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Advances in Brief

Both the Cytosols and Detergent Extracts of Breast Cancer Tissues Are Suited to Evaluate the Prognostic Impact of the Urokinase-Type Plasminogen Activator and Its Inhibitor, Plasminogen Activator Inhibitor Type 1[1]

Fritz Jänicke, Lothar Pache, Manfred Schmitt, Kurt Ulm, Christoph Thomssen, Anita Prechtl, and Henner Graeff

Frauenklinik and Poliklinik [F. J., L. P., M. S., C. T., A. P., H. G.] and Institut für Medizinische Statistik und Epidemiologie [K. U.] der Technischen Universität München, Klinikum rechts der Isar, Ismaningerstrasse 22, D-81675 Munich, Germany

Abstract

The serine protease urokinase-type plasminogen activator (uPA) plays a key role in tumor-associated proteolysis in malignant solid tumors. Proteolytic activity of uPA is controlled by its naturally occurring plasminogen activator inhibitor type 1. As an initial observation, a correlation of enzymatic uPA activity in breast cancer cytosols with prognosis was described in 1988 (Duffy *et al.*, Cancer (Phila.), *62:* 531–533, 1988). A pronounced prognostic impact of uPA, independent of classical risk parameters, was then first demonstrated in detergent-extracted (Triton X-100) breast cancer tissues by applying enzyme-linked immunosorbent assay techniques (Jänicke *et al.*, Lancet, *2:* 1049, 1989; Fibrinolysis, 4:69–78, 1990; Duffy *et al.*, Cancer Res., *50:* 6827–6829, 1990). In addition, not only uPA but also plasminogen activator inhibitor type 1 were shown to be of prognostic value in breast cancer (Jänicke *et al.*, Semin. Thromb. Hemostasis, *17:* 303–312, 1991; Breast Cancer Res. Treat., *24:* 195–208, 1993). Subsequently, the prognostic value of uPA and plasminogen activator inhibitor type 1 was also confirmed in studies using archived "cytosol fractions" of breast cancer tissues (Foekens *et al.*, Cancer Res., *52:* 6101–6105, 1992; Spyratos *et al.*, J. Natl. Cancer Inst., *84:* 1266–1272, 1992; Grondahl-Hansen *et al.*, Cancer Res., *53:* 2513–2521, 1993; Sumiyoshi *et al.*, Int. J. Cancer, *50:* 345–348, 1992). A direct comparison of both methods with regard to prognosis, however, was lacking. We therefore prepared both the detergent-treated tissue extracts and the cytosol fractions from the same breast cancer specimens to allow a direct comparison of both methods.

In 247 breast cancer patients investigated, the Triton X-100-extracted tissues revealed about twice as much uPA antigen (uPA_{Tx}: median, 2.32 ng/mg protein) than the cytosol fractions (uPA_{cyt}: median, 1.07 ng/mg protein). In contrast, the presence of Triton X-100 did not result in an increase of PAI-1 ($PAI\text{-}1_{Tx}$: median, 6.34 ng PAI-1/mg protein) compared to the cytosol fractions ($PAI\text{-}1_{cyt}$: median, 7.15 ng PAI-1/mg protein). Good correlations between uPA_{Tx} and uPA_{cyt} ($R = 0.72$) and between $PAI\text{-}1_{Tx}$ and $PAI\text{-}1_{cyt}$ ($R = 0.88$) were observed. Furthermore, PAI-1 and uPA are moderately correlated with each other (uPA_{Tx} *versus* $PAI\text{-}1_{Tx}$: $R = 0.40$; uPA_{cyt} *versus* $PAI\text{-}1_{cyt}$: $R = 0.39$). The prognostic power of uPA showed its best advantage in Triton X-100-extracted tissues ($RR = 3.22$), most pronounced in the subgroups of node-negative and premenopausal patients, respectively. The prognostic value of PAI-1 was not influenced by the extraction procedure ($RR = 3.15$). As uPA and PAI-1 are both strong independent prognostic parameters (multivariate analysis), simultaneous determination of both factors is recommended to yield optimal prognostic information, preferentially in Triton X-100 extracts.

Introduction

Disseminated disease in breast cancer patients is based on the capacity of tumor cells for invasion and early hematogenic metastasis. Evidence has accumulated that the action of tumor-associated proteases is a prerequisite for invasion and metastasis in solid tumors causing dissolution of the surrounding tumor matrix and basement membranes (1). A certain serine protease, the uPA[2] seems to play a key role in the regulation of these proteolytic processes. The proteolytic activity of uPA is controlled by PAI-1, a naturally occurring inhibitor. The clinical relevance of these proteolytic factors is supported by the fact that uPA and PAI-1 antigen contents of breast cancer tissue are strong prognostic factors, independent of classical risk parameters (2–9). It was shown by multivariate analysis that the prognostic impact of uPA in breast cancer is as strong as that of lymph node status (3–5, 9). Moreover, in the important subgroup of axillary node-negative patients, uPA and PAI-1 were even superior to hormone receptor status, cathepsin D, and tumor size with regard to their prognostic impact (4, 5).

First, a correlation of uPA enzymatic activity in breast cancer cytosols and prognosis was observed by Duffy *et al.* in 1988 (10). Jänicke *et al.*, in extension of this preliminary observation, measured uPA antigen in detergent-extracted (Triton X-100) breast cancer tissues by ELISA. In their prospective study a strong independent prognostic impact of this proteolytic factor for disease-free survival was demonstrated (2). In 1991 Jänicke *et al.* (9) for the first time reported that high levels of the uPA inhibitor PAI-1 are also correlated with poor prognosis in breast cancer (9). This initial observation was later confirmed by Grondahl-Hansen *et al.* (7). Subsequently, various groups have assessed the prognostic impact of uPA and PAI-1 antigen also in archived cytosol fractions of breast cancer tissues which were obtained by mechanical disintegration of the tumor (5–8). A direct comparison of both methods with regard to prognosis, however, is lacking. Therefore in this investigation both the detergent-treated tissue extracts and the cytosol fractions were prepared from the same breast cancer specimens to allow a direct comparison of both methods.

Materials and Methods

Patients. In a prospective study 247 patients with breast cancer were enrolled. Treatment was by modified radical mastectomy or by breast-conserving surgery, including axillary lymph node dissection. Premenopausal patients with lymph node involvement received adjuvant chemotherapy, while postmenopausal patients received adjuvant hormone therapy with tamoxifen. No adjuvant therapy was given to node-negative patients. Detailed clinical data

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[2] The abbreviations used are: uPA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; TBS, 0.02 M Tris-HCl-0.125 M NaCl, pH 8.5; ELISA, enzyme-linked immunosorbent assay; RR, relative risk.

Table 1 *Antigen content of uPA and PAI-1 in cytosol fractions and tissue extracts (with Triton X-100) of 247 breast cancer patients*

Antigen	Triton X-100 extract			Cytosol fraction			P^b
	Median (range)	Mean ± SD	Cut-off[a]	Median (range)	Mean ± SD	Cut-off[a]	
uPA (ng/mg protein)	2.32 (0.13–15.17)	3.06 ± 2.52	2.97	1.07 (0.02–9.08)	1.67 ± 1.63	1.56	<0.001
PAI-1 (ng/mg protein)	6.34 (0.02–168.4)	10.88 ± 17.1	13.6	7.15 (0.03–116.94)	12.07 ± 17.48	10.1	<0.05

[a] Optimal cutoff values determined by CART analysis (see "Materials and Methods").
[b] Differences determined by Mann-Whitney *U* test.

and characteristics of the patients analyzed in this report are published elsewhere (4). Seventeen of 247 patients (7%) had metastatic disease upon diagnosis and these patients were excluded from the prognostic evaluation. One patient was not available for follow-up. The remaining 229 patients were followed by clinical visits every 3 months for 12–54 months (median, 30 months). Within this time of observation, 48 patients relapsed and 29 patients died.

Tissue Extraction and Laboratory Assays. Breast cancer tissue specimens were obtained at surgery, selected by the pathologist and stored in liquid nitrogen until extraction. Specimens from patients with benign breast disease or normal mammary gland tissue served as controls. Deep-frozen specimens of 200–500 mg wet weight were pulverized by using the "Mikro-Dismembrator" (Braun-Melsungen, Melsungen, Germany) set to 30 s at maximal power. The resulting powder was immediately suspended in 1.8 ml TBS, and then the samples were split into two aliquots of 0.9 ml each. One aliquot received additional 0.1 ml of TBS, the other 0.1 ml 10% (w/v) of the nonionic detergent Triton X-100 in TBS (Sigma, Munich, Germany). Both aliquots were incubated at 4°C for 12 h under gentle shaking. The suspensions were then subjected to ultracentrifugation (100,000 × *g*, 45 min, 4°C) in order to separate cell debris, nuclei, and cell membranes. The supernatants which contain uPA and PAI-1 were divided into aliquots of 50 μl each and were stored in liquid nitrogen until use. uPA and PAI-1 were determined by ELISA [American Diagnostica, Greenwich, CT; no. 894 (uPA) and no. 821 (PAI-1)]. Antigen concentrations were calculated per mg of protein. Triton X-100 up to a concentration of 1% did not affect the ELISAs. For both assays the inter- and intraassay variations are very low (<10%) and were not statistically different for cytosol and detergent extracts. Hormone receptor determination was performed by using the dextran-coated charcoal technique. Specimens were considered estrogen or progesterone receptor positive if they contained more than 20 fmol/mg protein. Protein content was determined by the BCA protein assay reagent kit manufactured by Pierce (Rockford, IL). Triton X-100 up to 1% did not interfere with the protein determination.

Statistical Analysis. To determine the relative prognostic impact of uPA and PAI-1 in relation to the effect of known prognostic factors in a prospective fashion, disease-free survival was analyzed according to Cox's proportional hazard model (11), using the BMDP software package (BMDP Statistical Software, Los Angeles, CA) and by the CART (Classification and Regression Trees) technique (12, 13). Statistical analysis included continuous as well as discrete covariates, all of which were considered as fixed (not time dependent). Determination of the optimal cut-off for uPA and PAI-1 to discriminate low and high levels was performed by using CART. The value with maximal log-rank test (14) was taken for the discrimination of high and low levels. The 95% confidence interval for this cut-off was calculated by a test-based method (15) and Bootstrap (16) techniques. Curves for disease-free survival were calculated according to the Kaplan-Meier method (17). The relative risk of the prognostic variables after discrimination of high and low were estimated by the Cox model and Pearson correlation coefficients were calculated. The relation of uPA and PAI-1 to established prognostic factors was analyzed by the Mann-Whitney *U* test or the Kruskal-Wallis test.

Results and Discussion

Extraction of breast cancer tissue by means of the nonionic detergent Triton X-100 at pH 8.5 yields about twice as much uPA antigen as mechanical disruption without the detergent (cytosol fraction). On the average, a median value of 2.32 ng uPA/mg protein was obtained when the tissues were subjected to 1% Triton X-100 extraction (uPA_{Tx}) compared to only 1.07 ng uPA/mg protein in the cytosol fraction (uPA_{cyt}) (Table 1). This difference is statistically significant at a high confidence level. Camiolo *et al.* (18) also achieved a high extraction efficiency for uPA by using the nonionic detergent Triton X-100 with the use of an acidic buffer system. However, prognostic data for breast cancer patients regarding uPA extracted at low pH have not been demonstrated. A good correlation of $R = 0.72$ (Pearson) between uPA_{Tx} and uPA_{cyt} was found in the total group of 227 patients investigated (Fig. 1) comprising 100 node-negative ($R = 0.72$) and 123 node-positive ($R = 0.74$) breast cancer patients. In four patients the axillary status was unknown.

The presence of Triton X-100 during tissue extraction did not result

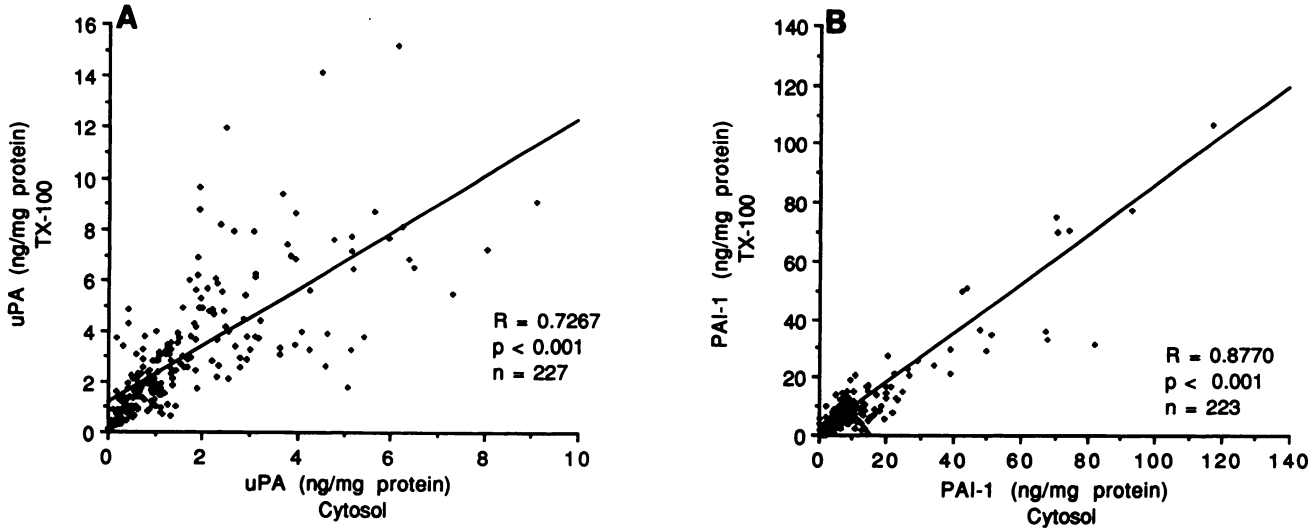

Fig. 1. Linear correlation of data sets obtained by cytosol fractions (uPA_{cyt}, $PAI\text{-}1_{cyt}$) or Triton X-100 extracts (uPA_{Tx}, $PAI\text{-}1_{Tx}$). There exists a high correlation between antigen values determined in cytosol fractions or Triton X-100 tissue extracts for both uPA and PAI-1. Evidently, the lower degree of correlation for uPA in comparison to PAI-1 ($R = 0.73$ *versus* $R = 0.88$) is due to a higher degree of scatter between uPA_{cyt} and uPA_{Tx}.

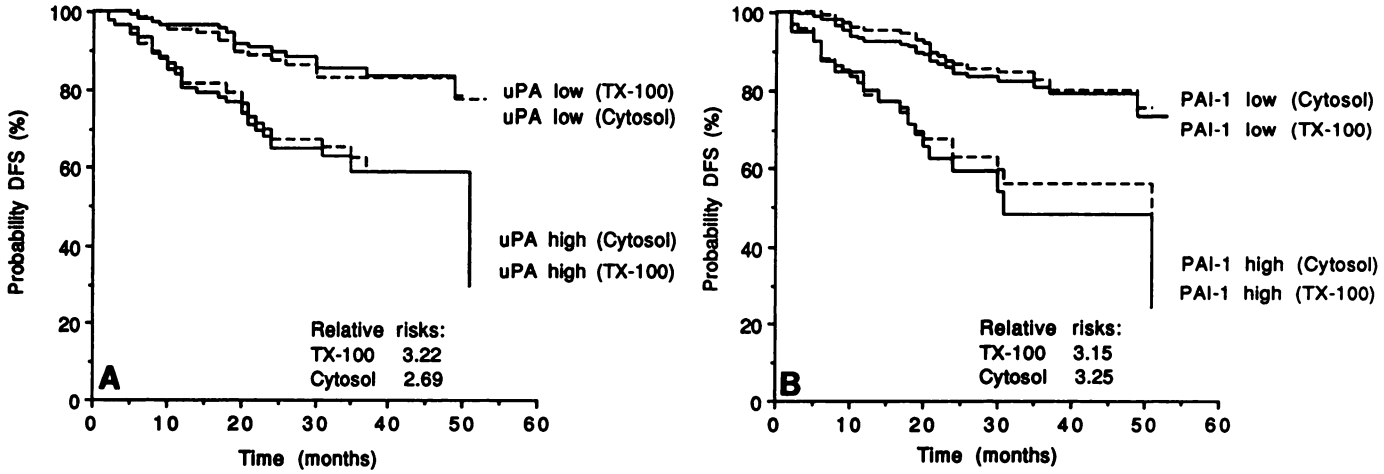

Fig. 2. Disease-free survival as a function of uPA or PAI-1 in breast cancer patients. Patients with either high uPA_{cyt} or uPA_{Tx} (*A*; $n = 229$) or high $PAI\text{-}1_{cyt}$ or $PAI\text{-}1_{Tx}$ (*B*; $n = 227$) have a significantly lower rate of disease-free survival than patients with low uPA or PAI-1. The differences in disease-free survival between the high and low risk groups are highly statistically significant ($P < 0.001$). Cutoff for uPA (per mg protein): uPA_{cyt}, 1.56; uPA_{Tx}, 2.97. Cutoff for PAI-1 (per mg protein): $PAI\text{-}1_{cyt}$, 10.1; $PAI\text{-}1_{Tx}$, 13.6. Note: the curves obtained when cytosol fractions were analyzed are almost superimposable with those curves obtained when the Triton X-100-extracted tissue samples were analyzed.

in an increase of PAI-1 antigen (Table 1). In contrast, median PAI-1 antigen content in the cytosol fraction ($PAI\text{-}1_{cyt}$: 7.15 ng/mg protein) was higher than PAI-1 in the Triton X-100 extract ($PAI\text{-}1_{Tx}$: 6.34 ng/mg protein) although no change in PAI-1 antigen yield was observed when related to tissue wet weight. This difference is based on the fact that 1% Triton X-100 treatment of our breast cancer specimens resulted in an increase of about 12% in tissue protein which leads to the apparent decrease in $PAI\text{-}1_{Tx}$ per mg protein. A quite close correlation ($R = 0.88$) was also observed between $PAI\text{-}1_{Tx}$ and $PAI\text{-}1_{cyt}$ (Fig. 1). In addition, PAI-1 and uPA are moderately correlated with each other (uPA_{Tx} *versus* $PAI\text{-}1_{Tx}$: $R = 0.40$; $P < 0.001$; uPA_{cyt} *versus* $PAI\text{-}1_{cyt}$: $R = 0.39$; $P < 0.001$).

Is the increase of uPA_{Tx} over uPA_{cyt} extracted from breast cancer tissue associated with a higher prognostic value for disease-free survival? (*a*) To discriminate between high- and low-risk patients the optimal cut-off values were determined by CART-analysis and found to be different for uPA_{Tx} (2.97 ng/mg protein) and uPA_{cyt} (1.56 ng/mg protein). For the high-risk group with $uPA_{Tx} > 2.97$, 88 patients (38%) were allocated. The high-risk group defined by $uPA_{cyt} > 1.56$ comprised 83 patients (37%), indicating a high degree of concordance of patient distribution. (*b*) Time-dependent observation of relapse formation (Kaplan-Meier analysis) revealed almost superimposable curves for disease-free survival (Fig. 2*A*).

Nevertheless, a closer look at the data brings to light that the *RR* to relapse is somewhat different for uPA_{Tx} (*RR* = 3.22; 95% confidence interval: 1.8–5.8) than for uPA_{cyt} (*RR* = 2.69; 95% confidence interval: 1.5–4.8). This difference in prognostic impact is even more evident in particular subgroups of patients (Table 2), especially in the clinically important group of node-negative patients (RR = 5.1 for uPA_{Tx} *versus* 2.48 for uPA_{cyt}) and in pre/perimenopausal patients (RR = 3.46 for uPA_{Tx} *versus* 2.07 for uPA_{cyt}). In this respect it is worth mentioning that prognostic discrimination within the group of pre/perimenopausal patients was possible only if uPA_{Tx} was determined ($P < 0.001$) (Table 2). This finding is endorsed by the results of Foekens *et al.* (5) who found in their group of 270 pre/perimenopausal patients no significant prognostic influence of uPA_{cyt}. Evidently, determination of uPA_{Tx} gives improved prognostic information. One might speculate that by treatment of tumor tissues with the non-ionic detergent Triton X-100, uPA which is bound to the uPA receptor or to thus far unidentified cellular components is released, which in the cytosol preparation escapes detection. This would be in line of the proven importance of the uPA/uPA receptor interaction in tumor invasion and metastasis (19).

Similarly, as shown for uPA, almost superimposable Kaplan-Meier curves (disease-free survival) were computed for PAI-1 (Fig. 2*B*). In contrast to uPA, however, for PAI-1 the calculation of the relative risk to relapse did not differ for $PAI\text{-}1_{Tx}$ (RR = 3.15) and $PAI\text{-}1_{cyt}$ (RR = 3.25). This was also true for subgroups based on nodal, hormone receptor, and menopausal status.

It seems somewhat contradictory that the uPA inhibitor PAI-1 is also of independent value for poor prognosis and that its ranking in multivariate analysis is close in order to that of uPA (4, 9), since one would expect PAI-1 to act protectively by blocking the enzymatic activity of free and receptor-bound uPA. Recent reports, however, documented a specific role of PAI-1 in uPA receptor clearance. Once enzymatically active uPA is bound to tumor cell surface receptor (uPA-R), PAI-1 might bind to and inactivate uPA and this ternary complex (uPA-R/uPA/PAI-1) is subsequently internalized (20).

Table 2 *Prognostic value of uPA in subgroups of patients related to mode of extraction*

		Triton X-100 extract (uPA_{Tx})[a]		Cytosol fraction (uPA_{cyt})[b]	
Subgroup	No.	Relative risk (95% CI)[c]	*P*	Relative risk (95% CI)	*P*
All patients	229	3.22 (1.8–5.8)	0.0001	2.69 (1.5–4.8)	0.0003
Node-negative	101	5.1 (1.4–18.9)	0.01	2.48 (0.8–7.8)	0.069
Node-positive	128	2.74 (1.4–11.3)	0.002	2.60 (1.3–5.0)	0.003
Hormone receptor-positive	170	3.72 (1.8–7.9)	0.0002	3.13 (1.5–6.5)	0.001
Hormone receptor-negative	59	1.79 (0.7–4.5)	0.213	1.43 (0.6–3.6)	0.429
Pre-/perimenopausal	90	3.46 (1.6–7.6)	0.001	2.07 (0.95–4.5)	0.06
Postmenopausal	139	3.12 (1.3–7.6)	0.008	3.21 (1.4–7.6)	0.005

[a] Cutoff, 2.97 ng/mg.
[b] Cutoff, 1.56 ng/mg.
[c] CI, confidence interval.

Table 3 *Multivariate analysis (disease-free survival) for 229 breast cancer patients*

Variable	RR (95% CI)[a]	*P*
Triton X-100 extract		
uPA_{Tx}	3.0 (1.7–5.4)	0.0002
Lymph node status (positive *vs.* negative)	2.9 (1.5–5.5)	0.0006
Progesterone receptor status (negative *vs.* positive)	2.2 (1.2–4.0)	0.0061
$PAI\text{-}1_{Tx}$	1.9 (0.99–3.5)	0.0536
Estrogen receptor status (negative *vs.* positive)		0.4137
Cytosol fraction		
Lymph node status (positive *vs.* negative)	2.7 (1.4–5.2)	0.0015
$PAI\text{-}1_{cyt}$	2.5 (1.4–4.4)	0.0023
Progesterone receptor status (negative *vs.* positive)	2.2 (1.2–6.0)	0.0089
uPA_{cyt}	1.9 (0.99–3.5)	0.0539
Estrogen receptor status (negative vs. positive)		0.5265

[a] CI, confidence interval.

Thereby the tumor cell is able to polarize proteolytic activity on the cell surface and thus directed invasion of tumor cells is facilitated. The process of cell migration would thus need a balanced proteolysis in which a finely tuned protease-antiprotease equilibrium must be achieved in order to limit extracellular proteolysis to the close vicinity of the cell surface and to prevent unnecessary extracellular matrix degradation. In cancer, increased PAI-1 tumoral secretion could thus favor cancer cell migration.

Both uPA and PAI-1 are strong independent prognostic factors for disease-free survival in breast cancer as determined by multivariate analysis (Cox regression), irrespective of the extraction procedure (Table 3). The most important information derived from this regression analysis is that, depending on the mode of extraction, either uPA (uPA_{Tx}) or PAI-1 ($PAI\text{-}1_{cyt}$) carries a higher weight for prognosis (expressed as relative risk, *RR*). Nevertheless, neither uPA_{Tx} entirely substitutes for $PAI\text{-}1_{Tx}$ nor $PAI\text{-}1_{cyt}$ for uPA_{cyt} (Table 3). For example, the additive prognostic value of uPA and PAI-1 has recently been reported for our node-negative patients, using Triton X-100-extracted tissues (4). Therefore we recommend always measuring uPA as well as PAI-1 to yield optimal prognostic information. In such retrospective studies where stored frozen tumor tissues are not available anymore, determination of uPA and PAI-1 in stored cytosols might be satisfactory for the estimation of prognosis in certain subgroups of patients (see Table 2). In prospective studies, however, determination of uPA and PAI-1 should also be performed in detergent-extracted tissues to gain optimal prognostic discrimination within the subgroup of node-negative or premenopausal patients.

Should recommendations already be given to use definite cut-off values for uPA and PAI-1 for clinical decision making in breast cancer? In our group of patients the cut-off values for uPA and PAI-1 tend to be stable when analyzed after 12, 24, and 30 months of follow-up and are not significantly different in patient subgroups (see Tables 1 and 2). A direct comparison of these cut-offs with values published by other investigators in different sets of patients is not possible at present due to differences in tissue extraction and assay procedures. To harmonize extraction procedures, assays, and statistical approaches and thereby cut-offs, a multicentric study has started this year as part of the newly established European Concerted Action, "clinical relevance of proteases in tumor invasion and metastasis," supported by the Commission of European Communities (BIOMED-1 program). Clinical recommendations on the use of extraction procedures, assays, and cut-off values will be based on these results.

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