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Comparison of potential biological markers cathepsin B, cathepsin L, stefin A and stefin B with urokinase and plasminogen activator inhibitor-1 and clinicopathological data of breast carcinoma patients

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Abstract

Cysteine, serine and metalloproteinases and their respective inhibitors are involved in tumor cell invasion and may have prognostic value for the outcome of malignant disease. The aim of the study was to compare the expression of new potential biological tumor markers, the lysosomal cysteine proteinases and their endogenous inhibitors, with that of the serine proteinases and their inhibitors in breast cancinoma and to relate their levels to the clinicopathological factors of the disease. Enzyme-linked immunosorbent assays (ELISAs) were used to measure cysteine cathepsin B (CatB) and cathepsin L (CatL) and their inhibitors, stefin A (StA) and stefin B (StB), together with urokinase (u-PA) and plasminogen activator inhibitor-1 (PAI-1), in 150 cytosols of primary invasive breast carcinoma. A good correlation was found between the levels of the two cysteine proteinases but only a moderate one between those of the cysteine and serine proteinases. u-PA and PAI-1 levels correlated positively with histological grade and negatively with estrogen receptor (ER) status. PAI-1 correlated with most clinicopathological factors that indicate the progression of the disease, while cathepsins and stefins were independent of these factors. In the total group of patients, high u-PA and PAI-1 and low StB levels correlated significantly with shorter disease-free survival (DFS), while CatB, CatL and StA did not. In lymph node negative patients, high CatB and CatL were also associated with shorter DFS, while u-PA remained the most significant of all these biological markers. In conclusion, this retrospective study showed u-PA to be of better prognostic relevance than the cysteine proteinases, though CatB and CatL were relevant for prognosis in lymph node negative breast cancer patients.

Keywords: Cathepsin B; Cathepsin L; Stefin A; Stefin B; Urokinase (u-PA); PAI-1; Breast cancer

1. Introduction

Metastatic spread is mediated by the most malignant tumor cells, which detach from the primary tumor, invade surrounding tissue and enter the lymph or blood circulation or both to form distant metastases. The processes involve degradation of extracellular matrix (ECM) or basement membrane (BM) or both, which is mediated by a variety of proteolytic enzymes. These act either alone or in a cascade of proteolytic events, in which aspartic, cysteine, serine and metalloproteases are involved [1,2].

The lysosomal cysteine proteinases, cathepsin B (CatB) and cathepsin L (CatL), degrade several components of ECM in vitro [3,4] and in vivo [5] and can also activate pro-enzymes, such as pro-(u-PA) to their active forms [6]. Previous studies have shown that the increase in activities and protein and mRNA levels of CatB and CatL in several human tumors [7], such as breast [8–13], head and neck carcinoma [14] and lung cancer [15] correlated with shorter disease-free survival (DFS) and decreased overall survival (OS) [16] The activities of cysteine proteinases are regulated by their endogenous inhibitors, stefin A (StA) and stefin B (StB), cystatins and kininogens [17]. Altered levels of the inhibitors have been found to be associated with several pathological states, including cancer [18]. Lower mRNA and protein levels of StA in breast tumors were found in

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patients with clinical indicator of bad prognosis [8] and down-regulation of StB was reported in carcinoma of the esophagus [19]. Such clinical studies strongly suggest that cysteine cathepsins and their inhibitors are also potential biological markers and prognosticators in breast carcinoma ([8–13], for review see [16]).

The main physiological role of urokinase (u-PA), a serine protease, is to convert the inactive pro-enzyme plasminogen to plasmin [20–22] which can itself activate certain metalloproteases or directly degrade a variety of protein components of the ECM and BM. This leads to the suggestion that the enzyme participates in tissue re-modeling in various physiological and pathophysiological conditions, including cancer. Activation of the enzyme is initiated with pro-(u-PA) binding to specific plasminogen activator receptor (u-PAR) on the cell membrane. The u-PA–u-PAR complex also binds plasminogen activator inhibitor-1 (PAI-1), which controls the u-PA activity. On the other hand, u-PAR and PAI-1 interact with cell adhesion molecules and their receptors and the system is thereby associated with cell migration mechanisms. In breast tumors, increased levels of u-PA, PAI-1 and u-PAR play a crucial role in tumor cell surface associated proteolysis and have been shown to be prognostic for the outcome of the disease [23–26]. A high prognostic impact was found for u-PA and its inhibitor PAI-1 in the cytosols and in detergent extracts of breast cancer tissues [24]. They have a particularly significant impact on prognosis in lymph node negative breast tumor patients [25].

Although these proteolytic components have been found to be relevant for prognosis of the outcome of the disease in several studies of breast carcinoma patients, the prognostic significance of CatB, CatL, StA and StB [9–13,16] and u-PA and PAI-1 [20–26] has so far not been measured nor have the two groups been correlated in these tumors. The aim of this study was: firstly, to determine any correlation between the amounts of these proteins in biopsies of invasive ductal breast carcinoma; secondly, to correlate the levels of cysteine and serine proteases and their inhibitors with clinical and histopathological parameters indicating disease progression; and thirdly, to evaluate their prognostic relevance for DFS.

2. Materials and methods

2.1. Characteristics of patients and tumors

The study was carried out with ethical committee approval (Ministry of Health, Slovenia). One hundred and fifty female patients, diagnosed with primary invasive ductal breast carcinoma and treated at the Institute of Oncology in Ljubljana over the period 1991–1995, were included in the study. The selection criteria of the tumors was based on the availability of the stored cytosol (at 20 °C) remaining after routine estrogen receptor (ER) and progesterone receptor (PR) analysis. The characteristics of patients are presented in Table 1. The median age was 62 years, ranging from 33 to 91 years. The median size of the tumors was 3.0 cm, ranging between 1 and 11 cm in diameter. Fifty-nine percent of the patients had metastases in the axillary lymph nodes and 76% of patients were TNM stage IIA/IIB. Fifty-eight percent of the tumors were grade III, 60% of the tumors expressed ER and almost half tumors expressed PR.

Table 1
Characteristics of breast cancer patients

Clinical and histopathological characteristics of patients	Number of patients (*n*)	Percentage
All patients	150	100
Relapse	44	29
Local recurrence	2	1
Distant metastasis	42	28
Age (years)		
Median	62	
Min–max	33–91	
Tumor size (median 3.0 cm)		
≤2.0 cm	30	20
2.1–5.0 cm	99	66
>5.1 cm	21	14
Lymph nodes		
Negative	56	37
Positive	88	59
Unknown	6	4
ER		
>10 fmol/mg	90	60
<10 fmol/mg	60	40
PR		
>10 fmol/mg	69	46
<10 fmol/mg	81	54
Histological grade		
1	11	7
2	49	33
3	88	58
Unknown	2	2
Stage TNM		
I	16	10
IIA/IIB	114	76
IIIA/IIIB	13	9
IV	7	5
Median (min–max) follow-up (months)	45 (1–72)	
Median (min–max) DFS (months)	34 (1–71)	

2.2. Treatment and follow-up

Primary treatment of patients was radical surgery (mastectomy in 88% of cases) and breast conserving surgery (in 12% of cases), including axillary lymph node dissection. No radiotherapy was given to the patients included in this study. Lymph node positive patients were treated additionally with systemic adjuvant therapy: chemotherapy in 30% of cases, hormone therapy in 40% (only patients with positive hormone receptor status) and both, chemotherapy and hormone therapy in 26% of patients. Four percent of lymph

node positive patients received no additional therapy. In the lymph node negative population, 21% of patients received no adjuvant therapy, while 79% received systemic adjuvant therapy: 23% chemotherapy, 59% hormone therapy and 18% both chemotherapy and hormone therapy (patients with positive hormone receptor status). Chemotherapy consisted of six courses of CMF (cyclophosphamide, methotrexate and 5-fluorouracil, repeated every 21 days) and hormone therapy consisted of tamoxifen (30 mg p.o. daily). The patients were followed up by clinical check-ups every 3–6 months during the first 5 years and once every year after that. The median follow-up time was 45 months. The end point of the study was the recurrence of the breast cancer. The DFS was the time from the surgery of the primary tumor to the reported recurrence of the disease. The median DFS time was 34 months (Table 1).

2.3. Tissues

In 83 patients (55%), normal breast parenchyma were obtained from the same breast and taken as control tissue. Pathological staging was performed according to TNM [27]. Histological grading was carried out according to Nottingham scheme [28]. Tumor and control tissue specimens were obtained at surgery and stored in liquid nitrogen until extraction. Frozen specimens were pulverized using a micro-dismembrator (Braun Biotech International). The resulting frozen powder was suspended in EORTC buffer (1 mM dipotassium hydrogen phosphate, 1.5 mM dipotassium chloride EDTA, 3 mM sodium azide, 10 mM monothioglycerol, 10% glycerol, pH 7.4) as described for the determination of steroid receptors [29]. The homogenates were centrifuged (40,000 $\times$ g, 1 h, 4 °C) to separate cell debris, nuclei and cell membranes. The supernatants also named cytosols, were aliquoted (100 μl) and stored at −70 °C for several years before being used for analysis of protease components.

2.4. Enzyme-linked immunosorbent assays (ELISAs)

ELISAs for human CatB, CatL, StA and StB were developed at the Department of Biochemistry and Molecular Biology (Jožef Stefan Institute, Slovenia) and are commercially available from KRKA d.d. (Novo mesto, Slovenia). The ELISAs were performed as suggested by the manufacturer. Their characteristics, including linearity, specificity and sensitivity have been described [30]. CatB and CatL purified from human tissue and recombinant human StA and StB were used as standards. ELISAs from Monozyme (Horsholm, Denmark) were used for u-PA and PAI-1 determinations and carried out as described [24]. Single-chain recombinant human u-PA and diluted human plasma were used as standards. As internal controls, two pools of normal breast cytosols and tumor cytosols were prepared and stored in aliquots at −20 °C and assayed along with the samples.

2.4.1. CatB

For CatB, immunoselective sheep and rabbit polyclonal antibodies (Abs) were used as capture and detection Abs, respectively. Tumor samples at 1:25 (v/v) dilution and control samples at 1:20 (v/v) dilution were used in the assay.

2.4.2. CatL

For CatL, a sandwich ELISA was used, with sheep anti-CatL immunoselective IgG as capture Abs and horseradish-peroxidase-conjugated Abs for detection. Control samples were not diluted, while tumor samples were diluted 1:10 (v/v).

2.4.3. StA and StB

For StA, monoclonal Ab C5/2 was used as a capture Ab and monoclonal A2/2 Ab, conjugated with horseradish-peroxidase, for detection. Tumor and control samples were used at 1:10 (v/v) dilution. For StB, monoclonal Ab A6/2 was used as a capture Ab and horseradish-peroxidase-conjugated monoclonal Ab E7/1 for detection. Tumor samples were used at 1:20 (v/v) dilution and control samples at 1:10 (v/v) dilution.

2.4.4. u-PA

Polyclonal anti-human u-PA was used as capture Ab and a mixture of three biotinylated monoclonal anti-human u-PA Abs was used for detection. Control samples were not diluted, while tumor samples were diluted 1:5 (v/v).

2.4.5. PAI-1

For PAI-1, sandwich ELISA was used with monoclonal capture and detection Abs. Control samples were not diluted, while tumor samples were diluted 1:5 (v/v).

2.4.6. Protein determination

Protein concentration of the cytosols was determined by Bio-Rad protein assay (Bio-Rad, Richmond, CA) using bovine serum albumin as standard.

2.4.7. ER and PR

Hormone receptor status was determined according to the protocol of DDV Diagnostika (Marburg, Germany). Tumors with ER and PR levels above 10 fmol/mg protein were considered hormone receptor positive.

2.5. Statistical methods

Wilcoxon's rank test was used for analysis of differences in the levels of the variables (tumor and control tissues). Correlation between biochemical parameters was calculated by Spearman's method. Their relation to clinical and histopathological factors was determined by the Kruskal–Wallis test. Survival curves were estimated using the univariate Kaplan–Meier method [31] and compared by log-rank test. P-values below 0.05 were considered statistically significant. Cut-off values for continuous variables

were calculated using isotonic regression analysis. Comparison between randomly selected samples, comprising an experimental set and a validation set, resulted in the cut-off value, which was used for the total and lymph node negative group of patients and this was also obtained using Cox proportional hazards model [32].

3. Results

3.1. Cathepsins, stefins, u-PA and PAI-1 in tumor and control cytosols

The results of measurements of CatB, CatL, StA, StB, u-PA and PAI-1 in matched pairs of breast tumor and control cytosols showed very significantly higher levels ($P < 0.001$) in protein concentrations of CatB (8.5-fold), CatL (5.2-fold), StA (1.9-fold), StB (3.9-fold), u-PA (5.7-fold) and PAI-1 (12.3-fold) in tumor tissue than in cytosols from normal breast parenchyma.

The above results were used to compare the enzyme: inhibitor ratios between tumors and control tissues. The ratios in tumors were greater by 4.4-fold for CatB:StA, 2.15-fold for CatB:StB, 2.9-fold for CatL:StA and 1.3-fold for CatL:StB than those in control tissue. Although both u-PA and PAI-1 levels were higher in tumors, the increase in inhibitor was markedly greater than in proteinase, in contrast to what is observed for cathepsins and stefins.

3.2. Correlation between cathepsins, stefins, u-PA and PAI-1 levels in breast tumor cytosols

Levels of CatB, CatL, StA, StB, u-PA and PAI-1 were correlated by non-parametric Spearman's test. The strongest correlation was found between CatB and CatL ($r = 0.65$). u-PA also correlated with PAI-1 ($r = 0.41$) and CatB weakly with StB ($r = 0.32$). The correlation between CatB and u-PA was also weak ($r = 0.32$) and no correlation was found between u-PA and CatL.

3.3. Association with histopathological parameters

There was no significant association between CatB, CatL, StA, StB levels and factors prognostic for disease progression, such as lymph node status, ER, PR, grade, tumor size or TNM stage. In contrast, significantly ($P < 0.001$) higher u-PA levels were observed in histological grade 3 than in 1 and 2. Also, significantly ($P < 0.01$) higher concentration of u-PA were found in ER negative tumors ($P < 0.01$). Significantly elevated PAI-1 levels were observed in patients, who had all the above clinical and histopathological factors indicating poor prognosis, except the tumor size. In general, the ratios between the enzymes and the inhibitors in tumors were not related to the clinical and histopathological indicators of breast tumor progression, the only exception being the increased ratio of u-PA:PAI-1 in high grade tumors ($P < 0.02$).

3.4. Prognostic relevance of clinical and histopathological parameters

Using Kaplan–Meier univariate analysis, we found that lymph node involvement and histological grade had the strongest prognostic impact for DFS (both $P < 0.001$). In this study, ER, PR and tumor size were not significantly associated with the DFS (results not shown).

3.5. Prognostic relevance of cathepsins, stefins, u-PA and PAI-1

The prognostic significance of the biochemical parameters is shown in Table 2. High levels of u-PA and PAI-1

Table 2
Prognostic significance of cathepsins, stefins, u-PA and PAI-1 in total group of patients and lymph node negative patients[a]

Prognostic parameter (cut-off value)	Number of patients (n_1/n_2)[b]	Number of events (n_1/n_2)[b]	DFS (P)
Total group of patients			
CatB (not found)	NS[c]	–	NS
CatL (not found)	NS	–	NS
StA (21 ng/mg)	79/70	–	NS
StB (67 ng/mg)	98/51	24/20	0.059
u-PA (0.8 ng/mg)	50/98	20/24	0.001
PAI-1 (1.7 ng/mg)	34/114	14/30	0.021
Lymph node negative patients			
CatB (430 ng/mg)	30/24	2/7	0.049
CatL (70 ng/mg)	22/31	1/8	0.034
StA (21 ng/mg)	33/23	3/6	0.058
StB (67 ng/mg)	40/16	1/5	0.070
u-PA (1 ng/mg)	15/40	5/4	0.003
PAI-1 (1.5 ng/mg)	13/43	–	NS

[a] Kaplan–Meier statistical analysis (log-rank test). Cut-off values were obtained as described in Section 2.5.
[b] n_1: Number of patients below cut-off; n_2: number of patients above cut-off value.
[c] NS: Not significant.

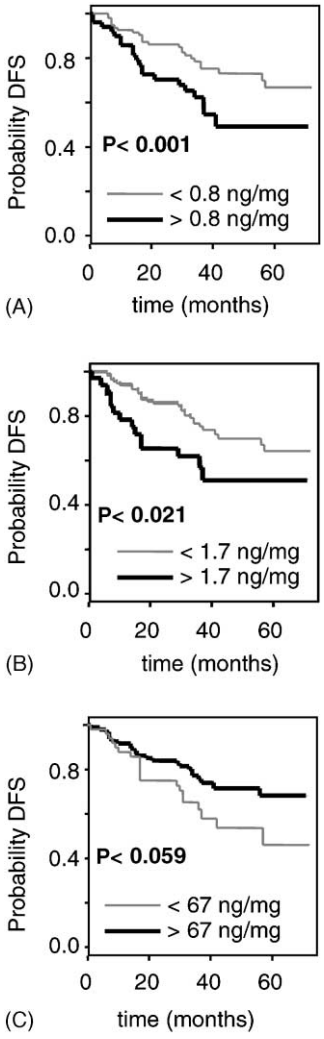

Fig. 1. Impact of the proteases and inhibitors levels in cytosolic extracts on DFS in total group of patients. Patients were divided into two groups with levels of the indicated parameter above and below the respective cut-off values. *P*-values were calculated by log-rank test as discussed in Section 2.5. A: u-PA; B: PAI-1; C: StB.

showed the most significant association with shorter DFS ($P < 0.001$ and $P < 0.021$, respectively) (Fig. 1A and B). Cathepsins and StA levels were not significantly related to DFS. However, patients with high levels of StB showed longer DFS than patients with low StB levels ($P < 0.059$), although with borderline significance (Fig. 1C). We have also stratified the entire population into the sub-groups of lymph node negative and positive patients and determined the prognostic significance for all the proteolytic parameters (Table 2). In lymph node negative patients, u-PA remains the most significant, although we observed significant increases in prognostic relevance of CatB ($P < 0.049$), CatL ($P < 0.034$) and StA ($P < 0.058$), compared with that in the total group of patients. No prognostic significance was observed in lymph node positive patients (results not shown). It is noteworthy that PAI-1, which has a strong prognostic impact in the total group of patients, had no such an impact in the lymph node negative patients.

Table 3
Prognostic significance of ratios between enzymes and inhibitors[a]

Prognostic parameter (cut-off value)	Number of patients (n_1/n_2)[b]	DFS (*P*)
CatB:StA (32)	27/121	0.040
CatB:StB (4.3)	97/51	0.011
CatL:StA (3)	68/77	NS[c]
CatL:StB (0.5)	114/29	0.072
u-PA:PAI-1 (10.83)	61/89	0.032

[a] Kaplan–Meier statistical analysis (log-rank test).
[b] n_1: Number of patients below cut-off; n_2: number of patients above cut-off value.
[c] NS: Not significant.

We also evaluated the prognostic significance of the enzyme to inhibitor ratios, such as CatB:StA, CatB:StB, CatL:StA and CatL:StB and u-PA:PAI-1. The ratio CatB:StB was the most significant for DFS, followed by u-PA:PAI-1 and CatB:StA (Table 3).

4. Discussion

One of the proposed roles of CatB and CatL in the pathophysiological mechanisms of breast tumor progression is the processing of proteins crucial for cell de-differentiation and development of metastatic cell phenotype [7,33]. Since more of the lysosomal enzymes in the tumor cells are associated with the cell surface or secreted than in normal cell [34,35], they may activate protease precursors bound to the plasma membrane or when released extracellularly. The activation of pro-(u-PA) by CatB and CatL has been reported [6,36] and we might expect a correlation between cysteine cathepsins and u-PA. However, in this study, we found a poor ($r = 0.32$) correlation between u-PA and CatB, similar to that reported by Duffy et al. [22] ($r = 0.24$) and no correlation with CatL. This does not support a hypothesis of a close association in regulation between the cysteine cathepsins and u-PA. In contrast, both cathepsins correlated well ($r = 0.65$) with each other, which could indicate their coordinated up-regulation in tumors. Correlation between the enzymes and their inhibitors was highest between increased PAI-1 levels ($r = 0.41$), as reported previously [24,26], possibly indicating their interaction in tumor tissues. We observed a moderate correlation between cathepsins and stefins, the highest being between CatB and StB ($r = 0.32$). This does not, however, allow speculation about their specific interaction, since numerous interactions are possible between cysteine cathepsins and members of the cystatin superfamily and the pathophysiological relevance of which is not so far known.

This retrospective study has confirmed our previous findings, that in breast tumors, the increase in expression of cysteine cathepsins is far greater than the increase in expression of their intracellular inhibitors StA and StB, compared

with the control tissue [11]. This supports our initial hypothesis that the relatively lower levels of the inhibitors are partially responsible for highly elevated proteolytic activity of CatB and CatL in breast tumor tissue [8]. Inversely, a much greater increase in PAI-1 than u-PA was observed in tumors, resulting in a lower u-PA:PAI-1 ratio and suggesting a possible decrease in tumors. u-PA activity in tumors. u-PA and PAI-1 are also associated with most of the clinical and histopathological factors, as reported [21,24,26] while the cysteine cathepsins and stefins appear to be independent of other clinical parameters of cancer progression. Similar findings were reported for CatB and CatL by Thomssen et al. [10], whereas, Foekens et al. [12] found marginal associations of cysteine cathepsins with clinical parameters. Our data, therefore, show that the regulation of the two proteolytic systems during tumor progression is different.

The separate correlations of serine [20–26] and cysteine [8–13,16] proteinases to prognostic impact have been reported. Here, we have aimed to estimate the prognostic impact of these two proteolytic systems relative to each other. Due to the small number of patient events and the heterogeneity of therapeutical protocols, the cut-off and the significance (*P*-values) found in this study are similar, but not identical to those found in the previous studies. However, we believe that the comparisons are valid, as the proteolytic enzymes measured in our study are presumably not related to the response to therapy. With respect to prognosis, we showed that u-PA and PAI-1 were of markedly higher prognostic significance in the total population of patients than the cysteine proteinases and their inhibitors. u-PA and PAI-1 have been suggested as new biological prognostic factors for breast cancer in numerous studies [20–26]. Their prognostic potentials were found to be comparable with that of the axillary nodal status [37,38]. Furthermore, multi-centre studies are currently in progress to standardize the protocols for their clinical application [39].

In contrast, the relative prognostic impact of cysteine cathepsins and their inhibitors in breast carcinoma has not been intensively studied. Here, we found that increased levels of CatB and CatL were significantly related to poor prognosis only in lymph node negative patients, suggesting that CatB and CatL may be active in the early spread of local breast carcinoma. This is not in complete agreement with previous studies. For example, in multivariate analysis for DFS, Thomssen et al. [10] found the prognostic impact of CatL (but not of CatB), comparable to that of axillary lymph node status and grading in a total patient population. Similarly, Foekens et al. [12] reported that high levels of both CatB and CatL have strong ($P < 0.0001$) and independent prognostic relevance on early relapse and death in all patients. In our earlier study of 282 patients [13], cytosolic CatB had a stronger impact for relapse and death than CatL and, similarly to this study, the prognostic significance was markedly increased in lymph node negative patients. This would suggest that cysteine cathepsins should be used for prognosis in this specific group of patients, who are at lower risk of relapse. The discrepancies regarding the prognostic impact of cysteine proteinases in different studies are related to different study designs [12], such as different sizes of patient population and uncontrolled biological differences of patients, such as menopausal status and cyclic variation in cathepsin expression in pre-menopausal women [40]. Also, methodologies for preparation of cytosols and the storage of tumor tissues, affecting the stability of CatB and CatL, are different [41], emphasizing the need to introduce unified protocols and external references in future clinical studies of cathepsins.

This is also the first report of a correlation, although at borderline significance, of higher levels of the two stefins with poor prognosis for DFS (Table 2) in a lymph node negative population of breast carcinoma patients. This is in agreement with our pilot study of 60 matched pairs and control tissue where we found an inverse correlation between the increase in tumor concentration of StA with prognosis [11]. Similarly, the activity of cysteine protease inhibitors and levels of StA mRNA were both down-regulated in the majority of breast tumor tissues in patients exhibiting poor clinical prognosis [8,9]. However, these findings contrast with the immunohistochemical study, where intense StA staining indicated bad prognosis [42] and with the study on extracellular levels of StA and StB in sera of colorectal cancer patients, which indicated the association of high levels of inhibitors with poor prognosis [43]. In the present study, low levels of StB indicated bad prognosis in the total and lymph node negative populations of patients, although at borderline significance. Lower levels of StB have also been found to be prognostic for survival of lung carcinoma [44] and head and neck carcinoma patients [14].

The ratios between the enzymes and their respective inhibitors have also been found to be significant for prognosis of patient survival [11,45]. In our study, the increased ratios CatB:StA and CatB:StB related to poor prognosis more significantly than the levels of cathepsins and stefins alone, suggesting that the balance between the enzymes and the inhibitors is relevant for tumor malignancy.

In conclusion, this pilot study showed that in breast tumors the balance of enzymes and inhibitors in both cysteine and serine dependent proteolytic systems is disturbed so that they are regulated in a non-coordinated fashion. With respect to their clinical relevance, u-PA and PAI-1 appear to have a higher prognostic impact in the total group of patients than the cysteine cathepsins and stefins. However, CatB and CatL and their increased ratios to stefins may be useful for prognosis in lymph node negative patients, who are at lower risk for relapse and death after removal of the primary tumor.

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