# The Urokinase System of Plasminogen Activation and Prognosis in 2780 Breast Cancer Patients[1]

**John A. Foekens,[2] Harry A. Peters, Maxime P. Look, Henk Portengen, Manfred Schmitt, Michael D. Kramer, Nils Brünner, Fritz Jänicke, Marion E. Meijer-van Gelder, Sonja C. Henzen-Logmans, Wim L. J. van Putten, and Jan G. M. Klijn**

*Division of Endocrine Oncology, Department of Medical Oncology [J. A. F., H. A. P., M. P. L., H. P., M. E. M-v. G., J. G. M. K.], and Departments of Statistics [W. L. J. v. P.] and Pathology [S. C. H-L.], Rotterdam Cancer Institute (Daniel den Hoed Kliniek)/Academic Hospital, NL-3075 Rotterdam, the Netherlands; the Frauenklinik der Technischen Universität München, Klinikum rechts der Isar, D-81675 München, Germany [M. S.]; Institut für Immunologie und Serologie der Universität, D-69120 Heidelberg, Germany [M. D. K.]; Finsen Laboratory, Rigshospitalet, DK-2100 Copenhagen, Denmark [N. B.]; and Frauenklinik und Poliklinik, Universitäts-Krankenhaus der Universität Hamburg, D-20246 Hamburg, Germany [F. J.]*

## ABSTRACT

**The antigen levels of components of the urokinase-type plasminogen activator (uPA) system of plasminogen activation are correlated with prognosis in several types of cancers, including breast cancer. In the present study involving 2780 patients with primary invasive breast cancer, we have evaluated the prognostic importance of the four major components of the uPA system [uPA, the receptor uPAR (CD87), and the inhibitors PAI-1 and PAI-2]. The antigen levels were determined by ELISA in cytosols prepared from primary breast tumors. The levels of the four factors significantly correlated with each other; the Spearman rank correlation coefficients ($r_s$) ranged from 0.32 (between PAI-2 and PAI-1 or uPAR) to 0.59 (between uPA and PAI-1). The median duration of follow-up of patients still alive was 88 months. In the multivariate analyses for relapse-free survival (RFS) and overall survival (OS), we defined a basic model including age, menopausal status, tumor size and grade, lymph node status, adjuvant therapy, and steroid hormone receptor status. uPA, uPAR, PAI-1, and PAI-2 were considered as categorical variables, each with two cut points that were established by isotonic regression analysis. Compared with tumors with low levels, those with intermediate and high levels showed a relative hazard rate (RHR) and 95% confidence interval (95% CI) of 1.22 (1.02–1.45) and 1.69 (1.39–2.05) for uPA, and 1.32 (1.14–1.54) and 2.17 (1.74–2.70) for PAI-1, respectively, in multivariate analysis for RFS in all patients. Compared with tumors with high PAI-2 levels, those with intermediate and low levels showed a poor RFS with a RHR (95% CI) of 1.30 (1.14–1.48) and 1.76 (1.38–2.24), respectively. Similar results were obtained in the multivariate analysis for OS in all patients. Furthermore, uPA and PAI-1 were independent predictive factors of a poor RFS and OS in node-negative and node-positive patients. PAI-2 also added to the multivariate models for RFS in node-negative and node-positive patients, and in the analysis for OS in node-negative patients. uPAR did not further contribute to any of the multivariate models. A prognostic score was calculated based on the estimates from the final multivariate model for RFS. Using this score, the difference between the highest and lowest 10% risk groups was 66% in the analysis for RFS at 10 years and 61% in the analysis for OS. Moreover, separate prognostic scores were calculated for node-negative and node-positive patients. In the 10% highest risk groups, the proportion of disease-free patients was only 27 ± 6% and 9 ± 3% at 10 years for node-negative and node-positive patients, respectively. These proportions were 86 ± 4% and 61 ± 6% for the corresponding 10% lowest risk groups of relapse. We conclude that several components of the uPA system are potential predictors of RFS and OS in patients with primary invasive breast cancer. Knowledge of these factors could be helpful to assess the individual risk of patients, to select various types of adjuvant treatment and to identify patients who may benefit from targeted therapies that are currently being developed.**

## INTRODUCTION

Cancer cell invasion and metastasis result from a coordinated interaction between proteolytic enzymes degrading the ECM[3] and the adhesive proteins playing a role in cell attachment and migration. Data from preclinical and clinical studies point toward a central role for the uPA system in these processes (reviewed in Refs. 1–4). The serine protease uPA, which binds to a specific cell surface receptor uPAR (5, 6), facilitates the conversion of plasminogen into the serine protease plasmin. This wide-spectrum protease is able to degrade most components of the ECM directly or indirectly through activation of metalloproteinases, which subsequently degrade collagens and other matrix proteins (reviewed in Ref. 7). The activity of uPA can be inhibited by the serpin inhibitors PAI-1 and PAI-2 (8). In addition, most components of the uPA system of plasminogen activation have been linked to cell adhesion and migration through both proteolytic and nonproteolytic mechanisms (reviewed in Refs. 1–3). Cell migration requires the interaction of cell-bound adhesion receptors, such as integrins and uPAR, with their ECM-associated ligands such as vitronectin (9–12). Binding of uPA, or fragments of uPA containing only the receptor binding domain, enhance binding of uPAR to vitronectin. PAI-1 can inhibit integrin and uPAR binding to vitronectin, thus directing a stepwise cell migration by allowing tumor cells to be attached or alternatively being detached from the ECM (10, 11, 13, 14).

Duffy *et al.* (15) were the first to link increased levels of uPA activity in breast tumor extracts with a high rate of relapse in patients with breast cancer. This important finding of an association between uPA and a poor prognosis has been confirmed by various research groups measuring uPA antigen levels (16) in breast tumors, as well as in a variety of other cancer types (reviewed in Refs. 4, 17). Interestingly, immunocytologically detected uPA-positive tumor cells in bone marrow from primary breast cancer patients were predictive of a poor prognosis (18). Moreover, as reported by Jänicke *et al.* (19), surprisingly at first, increased levels of the inhibitor PAI-1 were associated with a poor prognosis in primary breast cancer (4, 17), and like uPA, also in recurrent breast cancer treated with tamoxifen (20). These findings can now partly be explained by the recently ascribed role of PAI-1 in tumor cell adhesion and migration (1–3). As would be expected, high tumor levels of uPAR were associated with a poor prognosis (21, 22), and high levels of PAI-2 were associated with a favorable prognosis in patients with breast cancer (23, 24).

Because simultaneous measurement of the different components of the uPA system of plasminogen activation may provide more powerful prognostic information, we determined the levels of uPA, uPAR,

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[2] To whom requests for reprints should be addressed, at Josephine Nefkens Institute, Dr. Molewaterplein 50, Room Be426, 3015 GE Rotterdam, the Netherlands. Phone: (31)-10-4088 369; Fax: (31)-10-4088-377/365; E-mail: foekens@bidh.azr.nl.

[3] The abbreviations used are: ECM, extracellular matrix; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; PAI, plasminogen activator inhibitor; RFS, relapse-free survival; OS, overall survival; RHR, relative hazard rates; CI, confidence interval; df, degrees of freedom; $\Delta\chi^2$, increase in $\chi^2$; CV, coefficient of variation; ER, estrogen receptor; PgR, progesterone receptor; IRA, isotonic regression analysis.

PAI-1, and PAI-2 in breast tumors of 2780 patients and have correlated their levels with RFS and OS.

## MATERIALS AND METHODS

**Patients and Tissues.** Analysis of RFS and OS was performed in 2780 patients with primary, operable, invasive breast cancer. The selection of samples was based on the availability of 3486 stored cytosol extracts (in liquid nitrogen), which remained after routine ER and PgR analysis. Exclusion criteria were: patient tissue that was taken from a biopsy only (such as inoperable T4 tumors or tissue that was not obtained from the primary breast tumor); previous diagnosis of carcinoma, with the exception of basal skin carcinoma and cervical cancer stage I; metastatic disease at diagnosis (M1 patients) or evidence of disease within 1 month of primary surgery; neoadjuvant therapy; and insufficient follow-up documentation. In the case of mastectomy after an initial lumpectomy because of residual disease, mastectomy is considered the primary treatment. Median age of the patients at surgery (modified mastectomy, 1488 patients; breast conserving treatment, 1292 patients) was 57 years (range, 22–94 years). Radiotherapy was given to 75% of the patients: on the breast/thoracic wall in 1762 patients and/or on the axilla in 747 patients; and/or parasternal and/or supraclavicular lymph nodes in 873 patients. None of the 1405 node-negative patients received systemic adjuvant therapy. Of the 1375 node-positive patients, adjuvant chemotherapy (mainly cyclophosphamide/methotrexate/5-fluorouracil) was given to 446 patients (mainly premenopausal patients), whereas 208 patients received adjuvant hormonal therapy (mainly postmenopausal patients), either alone (185 patients) or in combination with chemotherapy (23 patients). All patients were examined routinely every 3–6 months during the first 5 years of follow-up and once a year thereafter. Of the 2780 patients included, 1297 patients (47%) showed evidence of disease during follow-up and count as failures in the analysis for RFS. One-hundred and seventy-two patients (6%) died without evidence of disease and were censored at last follow-up in the analysis of RFS. Nine-hundred and twelve patients (33%) died after a previous relapse. A total of 1084 (172 + 912) patients (39%) were failures in the analysis for OS. The median follow-up period of patients alive ($n$ = 1,696) was 88 months (range, 1–207 months). Further characteristics of patients and tumors are listed in Table 1.

**Assay of uPA, uPAR, PAI-1, PAI-2, ER, and PgR in Tumor Tissue Extracts.** Tumor tissues were stored in liquid nitrogen and pulverized in the frozen state with a microdismembrator as recommended by the European Organization for Research and Treatment of Cancer for processing of breast tumor tissue for cytosolic ER and PgR determinations (25). The resulting tissue powder was suspended in European Organization for Research and Treatment of Cancer receptor buffer (10 mM $K_2HPO_4$, containing 1.5 mM dipotassium chloride EDTA, 3 mM $NaN_3$, 10 mM monothioglycerol, and 10% v/v glycerol, pH 7.4). The suspension was centrifuged for 30 min at 100,000 × $g$ at 4°C to obtain the supernatant fraction (cytosol). ER and PgR levels were determined by ligand binding assay or enzyme immunoassay, as described before (26).

uPA, uPAR, PAI-1, and PAI-2 levels were determined in breast tumor cytosols with ELISAs. For uPA and PAI-1, ELISA (27, 28) reagents have been used that are commercially available in assay kits (American Diagnostica, Greenwich, 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 (22, 24, 29). To enable the assessment of the between-assay variations (% CV), in each assay-run an aliquot of a pooled breast cancer cytosol sample was analyzed. The between-assay CV was 12.7, 21.2, 14.3, and 8.4% for the uPA, uPAR, PAI-1, and PAI-2 assays, respectively. The within-

Table 1 *Relationships of uPA, uPAR, PAI-1, and PAI-2 with patient and tumor characteristics*

| Characteristic | Frequency[b] | Median value (interquartile range)[a] | | | |
|---|---|---|---|---|---|
| | | uPA | uPAR | PAI-1 | PAI-2 |
| All patients | 2780 | 0.75 (0.28–1.49)[c] | 0.94 (0.59–1.38)[c] | 15.2 (8.4–25.3)[c] | 2.35 (1.32–4.72)[c] |
| Age at surgery (yr) | | | | | |
| ≤40 | 324 | 0.76 (0.34–1.38) | 1.00 (0.67–1.51) | 15.5 (8.3–25.3) | 1.98 (1.18–4.23) |
| 41–55 | 998 | 0.76 (0.28–1.47) | 0.95 (0.59–1.39) | 13.8 (7.6–23.1) | 2.00 (1.12–3.90) |
| 56–70 | 943 | 0.79 (0.28–1.57) | 0.95 (0.61–1.35) | 15.4 (8.7–26.5) | 2.64 (1.42–5.52) |
| >70 | 515 | 0.64 (0.26–1.41) | 0.85 (0.54–1.28) | 16.5 (9.0–27.5) | 2.84 (1.65–5.90) |
| *P* | | 0.16[d] | <0.0005[e] | <0.001[d] | <0.0001[e] |
| Menopausal status | | | | | |
| Premenopausal | 1107 | 0.77 (0.29–1.44) | 0.96 (0.62–1.42) | 14.2 (7.9–23.2) | 1.95 (1.12–3.80) |
| Postmenopausal | 1673 | 0.73 (0.27–1.51) | 0.94 (0.58–1.35) | 15.7 (8.8–26.7) | 2.68 (1.47–5.62) |
| *P* | | 0.92[f] | 0.04[f] | 0.0002[f] | <0.0001[f] |
| Tumor size | | | | | |
| $T_1$ | 1184 | 0.73 (0.24–1.41) | 0.92 (0.57–1.31) | 13.5 (7.8–23.0) | 2.49 (1.37–5.31) |
| $T_2$ | 1334 | 0.80 (0.32–1.58) | 0.96 (0.61–1.43) | 16.2 (8.9–26.8) | 2.31 (1.33–4.38) |
| $T_{3/4}$ | 262 | 0.62 (0.24–1.29) | 0.97 (0.59–1.57) | 15.2 (8.7–25.3) | 1.96 (1.09–3.58) |
| *P* | | <0.001[d] | <0.005[e] | 0.0001[d] | <0.001[e] |
| Nodal status | | | | | |
| $N_0$ | 1405 | 0.73 (0.24–1.43) | 0.91 (0.56–1.33) | 14.3 (7.8–23.4) | 2.47 (1.39–5.01) |
| $N_{1–3}$ | 725 | 0.76 (0.30–1.49) | 0.96 (0.66–1.40) | 15.3 (8.6–25.8) | 2.47 (1.36–5.03) |
| $N_{>3}$ | 650 | 0.77 (0.31–1.57) | 0.97 (0.62–1.48) | 16.5 (9.2–27.0) | 2.02 (1.12–3.71) |
| *P* | | 0.11[e] | <0.005[e] | <0.0001[e] | <0.0001[d] |
| Grade | | | | | |
| Well | 43 | 0.24 (0.11–0.75) | 0.66 (0.38–1.02) | 8.6 (3.7–13.3) | 1.87 (1.24–4.90) |
| Moderate | 530 | 0.81 (0.32–1.42) | 0.98 (0.64–1.36) | 15.0 (8.6–24.1) | 2.73 (1.45–6.69) |
| Poor | 1540 | 0.78 (0.29–1.57) | 0.96 (0.61–1.42) | 15.8 (9.0–26.3) | 2.33 (1.32–4.53) |
| *P* | | 0.0002[d] | 0.005[d] | <0.0002[e] | <0.0005[d] |
| ER positive[g] | | | | | |
| No | 602 | 0.99 (0.40–1.78) | 1.21 (0.75–1.81) | 17.5 (9.3–31.3) | 2.82 (1.59–6.14) |
| Yes | 2102 | 0.66 (0.24–1.39) | 0.91 (0.57–1.26) | 14.4 (8.2–24.2) | 2.21 (1.26–4.39) |
| *P* | | <0.0001[f] | <0.0001[f] | <0.0001[f] | <0.0001[f] |
| PgR positive[g] | | | | | |
| No | 794 | 0.92 (0.35–1.77) | 1.04 (0.62–1.62) | 17.3 (9.4–30.6) | 2.63 (1.45–5.46) |
| Yes | 1866 | 0.66 (0.24–1.37) | 0.92 (0.58–1.28) | 14.1 (8.0–23.7) | 2.24 (1.26–4.57) |
| *P* | | <0.0001[f] | <0.0001[f] | <0.0001[f] | 0.0002[f] |

[a] All values in ng/mg protein.
[b] Because of unknown values, the numbers do not always add up to 2780.
[c] The full range in ng/mg of protein were: 0–24.4 for uPA; 0–37.0 for uPAR; 0–479 for PAI-1; and 0–829 for PAI-2.
[d] *P* for Kruskal-Wallis test.
[e] *P* for Wilcoxon-type test for trend.
[f] *P* for Wilcoxon Rank-Sum test.
[g] Cutoff point used for ER and PgR: 10 fmol/mg of protein.

Table 2 *Cox multivariate analysis of RFS and OS in 2780 patients*

| Factor | RFS | | OS | |
|---|---|---|---|---|
| | RHR[a] | *P* | RHR[a] | *P* |
| Basic model | | | | |
| Age and menopausal status[b] | | <0.0001 | | <0.0001 |
| Age premenopausal[c] | 0.68 (0.60–0.77) | | 0.75 (0.65–0.88) | |
| Age postmenopausal[c] | 0.94 (0.84–1.00) | | 1.24 (1.13–1.35) | |
| Post- *vs.* premenopausal | 1.43 (1.16–1.77) | | 1.35 (1.05–1.74) | |
| Tumor size | | <0.0001 | | <0.0001 |
| 2–5 cm *vs.* ≤2 cm | 1.43 (1.26–1.62) | | 1.45 (1.26–1.67) | |
| >5 cm *vs.* ≤2 cm | 1.85 (1.53–2.24) | | 1.87 (1.53–2.29) | |
| Nodal status | | <0.0001 | | <0.0001 |
| $N_{1–3}$ *vs.* $N_0$ | 2.02 (1.72–2.37) | | 2.08 (1.75–2.47) | |
| $N_{>3}$ *vs.* $N_0$ | 3.75 (3.22–4.36) | | 3.53 (2.99–4.17) | |
| Grade | | <0.0001 | | <0.005 |
| Moderate *vs.* poor | 0.72 (0.62–0.85) | | 0.74 (0.62–0.88) | |
| Well *vs.* poor | 0.61 (0.34–1.08) | | 0.65 (0.36–1.19) | |
| Missing *vs.* poor | 0.73 (0.56–0.96) | | 0.91 (0.78–1.05) | |
| Adjuvant therapy (yes *vs.* no) | 0.61 (0.52–0.71) | <0.0001 | 0.76 (0.64–0.89) | <0.001 |
| ER/PgR status[d] | | 0.42 | | <0.0001 |
| +/− *vs.* −/− | 0.97 (0.79–1.19) | | 0.82 (0.66–1.01) | |
| −/+ *vs.* −/− | 0.81 (0.61–1.08) | | 0.63 (0.46–0.86) | |
| +/+ *vs.* −/− | 0.91 (0.79–1.06) | | 0.63 (0.54–0.74) | |
| Missing *vs.* −/− | 0.80 (0.58–1.10) | | 0.65 (0.47–0.90) | |
| Additions to basic model: | | | | |
| +uPA[e,f] | | <0.0001 | | <0.0001 |
| Intermediate *vs.* low | 1.29 (1.10–1.52) | | 1.37 (1.15–1.64) | |
| High *vs.* low | 1.93 (1.64–2.28) | | 2.15 (1.79–2.59) | |
| +uPAR[e,g] | | <0.0001 | | <0.0001 |
| Intermediate *vs.* low | 1.10 (0.95–1.28) | | 1.01 (0.85–1.19) | |
| High *vs.* low | 1.36 (1.17–1.59) | | 1.32 (1.12–1.56) | |
| +PAI-1[e,h] | | <0.0001 | | <0.0001 |
| Intermediate *vs.* low | 1.50 (1.31–1.71) | | 1.43 (1.23–1.66) | |
| High *vs.* low | 2.52 (2.06–3.08) | | 2.64 (2.13–3.26) | |
| +PAI-2[e,i] | | 0.10 | | 0.99 |
| Intermediate *vs.* high | 1.12 (0.99–1.27) | | 1.00 (0.88–1.15) | |
| Low *vs.* high | 1.24 (0.99–1.57) | | 0.99 (0.76–1.29) | |

[a] Numbers in parentheses, 95% CI.
[b] Age and menopausal status (at surgery) combined.
[c] Age in decades tested separately for pre- and postmenopausal patients.
[d] Cut points used: 10 fmol/mg of protein for both.
[e] Added separately to the basic multivariate model.
[f] Low, ≤0.19 ($n$ = 560); intermediate, >0.19 and ≤1.21 ($n$ = 1313); high, >1.21 ng/mg of protein ($n$ = 907).
[g] Low, ≤0.57 ($n$ = 663); intermediate, >0.57 and ≤1.13 ($n$ = 1100); high, >1.13 ng/mg of protein ($n$ = 1017).
[h] Low, ≤9.33 ($n$ = 815; intermediate, >9.33 and ≤45.28 ($n$ = 1731); high, >45.28 ng/mg of protein ($n$ = 229).
[i] Low, ≤0.62 ($n$ = 165); intermediate, >0.62 and ≤4.05 ($n$ = 1804); high, >4.05 ng/mg of protein ($n$ = 811).

assay CVs of samples measured in duplicate were all <5%. During the period of the assays (>3 years), there was no significant change in the levels of any of the four factors in the breast cancer cytosol pool. These results suggest that the factors were stable during long-term storage of the cytosols.

**Statistics.** The strength of the associations between uPA, uPAR, PAI-1, and PAI-2 was tested with Spearman rank correlation ($r_s$). The associations of these factors with other variables were tested with the nonparametric Wilcoxon Rank-Sum test or Kruskal-Wallis test, followed by a Wilcoxon-type test for trend across ordered groups where appropriate. RFS and OS probabilities were calculated by the actuarial method of Kaplan and Meier (30). In our search for the best categorization of uPA, uPAR, and PAI-1, we have used IRA using RFS as end point (28, 31). After it had been established that in a test for trend using log-transformed variables increasing levels were significantly associated with RFS, an IRA was performed after correction for age/menopausal status, tumor size and grade, lymph node status, adjuvant therapy, and ER and PgR status. These factors defined the basic multivariate model for all patients that we incorporated in our analyses. Increasing PAI-2 levels were significantly associated with a favorable prognosis, however, only when uPA was also added to the basic multivariate model. For node-positive patients, nodal status was included as N>3 *versus* N1–3, and for node-negative patients, nodal status and adjuvant therapy were not applicable. The univariate and multivariate analyses, including tests for interactions, were performed using the Cox proportional hazards model (32). The assumption of proportional hazards was verified graphically. The associated likelihood ratio test was used to test for differences between models with variables included and excluded. In the multivariate analyses, the missing values for ER ($n$ = 76), PgR ($n$ = 120), and tumor grade ($n$ = 677) were treated as separate groups to allow inclusion of all patients in all models. The results from Cox analyses, including the basic model and the components of the uPA system that significantly added to the model, were used to classify patients into risk groups using as cut points the 10, 25, 50, 75, and 90 percentiles of the calculated linear score from the Cox analyses. The resulting risk groups are visualized by Kaplan-Meier curves. All computations were done with the STATA statistical package, release 6.0 (STATA Corp., College Station, TX). All *P*s are two-sided and relate to all available data during the total period of follow-up.

## RESULTS

**Levels and Associations.** Levels of uPA, uPAR, PAI-1, and PAI-2 expressed as ng/mg of protein were measured in cytosols of primary breast tumors using specific ELISAs. The levels determined in 2780 patients (Table 1) are comparable with those obtained previously in fewer patients, with reported median levels in ng/mg of protein of 0.7 for uPA (27), 0.87 for uPAR (22), 15.2 for PAI-1 (28), and 2.26 for PAI-2 (24). For all four variables, the distribution was approximately log normal.

The Spearman rank correlation coefficients ($r_s$) between the various factors of the uPA system varied from $r_s$ = 0.32 (between PAI-2 and PAI-1 or uPAR) to $r_s$ = 0.59 (between uPA and PAI-1). Table 1 shows their relationships with patient and tumor characteristics. Levels of all four parameters were higher in ER- or PgR-negative tumors as compared with hormone receptor-positive tumors. The levels of uPA were not significantly related with age, menopausal status, or lymph node status. Well-differentiated and $T_{3/4}$ tumors had the lowest

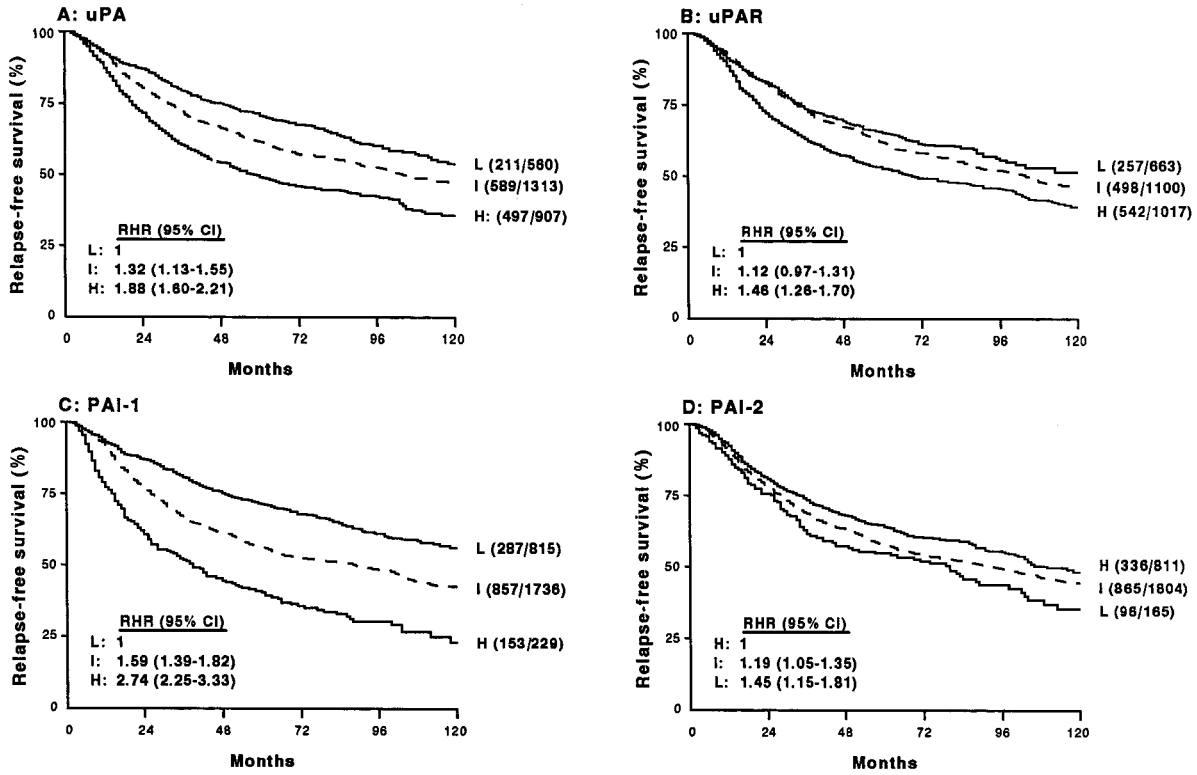


Fig. 1. RFS according to uPA, uPAR, PAI-1, or PAI-2 in all 2780 patients. *A*, RFS as a function of uPA values; *B*, RFS as a function of uPAR values; *C*, RFS as a function of PAI-1 values; *D*, RFS as a function of PAI-2 values. Numbers in parentheses refer to the number of failures/total number of patients per group. *L*, low values; *I*, intermediate values; *H*, high levels, of the respective variable. For cut points, see the legend to Table 2.

uPA levels, whereas moderately differentiated and $T_2$ tumors had the highest levels. In general, uPAR followed the same pattern of distribution over the various patient groups, and all of the relationships were statistically significant, *i.e.*, the negative relationships of uPAR with age and menopausal status, and the positive associations between uPAR and tumor size and nodal status (Table 1). As was also observed for uPA, the highest uPAR and PAI-2 levels were found in moderately differentiated tumors. PAI-1 showed a positive relationship with tumor size, nodal status, tumor grade, age, and menopausal status. PAI-2 showed positive relationships with age and menopausal status, and negative relationships with nodal status and tumor size (Table 1).

**Multivariate Analysis.** To study which factors of the urokinase system add significantly to the prognostic information already provided by traditional prognostic factors, we first designed a basic multivariate model for all patients (Table 2). This model, which included age, menopausal status, tumor size and grade, the number of positive lymph nodes, adjuvant therapy, and ER/PgR status, was significantly associated with RFS ($\chi^2 = 517$; df = 15; $P < 0.0001$) and OS ($\chi^2 = 519$; df = 15; $P < 0.0001$). For both uPAR and PAI-2, the results of the IRA, after correction for the basic multivariate model, suggested that these variables could be considered as categorical variables with two cut points. The cut points chosen were 0.57 and 1.13 ng/mg of protein for uPAR and 0.62 and 4.05 ng/mg of protein for PAI-2. On the other hand, for uPA and PAI-1 the results were less clear and did not reveal a clear indication for cut points. However, to enable visualization of their relationships with RFS and for reasons of uniformity with the analyses of uPAR and PAI-2, we categorized uPA and PAI-1 at the levels of the two largest steps in the IRA. These levels were 0.19 and 1.21 ng/mg protein for uPA and 9.33 and 45.28 ng/mg protein for PAI-1. When each variable was added separately to the basic model for RFS, the addition of uPA resulted in a $\Delta\chi^2$ of 73.6 (df = 2), a $\Delta\chi^2$ of 19.9 (df = 2) for uPAR, a $\Delta\chi^2$ of 81.4 (df = 2) for PAI-1, and a $\Delta\chi^2$ of 4.6 (df = 2) for PAI-2. The RHRs and their 95% CI from the Cox multivariate analysis are listed in Table 2. High levels of uPA, uPAR, or PAI-1 were significantly associated with an early relapse, whereas PAI-2 did not significantly contribute to the basic multivariate model. Similar results were obtained in the analyses for OS (Table 2). The Kaplan-Meier curves for RFS as a function of the levels of uPA, uPAR, PAI-1, and PAI-2 are shown in Fig. 1. There were no statistically significant interactions between any of the prognostic variables in multivariate analysis for RFS or OS. Separate Cox multivariate analyses for RFS and OS were subsequently performed to establish whether combinations of uPA, uPAR, PAI-1, and PAI-2 would increase the prognostic strength of the classical prognostic factors already included in the basic model. When the four factors of the uPA system were added as log-transformed continuous variables to the basic model for RFS in all patients, high levels of uPA and PAI-1 were associated with a poor prognosis, whereas high levels of PAI-2 were associated with a favorable prognosis in the final model for RFS (for all, $P < 0.001$). The RHRs and 95% CIs for the components of the uPA system that are included as categorical variables in the final model for all patients and in subgroups of node-negative and node-positive patients are shown in Table 3. uPAR did not contribute to any of the models. Only in the analysis for OS in node-positive patients did PAI-2 not appear to be an independent prognostic variable. In all other analyses, uPA, PAI-1, and PAI-2 significantly added to the prognostic strength provided by the classical prognostic factors included in the basic model (Table 3). The increase in $\chi^2$ caused by the addition of uPA, PAI-1, and PAI-2 to the basic model was 136 (df = 6) in the analysis for RFS and 120 in the analysis for OS. In node-negative patients with a $\chi^2$ of 76 (df = 12) for the model including age and menopausal status, tumor size and grade, and ER/PgR status, the $\Delta\chi^2$ as a result of the addition of uPA, PAI-1, and PAI-2 was 71 (df = 6) in analysis for RFS, and

Table 3 *Cox multivariate analysis in all patients and in nodal subgroups of patients*

| | RFS | | OS | |
|---|---|---|---|---|
| Factor[a] | RHR[b] | *P* | RHR[b] | *P* |
| All patients: | | | | |
| +uPA | | <0.0001 | | <0.0001 |
| Intermediate *vs.* low | 1.22 (1.02–1.45) | | 1.32 (1.08–1.61) | |
| High *vs.* low | 1.69 (1.39–2.05) | | 1.92 (1.54–2.38) | |
| +PAI-1 | | <0.0001 | | <0.0001 |
| Intermediate *vs.* low | 1.32 (1.14–1.54) | | 1.18 (0.99–1.40) | |
| High *vs.* low | 2.17 (1.74–2.70) | | 2.00 (1.58–2.55) | |
| +PAI-2 | | <0.0001 | | 0.02 |
| Intermediate *vs.* high | 1.30 (1.14–1.48) | | 1.16 (1.01–1.33) | |
| Low *vs.* high | 1.76 (1.38–2.24) | | 1.43 (1.09–1.89) | |
| Node-negative patients: | | | | |
| +uPA | | <0.0001 | | <0.0001 |
| Intermediate *vs.* low | 1.31 (0.98–1.76) | | 1.46 (1.03–2.07) | |
| High *vs.* low | 2.02 (1.47–2.77) | | 2.45 (1.67–3.59) | |
| +PAI-1 | | <0.002 | | 0.003 |
| Intermediate *vs.* low | 1.34 (1.04–1.73) | | 1.09 (0.81–1.47) | |
| High *vs.* low | 2.03 (1.39–2.97) | | 1.71 (1.13–2.61) | |
| +PAI-2 | | <0.0001 | | <0.05 |
| Intermediate *vs.* high | 1.45 (1.17–1.81) | | 1.19 (0.93–1.52) | |
| Low *vs.* high | 2.57 (1.71–3.86) | | 1.89 (1.16–3.10) | |
| Node-positive patients: | | | | |
| +uPA | | <0.001 | | <0.0001 |
| Intermediate *vs.* low | 1.18 (0.94–1.47) | | 1.27 (1.00–1.61) | |
| High *vs.* low | 1.52 (1.19–1.94) | | 1.72 (1.32–2.24) | |
| +PAI-1 | | <0.0001 | | <0.0001 |
| Intermediate *vs.* low | 1.34 (1.11–1.63) | | 1.23 (1.00–1.52) | |
| High *vs.* low | 2.26 (1.72–2.98) | | 2.13 (1.59–2.86) | |
| +PAI-2 | | <0.002 | | 0.20 |
| Intermediate *vs.* high | 1.23 (1.04–1.45) | | 1.15 (0.97–1.36) | |
| Low *vs.* high | 1.47 (1.09–1.99) | | 1.26 (0.90–1.75) | |

[a] Low, intermediate, and high for the respective factors; see legend to Table 2.
[b] Numbers in parentheses, 95% CI; corrected for the basic model including age and menopausal status, tumor size and grade, nodal status, adjuvant therapy, and ER/PgR status. For node-positive patients, nodal status was included as $N_{>3}$ *vs.* $N_{1-3}$; for node-negative patients, nodal status and adjuvant therapy were not applicable.

in the analysis for OS, the $\chi^2$ increased from 82 to 133 by the addition of the three factors. In node-positive patients, the $\chi^2$ increased from 221 (df = 14) for the model including age and menopausal status, tumor size and grade, the number of positive lymph nodes, adjuvant therapy, and ER/PgR status to 295 in the analysis for RFS, and from 207 to 297 in the analysis for OS.

**Prognostic Scores.** In further analyses, we established prognostic scores based on the regression coefficients of the variables included in the final Cox multivariate models for all patients and separately for node-negative and node-positive patients. Kaplan-Meier curves were constructed for patients with the 10% lowest risk (group a), between 10% and 25% (group b), between 25% and 50% (group c), between 50% and 75% (group d), between 75% and 90% (group e), and the 10% highest risk for failure (group f). The actuarial RFS curve for all patients as a function of the prognostic score shows a significant separation for the different groups. At 10 years of follow-up, the difference between patients in the 10% highest and lowest risk groups was 66% in analysis for RFS (Fig. 2*A*) and 61% in analysis for OS (Fig. 2*B*).

The actuarial RFS curves as a function of the prognostic score for node-negative and node-positive patients are shown in Fig. 3, *A* and *B*, respectively. For node-negative patients, at 10 years the difference between the 10% lowest risk group (86 ± 4% relapse free) and the 10% highest risk group (27 ± 6% relapse free) was 59%. For node-positive patients, at 10 years the difference in RFS between the two extreme groups was 52% (61 ± 6% and 9 ± 3% for the 10% lowest and highest risk groups, respectively).

## DISCUSSION

Determination of the tumor antigen levels of components of the uPA system may help to predict the time to disease recurrence and the overall survival rate in patients with primary breast cancer. This is of particular importance for patients with node-negative disease, who as a group have a relatively favorable prognosis. The goal of the present investigation was to evaluate a possible combined prognostic value of the four major components of the uPA system of plasminogen activation, uPA, uPAR, PAI-1, and PAI-2, in patients with primary invasive breast cancer.

Immunohistochemistry and *in situ* hybridization on breast cancer tissues showed that uPA, uPAR, PAI-1, and/or PAI-2 are expressed and synthesized by both tumor cells and host cells, including (myo)fibroblasts, endothelial cells, and phagocytic cells (reviewed in Refs. 1 and 4). A differential expression of the four components by the various cell types and an interplay between these cells may be necessary for the function of the system in various cellular processes at different stages of tumor progression (1). It has been shown that, in general, elevated antigen levels of uPA, uPAR, and PAI-1 determined in tumor extracts are associated with poor prognosis in a variety of cancer types. In contrast, increased PAI-2 antigen levels are associated with a favorable prognosis (reviewed in Refs. 1, 4, and 17). The association of a high tumor level of PAI-1 with a poor prognosis in patients with primary breast cancer (19) has been explained by its inhibition of uPA activity and thus preventing degradation of tumor stroma, allowing new ECM to be formed (33), but it can also be attributed to its recently ascribed role in tumor cell adhesion and migration (10, 11, 13, 14). Furthermore, PAI-1 has been shown to be involved in uPAR clearance from the cell surface by promoting internalization of the formed ternary uPA:uPAR:PAI-1 complex (3, 34). Besides its antiproteolytic function, PAI-1 is necessary for focalized and optimal invasiveness (35), associated with angiogenic activity (36), and is essential for tumor cell invasion and tumor vascularization in PAI-1-deficient mice (37). Together these observations strongly imply that PAI-1 plays a primary role in tumor progression. Because we showed previously that the uPAR level in the cytosol was

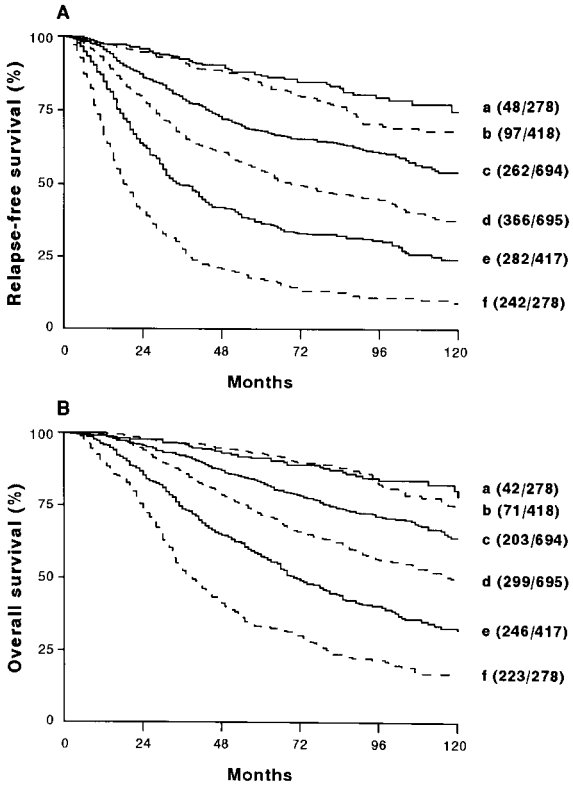


Fig. 2. RFS and OS according to prognostic score in all 2780 patients. *A*, RFS and *B*, OS, as a function of the total prognostic score, which was derived from the estimates of the coefficients of the variables from the Cox multivariate analysis for RFS. Included in the multivariate analysis were: age premenopausal (in decades), age postmenopausal (in decades), postmenopausal status (post- *versus* premenopausal), lymph node status ($N_{1-3}$ *versus* $N_0$, $N_{>3}$ *versus* $N_0$), tumor size ($T_2$ *versus* $T_1$, $T_{3/4}$ *versus* $T_1$), tumor grade (well *versus* poor, moderate *versus* poor, missing *versus* poor), ER/PgR status (−/+ *versus* −/−, +/− *versus* −/−, +/+ *versus* −/−, missing *versus* −/−), adjuvant therapy (yes *versus* no), uPA (>0.19–1.21 ng/mg of protein *versus* ≤0.19 ng/mg of protein, >1.21 ng/mg of protein *versus* ≤0.19 ng/mg of protein), PAI-1 (>9.33–45.28 ng/mg of protein *versus* ≤9.33 ng/mg of protein, >45.28 ng/mg of protein *versus* ≤9.33 ng/mg of protein), and PAI-2 (>0.62–4.05 ng/mg of protein *versus* ≤0.62 ng/mg of protein, >4.05 ng/mg of protein *versus* ≤0.62 ng/mg of protein). *a*, the lowest 10% risk group of patients; *b*, the 10–25% risk group; *c*, the 25–50% risk group; *d*, the 50–75% risk group; *e*, the 75–90% risk group; and *f*, the >90% risk group. Numbers in parentheses refer to the number of failures/total number of patients per group.

a much stronger prognostic factor than the uPAR level in the detergent extract, which also contains plasma membrane components (22), uPAR was measured only in the cytosols in the present study. The higher prognostic value of uPAR in cytosols was explained by its presentation as a water-soluble degradation product that has lost its lipid anchor because of the action of either proteases or phospholipases. The soluble uPAR may have been formed as a result of plasmin generation, which on cell surfaces happens in close vicinity to uPAR (38). Such a soluble form of uPAR has been detected in ascites fluid from patients with ovarian cancer (39). It was also found to be elevated in the blood of cancer patients (40, 41) and patients with paroxysmal nocturnal hemoglobinuria (42), when compared with healthy controls. Recently, high plasma levels of soluble uPAR were found to be correlated with a poor prognosis in patients with colorectal cancer (43).

We showed that the interrelationships between uPA, uPAR, PAI-1, and PAI-2 were all positive and statistically significant, with Spearman correlations ranging from 0.32 to 0.59. Similar positive relationships between two or more of these factors have been reported before (21–22, 24, 28, 44–46). In the present study, all four variables were negatively related with ER and PgR. The levels of uPA were in general not significantly related with poor prognostic characteristics. However, the levels of uPAR and PAI-1 were in most analyses weakly, although significantly, associated with poor prognostic features, whereas PAI-2 levels were associated with favorable prognostic characteristics. Because most relationships were weak yet statistically significant, probably as a result of the large numbers included, several of the observed associations may hardly be of biological relevance. With respect to prognosis, we show that high tumor levels of uPA, uPAR, and PAI-1 were associated with poor, and of PAI-2 with a favorable, RFS and OS for patients with primary breast cancer. Moreover, when added separately to the basic multivariate model including traditional prognostic factors, uPA, uPAR, and PAI-1 all gave additional prognostic information. PAI-1 appeared to be the second strongest prognostic factor after nodal status, superior to the established factors such as tumor size (Table 2). In contrast, PAI-2 levels were not significantly associated with prognosis in multivariate analysis when corrected for the contribution of traditional prognostic factors. PAI-2 was significantly associated with a favorable prognosis when added to the multivariate models together with uPA and PAI-1. This may seem odd, however, we have shown before that PAI-2 was only of prognostic value in tumors with high levels of uPA and not in those with low uPA levels (24). This phenomenon of PAI-2 being an independent favorable prognostic factor in the presence of uPA could be attributable to the positive association between the levels of PAI-2 and the other three components of the uPA system that are related to a poor prognosis. When combining the various factors of the urokinase system in multivariate analyses for RFS and OS, uPAR did not

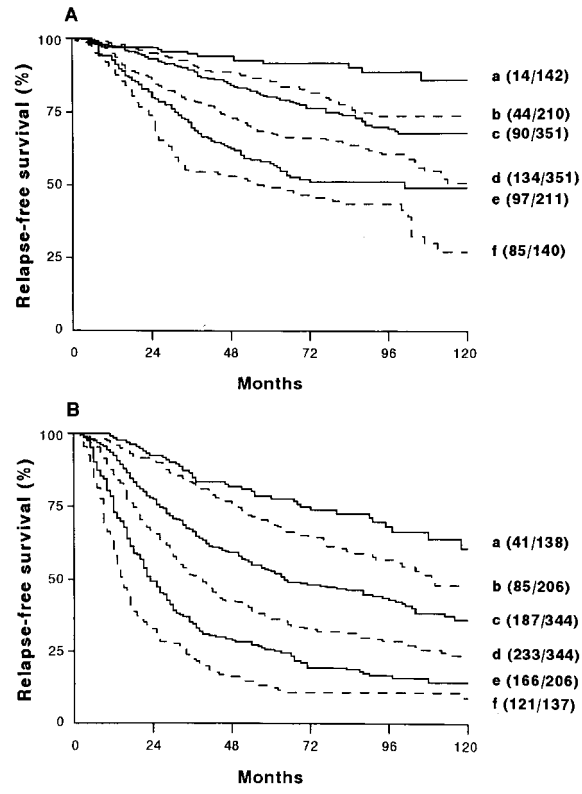


Fig. 3. RFS according to prognostic score in node-negative and node-positive patients. *A*, RFS in node-negative patients, and *B*, in node-positive patients, as a function of the prognostic score based on variables comprising the basic model and uPA, PAI-1, and PAI-2. The prognostic score was derived from the estimates of the coefficients of the variables of the basic model and those of uPA, PAI-1, and PAI-2, from Cox multivariate analyses for RFS in node-negative and node-positive patients, respectively (see Table 3). uPA, PAI-1, and PAI-2, were included as categorical variables as described in the legend to Fig. 2. *a*, the lowest 10% risk group of patients; *b*, the 10–25% risk group; *c*, the 25–50% risk group; *d*, the 50–75% risk group; *e*, the 75–90% risk group; and *f*, the >90% risk group. Numbers in parentheses refer to the number of failures/total number of patients per group.

further contribute to the models in which uPA and/or PAI-1 were included.

It has been argued recently by Powles (47) that "the time has come to individualize adjuvant chemotherapy, basing it more on the biological characteristics of individual tumors rather than on the widespread treatment of large groups of patients." In this line of thought, we aimed to establish a prognostic score based on the contribution of various traditional and tumor cell biological prognostic factors. Using a score based on the traditional prognostic variables and the independent biological variables uPA, PAI-1, and PAI-2, we were able to obtain survival curves that showed a wide separation between patients in the various risk groups of patients, as well as in nodal subgroups of patients. It should, however, be emphasized that the largest power of the models in all patients are derived from the classical prognostic factors, particularly nodal status and tumor size. In analysis for RFS in all patients, the $\chi^2$ of the basic model containing the classical prognostic factors was 517 (df = 15). The independent biological factors uPA, PAI-1, and PAI-2 only added moderately to this model (increase in $\chi^2$ of 136, df = 6). Importantly, for node-negative patients, this increase in $\chi^2$ caused by the addition of the three factors was 71 (df = 6). This is relatively high compared with the $\chi^2$ of 76 (df = 12) already provided by the classical prognostic factors.

In the present study, the levels of the components of the urokinase system of plasminogen activation were determined with specific ELISAs performed on tumor extracts, which does not discriminate between the cell type that expresses the specific factor. Nevertheless, the measured levels correlated with prognosis in several cancer types in many published studies. An advantage of using ELISAs is that the assays can easily be subjected to external quality control programs (48). With respect to immunohistochemical assessment of the components of the uPA system, discrepant results on the localization of the different factors have been published by various groups. It has been argued by Andreasen *et al.* (1) that to ascertain the specificity of immunohistochemical stainings and to obtain conclusive results, special care should be taken regarding a number of control experiments and that, although immunohistochemical studies may reveal where the various components are present, the localization of the active components remains elusive (1).

Several of the components of the uPA system are potential targets for antiangiogenic, anti-invasive, and/or antimetastatic therapy, and various different approaches to interfere with the expression or reactivity of uPA or uPAR at the gene or protein level have been proven successful. Such therapeutic approaches include the application of antisense oligonucleotides, antibodies, enzyme inhibitors, and recombinant and synthetic uPA and uPAR analogues (reviewed in Refs. 1, 3, and 4). Because the uPA system plays an important role in tumor cell adhesion and migration as well, treatments aimed at interfering with the nonproteolytic properties of this multifactorial system may also prove beneficial.

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## REFERENCES

1. Andreasen, P. A., Kjøller, L., Christensen, L., and Duffy, M. J. The urokinase-type plasminogen activator system in cancer metastasis: a review. Int. J. Cancer, *72:* 1–22, 1997.
2. Chapman, H. A. Plasminogen activators, integrins, and the coordinated regulation of cell adhesion and migration. Curr. Opin. Cell Biol., *9:* 714–724, 1997.
3. Conese, M., and Blasi, F. The urokinase/urokinase-receptor system and cancer invasion. Ballière Clin. Hematol., *2:* 365–389, 1995.
4. Schmitt, M., Harbeck, N., Thomssen, C., Wilhelm, O., Magdolen, V., Reuning, U., Ulm, K., Höfler, H., Jänicke, F., and Graeff, H. Clinical impact of the plasminogen activation system in tumor invasion and metastasis: prognostic relevance and target for therapy. Thromb. Haemostasis, *78:* 285–296, 1997.
5. Stoppelli, M. P., Corti, A., Soffientini, A., Cassani, G., Blasi, F., and Assoian, R. K. Differentiation enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes. Proc. Natl. Acad. Sci. USA, *82:* 4939–4943, 1985.
6. Vassalli, J. D., Baccino, D., and Berlin, D. A cellular binding site for the $M_r$ 55,000 form of the human plasminogen activator urokinase. J. Cell. Biol., *100:* 86–92, 1985.
7. Mignatti, P., and Rifkin, D. B. Biology and biochemistry of proteases in tumor invasion. Physiol. Rev., *73:* 161–195, 1993.
8. Andreasen, P. A., Georg, B., Lund, L. R., Riccio, A., and Stacey, S. N. Plasminogen activator inhibitors: hormonally regulated serpins. Mol. Cell. Endocrinol., *68:* 1–19, 1990.
9. Wei, Y., Waltz, D. A., Rao, N., Drummond, R. J., Rosenberg, S., and Chapman, H. A. Identification of the urokinase receptor as an adhesion receptor for vitronectin. J. Biol. Chem., *269:* 32380–32388, 1994.
10. Deng, G., Curriden, S. A., Wang, S., Rosenberg, S., and Loskutoff, D. J. Is plasminogen activator inhibitor-1 the molecular switch that governs urokinase receptor-mediated cell adhesion and release? J. Cell. Biol., *134:* 1563–1571, 1996.
11. Kanse, S., Kost, C., Wilhelm, O., Andreasen, P. A., and Preissner, K. T. The urokinase receptor is a major vitronectin-binding protein on endothelial cells. Exp. Cell Res., *224:* 344–353, 1996.
12. Wei, Y., Lukashev, M., Simon, D. I., Bodary, S. C., Rosenberg, S., Doyle, M. V., and Chapman, H. A. Regulation of integrin function by the urokinase receptor. Science (Washington DC), *273:* 1551–1555, 1996.
13. Stefansson, S., and Lawrence, D. A. The serpin PAI-1 inhibits cell migration by blocking integrin $\alpha_v\beta_3$ binding to vitronectin. Nature (Lond.), *383:* 441–443, 1996.
14. Kjøller, L., Kanse, S., Kirkegaard, T., Rodenburg, K. W., Rønne, E., Goodman, S. L., Preissner, K. T., Ossowski, L., and Andreasen, P. A. Plasminogen activator inhibitor-1 represses integrin- and vitronectin-mediated cell migration independently of its function as an inhibitor of plasminogen activation. Exp. Cell Res., *232:* 420–429, 1997.
15. Duffy, M. J., O'Grady, P., Devaney, D., O'Siorain, L., Fennelly, J. J., and Lijnen, H. J. Urokinase-plasminogen activator, a marker for aggressive breast carcinomas: preliminary report. Cancer (Phila.), *62:* 531–533, 1988.
16. Jänicke, F., Schmitt, M., Hafter, R., Hollreider, A., Babic, R., Ulm, K., Gössner, W., and Graeff, H. Urokinase-type plasminogen activator (uPA) antigen is a predictor of early relapse in breast cancer. Fibrinolysis, *4:* 69–78, 1990.
17. Duffy, M. J. Proteases as prognostic markers in cancer. Clin. Cancer Res., *2:* 613–618, 1996.
18. Solomayer, E-F., Diel, I. J., Bode, S., Meyberg, G., Sillem, M., Gollan, C., Kramer, M. D., Krainick, U., and Bastert, G. Prognostic relevance of urokinase plasminogen activator detection in micrometastatic cells in bone marrow of patients with primary breast cancer. Br. J. Cancer, *76:* 812–818, 1997.
19. Jänicke, F., Schmitt, M., and Graeff, H. Clinical relevance of the urokinase-type and the tissue-type plasminogen activators and their inhibitor PAI-1 in breast cancer. Semin. Thromb. Hemostasis, *17:* 303–312, 1991.
20. Foekens, J. A., Look, M. P., Peters, H. A., van Putten, W. L. J., Portengen, H., and Klijn, J. G. M. Urokinase-type plasminogen activator and its inhibitor PAI-1: predictors of poor response to tamoxifen therapy in recurrent breast cancer. J. Natl. Cancer Inst., *87:* 751–756, 1995.
21. Duggan, C., Maguire, T., McDermott, E., Higgins, N., Fennelly, J. J., and Duffy, M. J. Urokinase plasminogen activator and urokinase plasminogen activator receptor in breast cancer. Int. J. Cancer, *61:* 597–600, 1995.
22. Grøndahl-Hansen, J., Peters, H. A., van Putten, W. L. J., Look, M. P., Pappot, H., Rønne, E., Danø, K., Klijn, J. G. M., Brünner, N., and Foekens, J. A. Prognostic significance of the receptor for urokinase plasminogen activator in breast cancer. Clin. Cancer Res., *1:* 1079–1087, 1995.
23. Bouchet, C., Spyratos, F., Martin, P. M., Hacène, K., Gentile, A., and Oglobine, J. Prognostic value of urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitors PAI-1 and PAI-2 in breast carcinomas. Br. J. Cancer, *69:* 398–405, 1994.
24. Foekens, J. A., Buessecker, F., Peters, H. A., Krainick, U., van Putten, W. L. J., Look, M. P., Klijn, J. G. M., and Kramer, M. D. Plasminogen activator inhibitor-2: prognostic relevance in 1012 patients with primary breast cancer. Cancer Res., *55:* 1423–1427, 1995.
25. EORTC Breast Cancer Cooperative Group. Revision of the standards for the assessment of hormone receptors in human breast cancer. Eur. J. Cancer, *16:* 1513–1515, 1980.
26. Foekens, J. A., Portengen, H., van Putten, W. L. J., Peters, H. A., Krijnen, H. L. J. M., Alexieva-Figusch, J., and Klijn, J. G. M. Prognostic value of estrogen and progesterone receptors measured by enzyme immunoassays in human breast tumor cytosols. Cancer Res., *49:* 5823–5828, 1989.
27. Foekens, J. A., Schmitt, M., van Putten, W. L. J., Peters, H. A., Bontenbal, M., Jänicke, F., and Klijn, J. G. M. Prognostic value of urokinase-type plasminogen activator in 671 primary breast cancer patients. Cancer Res., *52:* 6101–6105, 1992.
28. Foekens, J. A., Schmitt, M., van Putten, W. L. J., Peters, H. A., Kramer, M. D., Jänicke, F., and Klijn, J. G. M. Plasminogen activator inhibitor-1 and prognosis in primary breast cancer. J. Clin. Oncol., *12:* 1648–1658, 1994.

29. Rønne, E., Høyer-Hansen, G., Brünner, N., Pedersen, H., Rank, F., Osborne, C. K., Clark, G. M., Danø, K., and Grøndahl-Hansen, J. Urokinase receptor in breast cancer tissue extracts. Enzyme-linked immunosorbent assay with a combination of mono- and polyclonal antibodies. Breast Cancer Res. Treat., *33:* 199–207, 1995.
30. Kaplan, E. L., and Meier, P. Nonparametric estimation from incomplete observations. J. Am. Stat. Assoc., *53:* 457–481, 1958.
31. Barlow, R. E., Bartholomew, D. J., Bremner, J. M., and Brunk, H. D. Statistical interference under order restrictions. London: John Wiley & Sons, Inc., 1972.
32. Cox, D. R. Regression models and life tables. J. R. Stat. Soc., *34:* 187–220, 1972.
33. Sier, C. M., Vloedgraven, H. J. M., Ganesh, S., Griffioen, G., Quax, P. H., Verheijen, J. H., Dooijewaard, G., Welvaart, K., van de Velde, C. J., Lamers, C. B., and Verspaget, H. W. Inactive urokinase and increased levels of its inhibitor type 1 in colorectal liver metastasis. Gastroenterology, *107:* 1449–1456, 1994.
34. Conese, M., and Blasi, F. Urokinase/urokinase receptor system: internalization/degradation of urokinase-serpin complexes: mechanism and regulation. Biol. Chem. Hoppe-Seyler, *376:* 143–155, 1995.
35. Liu, G., Shuman, M. A., and Cohen, R. L. Co-expression of urokinase, urokinase receptor and PAI-1 is necessary for optimum invasiveness of cultured lung cancer cells. Int. J. Cancer, *60:* 501–506, 1995.
36. Barbareschi, M., Gasparini, G., Morelli, L., Forti, S., and Dalla Palma, S. Novel methods for the determination of the angiogenic activity of human tumors. Breast Cancer Res. Treat., *36:* 181–192, 1995.
37. Bajou, K., Noël, A., Masson, V., Masson, V., Brünner, N., Holst-Hansen, C., Skobe, M., Fusenig, N. E., Carmeliet, P., Collen, D., and Foidart, J. M. Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. Nat. Med., *4:* 923–928, 1998.
38. Danø, K., Behrendt, N., Brünner, N., Ellis, V., Plough, M., and Pyke, C. The urokinase receptor: protein structure and role in plasminogen activation and cancer invasion. Fibrinolysis, *8:* 189–203, 1994.
39. Pedersen, N., Schmitt, M., Rønne, E., Nicoletti, M. I., Hoyer-Hansen, G., Conese, M., Giavazzi, R., Danø, K., Kuhn, W., Jänicke, F., and Blasi, F. A ligand-free, soluble urokinase-receptor is present in the ascitic fluid from patients with ovarian cancer. J. Clin. Investig., *92:* 2160–2167, 1993.
40. Pappot, H., Høyer-Hansen, G., Rønne, E., Hanssen, H. H., Brünner, N., Danø, K., and Grøndahl-Hansen, J. Elevated plasma levels of urokinase plasminogen activator receptor in non-small cell lung cancer patients. Eur. J. Cancer, *33:* 867–872, 1997.
41. Stephens, R. W., Pedersen, A. N., Nielsen, H. J., Hamers, M. J., Hoyer-Hansen, G., Rønne, E., Dybkjaer, E., Danø, K., and Brünner, N. ELISA determination of soluble urokinase receptor in blood from healthy donors and cancer patients. Clin. Chem., *43:* 1868–1876, 1997.
42. Rønne, E., Pappot, H., Grøndahl-Hansen, J., Hoyer-Hansen, G., Plesner, T., Hansen, N. E., and Danø, K. The receptor for urokinase plasminogen activator is present in plasma from healthy donors and elevated in patients with paroxysmal nocturnal hemoglobinuria. Br. J. Hematol., *89:* 576–581, 1995.
43. Stephens, R. W., Nielsen, H. J., Christensen, I. J., Thorlacius-Ussing, O., Sørensen, S., Danø, K., and Brünner, N. Plasma urokinase receptor levels in patients with colorectal cancer: relationship to prognosis. J. Natl. Cancer Inst., *91:* 869–874, 1999.
44. Grøndahl-Hansen, J., Christensen, I. J., Briand, P., Pappot, H., Mouridsen, H. T., Blichert-Toft, M., Danø, K., and Brünner, N. Plasminogen activator inhibitor type 1 in cytosolic tumor extracts predicts prognosis in low-risk breast cancer patients. Clin. Cancer Res., *3:* 233–239, 1997.
45. Jänicke, F., Pache, L., Schmitt, M., Ulm, K., Harbeck, N., Höfler, H., and Graeff, H. Urokinase (uPA) and its inhibitor PAI-1 are strong and independent prognostic factors in node-negative breast cancer. Breast Cancer Res. Treat., *24:* 195–208, 1993.
46. Bouchet, C., Hacène K., Martin, P. M., Becette, V., Tubiana-Hulin, M., Lasry, S., Oglobine, J., and Spyratos, F. Dissemination risk index based on plasminogen activator system components in primary breast cancer. J. Clin. Oncol., *17:* 3048–3057, 1999.
47. Powles, T. J. Adjuvant therapy for early breast cancer: a time to refine. J. Natl. Cancer Inst., *89:* 1652–1654, 1997.
48. Sweep, C. G. J., Geurts-Moespot, J., Grebenshikov, N., de Witte, J. H., Heuvel, J. J. T. M., Schmitt, M., Duffy, M. D., Jänicke, F., Kramer, M. D., Foekens, J. A., Brünner, N., Brugal, G., Pedersen, A. N., and Benraad, T. J. External quality assessment of trans-European multicentre antigen determinations (enzyme-linked immunosorbent assay) of urokinase-type plasminogen activator (uPA) and its type 1 inhibitor (PAI-1) in human breast cancer tissue extracts. Br. J. Cancer, *78:* 1434–1441, 1998.