Pooled Analysis of Prognostic Impact of Urokinase-Type Plasminogen Activator and Its Inhibitor PAI-1 in 8377 Breast Cancer Patients

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Background: **Urokinase-type plasminogen activator (uPA) and its inhibitor (PAI-1) play essential roles in tumor invasion and metastasis. High levels of both uPA and PAI-1 are associated with poor prognosis in breast cancer patients. To confirm the prognostic value of uPA and PAI-1 in primary breast cancer, we reanalyzed individual patient data provided by members of the European Organization for Research and Treatment of Cancer–Receptor and Biomarker Group (EORTC-RBG).** ***Methods:*** **The study included 18 datasets involving 8377 breast cancer patients. During follow-up (median 79 months), 35% of the patients relapsed and 27% died. Levels of uPA and PAI-1 in tumor tissue extracts were determined by different immunoassays; values were ranked within each dataset and divided by the number of patients in that dataset to produce fractional ranks that could be compared directly across datasets. Associations of ranks of uPA and PAI-1 levels with relapse-free survival (RFS) and overall survival (OS) were analyzed by Cox multivariable regression analysis stratified by dataset, including the following traditional prognostic variables: age, menopausal status, lymph node status, tumor size, histologic grade, and steroid hormone-receptor status. All *P* values were two-sided.** ***Results:*** **Apart from lymph node status, high levels of uPA and PAI-1 were the strongest predictors of both poor RFS and poor OS in the analyses of all patients. Moreover, in both lymph node-positive and lymph node-negative patients, higher uPA and PAI-1 values were independently associated with poor RFS and poor OS. For (untreated) lymph node-negative patients in particular, uPA and PAI-1 included together showed strong prognostic ability (all *P*<.001).** ***Conclusions:*** **This pooled analysis of the EORTC-RBG datasets confirmed the strong and independent prognostic value of uPA and PAI-1 in primary breast cancer. For patients with lymph node-negative breast cancer, uPA and PAI-1 measurements in primary tumors may be especially useful for designing individualized treatment strategies. [J Natl Cancer Inst 2002;94:116–28]**

Breast cancer is the most common malignancy in women in the Western world. To improve the survival of patients with primary breast cancer, adjuvant systemic treatment aimed at the eradication of occult metastases has been shown to be beneficial *(1)*. For patients with lymph node-negative disease, however, the clinical benefits of adjuvant systemic treatment are relatively small because primary locoregional treatment cures 60%–70% of these patients. As a result, a large number of patients would be subjected to unnecessary and toxic side effects were adjuvant therapy to be given to all breast cancer patients. To avoid a burdening of patients not in need of systemic adjuvant treatment, strong prognostic markers are warranted to distinguish between patients with low and high risks of disease recurrence. The use of traditional prognostic factors, such as age, menopausal status, tumor size, tumor grade, and steroid hormone-receptor status, is not sufficient to make such a classification.

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See "Notes" following "References."

To facilitate refinement of risk-adapted individualized treatment, major effort has been devoted to the study of factors that govern tumor spread and metastasis. From these studies, it has become clear that the ability of cancer cells to invade the extracellular matrix, to intravasate into lymphatics and blood vessels, and to form metastases at distant sites depends on the coordinated interaction of two main protease systems, the matrix metalloproteinase and the plasminogen activator systems. The latter system comprises the serine protease urokinase-type plasminogen activator (uPA), its receptor (uPAR, CD87), and its main inhibitor (PAI-1). These components play a central role in the processes leading ultimately to the development of metastases [for reviews, *see* references *(2–4)*]. Plasminogen activation results in the formation of the broad-spectrum serine protease plasmin, which in turn activates the proenzyme of uPA to proteolytic active uPA and is the activator of several matrix metalloproteinases *(2–5).* When compared with normal tissues, uPA, uPAR, and PAI-1 all have been shown to be at increased levels in malignant solid tumors in a variety of human cancers, including breast cancer *(2–4).* As early as 1988, elevated uPA levels in primary breast tumor tissue were shown to be associated with a poor prognosis of the patient *(6).* In the early 1990s, it became evident that high antigen levels of PAI-1 in primary breast tumors also were associated with a poor prognosis *(7).* This finding of a relationship between a protease inhibitor and an unfavorable prognosis was initially surprising but is now explained by the crucial role of PAI-1 in tumor cell adhesion, cell migration, and angiogenesis *(8–10).* Following the initial reports *(6,7)*, many independent studies have demonstrated that elevated tumor antigen levels of uPA and PAI-1 are associated with poor disease outcome in breast cancer [for reviews, *see* references *(2–4,11,12)*]. Furthermore, it has been shown that high tumor levels of uPA and PAI-1 predict a poor outcome for patients who were treated with tamoxifen for advanced disease *(13).*

The main purpose of this study was to investigate whether the prognostic impact of uPA and PAI-1, as published in different reports from various authors, could be confirmed by a reanalysis, conducted in a uniform manner, of the individual patient data. To this end, the European Organization for Research and Treatment of Cancer–Receptor and Biomarker Group (EORTC-RBG) performed a pooled analysis with the use of 18 datasets originating from nine European countries. The prognostic relevance of uPA and PAI-1 in the analyses of relapse-free survival (RFS) and overall survival (OS) was studied for all patients and in subgroups of lymph node-negative and lymph node-positive patients by stratified Cox multivariable regression analysis including the traditional prognostic factors.

METHODS

Datasets

The members of the EORTC-RBG proposed to share their data on uPA and/or PAI-1 levels in primary breast cancer tumors, on classical factors, and on the follow-up information. All centers with published *(14–25)* (n = 11) and unpublished (n = 7) datasets agreed to participate and sent their data to Rotterdam for statistical reanalysis. All published studies reported on the prognostic value of uPA and/or PAI-1. All of the participating laboratories are involved in ongoing quality-assurance programs for measurement of biologic variables in tumor tissue *(26).* Studies were approved by the local review boards. The following inclusion and exclusion criteria were used: 1) All patients underwent primary surgery for breast cancer; 2) surgery took place before January 1, 1996; 3) there was no distant spread at or within 1 month of surgery; 4) patients with residual disease diagnosed within 1 month after primary surgery were excluded; 5) patients with noninvasive breast cancer or those who received neoadjuvant therapy were excluded; 6) male breast cancer patients and female patients who previously experienced another cancer (except basal cell skin cancer or early-stage cervical cancer stage Ia/Ib) and male breast cancer patients were excluded; 7) biochemical variables were determined in primary tumor tissue extract; and 8) uPA and PAI-1 antigen levels were determined by immunoassays. Staging of the tumors was according to the International Union Against Cancer TNM classification *(27).* Patients with missing values for postsurgical histopathologic tumor size (pT), lymph node status, and/or both estrogen receptor (ER) and progesterone receptor (PgR) status and/or both uPA and PAI-1 were excluded.

Of the 8377 patients included, the dates of primary surgery ranged from September 1978 through December 1995. The patients were born between November 1891 and September 1969. Of the 18 cooperating laboratories, 17 were able to provide data for the analysis of uPA, and 15 provided data for PAI-1. For the datasets labeled A through R (*see* Fig. 2), the levels of uPA and PAI-1 were determined in either cytosolic tumor extracts (datasets A–D and K–Q) or Triton X-100-treated tumor extracts (datasets E–J and R). All assays used were commercially available immunoassays. Enzyme-linked immunosorbent assay (ELISA) kits were obtained from American Diagnostica, Inc. (Greenwich, CT; datasets A–I), Monozyme (Horsholm, Denmark; datasets P [only for PAI-1] and Q), and Oncogene Science (Cambridge, MA; dataset R). Luminometric Immuno Assay (LIA) kits were obtained from AB Sangtec Medical (Bromma, Sweden; datasets M–O and P [only for uPA]), and an in-house assay was obtained as described (datasets J–L) *(28).* The protein assays used for the various datasets consisted of Bio-Rad (Hercules, CA; datasets A, D, F, L, M, Q, and R), Pierce (Rockford, IL; datasets B, C, E, and G–J), or according to Lowry et al. *(29;* datasets K and N–P). With the use of these various ELISAs, LIA, and protein assays, it was found in the different datasets that median levels of uPA ranged from 0.2 to 5.1 ng/mg of protein and that for PAI-1 they ranged from 1.3 to 26.7 ng/mg of protein. These wide ranges of median values highlighted the need to model the underlying biologic relationship between uPA and PAI-1 measurements in different datasets so as to allow a uniform analysis, which was accomplished by introducing fractional ranks as explained below. Histologic characteristics of the tumors, patients' ages, and adjuvant treatments are listed in Table 1. Three percent of the patients were less than 35 years old, and 40% were premenopausal. Forty-four percent of the patients had small tumors (pT1), and 56% were lymph node negative. The histologic grade of one third of the patients was unknown. Forty-five percent of the patients received some form of systemic adjuvant treatment. Thirty-five percent (n = 2955) of the patients experienced disease recurrence, and 27% (n = 2254) of the patients died within 10 years.

Statistical Analysis

Relationships between uPA and PAI-1 as well as patient and tumor characteristics were investigated with the use of nonparametric methods, i.e., Spearman rank correlations for continuous

Table 1. Patient and tumor characteristics

Factor	No. of patients	%	uPA			PAI-1		
			Total/high†	% high†	Two-sided *P*	Total/high†	% high†	Two-sided *P*
Total	8377	100	8175/4086	50		6682/3337	50	
Age in categories, y					.55‡			.03‡
<35	243	3	239/116	49		212/118	56	
35–55	3799	45	3720/1841	49		3104/1485	48	
56–70	3084	37	2998/1546	52		2357/1192	51	
>70	1251	15	1218/583	48		1009/542	54	
Menopausal status					.52§			.02§
Premenopausal	3338	40	3278/1618	49		2749/1336	49	
Postmenopausal	5039	60	4897/2468	50		3933/2001	51	
Tumor size					<.001‖			<.001‖
pT1, ⩽2 cm	3650	44	3540/1694	48		2857/1289	45	
pT2, >2–5 cm	4120	49	4035/2131	53		3266/1760	54	
pT3, >5 cm	444	5	438/180	41		407/204	50	
pT4	163	2	162/81	50		152/84	55	
Lymph nodes involved					.61‖			<.001‖
0	4676	56	4497/2270	50		3662/1740	48	
1–3	2092	25	2079/1005	48		1692/890	53	
4–10	1166	14	1160/583	50		952/513	54	
>10	443	5	439/228	52		376/194	52	
Histologic grade					<.001‖			<.001‖
I	583	7	530/209	39		439/157	36	
II	2237	27	2156/1065	49		1811/866	48	
III	2786	33	2728/1480	54		2490/1385	56	
Unknown	2771	33	2761/1332	48		1942/929	48	
Adjuvant treatment					.44‖			<.001‖
No	4286	51	4127/2061	50		3844/1891	49	
Unknown	320	4	320/145	45		44/20	45	
Yes	3771	45	3728/1880	50		2794/1426	51	
Steroid hormone-receptor status¶					<.001§			<.001§
Low	1697	20	1665/1052	63		1306/825	63	
High	6680	80	6510/3034	47		5376/2512	47	

*uPA = urokinase-type plasminogen activator; PAI-1 = plasminogen activator inhibitor; pT = postsurgical histopathologic tumor size.

†Above the overall median rank (rescaled ranks pooled for all datasets) for all tumors with data on uPA (8175 patients) or PAI-1 available (6682 patients).

‡Spearman rank correlation.

§Mann–Whitney *U* test.

‖Kruskal–Wallis test, followed by a nonparametric test for trend when appropriate.

¶Low and high steroid hormone-receptor status as defined in the "Methods" section.

variables and the Wilcoxon rank-sum test or the Kruskal–Wallis test for ordered variables. In the latter tests, patient and tumor characteristics were used as grouping variables. A nonparametric test for trend was used when appropriate. For all survival analyses, a Cox proportional hazards model was used. The likelihood ratio test was used to test for differences between models. The endpoint for RFS was defined as any recurrence of breast cancer or contralateral breast cancer; for OS, it was defined as death from any cause. Patients who died without relapse were censored at the last date of follow-up for the analysis of RFS. Patients with events after 120 months were censored at 120 months. The rationale was that, after 10 years of observation, patients frequently are redirected to their general practitioner for checkups and mammography and cease to be patients of the outpatient breast cancer clinic. This would have an impact on the availability and validity of follow-up data after 10 years.

The median follow-up of patients alive ranged from 46 months to more than 120 months in the different datasets. Five datasets had a median follow-up of patients alive of less than 60 months (46, 48, 53, 55, and 58 months, respectively). A base model was defined with the use of traditional prognostic factors and adjuvant systemic therapy. Age was included as a linear variable for both premenopausal and postmenopausal age combined with an indicator variable for menopausal status. This proved to be the best way of introducing age into the model, as it allows for the sharp decrease in hazard rates with age for the youngest patients. As for tumor status, pT3 and pT4 were treated as one category because the estimates from the Cox regression were similar in univariate and multivariable analyses with RFS as the endpoint. Lymph node status was the number of involved lymph nodes categorized as 0, 1–3, 4–10, and more than 10. Low hormone-receptor status was defined as either both ER low and PgR low or either ER low or PgR low and the other unknown; high hormone-receptor status was defined as at least one of ER or PgR high. The cutoff points as determined by each individual laboratory for its dataset in defining high and low for ER and PgR, or the categorization as it was provided, was used. Histologic grade was included as well differentiated (grade I), moderately differentiated (grade II), undifferentiated (grade III), or unknown. Grade III was used as the reference category in the survival analyses. Adjuvant therapy was included as none, unknown, or any therapy. Some datasets did not have sufficient numbers of events to allow a multivariable analysis. Cox univariate and multivariable regression analyses with either RFS or

OS as endpoint were used; the sets were pooled and stratified by dataset. All multivariable analyses included the base model, which was defined as all variables except uPA and PAI-1. The proportional hazards assumption was checked on the full models. The assumption was not violated by uPA or PAI-1; however, some traditional prognostic factors, especially hormone-receptor status and lymph node status, did. Because the purpose of this study was to show the prognostic impact of uPA and PAI-1, we chose to keep the base model as defined. To visualize uPA and PAI-1 in Kaplan–Meier curves, for each dataset, the uPA and PAI-1 data were divided into fifths with the use of their 20^{th}, 40^{th}, 60^{th}, and 80^{th} percentiles. The log-rank test for trend was used to test for differences.

Despite the use of different extraction and assay methods, measurements are highly correlated *(30–32)*; i.e., high levels of uPA and PAI-1 with the use of one method are high according to another method as well. However, because the method of determination varied between laboratories, a common scale was required. For each dataset, we ranked the uPA and PAI-1 measurements and divided the ranks by the number of patients. Thus, the uPA and PAI-1 levels were converted to fractional ranks (between 0 and 1). In this way, equal fractional ranks are comparable across datasets with different numbers of patients included. Effectively, we assume that the use of different assays did not influence the ranks of uPA and PAI-1 levels profoundly. As an alternative to dichotomization in each dataset, ranking and rescaling also have the advantage of allowing us to analyze uPA and PAI-1 as continuous variables. The hazard ratios (HRs) for the ranked variables represent the differences between the extremes. For a continuous variable, the hazard increases per unit with the HR, which, in the case of fractional ranks, is the full range from 0 to 1. For categorized variables, such as uPA and PAI-1 when divided into fifths, dummy variables were introduced. The lower fifth was considered the reference category. The HRs for the remaining four groups were calculated compared with the reference category.

The base model was used to investigate the best fit of ranked uPA and PAI-1 in relation to RFS. The effect of ranked uPA and PAI-1 was modeled with the use of spline regression and fractional polynomials with relapse as the endpoint in a Cox proportional hazards model, stratified by dataset, and adjusted for the base model. For uPA, spline regression with four knots (0.2, 0.4, 0.6, and 0.8) showed the best fit. Fractional polynomials or five knots in spline transformation did not result in a statistically significantly better fit. The spline-transformed ranked uPA values, rescaled between 0 and 1, were used for survival analyses. The ranks of PAI-1 did not need transformation because the linear variable showed the best fit. For visualization of the individual datasets, the HRs and 95% confidence intervals (CIs) corrected for the base model were presented as a forest plot *(33)*. The combined HR and its 95% CI were calculated with the use of a random effects model *(34)*. In random effects models, the between-studies variance is estimated and used to modify the weight used to calculate the summary estimate. It is assumed that, in addition to sampling variation, the true effect varies between studies. The random effects model will in general be more conservative (wider CIs) than the fixed effects model, which is based on the assumption that the true effect does not differ between studies. Using the random effects model, we estimated a mean effect (of the differing effects) around which the true effect is assumed to vary.

With the use of the base model and the final transformations of uPA and PAI-1, interactions were investigated between the prognostic variables (age and menopausal status, tumor size, lymph node status, hormone-receptor status, histologic grade, and adjuvant treatment) and uPA or PAI-1 with the use of the Cox proportional hazards model, stratified by dataset with RFS as the endpoint. For all interactions, a P value less than .01 was considered statistically significant. The results suggest the analysis of the subsets of lymph node-negative and lymph node-positive patients. To visualize the prognostic value of uPA and PAI-1 in Kaplan–Meier curves for lymph node-negative and lymph node-positive patients, prognostic scores were calculated with both uPA and PAI-1 added to the base model and stratified for datasets. The scores (exponentiated linear prediction) then were divided into fifths (i.e., with the use of the 20^{th}, 40^{th}, 60^{th}, and 80^{th} percentiles). All statistical analyses were performed with the use of Stata Statistical Software (release 6.0; Stata Corp., College Station, TX). Two-sided P values are given.

RESULTS

Associations of uPA and PAI-1 With Patient and Tumor Characteristics

The patient and tumor characteristics of all 8377 patients included in the pooled analysis are presented in Table 1. The relationships between uPA (8175 patients) or PAI-1 (6682 patients) and patient and tumor characteristics are shown. The uPA and PAI-1 data are expressed as percentages of tumors above the median rank value of 0.5. There were no statistically significant relationships between uPA and age, menopausal status, or lymph node status. PAI-1 values were correlated with age and were found to be higher in postmenopausal and lymph node-positive patients. The slightly higher PAI-1 levels in patients who received adjuvant treatment compared with those who did not are probably a reflection of the association between treatment and lymph node status. Both the Spearman correlation coefficient between PAI-1 and age and its P value were .03. We considered this correlation and the relationship between PAI-1 and menopausal status to be of no clinical relevance. uPA and PAI-1 were positively correlated with histologic grade, negatively correlated with hormone-receptor status, and found to be highest in pT2 and pT4 tumors. The Spearman correlation coefficient between the ranked uPA and PAI-1 values was .57 ($P<.001$; $n = 6480$).

Survival Analysis in All Patients as a Function of uPA and PAI-1 Status

Relapse-free survival. In Cox univariate analysis for RFS (data not shown), young premenopausal age, postmenopausal status, tumor size, number of lymph nodes involved, low steroid hormone-receptor level, and poor grade were all statistically significantly associated with a poor disease outcome (all $P<.001$). Adjuvant treatment was associated with a poor RFS as a result of its association with poor prognostic features such as young premenopausal age and axillary lymph node involvement at diagnosis. Higher values of uPA and PAI-1 as continuous values were associated with a poor RFS ($\chi^2 = 189.5$ and 247.1, respectively; $df = 1$). For visualization of the associations of uPA and PAI-1 with RFS in Kaplan–Meier curves (Fig. 1,

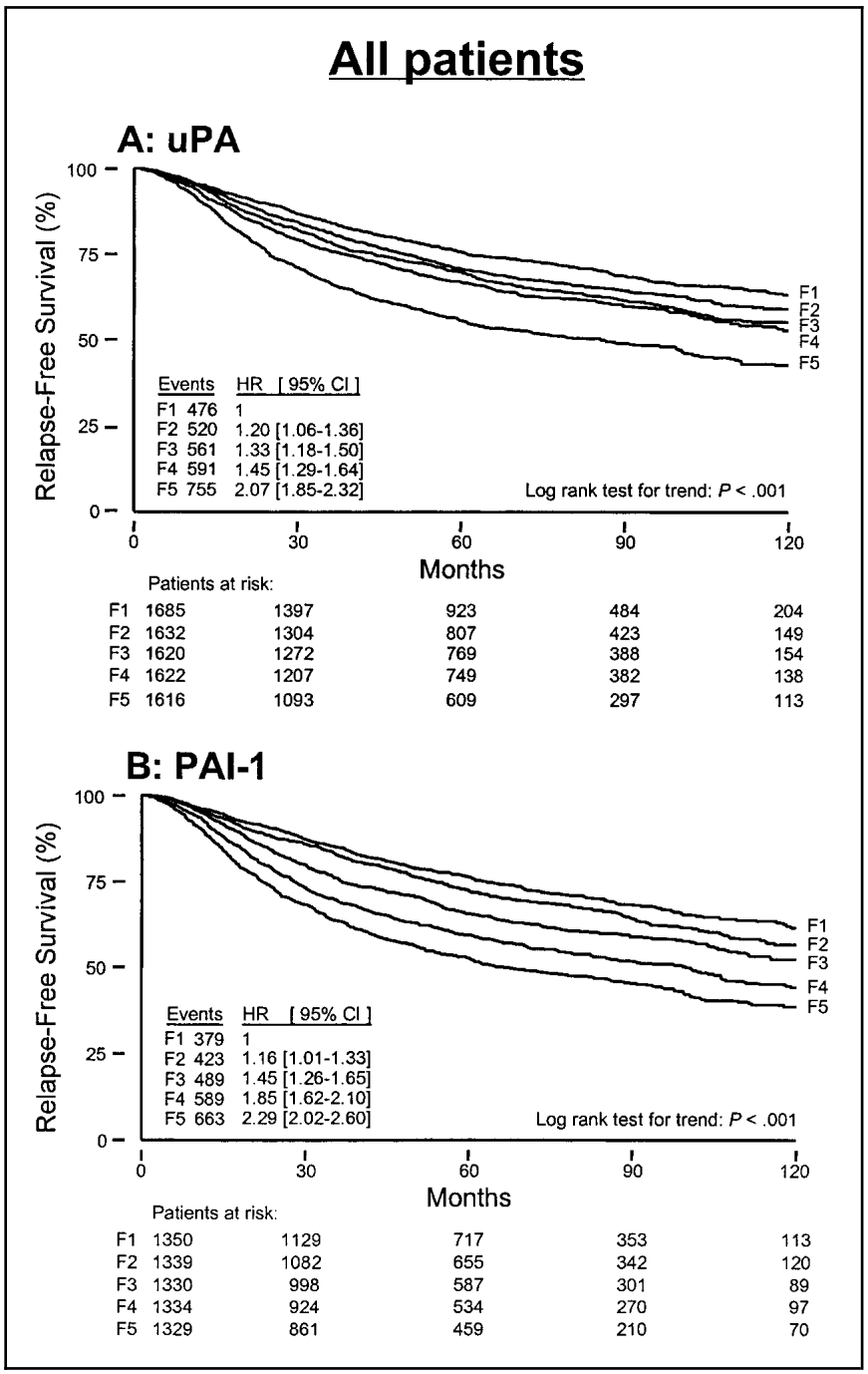

Fig. 1. Relapse-free survival (**A** and **B**) and overall survival probabilities (**C** and **D**) as a function of urokinase-type plasminogen activator (uPA) (**A** and **C**) and its inhibitor (PAI-1) (**B** and **D**) values. For each dataset, original uPA and PAI-1 values were divided into five groups (F1–F5) with the use of their 20th, 40th, 60th, and 80th percentiles. Events indicate the number of failures in each group. Patients at risk at 0, 30, 60, 90, and 120 months are indicated. The survival probabilities (and their 95% confidence intervals) at 60 and 120 months, respectively, are as follows: **panel A**—curve F1 = 75.6 (73.4 to 77.7) and 63.2 (60.0 to 66.2), curve F2 = 70.7 (68.3 to 73.0) and 59.2 (55.9 to 62.2), curve F3 = 69.7 (67.3 to 72.0) and 55.2 (51.8 to 58.4), curve F4 = 66.8 (64.3 to 69.1) and 52.8 (49.3 to 56.2), and curve F5 = 55.6 (53.0 to 58.1) and 42.7 (39.4 to 46.0); **panel B**—curve F1 = 76.2 (73.7 to 78.5) and 61.5 (57.6 to 65.2), curve F2 = 72.4 (69.7 to 74.8) and 56.6 (52.7 to 60.3), curve F3 = 65.6 (62.8 to 68.2) and 52.3 (48.4 to 56.0), curve F4 = 59.2 (56.3 to 61.9) and 44.2 (40.4 to 47.9), and curve F5 = 52.6 (49.7 to 55.3) and 38.6 (34.8 to 42.3); **panel C**—curve F1 = 86.8 (85.0 to 88.4) and 70.8 (67.7 to 73.8), curve F2 = 82.1 (80.0 to 84.0) and 66.3 (63.0 to 69.3), curve F3 = 79.6 (77.5 to 81.6) and 63.6 (60.3 to 66.6), curve F4 = 77.5 (75.3 to 79.5) and 62.0 (58.8 to 65.0), and curve F5 = 69.1 (66.6 to 71.4) and 50.8 (47.5 to 54.0); and **panel D**—curve F1 = 87.4 (85.5 to 89.2) and 71.1 (67.1 to 74.7), curve F2 = 83.1 (80.9 to 85.1) and 65.8 (62.0 to 69.3), curve F3 = 78.6 (76.2 to 80.8) and 58.1 (54.1 to 61.9), curve F4 = 73.9 (71.4 to 76.3) and 54.5 (50.7 to 58.1), and curve F5 = 62.2 (59.3 to 64.8) and 44.8 (41.0 to 48.6). HR = hazard ratio.

A and B), both variables were divided into fifths (F1 through F5) (χ^2 = 176.2 for uPA and χ^2 = 228.4 for PAI-1; *df* = 4; both *P*<.001).

In Cox multivariable analysis for RFS, all classical prognostic variables were statistically significantly associated with RFS (all *P*<.001), with adjuvant treatment now being a favorable prognostic factor as a result of the inclusion of the traditional prognostic variables. This multivariable model, with adjuvant treatment included, was defined as the base model (Table 2). The HRs and 95% CIs corrected for the base model of each individual dataset are shown in Fig. 2. The areas of the boxes are inversely proportional to the variance and, as such, are related to the number of patients included in the individual datasets *(33)*. Fig. 2, A, shows RFS for ranked uPA; Fig. 2, B, shows RFS for ranked PAI-1. For uPA, six of the 17 CIs include the value 1; for PAI-1, seven of 15 CIs include the value 1, implying no statistical significance (*P*>.05) for those (mainly small) datasets. Not all datasets had sufficient numbers of events. Only one HR is estimated to be less than 1 (set J with 88 patients in Fig. 2, A). The diamonds present the combined estimates of the HRs and 95% CIs. Adding uPA and PAI-1 separately as continuous variables to the base model for RFS resulted in an increase in χ^2 ($\Delta\chi^2$) of 168.5 and 176.1 (both with 1 *df*; *P*<.001). For both, the HR (95% CI) was 2.58 (2.24 to 2.97) (Table 3). When uPA and PAI-1 were added simultaneously as continuous variables to the base model, stratified by dataset, their respective HRs and 95% CIs for RFS were 1.70 (1.42 to 2.04) and 2.00 (1.69 to 2.37). This resulted in a $\Delta\chi^2$ of 203.6 with one additional *df* (*df* = 2). Because an increase of 3.84 for one more *df* is necessary to obtain statistical significance, the increase in χ^2 of

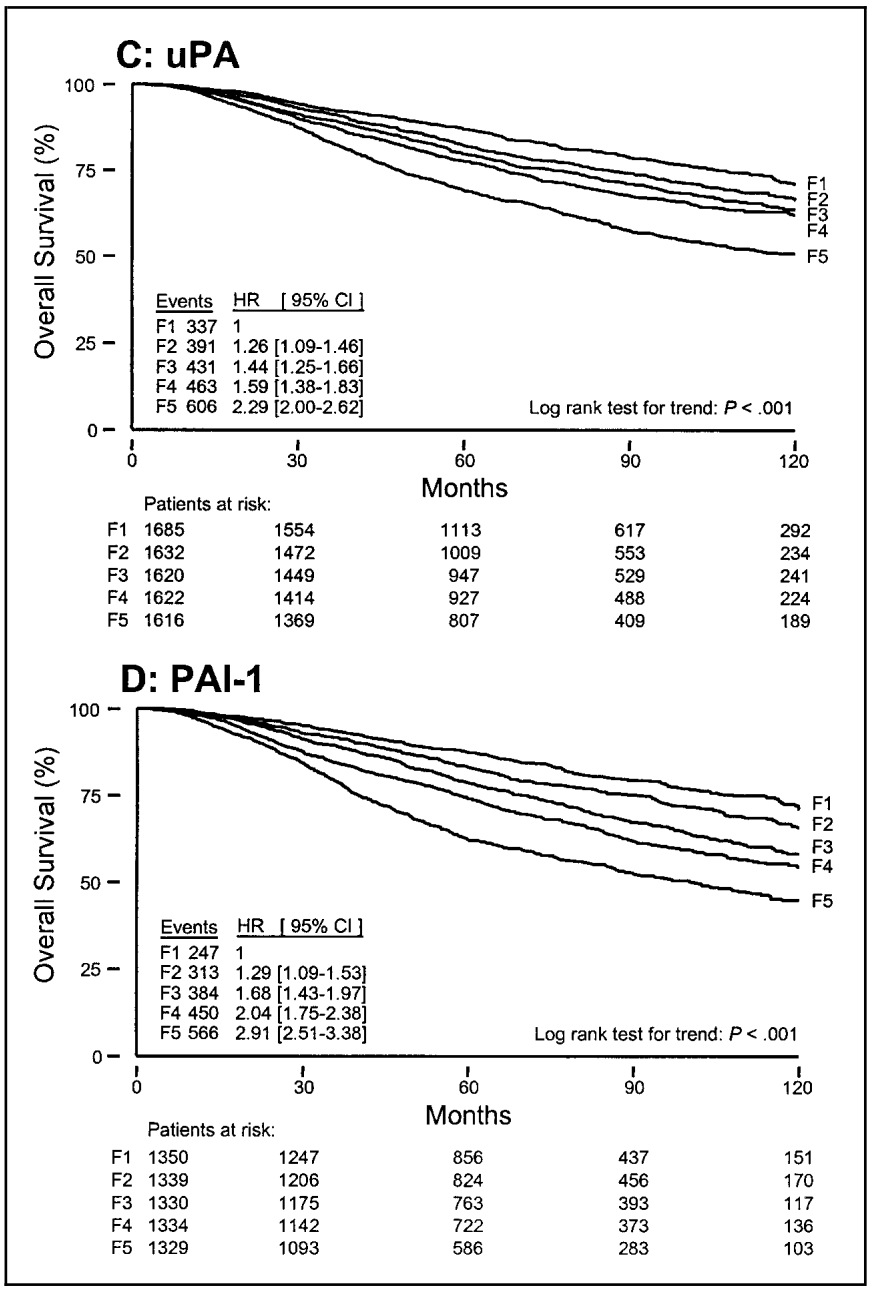

Fig. 1. Continued.

27.5 (203.6 minus 176.1) implies that both factors independently added to the prognostic strength provided by the traditional prognostic factors, of which nodal status is the strongest factor (Table 2). Thus, the model containing both uPA and PAI-1 is preferable over a model with only one of these factors (Table 3).

Overall survival. In the Cox univariate and multivariable analyses for OS, the same factors that were of prognostic value in the analysis of RFS showed statistically significant associations in the same directions (all $P<.001$). Again nodal status is the strongest prognostic factor in the model (data not shown). As in the analysis for RFS, in the multivariable analysis for OS it was shown that both uPA and PAI-1 were independent of the traditional prognostic factors included in the base model and, furthermore, that inclusion of both factors resulted in a better fit ($\Delta\chi^2 = 212.9$; $df = 2$) than that achieved with the inclusion of either factor alone ($\Delta\chi^2 = 149.5$ and 192.4 for uPA and PAI-1, respectively; $df = 1$) (Table 3). The Kaplan–Meier curves presented in Fig. 1, C and D, illustrate the relationships of uPA and PAI-1 as categorical variables divided into fifths ($\chi^2 = 171.9$ and $\chi^2 = 259.4$, [$df = 4$], respectively; both $P<.001$).

Interaction. The prognostic variables included in the base model were tested separately for interaction with uPA and PAI-1, with relapse as the endpoint. Significant interactions were observed between uPA and PAI-1 values with lymph node status ($\Delta\chi^2 = 13.6$, $df = 3$, and $P = .004$, and $\chi^2 = 14.4$, $df = 3$, and $P = .002$, respectively) and of uPA with tumor size ($\Delta\chi^2 = 9.3$, $df = 2$, and $P = .009$). The other interactions tested were not statistically significant, including those between uPA and PAI-1 ($\Delta\chi^2 = 1.9$, $df = 1$, and $P = .17$). The interaction of lymph node status with uPA and PAI-1 suggested the analysis of the prognostic value of uPA and PAI-1 in the clinically important subgroups of lymph node-negative and lymph node-positive patients.

Table 2. Multivariable stratified Cox regression analysis for relapse-free survival: base model*

Factor	No. of patients	Events	HR†	95% CI†	χ^2	*df*	Two-sided *P*‡
Total	8377	2955			1173	14	<.001
Age and menopausal status§					74.6	3	<.001
Age premenopausal	3338	1258	0.70	0.64 to 0.76			
Age postmenopausal	5039	1697	0.96	0.91 to 1.02			
Postmenopausal vs. premenopausal			1.35	1.18 to 1.54			
Tumor size, cm					98.8	2	<.001
⩽2	3650	953	1				
>2–5	4120	1651	1.43	1.31 to 1.55			
>5‖	607	351	1.79	1.57 to 2.04			
Lymph nodes involved					626.5	3	<.001
0	4676	1211	1				
1–3	2092	760	1.86	1.67 to 2.08			
4–10	1166	662	3.27	2.91 to 3.66			
>10	443	322	5.54	4.81 to 6.39			
Steroid hormone-receptor status¶					24.6	1	<.001
Low	1697	689	1				
High	6680	2266	0.79	0.73 to 0.87			
Histologic grade					71.4	3	<.001
III	2786	1259	1				
Unknown	2771	959	0.79	0.71 to 0.89			
II	2237	645	0.73	0.66 to 0.81			
I	583	92	0.48	0.38 to 0.60			
Adjuvant therapy					66.7	2	<.001
No	4286	1516	1				
Unknown	320	80	0.98	0.70 to 1.37			
Yes	3771	1359	0.66	0.60 to 0.73			

*Stratified for dataset.

†HR = hazard ratio; CI = confidence interval.

‡Likelihood ratio test.

§Age and menopausal status combined; age tested separately for premenopausal and postmenopausal patients.

‖Postsurgical histopathologic tumor size pT3 and pT4 combined.

¶Low and high steroid hormone-receptor status as defined in the "Methods" section.

Survival Analysis as a Function of uPA and PAI-1 Status According to Lymph Node Status of the Patients

Lymph node-negative patients. Until recently, systemic adjuvant treatment for lymph node-negative patients was not standard practice in many European countries. Of the 4676 lymph node-negative patients in the pooled dataset, 3362 (72%) did not receive systemic adjuvant therapy. To study the impact of uPA and PAI-1 on RFS and OS, we analyzed the prognostic value of uPA and PAI-1 in all 3483 lymph node-negative patients for whom both uPA and PAI-1 data were available. Table 4 (*upper part*) shows the results of adding uPA and PAI-1, separately or combined, to the base model for RFS. Except for the lack of a statistically significant association of steroid hormone-receptor status and adjuvant treatment with RFS, all traditional prognostic variables were statistically significantly associated with RFS and OS. uPA and PAI-1 were statistically significantly associated with poor RFS (Table 4, *upper part*) when added separately to the base model (all $P<.001$). Furthermore, the addition of both uPA and PAI-1 to the base model for RFS resulted in a statistically significantly better fit ($\Delta\chi^2 = 116.8$; $df = 2$) than the addition of either factor alone ($\Delta\chi^2 = 94.8$ for uPA, $\Delta\chi^2 = 83.1$ for PAI-1; $df = 1$). It is remarkable that the $\Delta\chi^2$ associated with the simultaneous addition of uPA and PAI-1 was of a similar magnitude as the χ^2 of 129.3 of the base model with all of the traditional prognostic factors. In the analysis for OS, the addition of uPA and PAI-1 together resulted in a $\Delta\chi^2$ of 95.3 ($df = 2$). This compared with 69.2 ($df = 1$) for uPA and 74.2 ($df = 1$) for PAI-1, implying a statistically significantly better fit after the addition of both UPA and PAI-1 in the analysis for OS, as well. The HRs and 95% CIs were not different when the multivariable-stratified analysis for RFS and OS were restricted to the 2864 lymph node-negative patients who had not received adjuvant treatment and for whom both uPA and PAI-1 data were available. When both uPA and PAI-1 were added to the base model for RFS, a statistically significantly better fit was observed ($\Delta\chi^2 = 94.0$; $df = 2$) than that observed after the addition of either factor alone ($\Delta\chi^2 = 76.6$ for uPA, and $\Delta\chi^2 = 66.5$ for PAI-1; $df = 1$) (Table 4, *middle part*). Similar results were obtained in the analysis for OS (uPA and PAI-1 together, $\Delta\chi^2 = 81.6$, and $df = 2$; uPA or PAI-1 alone, $\Delta\chi^2 = 57.5$ and 65.6, respectively, and $df = 1$).

To show categorized results for uPA and PAI-1 in lymph node-negative patients, we calculated a prognostic score based on the estimates from the multivariable analysis for RFS for all lymph node-negative patients (Table 4, *upper part*). For visualization in Kaplan–Meier curves, the prognostic score was divided into fifths with the use of the 20^{th}, 40^{th}, 60^{th}, and 80^{th} percentiles. In the analysis for RFS, based on the prognostic score for all 3483 lymph node-negative patients with both uPA and PAI-1 data available, the difference in 10-year RFS between the lowest and highest risk groups was 34.5% (Fig. 3, A). In the analysis for OS, the survival difference between the extreme-risk groups was 28.2% at 10 years (Fig. 3, C). For the 2864 untreated

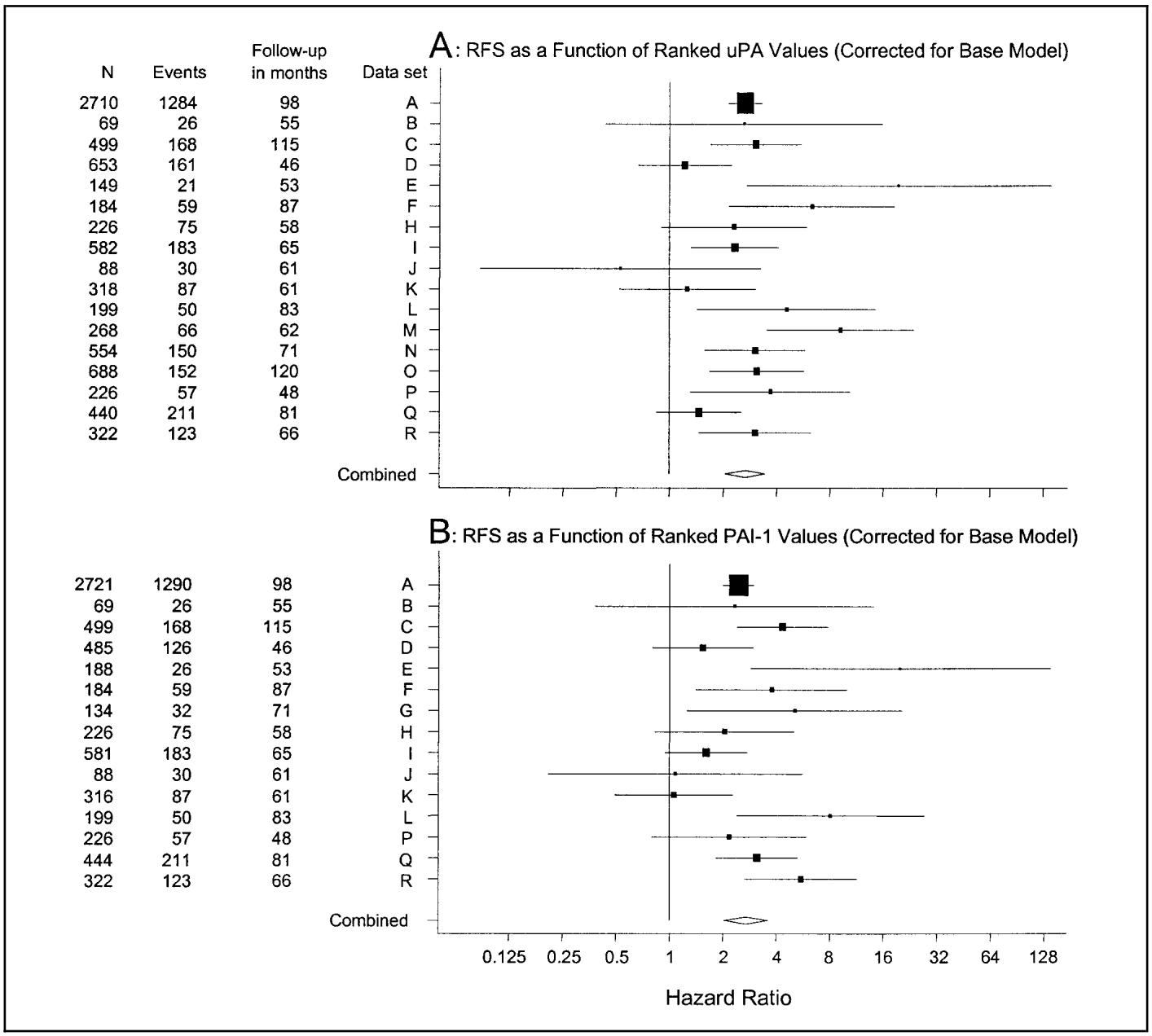

Fig. 2. Multivariable analysis for relapse-free survival (RFS) as a function of urokinase-type plasminogen activator (uPA) (**A**) and its inhibitor (PAI-1) (**B**) per dataset presented as a forest plot. All data are corrected for the base model, including age and menopausal status, tumor size, lymph node status, steroid hormone-receptor status, histologic grade, and adjuvant treatment. For each dataset, the hazard ratio (HR) of transformed ranked uPA or ranked PAI-1 values is plotted as a **solid square**, its area being inversely proportional to the variance of the estimated effect, and its 95% confidence interval (CI) is plotted as a **horizontal line.** Individual patient data were obtained from both published [in whole or in part; A *(14)*, C *(15)*, D *(16)*, F *(17)*, I *(18)*, M *(19)*, N *(20)*, O *(21)*, P *(22)*, Q *(23,24)*, and R *(25)*] and unpublished (B, E, G, H, J, K, and L) datasets. **Diamond** represents the combined random effects estimate (**middle of the diamond**) and its 95% CI (**extremes of the diamond**) of the combined estimates adjusted for the base model.

lymph node-negative patients, the differences in 10-year RFS and OS between the lowest and highest risk groups were of similar magnitude: 35.4% and 28.1%, respectively (Kaplan–Meier curves not shown).

Lymph node-positive patients. For the 2997 lymph node-positive patients with both uPA and PAI-1 data available, the results of adding uPA and PAI-1 separately or together to the base model for RFS are shown in Table 4 (*bottom part).* All traditional prognostic variables were statistically significantly associated with RFS and OS. Furthermore, uPA and PAI-1 were statistically significantly associated with poor RFS (Table 4, *bottom part*) and OS when added separately to the base model (for all, $P<.001$). In contrast to lymph node-negative patients, for lymph node-positive patients, the addition of both uPA and PAI-1 to the base model in the analysis for RFS did not result in a better fit ($\Delta\chi^2 = 88.4$; $df = 2$) than the addition of either factor alone ($\Delta\chi^2 = 52.5$ for uPA, and $\Delta\chi^2 = 87.1$ for PAI-1; $df = 1$) (Table 4, *bottom part*). However, in the OS analysis, the simultaneous addition of uPA and PAI-1 resulted in a better fit ($\Delta\chi^2 = 121.4$; $df = 2$), compared with the addition of uPA ($\Delta\chi^2 = 77.0$; $df = 1$) or PAI-1 alone ($\Delta\chi^2 = 105.9$; $df = 1$).

A prognostic score was calculated for lymph node-positive patients also. In the analysis for RFS, the difference in 10-year

Table 3. Multivariable stratified analysis for relapse-free survival and overall survival as a function of urokinase-type plasminogen activator (uPA) and its inhibitor PAI-1

Factor*	No. of patients	Events	HR†	95% CI†	χ^2	*df*	Two-sided *P*‡
Relapse-free survival							
uPA§	8175	2903	2.58	2.24 to 2.97	168.5	1	<.001
PAI-1§	6682	2543	2.58	2.24 to 2.97	176.1	1	<.001
uPA + PAI-1‖	6480	2491			203.6	2	<.001
uPA			1.70	1.42 to 2.04			
PAI-1			2.00	1.69 to 2.37			
Overall survival							
uPA§	8175	2228	2.73	2.33 to 3.19	149.5	1	<.001
PAI-1§	6682	1960	3.12	2.65 to 3.67	192.4	1	<.001
uPA + PAI-1‖	6480	1934			212.9	2	<.001
uPA			1.77	1.44 to 2.16			
PAI-1			2.34	1.93 to 2.83			

*uPA and its inhibitor, PAI-1, added alone or together, as continuous variable, to the base model presented in Table 2. Hazard ratios represent the difference between the extreme fractional ranks of uPA or PAI-1 levels (i.e., a fractional rank of 1 compared with a fractional rank of 0).

†HR = hazard ratio; CI = confidence interval.

‡Likelihood ratio test.

§uPA or PAI-1 added alone.

‖uPA and PAI-1 added togther.

Table 4. Multivariable stratified analysis for relapse-free survival in lymph node subgroups of patients*

Factor†	No. of patients	Events	HR‡	95% CI‡	χ^2	*df*	Two-sided *P*§
All lymph node-negative patients							
Base model	3483	970			129.3	11	<.001
uPA‖			3.42	2.68 to 4.37	94.8	1	<.001
PAI-1‖			2.87	2.28 to 3.60	83.1	1	<.001
uPA + PAI-1¶					116.8	2	<.001
uPA			2.37	1.78 to 3.16			
PAI-1			1.90	1.45 to 2.49			
Lymph node-negative patients without adjuvant treatment							
Base model	2864	829			105.7	9	<.001
uPA‖			3.34	2.56 to 4.36	76.6	1	<.001
PAI-1‖			2.77	2.17 to 3.55	66.5	1	<.001
uPA + PAI-1¶					94.0	2	<.001
uPA			2.34	1.71 to 3.21			
PAI-1			1.86	1.39 to 2.48			
Lymph node-positive patients							
Base model	2997	1521			476.0	13	<.001
uPA‖			2.10	1.72 to 2.56	52.5	1	<.001
PAI-1‖			2.41	2.00 to 2.90	87.1	1	<.001
uPA + PAI-1¶					88.4	2	<.001
uPA			1.34	1.06 to 1.71			
PAI-1			2.05	1.64 to 2.56			

*For patients for whom information on levels of both urokinase-type plasminogen activator (uPA) and its inhibitor (PAI-1) are available.

†uPA and PAI-1 added alone or together as continuous variable to the base models (lymph node status not included in the base models for lymph node-negative patients; adjuvant therapy not included in the base model for untreated lymph node-negative patients). Hazard ratios represent the difference between the extreme fractional ranks of uPA or PAI-1 levels (i.e., a fractional rank of 1 compared with a fractional rank of 0).

‡HR = hazard ratio; CI = confidence interval.

§Likelihood ratio test.

‖uPA or PAI-1 added alone.

¶uPA and PAI-1 added together.

RFS between the lowest and highest risk groups was 41.4% (Fig. 3, B). In the analysis for OS, the survival difference between the extreme-risk groups defined by the prognostic score was 45.9% at 10 years (Fig. 3, D).

Discussion

Identification of those prognostic and predictive factors that reflect the biology of breast cancer is important for refining our assessment of prognosis and the selection of patients who may benefit from adjuvant systemic therapy. In this respect, numerous cell biologic factors have been studied in the past decade. Except for the well-established clinical utility of steroid hormone receptors, ER and PgR, many studies on prognostic factors are hampered by inconsistent results. They are often inconclusive as a result of small numbers of patients and heterogeneity of the studies with respect to patient populations and laboratory techniques used. Therefore, guidelines have been established for the assessment of the clinical utility of prognostic factors *(35,36).* The studies reporting the impact of uPA and PAI-1 on primary breast cancer prognosis all have shown an unfavorable

clinical outcome with high levels of uPA and/or PAI-1, and virtually all of the criteria set by the guidelines have been met [for a review, *see* reference *(12)*]. More recently, it has been proposed that, for acceptance of new tumor-associated markers into clinical practice, studies should meet the Tumor Marker Utility Grading System criteria for level of evidence 1 (LOE-1) *(37,38).* According to these criteria, LOE-1 can be attained either by a prospective high-powered study of a tumor-associated marker or by a suitable meta-analysis of prospective or retrospective datasets *(37,38).* To substantiate the prognostic value of uPA and PAI-1 for patients with primary breast cancer, two LOE-1-type studies now have been carried out. The first is a prospective clinical trial in lymph node-negative breast cancer patients *(39)*, and the second is the pooled analysis presented in this article. This analysis is, to our knowledge, the first collaborative study on tumor biologic markers in breast cancer with the use of observational individual patient data.

In the various published studies on the prognostic significance of uPA and PAI-1, different variables were included in the final multivariable models, and often the numbers of events were too low to be conclusive *(12).* In this pooled analysis of individual patient data, we included the same variables and endpoints for all datasets. All analyses presented included the same traditional prognostic variables, defined as the base model. Our study population included the individual patient and laboratory data provided by the members of the EORTC-RBG. The EORTC-RBG designs and validates new laboratory assays and interacts on an international basis. It is important that all member laboratories are involved in quality-assurance programs for uPA and PAI-1 *(26,30).*

The 18 datasets included in these analyses were heterogeneous with respect to the immunoassays used, the method of tumor extraction, the protein determination, and the patient characteristics. The first three of these differences were overcome in part by transforming uPA and PAI-1 levels to fractional ranks for all individual datasets, while the introduction of a base model helped to decrease the heterogeneity between datasets as a result of the patient characteristics.

For uPA, spline-transformed ranked values with four knots, rescaled between 0 and 1, gave the best fit and were thus used in the analyses; increasing the number of the knots or analyzing by fractional polynomials did not result in a better fit. For PAI-1, no further transformation of the ranks was necessary; the ranked variable already provided the best fit. Note that, despite the heterogeneity between the individual datasets, the multivariable analysis with the use of ranked variables showed that the HRs for relapse in the individual datasets all point in the same direction, with the exception of one small study of 88 patients. In the various published studies on the prognostic value of uPA and/or PAI-1 in primary breast cancer [for review, *see* reference *(12)*], different subgroups were analyzed, such as lymph node-negative and lymph node-positive patients, premenopausal and postmenopausal patients, and steroid hormone-receptor subgroups of patients. In this study, we corrected for these traditional prognostic variables in the multivariable base model, including tumor grade and adjuvant treatment. We found a statistically significant interaction between uPA and PAI-1 with lymph node status in the analysis for RFS that led us to further investigate the subgroups of lymph node-negative and lymph node-positive patients. Analyzing subgroups ignores a large number of patients, and there is the risk of running into low numbers of events jeopardizing these analyses. For lymph node-negative and lymph node-positive patients with both uPA and PAI-1 data available, the numbers of events (970 and 1521, respectively) were sufficient, assuming that we need 15 events per category of a variable *(40).* In our final multivariable models, the required numbers of events would amount to approximately 200 for the lymph node-negative patients and 225 for the lymph node-positive patients.

The results presented here on more than 8000 female patients with primary breast cancer show unequivocally that high tumor levels of uPA and PAI-1 are associated with poor RFS and OS. Similar findings were observed in the clinically important subgroups of (untreated) lymph node-negative and lymph node-positive breast cancer patients. In the multivariable RFS and OS analyses of all patients, lymph node status was the strongest prognostic variable, followed by levels of uPA and PAI-1 together. Adding both uPA and PAI-1 to the model showed an effect twice as strong as tumor size or histologic grade, irrespective of the observed positive correlation between uPA and PAI-1 with size and grade of the tumor. Of particular interest was the observation that, in multivariable RFS analyses of lymph node-negative patients, the addition of uPA and PAI-1 together was of a similar prognostic magnitude as the base model itself, with the currently used prognostic factors included. When stratified by dataset, a prognostic score for the full model, including both uPA and PAI-1, distinguished between patients with a favorable and an unfavorable prognosis (a 34.5% difference in RFS and a 28.2% difference in OS at 10 years between the extreme-risk groups). In the most favorable 20% group of patients, at 10 years, the RFS and OS probabilities were 76.9% and 87.2%, respectively. For patients in this low-risk group, some form of adjuvant endocrine treatment could be considered, which may be less beneficial for patients with high uPA and PAI-1 levels *(13).* Some form of adjuvant chemotherapy could be offered to the remaining lymph node-negative patients. In lymph node-positive patients, with the use of the categorized prognostic score, one fifth of the patients showed a relatively good prognosis (60.9% RFS and 72.0% OS at 10 years). Furthermore, a large group of patients (40%) with a particularly poor prognosis (<30% RFS) could be identified. These latter patients could possibly benefit from an aggressive form of adjuvant chemotherapy. Moreover, patients with uPA-expressing tumors may be future candidates for receiving drugs targeting the plasminogen activation system, several of which are currently being developed and tested preclinically *(3,4).*

Despite the heterogeneity of patient samples included and the differences in tumor extraction methods and assays to measure uPA and PAI-1 levels, this meta-analysis shows unequivocally that high levels of uPA and PAI-1 are associated with a poor prognosis in primary breast cancer. The information that can be gained by measuring the tumor levels of uPA and PAI-1 is surely beyond that which is obtained by the currently used classical prognostic factors such as tumor stage and grade. This statement also holds for the clinically important subgroups of (untreated) lymph node-negative and lymph node-positive patients. In a routine setting, the levels of uPA and PAI-1 can be measured in as little as 50 mg of tumor tissue, with the use of assays that can be easily quality controlled *(26)* and standardized, making determinations of uPA and PAI-1 tumor levels feasible for almost all available primary breast tumors.

In spite of the strong data presented here, from this study, no

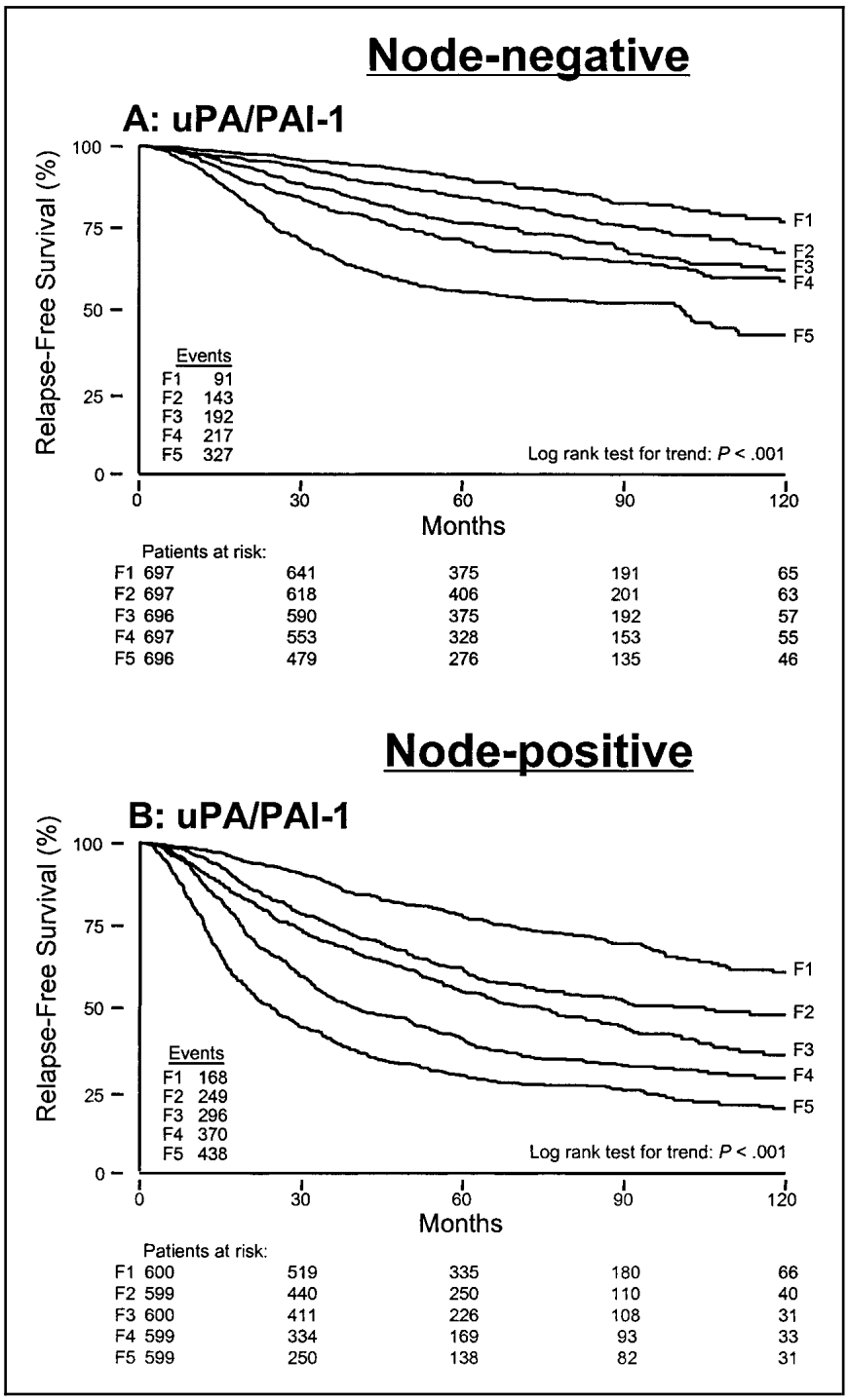

Fig. 3. Relapse-free survival (**A** and **B**) and overall survival (**C** and **D**) probabilities as a function of categorized prognostic scores in lymph node-negative (**A** and **C**) and lymph node-positive (**B** and **D**) breast cancer patients. Based on the stratified estimates for the full model (for hazard ratios of urokinase-type plasminogen activator [uPA] and its inhibitor [PAI-1], *see* Table 4), prognostic scores were calculated and divided into five groups (F1–F5) with the use of their 20^{th}, 40^{th}, 60^{th}, and 80^{th} percentiles. Events indicate the number of failures in each group. Patients at risk at 0, 30, 60, 90, and 120 months are indicated. The survival probabilities (and their 95% confidence intervals) at 60 and 120 months, respectively, are as follows: **panel A**—curve F1 = 90.0 (87.2 to 92.2) and 76.9 (71.2 to 81.6), curve F2 = 84.3 (81.1 to 86.9) and 67.6 (61.8 to 72.8), curve F3 = 76.4 (72.9 to 79.5) and 62.3 (57.0 to 67.2), curve F4 = 71.2 (67.4 to 74.6) and 58.8 (53.3 to 63.8), and curve F5 = 55.5 (51.6 to 59.3) and 42.4 (37.1 to 47.5); **panel B**—curve F1 = 77.5 (73.7 to 80.8) and 60.9 (55.3 to 66.1), curve F2 = 61.8 (57.6 to 65.8) and 47.9 (42.5 to 53.1), curve F3 = 54.8 (50.5 to 59.0) and 35.8 (30.3 to 41.4), curve F4 = 40.5 (36.3 to 44.6) and 28.9 (24.4 to 33.5), and curve F5 = 29.5 (25.7 to 33.3) and 19.5 (15.8 to 23.6); **panel C**—curve F1 = 95.0 (93.0 to 96.5) and 87.2 (82.4 to 90.8), curve F2 = 92.1 (89.6 to 94.0) and 75.7 (69.7 to 80.7), curve F3 = 90.8 (88.3 to 92.8) and 73.6 (68.1 to 78.2), curve F4 = 84.8 (81.7 to 87.3) and 69.4 (64.2 to 74.0), and curve F5 = 74.2 (70.6 to 77.4) and 59.0 (53.7 to 63.8); and **panel D**—curve F1 = 86.2 (83.1 to 88.9) and 72.0 (66.3 to 76.9), curve F2 = 74.6 (70.7 to 78.1) and 46.2 (40.2 to 52.0), curve F3 = 66.1 (62.0 to 69.9) and 44.0 (38.4 to 49.4), curve F4 = 55.4 (51.1 to 59.4) and 32.6 (27.9 to 37.5), and curve F5 = 42.3 (38.2 to 46.3) and 26.1 (22.0 to 30.4).

recommendations for specific treatments for the various risk groups of (subgroups of) patients can be made. To address this issue, data from large, prospective, randomized (multicenter) trials, where all laboratories use identical assays and are subject to external quality control, are needed. To this end, lymph node-negative patients stratified by their tumor uPA and PAI-1 levels have been randomly assigned to receive chemotherapy or observation *(39)*.

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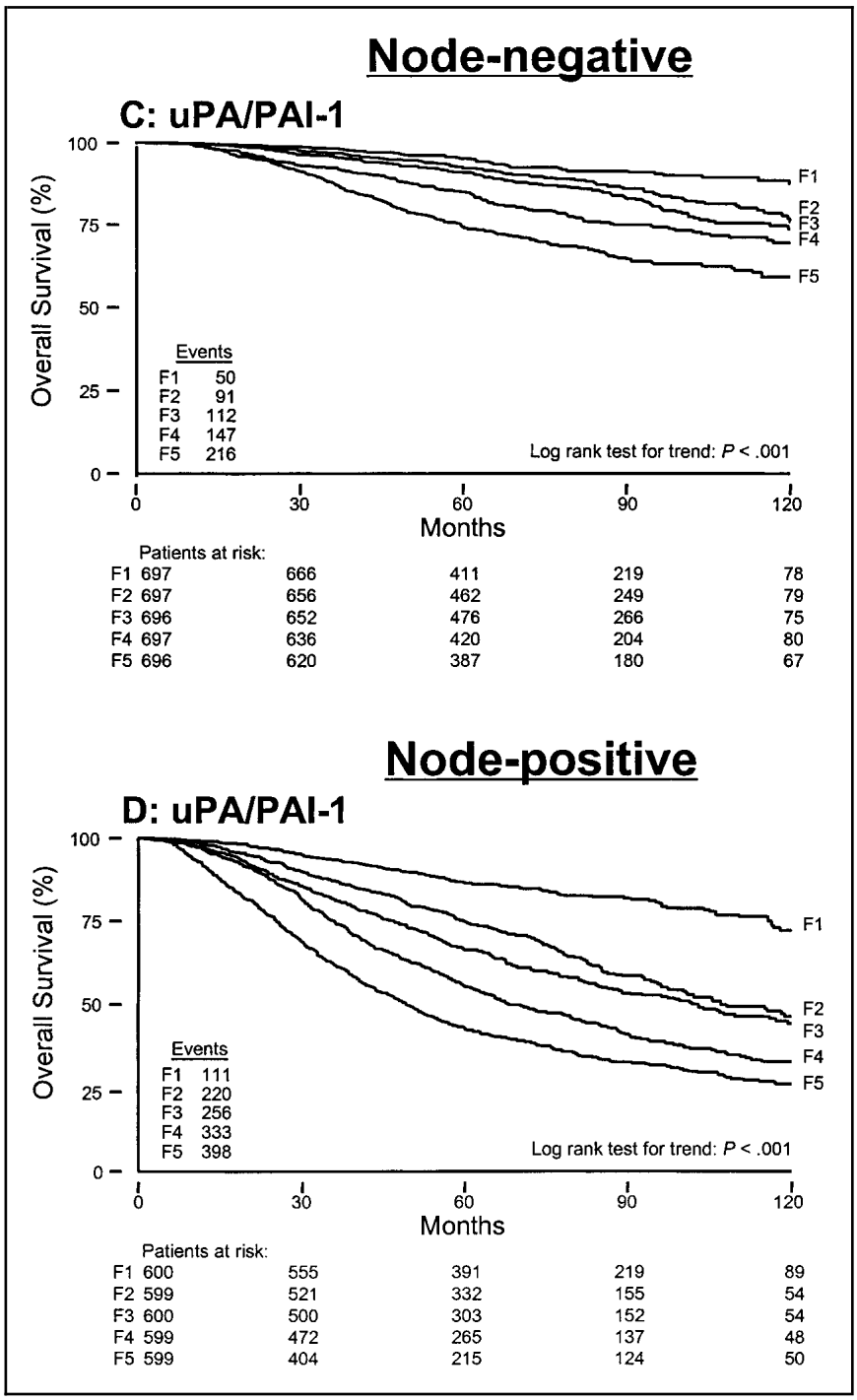

Fig. 3. Continued.

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NOTES

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