replaced by s-suPAR (data not shown). Table 3 summarizes the results of the univariate survival analyses. Dichotomizing the aa-s-suPAR levels by the 95% confidence limit demonstrated for patients with elevated s-suPAR a significantly shorter RFS (HR, 1.7; 95% CI, 1.1–2.8;  $P = 0.03$ ), and a significantly shorter OS (HR, 1.2; 95% CI, 1.2–3.3;  $P = 0.005$ ). Thus, there were significantly more relapses/deaths in this group of patients compared with the overall number of relapses/deaths in the patient population ( $P = 0.05$  and  $P = 0.003$ , respectively). A similar analysis but using the 95th percentile of the donors without age correction (4.3 ng/ml) showed that it was not possible to demonstrate a significant difference for RFS (HR, 1.5; 95% CI, 0.9–2.4;  $P = 0.09$ ), whereas there still was a significant difference for OS (HR, 1.9; 95% CI, 1.2–3.1;  $P = 0.008$ ).

**Association of c-suPAR with Survival.** No significant association was found between continuous c-suPAR and RFS

(186 patients, 53 relapses; HR, 1.1; 95% CI, 0.8–1.7;  $P = 0.55$ ), whereas a trend was found between increasing c-suPAR and shorter OS (186 patients, 48 deaths; HR, 1.5; 95% CI, 1.0–2.3;  $P = 0.06$ ). For comparison with data published previously (28), additional analyses of RFS and OS were performed using c-suPAR stratified according to the median c-suPAR level (0.44 ng/mg protein). No significant association was found between RFS and c-suPAR (HR, 1.1; 95% CI, 0.7–1.9;  $P = 0.67$ ), whereas high c-suPAR ( $\geq 0.44$  ng/mg protein) was significantly associated with shorter OS (HR, 2.4; 95% CI, 1.3–4.4;  $P = 0.004$ ; data not shown).

**Multivariate Analyses.** Multivariate Cox analyses of RFS (77 relapses) and OS (69 deaths) included aa-s-suPAR and age as continuous variables, lymph node status, tumor size, estrogen receptor status, and menopausal status as dichotomized variables, and if premenopausal then age dichotomized by 40 years. Scoring of the aa-s-suPAR as a continuous variable adequately fit a linear model in the analyses. As seen from Table 3, aa-s-suPAR was independently associated with RFS (HR, 1.3; 95% CI, 1.1–1.7;  $P = 0.01$ ) and OS (HR, 1.4; 95% CI, 1.1–1.8;  $P = 0.002$ ). Additional multivariate analyses of RFS (186 patients, 53 relapses) and OS (184 patients, 48 deaths) were performed including all of the above-mentioned variables to

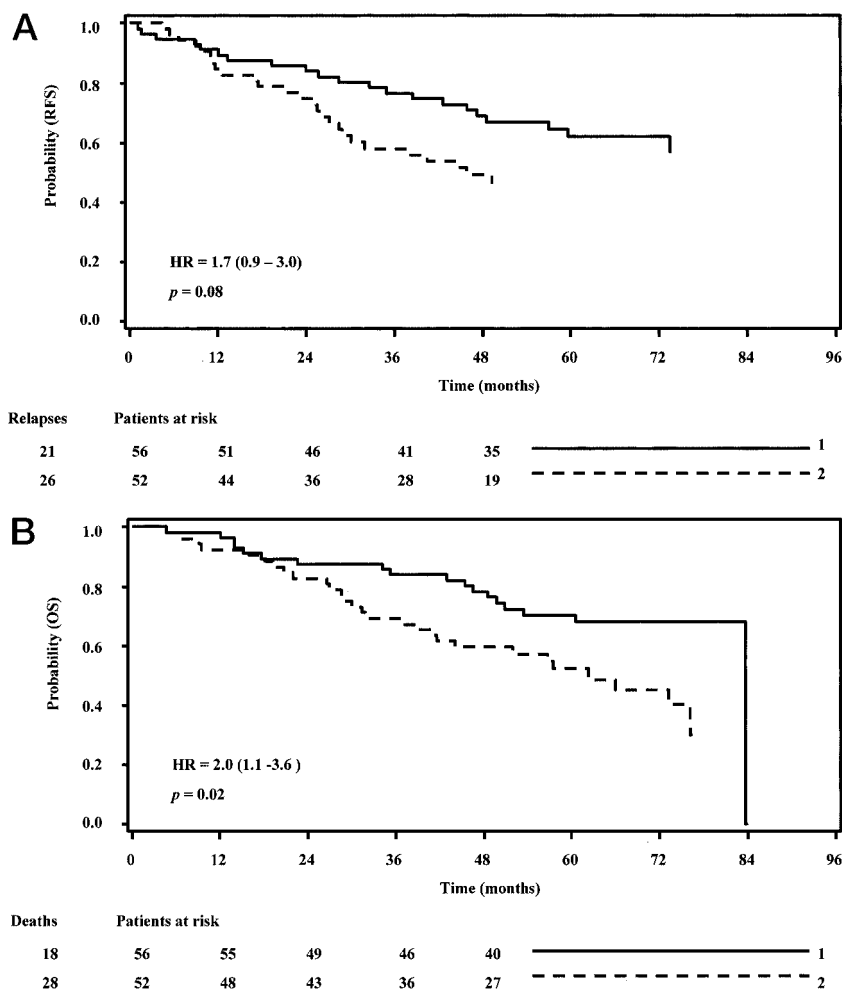


Fig. 4 Survival curves showing the association between aa-suPAR and RFS (a), and the aa-suPAR and OS (b) for the lymph node-positive patients. The patients have been divided into two groups by the mean aa-suPAR level. The numbers of events during the follow-up period and the numbers of patients at risk after each 12-month interval are indicated below each figure. The log-rank test was used to test for differences between the strata. HR and 95% CI were calculated by COX regression. b, the last patient at risk in the group with low aa-suPAR levels died after 83 months of follow-up.

gether with c-suPAR as a continuous variable. In these analyses together with nodal status and tumor size aa-s-suPAR was an independent marker for both RFS (HR, 1.4; 95% CI, 1.0–1.8;  $P = 0.02$ ) and OS (HR, 1.3; 95% CI, 1.0–1.8;  $P = 0.03$ ), whereas estrogen receptor level was rejected from the model. c-suPAR was not significantly associated with either RFS (HR, 1.0; 95% CI, 0.7–1.4;  $P = 0.84$ ) or OS (HR, 1.4; 95% CI, 0.9–2.2;  $P = 0.14$ ). Excluding aa-s-suPAR from the above analyses did not change the information given by c-suPAR (data not shown). In subsequent multivariate analyses of RFS and OS, the patients were dichotomized by the median c-suPAR level; c-suPAR level below the median was associated with longer survival (HR, 2.1; 95% CI, 1.1–3.9;  $P = 0.03$ ), whereas c-suPAR was not significantly associated with RFS (HR, 0.9; 95% CI, 0.5–1.6;  $P = 0.73$ ). Only minor changes were seen in all of the other parameters included in these multivariate analyses (data not shown). Tumor grade was not included in the multivariate analysis because of missing information on 45 of the 237 patients (19%) with ductal carcinomas. Table 3 summarizes the results of the multivariate analyses.

## DISCUSSION

To our knowledge this is the first study to report an association between survival and suPAR levels measured in preoperatively obtained sera from patients with primary breast cancer. The results obtained from this retrospective study show that there is a continuously increasing risk of breast cancer morbidity and mortality with increasing serum suPAR levels. Multivariate analyses of RFS and OS showed that aa-s-suPAR level was a statistically significant prognostic marker independent of ipsilateral axillary lymph node involvement, estrogen receptor status, menopausal status, and tumor size.

This study was based on a modified suPAR kinetic ELISA that with high specificity and sensitivity detects any forms of suPAR that contain the domain 3 epitope, which is recognized by the R2 monoclonal antibody used for coating (31). These forms include full-length suPAR, suPAR(2 + 3), and uPA/suPAR complexes with and without bound PAI-1, which would all be detected by the rabbit polyclonal anti-suPAR antibody used for primary detection. The primary modification by including heparin in the assay buffer was necessitated by variable

Table 3 Univariate and multivariate Cox regression analyses of RFS and OS

	Univariate analysis						Multivariate analysis					
	RFS			OS			RFS			OS		
	HR	(95% CI)	P	HR	(95% CI)	P	HR	(95% CI)	P	HR	(95% CI)	P
All patients <sup>a</sup>												
Positive lymph nodes												
No	1.0			1.0			1.0			1.0		
Yes	3.0	(1.9–4.7)	<0.0001	3.4	(2.3–6.2)	<0.0001	2.5	(1.6–4.0)	<0.0001	3.3	(2.0–5.5)	<0.0001
Tumor size												
≤2 cm	1.0			1.0			1.0			1.0		
>2 cm	3.4	(2.0–5.5)	<0.0001	3.1	(1.9–5.3)	<0.0001	3.1	(1.8–5.1)	<0.0001	2.4	(1.4–4.1)	0.002
Menopausal status												
Pre <sup>b</sup>	1.0			1.0			1.0			1.0		
Post	1.0	(0.6–1.6)	0.89	1.5	(0.9–2.7)	0.12	0.3	(0.1–1.1)	0.08	0.5	(0.1–2.2)	0.38
Estrogen receptor												
Positive <sup>c</sup>	1.0			1.0			1.0			1.0		
Negative	0.5	(0.3–0.8)	0.005	0.5	(0.3–0.8)	0.002	0.5	(0.3–0.8)	0.005	0.5	(0.3–0.8)	0.002
Premenopausal												
40 years ≤ age < 50 years	1.0			1.0			1.0			1.0		
age < 40 years	2.5	(1.1–5.6)	0.03	1.3	(0.4–3.9)	0.67	3.6	(1.5–9.0)	0.006	2.0	(0.6–6.7)	0.25
Age (years) <sup>d</sup>	0.99	(0.97–1.01)	0.47	1.02	(1.00–1.02)	0.07	1.01	(0.97–1.04)	0.79	1.02	(0.99–1.06)	0.19
aa-s-suPAR (ng/ml) <sup>e</sup>	1.4	(1.1–1.8)	0.003	1.6	(1.3–2.0)	<0.0001	1.3	(1.1–1.7)	0.01	1.4	(1.1–1.8)	0.002
Patients with cytosol available <sup>f</sup>												
log c-suPAR (ng/mg protein) <sup>g</sup>	1.1	(0.8–1.7)	0.55	1.5	(1.0–2.3)	0.06	1.0	(0.7–1.4)	0.84	1.4	(0.9–2.2)	0.14
aa-s-suPAR (ng/ml) <sup>e</sup>	1.5	(1.1–1.9)	0.003	1.6	(1.2–2.1)	0.007	1.4	(1.0–1.8)	0.02	1.3	(1.0–1.8)	0.03

<sup>a</sup> Patients ( $n = 271$ ); relapses ( $n = 77$ ), deaths ( $n = 69$ ).

<sup>b</sup> ≤50 years.

<sup>c</sup> ≥10 fmol/mg protein.

<sup>d</sup> Continuous scale; increasing 1 year.

<sup>e</sup> Continuous scale; increasing 1.0 ng/ml.

<sup>f</sup> Patients ( $n = 186$ ); relapses ( $n = 53$ ), deaths ( $n = 48$ ).

<sup>g</sup> Continuous scale; increasing 2.7 ng/ml.

ELISA signals significantly higher than the background in non-coated wells found when analyzing serum samples. In assays of citrate or EDTA plasma samples, the nonspecific signal was constantly low. The nonspecific signal in the assay of serum samples was significantly reduced and totally removed in assays of plasma samples by addition of 50 units/ml unfractionated heparin to the assay buffer. Theoretically, the negatively charged sulfated sugars in the heparin salt may have prevented substances in the blood, especially in serum, adhering to the positively charged surface of the plastic in the wells.

In a previous study we reported a median s-suPAR level of 2.6 ng/ml (range, 1.5–4.9 ng/ml) in 42 healthy women (35). Thus, the serum suPAR level in healthy women found in the present study (median, 3.0; range 1.3–6.4 ng/ml;  $n = 174$ ) is in agreement with the suPAR levels reported previously in healthy women. However, contrary to the finding in the present study, we found no correlation between donor age and s-suPAR in the previous study, but this is probably because of the lower number of healthy women in that study. Therefore, we suggest that use of s-suPAR in a clinical context would require a correction for age.

In the present study, we show that breast cancer patients, independent of stage of disease, have higher s-suPAR levels than healthy women. In addition, we confirm the association between c-suPAR levels and patient outcome found previously (28). The assay used in that study consisted of a rabbit polyclonal catching antibody raised against recombinant suPAR and

detecting with biotinylated R2, R3, and R5. R3 and R5 recognize different epitopes in domain 1 of suPAR, and R2 recognizes epitope on domain 3 (37, 38). The median suPAR level found in 505 cytosols was 0.87 ng/mg protein (range, 0.0–5.81 ng/mg protein), somewhat higher than the median c-suPAR (0.44; range, 0.07–2.83 ng/mg protein) found in the present study. The higher c-suPAR levels found by Grøndahl-Hansen *et al.* could be attributable to differences in the assays especially the specificities against different suPAR forms measured by the assays. Furthermore, it has been reported that the use of biotinylated antibodies in assays of suPAR in plasma samples results in a nonspecific signal because of interaction with unknown plasma proteins (39), and it could be speculated that a similar interaction takes place in assays of cytosols, thus explaining the higher c-suPAR levels in the first study. In addition, Grøndahl-Hansen *et al.* reported a HR of 1.47 (95% CI, 1.09–1.99), whereas we in this study found a HR of 2.4 (95% CI, 1.3–4.4). One possible explanation for this difference is that the study by Grøndahl-Hansen *et al.* contains 505 patients and, therefore, has a higher statistical power to determine the HR than the present study on only 186 cytosols. Furthermore, the patient populations are different in the two studies; more patients had large tumors, more patients had positive lymph nodes in the study by Grøndahl-Hansen *et al.*, and different treatment modalities were used. Recently, Foekens *et al.* (7) published a study on the levels of uPA, suPAR, PAI-1, and PAI-2 in breast tumor cytosols from 2780 patients with primary breast cancer. In this study, c-suPAR



determined by the same assay as used by Grøndahl-Hansen *et al.* was of independent prognostic value for both RFS and OS when included together with classical prognostic parameters in a multivariate analysis, but c-suPAR was excluded from both models when cytosolic uPA, PAI-1, and PAI-2 were included.

Surprisingly, no correlation was found between either aa-suPAR or s-suPAR and c-suPAR; thus, the increased s-suPAR concentration found in the breast cancer patients may not only be the result of an increased amount of uPAR being shed from the tumor tissue. Furthermore, the release of suPAR from the tumor to the circulation may not be proportional to the amount of uPAR present in the tumor tissue. In addition, some of the increase in blood suPAR level could be because of a systemic reaction to the malignant disease, including activation of blood monocytes and neutrophils.

The prognostic value of aa-suPAR was assessed by comparing the survival of the patients stratified into four groups with increasing aa-suPAR levels, and a significant association was observed between higher aa-suPAR levels and shorter survival, for both RFS and OS. It is of interest in the analysis of RFS to note that in the group of patients with low aa-suPAR, only 4 of the 36 patients (11%) experienced a relapse during the observation period compared with 40% (14 of 35) in the group with high aa-suPAR levels. Similarly, in the analysis of OS only 6 of the 36 patients (17%) in the low aa-suPAR group experienced an early death compared with 49% in the group with high aa-suPAR levels. The prognostic value of aa-suPAR was also tested in the subgroups of lymph-node positive and negative patients, and although only a limited number of relapses were registered during the observation period, aa-suPAR was statistically associated with RFS and with OS in the lymph node-negative patients. In the lymph node-positive patients, a nearly significant association was found between aa-suPAR and RFS, whereas aa-suPAR was significantly associated with OS. In multivariate analyses together with classical parameters with known prognostic value in breast cancer, aa-suPAR appeared to be an independent predictor of both relapse and early death. Similar results were obtained when replacing aa-suPAR with s-suPAR in these analyses. Additional multivariate analyses, including also c-suPAR as a continuous variable showed that c-suPAR, had no prognostic value in predicting relapse or early death, when only aa-suPAR was included in the model together with classic prognostic parameters. Similar results were obtained when including c-suPAR dichotomized by the median.

Numerous previous publications including one prospective randomized study have described the level of tumor tissue uPA and PAI-1 as strong and independent prognostic markers in primary breast cancer (6, 40–42). However, to perform these analyses, freshly frozen tumor tissue has to be available, and the proteins have to be extracted from the tissue before analysis. Furthermore, the distribution of these molecules may be heterogeneous in the tumor tissue, thus the pieces of tumor tissue used for these analyses may not always be representative for the entire malignant tumor. Our finding, that the concentration of suPAR in preoperatively obtained serum is significantly associated with patient outcome, may circumvent the problems encountered in obtaining representative samples of fresh tumor tissue.

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