Prognostic and predictive value of cathepsins D and L in operable breast cancer patients

M. JAGODIC, I. VRHOVEC, S. BORSTNAR, T. CUFER

Department of Medical Oncology, Institute of Oncology, e-mail: monika.jagodic@siol.net, SI-1000 Ljubljana, Slovenia

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None of the established prognostic factors in breast cancer (BC) is able to determine the final outcome with certainity. Tumor biological factors involved in tumor invasion and metastasis, such as cathepsins and proteins of u-PA system, have been put forward in the recent literature as strong novel prognostic factors in BC. We therefore evaluated prognostic and predictive value of cathepsin-D (CD) and cathepsin-L (CL) in 715 operable BC patients. CD and CL were determined in tumor extracts using immunoradiometric and ELISA assays, respectively. During follow-up (median 37 months), 151 (21%) patients relapsed. In a multivariate analysis of disease-free survival (DFS), CL ($p=0.04$), nodal status ($p<0.001$) and hormone receptor status ($p<0.001$) were the only independent significant prognostic factors. CL thus provided independent prognostic information on DFS and could also predict a response to adjuvant chemotherapy (ChT), while CD had no significant prognostic and predictive impact.

Key words: breast cancer, cathepsin D, cathepsin L, prognosis, treatment prediction

In operable BC patients, the oncologist's first concern is to find out whether or not the disease is likely to relapse after primary treatment. Evaluation of established prognostic factors, such as nodal status, tumor size, tumor grade, vascular/lymphatic tumor invasion, hormone receptor status, and menopausal status, is one way of predicting the natural course of the tumor. Nevertheless, no any of these factors is of absolute reliability. New prognostic factors are thus still required to improve prognostic evaluations.

In the 1990s, researchers came to the idea that different proteolytic factors such as CD, u-PA and PAI-1 could asses the prognosis in patients with different solid tumors [7, 18, 22, 28, 32], including BC [1, 7]. In many studies in the last decade, high CD antigen levels in primary BC have been linked with a poor patient outcome [8, 9, 11, 37]. Although early results with CD as a prognostic factor in BC were promising, the subsequent ones led to conflicting data [20, 31]. By using immunoradiometric assay to detect CD, most investigators usually found a relationship between high levels of the protease and poor prognosis when a univariate or/and multivariate analysis of survival was performed on total populations [2, 4, 8, 11, 12, 13, 14, 15, 27, 29, 33]. However, when subgroups of patients were studied different results were obtained in respect of nodal status [4, 9, 14, 15, 20, 27, 29]. In many published studies using different methods of assays, CD was recognized as an independent prognostic factor [2, 4, 8, 11, 13, 27, 29]. In other studies, CD did not have any significant prognostic value already in univariate analysis of survival [19, 21, 25, 26, 30, 31, 34]. Despite numerous studies, the prognostic value of CD for BC patients remains controversial; so, further, but better defined prospective studies with standardized methods of CD assay should be performed.

Much fewer publications can be found on the clinical relevance of CL in BC, which was proposed to be a promising proteolytic prognostic factor, although not enough studies were performed to draw a final conclusion. There were used different methods of CL assays, either ELISA test [10, 16, 23, 26, 36] or immunohistochemistry [6] or both of them [24] The independent prognostic significance of CL in total populations of BC patients was confirmed in three studies

Abbreviations: BC – breast cancer; CD – cathepsin D; CL – cathepsin L; DFS – disease-free survival; ChT – chemotherapy; ER – estrogen receptors; PR – progesterone receptors; RR – relative risk; HT – hormone therapy

[10, 16, 36]. On the other hand different researchers [6, 23, 24, 26] did not find any significant prognostic impact of CL in total populations of BC patients even in univariate analysis of survival and in three studies only a rather small number of patients was included [6, 24, 26]. In only three published studies [17, 23, 35], the prognostic impact of CL was analyzed in the subgroups of patients categorized by nodal status. In two of them, however CL failed statistical significance in node negative patients in a multivariate analysis of survival [17, 35].

Data on the predictive value of CD and CL for response to adjuvant systemic therapy are also still very scarce. FERNO et al [8] first reported that CD could be a predictive factor for the treatment effect of adjuvant tamoxifen on BC patients with positive lymph nodes. Later, BILLGREN et al [4] also confirmed that the patients with high levels of CD and positive estrogen receptors (ER) who received adjuvant tamoxifen had better DFS. There are also two reports [27, 30] about possible predictive value of high levels of CD for worse response to adjuvant ChT. Finally, HARBECK et al [17] reported about the predictive value of high levels of CD for a good response to adjuvant tamoxifen and adjuvant ChT, contrary to CL which retained a strong prognostic significance in treated patients.

To assess and rank clinical applicability of CD and CL, we studied their prognostic impact and predictive value within our large series of primary BC patients and compared them with those of established prognostic factors.

Patients and methods

Patients. A total of 715 patients with primary operable BC, undergoing curative surgical treatment between 1996 and 2000 at the Institute of Oncology Ljubljana and at the General hospital Celje, entered the study. Clinical and pathological data on the age of patients, stage of disease, menopausal status, kinds of primary treatment – surgical treatment, radiotherapy and adjuvant systemic therapy, histological type, size and grade of tumor, hormone receptor status, vascular/lymphatic tumor invasion, nodal status, values of cathepsins D and L in tumor extracts were determined. The stage of disease was determined according to the UICC-WHO criteria. Tumor grade was classified according to the Scarf-Bloom-Richardson classification. Estrogen receptors (ER) and progesterone receptors (PR) were assessed using the dextran-coated charcoal method recommended by the EORTC. The cut-off value for positivity of ER and PR was set at 10 fm/mg protein. Surgical treatment was performed in all patients first. In about two thirds of patients, modified radical mastectomy was performed. Other patients underwent quadrantectomy or some other kind of breast conserving surgery. Radiotherapy after surgery was given to more than one third of patients, including the majority of patients after conservative surgery and also the patients after modified radical mastectomy with bad prognosis. Ninety per cent of all patients received some kind of adjuvant systemic therapy according to the guidelines followed by our institute at that time. The patients with evidence of distant metastases at the time of primary treatment were not included in the study. Median age of the patients at the time of primary surgery was 58, with a range of 26–88 years. After completion of primary treatment, the patients were regularly monitored (median follow-up 37 months). Clinical and pathological characteristics of patients are presented in Table 1.

Tissue extraction and assays for CD and CL. For biochemical analysis of CD and CL, histologically confirmed tumor specimens were obtained from the tumor during surgery and immediately immersed in liquid nitrogen until extraction. Pulverization was performed on the frozen tissue with a micro-dismembranator (Braun, Melsungen, Germany). Each grounded tumor specimen was divided in two parts for preparing tumor cytosols and triton extracts. The resulting tissue powder from one part of each tumor specimen was suspended in phosphate buffer (1.7 mM KH_2PO_4, 5 mM Na_2HPO_4, 10% (v/v) glycerol, 1 mM monothioglycerol, pH 7.4). This suspension was then centrifuged for 90 min at 100,000 x g and at 4 ˚C to obtain the supernatant, i.e. cytosol. Total protein concentration in tissue cytosol was determined by the method of Bradford, viz. BIO-RAD method [5]. CD levels were determined in cytosol fraction of tumors by the commercially available immunoradiometric assay test (ELSA-CATH-D, CIS bio international, Gif-sur-Yvette, France). The antigen content of CD in cytosols was expressed as picomols of analyte per milligram of tissue protein. In order to obtain triton extracts, the second part of each grounded frozen tumor specimen was suspended in the following buffer-TBS: 0.02 M Tris-HCl, 0.125 M NaCl, 1% Triton X-100, pH 8.5. The resulting suspension was shaken for 3 hours at 4 ˚C and then centrifuged under the same conditions as for tissue cytosol. In triton extracts, we first determined protein content using the BCA method. The levels of CL were determined in triton extracts of tumors by commercially available ELISA test (Human Cathepsin L ELISA test, Krka, d.d., Novo mesto, Slovenia), which was developed at Jozef Stefan Institute, Ljubljana, Slovenia. The concentration of CL was expressed in picomols of analyte per milligram of tissue protein. The quality of the measurements was monitored by an ongoing quality assurance program for measurement of biological variables in tumor tissue.

Statistical analysis. Spearman rank correlation test was used to test the relationship between CD and CL. The statistical assessment of the correlation of CD and CL with other established prognostic factors was carried out using the Mann-Whitney test. We were trying to determine the optimal cut-off values of CD and CL that could best differ-

Table 1. Patient and tumor characteristics

Characteristic	Number	(%)
Menopausal status		
Premenopausal/perimenopausal	221/715	(31%)
Postmenopausal	479/715	(67%)
Unknown	15/715	(2%)
Histological tumor type		
Invasive ductal	604/715	(84%)
Invasive lobular	62/715	(9%)
Other invasive	40/715	(6%)
Unknown	9/715	(1%)
Tumor size		
PT1 (0–9 mm)	11/715	(2%)
PT2 (10–20 mm)	167/715	(23%)
PT3 (21–49 mm)	446/715	(62%)
PT4 (≥50 mm)	77/715	(11%)
Unknown	14/715	(2%)
Tumor grade (only invasive ductal)		
G I	75/715	(11%)
G II	287/715	(40%)
G III	338/715	(47%)
Unknown	15/715	(2%)
Lymph node status		
Negative	319/715	(45%)
Positive	382/715	(53%)
Unknown	14/715	(2%)
Hormone receptor status		
Negative	150/715	(21%)
Positive	531/715	(74%)
Unknown	34/715	(5%)
Vascular/lymphatic invasion		
Absent	500/715	(70%)
Present	215/715	(30%)
Unknown	0	

entiate the patients with favorable prognosis from those with unfavourable one by use of log-rank statistics, after CD and CL had been coded as binary variables. DFS was calculated by the Kaplan-Meier method according to all variables, and the differences were assessed with the log-rank test. Univariate and multivariate analyses were performed by applying the Cox proportional hazards model. All statistical analyses were performed using the SPSS software package (SPSS Inc., Chicago, IL, USA). All tests were performed at a significance level of α=0.05 and at a confidence interval of 95%.

Results

CD and CL levels. The range of levels of CD and CL, their median values, lower and upper quartile and cut-off values are shown in Table 2. For CD, the median value 50.3 pmol/mg was taken as cut-off for further analysis because an optimal cut-off discriminating low-risk and high-risk patients, was not found. In fact, optimal cut-off value for CL 5.35 pmol/mg was very close to median value.

Correlations between CD, CL and established prognostic factors. CD and CL significantly correlated with each other ($p<0.001$, $r=0.396$), although this correlation was not strong. The levels of CD were significantly higher in tumors larger than 2 cm, in G III tumors, in tumors with vascular/lymphatic invasion and in patients with positive axillary lymphnodes. CL was significantly associated with tumor size, histological tumor type, tumor grade and hormone receptor status. Correlations are shown in Table 3.

Prognostic significance of CD and CL. At the median follow-up of 37 months, it was discovered, that 151/715 (21%) of patients had relapse, 18/715 (2.5%) patients had local reccurences, 116/715 (16.2%) patients had distant metastases, and 17/715 (2.4%) had both. The three-year DFS of the whole group was 79%. There was no statistical difference ($p=0.209$) in DFS with regard to the values of CD above and under the median value. The three-year DFS was 78% in patients with CD values above the median value and 81% in the group with CD values below it (Fig. 1). On the other hand, there was a statistically significant ($p=0.0067$) difference in the levels of three-year DFS with regard to the values of CL. The patients with the levels of CL exceeding 5.35 pmol/mg protein had significantly poorer prognosis than the patients with CL values below that cut-off (75% vs. 84%, respectively) (Fig. 2). The Kaplan-Meier curves (Fig. 1 and 2) show the univariate impact of CD and CL on the three-year DFS.

Univariate survival analysis. In univariate Cox analysis of DFS, CL was a statistically significant prognostic factor, as well as the tumor size and grade, nodal status, hormone receptor status and vascular/lymphatic tumor invasion. CD, menopausal status and histological tumor type did not have any significant prognostic impact. The results of the univariate analysis for three-year DFS can be seen in Table 4.

Multivariate survival analysis. In multivariate analysis (Cox model) of survival, including all significant prognostic factors from univariate analysis of survival, CL ($p=0.04$, RR=1.48) retained a statistically significant independent prognostic impact, together with the nodal status ($p<0.001$, RR=1.93) and hormone receptor status ($p<0.001$, RR=0.38). The tumor size, the tumor grade and vascular/lymphatic invasion lost their independent prognostic value. The results of multivariate analysis including all significant prognostic factors are presented in Table 4.

Table 2. Summary of measurements of cathepsins D and L

Factor	Number of patients (%)	*Cut-off value	*Mini-mum	*1 st quartile	*Median	*3 rd quartile	*Maxi-mum
Cathepsin D	352 (50%)	low (<50.3)	4.3	33.9	50.3	69.92	297
	354 (50%)	high (≥50.3)					
Cathepsin L	349 (50%)	low (<5.35)	0.66	3.9	5.33	7.09	23.64
	346 (50%)	high (≥5.35)					

*in pmol/mg protein

Table 3. Correlations and associations between cathepsins D/L and established prognostic factors

Variable	Number of patients	*Median value (interquartile range) Cathepsin D	Cathepsin L
Menopausal status			
Pre/perimenopausal	221	47.2 (31.4–67.5)	5.36 (3.99–6.85)
Postmenopausal	479	50.95 (35.72–72.2)	5.33 (3.84–7.17)
p-value		0.192	0.828
Tumor size			
≤2 cm	178	45 (31.5–60.1)	4.79 (3.59–6.19)
>2 cm	523	52 (35.6–73.8)	5.47 (4.11–7.59)
p-value		0.013	0.009
Histological tumor type			
Invasive ductal	604	50.75 (35.52–71.6)	5.47 (4.03–7.45)
Other	102	42.4 (28.32–63.57	4.6 (3.34–5.76)
p-value		0.05	0.005
Tumor grade			
I+II	362	45.75 (32.52–66.05)	4.64 (3.51–6.06)
III	338	53.8 (38.4–73.4)	6.08 (4.78–8.27)
p-value		0.001	<0.01
Nodal status			
Negative	319	47.2 (32.3–61.4)	5.26 (3.74–6.97)
Positive	382	53.75 (37.02–73.8)	5.41 (4–7.26)
p-value		0.002	0.355
Hormone receptor status			
Negative	150	49 (29.7–68.1)	5.84 (4.35–8.57)
Positive	531	50.6 (34.8–70.8)	5.08 (3.84–6.61)
p-value		0.425	0.047
Vascular/lymphatic invasion			
Absent	500	48.8 (33.37–67.35)	5.33 (3.81–7.07)
Present	215	55.15 (34.97–77.22)	5.32 (4.11–7.38)
P-value		0.01	0.8

*The median values in pmol/mg protein

Univariate analysis of DFS in patient subgroups defined by adjuvant systemic therapy, CD and CL. Table 5 shows differences in the three-year DFS with regard to the values of CD/CL and kinds of adjuvant systemic therapy. Considering the adjuvant systemic therapy and values of CD, there was no significant difference in DFS in any subgroup of patients (Fig. 3). In the subgroups of patients defined by CL values and adjuvant systemic therapy, CL retained a statistically significant prognostic impact only in the subgroup of patients who received adjuvant hormone therapy (HT) alone or combined with ChT. The patients with high values of CL who received adjuvant ChT only had slightly better DFS than patients with low values of CL, although the difference was not statistically significant (Fig. 4). A risk for relapse is in fact equal in patients treated with adjuvant ChT alone regardless of CL values.

Discussion

To discuss briefly the methods, we determined the antigen levels of CD in cytosol fraction by the same commercially available immunoradiometric assay (ELSA-CATH-D kit, CIS bio international, Gif-sur-Yvette, France) as was performed by most other investigators. In addition to the measurements of other researchers, we also determined the concentrations of CL by use of ELISA. Our assay was developed by Jozef Stefan Institute, Ljubljana, Slovenia (Human Cathepsin L ELISA test, Krka, d.d., Novo mesto, Slovenia). According to the results of our preliminary analysis, the optimal results for CL determination by ELISA (Human Cathepsin L ELISA test, Krka, d.d., Novo Mesto, Slovenia) were obtained in triton extracts of tumors, contrary to other researchers, who used tumor cytosols. The results of CL determination in our tumor cytosols were not acceptable due to an amazing variability of results of repeated analyses. In Slovenia, in two smaller studies, LAH et al [23, 26] also used same ELISA kit as we did, but they performed the analysis in tumor cytosols and did not mention any problems associated with the CL determination.

According to the results of our study, CL determined in triton extracts of tumors, was an important independent prognostic factor, in addition to nodal status as the strongest prognostic factor, followed by hormone receptor status.

In concordance to the results of other studies [10, 16, 36],

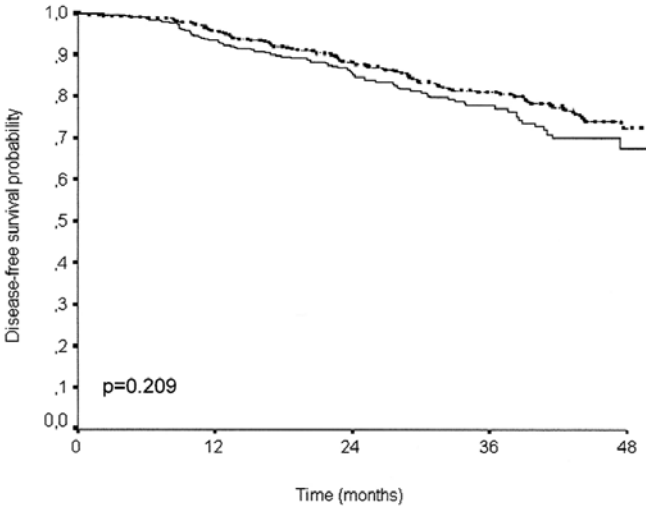

Figure 1. Disease-free survival with regard to cathepsin D.
▪▪▪▪▪▪▪ cathepsin D ≥50.3 pmol/mg,
——— cathepsin D ≤50.3 pmol/mg.

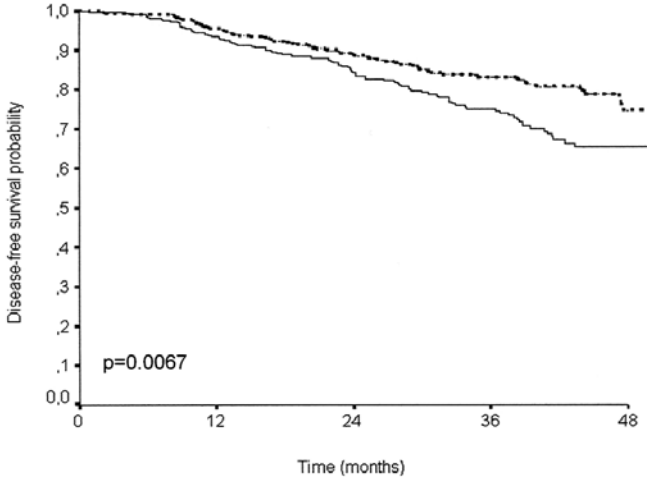

Figure 2. Disease-free survival with regard to cathepsin L.
▪▪▪▪▪▪▪ cathepsin L ≥5.35 pmol/mg,
——— cathepsin L ≤5.35 pmol/mg.

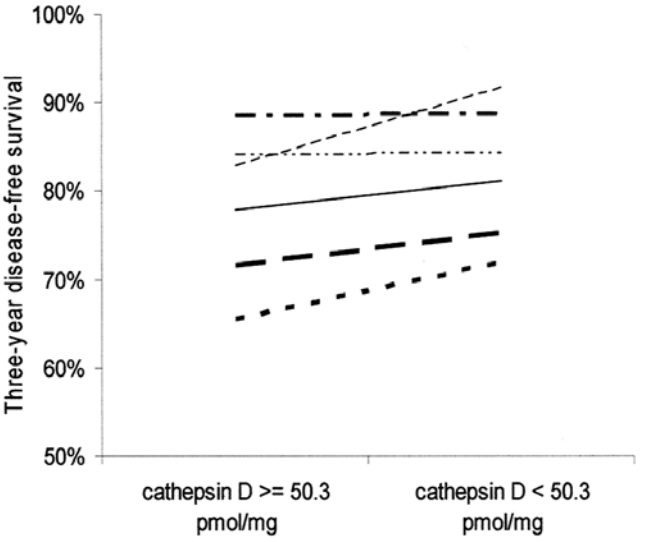

Figure 3. Disease-free survival with regard to the values of cathepsin D and adjuvant systemic therapy.
——— all, - - - - - - - without adjuvant systemic therapy, – · – · HT, – – – – ChT, – · · – · · HT or HT + ChT, —— — ChT or ChT + HT, Abbreviations: HT – hormone therapy, ChT – chemotherapy.

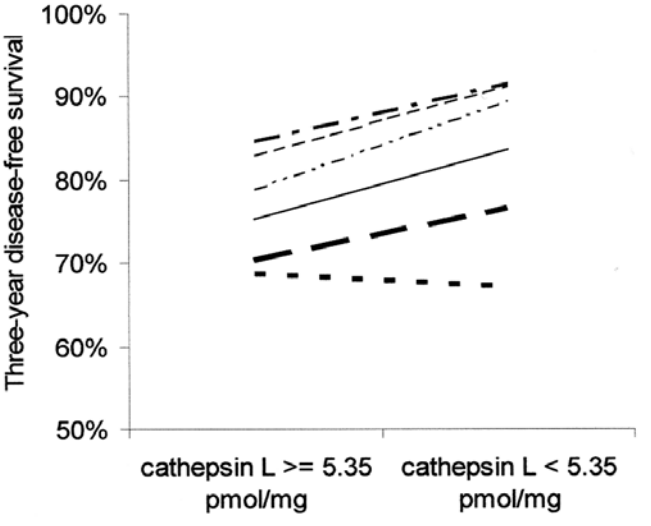

Figure 4. Disease-free survival with regard to the values of cathepsin L and adjuvant systemic therapy.
——— all, - - - - - - - without adjuvant systemic therapy, – · – · HT, – – – – ChT, – · · – · · HT or HT + ChT, —— — ChT or ChT + HT, Abbreviations: HT – hormone therapy, ChT – chemotherapy.

our study also proved that CL was an important independent prognostic factor. In all of these studies [10, 16, 36], the values of CL were determined by ELISA test. In patients with high levels of CL, three-year DFS was significantly worse than in patients with low levels of CL [10, 16, 36]. Multivariate analysis of survival in our study revealed that patients with high values of CL in tumors had nearly 1.5 times hig her RR than patients with low levels of it. Only one study [10] analyzing prognostic impact of CL was larger, including 1500 patients; the RR of 1.59 obtained by multivariate analysis of DFS was similar to ours. It is interesting, that LAH et al [23, 26] did not find any significant prognostic impact of CL in two studies including 60 and 282 BC patients, with the exception of those with negative lymph nodes [23]. They used the same ELISA kit (Krka d.d., Slovenia) as we did, but performed the analysis of CL content in tumor cytosols, not in triton extracts. Probably, the statistical power of the first study was too low to show a significant prognostic impact of CL [26]. In another study of 77 BC patients, using different ELISA kit (Bio-Ass Diesen, Germany) in tumor cytosols and immunohistochemistry, LAH

Table 4. Univariate and multivariate analysis of disease-free survival

Prognostic variable	Univariate analysis p value	Univariate analysis Relative risk (95% CI)	Multivariate analysis p value	Multivariate analysis Relative risk (95% CI)
Cathepsin D				
≥50.3 pmol/mg protein vs. <50.3 pmol/mg protein	0.21	1.23 (0.89–1.70)	–	–
Cathepsin L				
≥5.35 pmol/mg protein vs. <5.35 pmol/mg protein	0.007	1.57 (1.13–2.18)	0.04	1.47 (1.01–2.14)
Menopausal status				
Postmenopausal vs. pre/perimenopausal	0.365	0.85 (0.61–1.19)	–	–
Histologycal tumor type				
Others vs. invasive ductal	0.367	1.22 (0.79–1.89)	–	–
Pathological tumor size				
>20 mm vs. ≤20 mm	0.002	2.05 (1.31–3.21)	0.273	–
Tumor grade				
III vs. I + II	0.013	1.51 (1.09–2.10)	0.633	–
Nodal status				
Positive vs. negative	<0.001	2.11 (1.73–2.57)	<0.001	1.92 (1.53–2.42)
Hormone receptor status				
Positive vs. negative	<0.001	0.405 (0.28–0.56)	<0.001	0.38 (0.26–0.55)
Vascular/lymphatic invasion				
Present vs. absent	<0.001	1.94 (1.40–2.68)	0.211	–

CI – confidence interval

et al [24] again did not confirm any significant prognostic impact of CL. In one of studies, LAH et al [23] reported a cut-off value of CL 0.714 pmol/mg, only. This result seems to be paradoxally low in comparison with our 5.35 pmol/mg, determined in triton extracts. We would expect lower cut-off value of CL in triton extracts due to a larger proportion of different proteins extracted from a membrane by use of detergent; therefore the proportion of CL in extracted membrane proteins is smaller. In the largest study, FOEKENS et al [10] also reported a low cut-off value of CL of 0.22 pmol/mg determined in tumor cytosols by the same ELISA kit as applied in the study by LAH et al [23] and in ours. They also tried to explain such result by comparing it with the cut-off value of CL 13.428 pmol/mg determined by THOMSSEN et al [36] in tumor cytosols. In both studies by FOEKENS et al [10] and THOMSSEN et al [36] CL levels were not comparable despite the use of ELISA formats that were adopted for the quantification of total CL in tissue cytosols by both groups [10, 36]. These differences may be due to the recombinant antigen, used for the preparation of standards in the assay of FOEKENS et al [10], or the pretreatment of standards to ensure good parallelism with the samples upon dilution. Despite these differences, high levels of CL were associated with a poor relapse-free survival in both studies [10, 36]. In another study published in 1998, THOMSSEN et al [35] did not confirm the independent prognostic value of CL in 103 node negative patients. In both studies by THOMSSEN et al [35, 36] the determined cut-off points were almost equal (13.428 pmol/mg vs. 12.85 pmol/mg). As expected in our study CL cut-off point determined in triton extracts was more than half lower than the cut-off point in both studies by THOMSSEN et al [35, 36] using tumor cytosols.

As to the patient's characteristics, we observed a substantial difference between our study and others in a proportion of patients receiving some kind of adjuvant systemic therapy. In other studies [10, 16, 17, 36] only 12% to 59% of patients were treated with adjuvant systemic therapy. In our patients CL retained its significant prognostic impact despite of the fact that the majority of them received some kind of adjuvant systemic therapy. In the available literature, only HARBECK et al [17] reported about the predictive value of CL for response to adjuvant systemic therapy, which was not confirmed. In that study, CL maintained its strong prognostic impact in treated and untreated patients [17]. According to the results of our study, high values of CL could also predict a response to adjuvant ChT, because the three-year DFS in the patients with high values of CL, who received adjuvant ChT, was slightly better than DFS in equally treated patients but with low values of CL, although the difference in DFS was not statistically significant. In all other subgroups of our patients classified by adjuvant systemic therapy, DFS was better in the patients with low values of CL, although the differences in survival were not statistically significant, with the exception of the subgroup that received either HT alone or HT combined with ChT. In this subgroup of patients, high values of CL significantly reduced the three-year DFS. Data on the predictive value of CL are still too scarce to provide definitive conclusions.

In our study CD did not have any significant prognostic impact in univariate analysis of DFS already. This fact was not unexpected due to conflicting results of published stu-

dies, including many studies [19, 21, 25, 26, 30, 31, 34] with totally negative results. We could not define any uniform cut-off values of CD for a significant optimal prognostic separation of patients despite the use of different methods, e.g. log-rank test, also segregation of CD values by quartiles and tertiles. Most of the studies have dichotomized the values according to the median value, which enables comparisons of high levels with low levels independently of which methods of measurement have been used, so, we chose a median value for all further analyses. We focused our attention on the largest published studies also using immunoradiometric assay. In largest studies [4, 11, 13, 15, 30], including 710 to 2810 BC patients, independent prognostic value of CD for all included patients was confirmed in three [4, 11, 13] of them. RAVDIN et al [30] did not confirm a significant independent prognostic value of CD, determined by use of immunoradiometric assay in 1984 BC patients, as well as in their previous study [31] of 927 node negative BC patients, using Western blotting and immunohistochemistry for the CD determination instead of immunoradiometric assay. In the study of FOEKENS et al [12], CD was not an independent prognostic factor in 657 BC patients; however the primary objective of that study was focused on prognostic value of PAI-1. In other large studies [8, 15] an independent prognostic significance of CD was found only in the subgroup of patients with positive lymph nodes.

Table 5. Disease-free survival with regard to adjuvant systemic treatment and values of cathepsins D and L

Subgroup	Variable	No. of patients	3-year DFS	*p value	*RR (95% CI)
All	CD high	354	78%	0.21	1.22
	CD low	352	81%		(0.88–1.69)
Withouth adjuvant systemic therapy	CD high	27	83%	0.573	1.40
	CD low	51	92%		(0.42–4.61)
HT	CD high	116	88%	0.677	1.14
	CD low	107	89%		(0.52–2.48)
ChT	CD high	115	65%	0.234	1.31
	CD low	109	72%		(0.83–2.07)
HT or HT+ChT	CD high	211	84%	0.677	1.11
	CD low	192	84%		(0.67–1.84)
ChT or ChT+HT	CD high	210	71%	0.258	1.24
	CD low	194	75%		(0.85–1.80)
All	CL high	346	75%	0.007	1.57
	CL low	349	84%		(1.13–2.18)
Withouth adjuvant systemic therapy	CL high	30	83%	0.151	2.32
	CL low	49	91%		(0.71–7.65)
HT	CL high	96	85%	0.303	1.49
	CL low	121	91%		(0.69–3.24)
ChT	CL high	129	69%	0.759	1.07
	CL low	91	67%		(0.68–1.69)
HT or HT+ChT	CL high	186	79%	0.023	1.83
	CL low	209	89%		(1.08–3.08)
ChT or ChT+HT	CL high	219	70%	0.133	1.34
	CL low	179	76%		(0.91–1.97)

*univariate Cox analysis for DFS with a relative risk for relapse associated with CD/CL. CD – cathepsin D; CL – cathepsin L; ChT – chemotherapy; DFS – disease-free survival; HT – hormone therapy; No. – number; RR – relative risk; CI – confidence interval

Immunoradiometric assay has been found to yield acceptable and comparable results, as was shown in an External Quality Trial performed by EORTC Receptor Study group [3]. So, it is not easy to explain the divergent results obtained by immunoradiometric assay; since all investigators have used the same assay, and we can postulate that the varying results relate more to differences in the selected patient populations than to CD assays. In our opinion, there are many reasons due to which CD failed to show a significant prognostic impact in our present study. First, we observed a substantial difference between our and other studies in the number of patients treated with adjuvant systemic therapy. In the majority of large and also small studies, the proportion of patients who received adjuvant systemic therapy ranged from only 23% up to 72%. In our study, 90% of all patients received some kind of adjuvant systemic therapy. So, we suspected first that the effect of adjuvant systemic therapy given to the majority of our patients could totally diminish the prognostic strength of CD, as it happened in the study by HARBECK et al [17] Compared to our study, HARBECK et al [17] also looked at the influence of adjuvant systemic therapy within patient subgroups defined by adjuvant systemic therapy. They reported no significant difference in DFS with regard to CD values in the BC patients treated with adjuvant systemic therapy [17]. Similarly STONELAKE et al [34] and HAWKINS et al [19] did not find any significant prognostic impact of CD in the studies including BC patients almost all treated with some kind of adjuvant systemic therapy, although the effect of adjuvant systemic therapy on prognostic value of CD was not an

objective of their studies at all. With regard to CD values, the three-year DFS of our patients treated with adjuvant HT alone or of those treated with combined ChT and HT was almost equal, whereas in all other patients with high values of CD, it was worse. But there was no significant difference in DFS of our patients with regard to CD values, irrespective of whether they did or did not receive adjuvant systemic therapy, and irrespective of the kind of systemic treatment. So we could not confirm a predictive value of CD for a response to adjuvant systemic treatment. Other available data suggesting a possible predictive value of CD for response to adjuvant HT [4, 8, 17] and ChT [17, 27, 30] are too scarce for a definitive conclusion. In agreement with other investigators who also used immunoradiometric assay, we concluded that other differences in patient populations among studies, like a population size, age of patients, duration of follow-up, cut-off points and methods of their determination, other tumor characteristics and differences in nodal status, strongly influenced the results of our study on prognostic significance of CD, although the most prominent difference in patients characteristics, was a proportion of patients treated with adjuvant systemic therapy.

On the basis of the results of our study, we can conclude, that CL is a strong prognosticator of DFS in a multivariate analysis and could also predict a response to adjuvant ChT. Thus, CL deserves further research, especially the prognostic value of combinations of CL with proteolytic factors of u-PA protein system should be evaluated. On the other side, we did not find CD to be a clinically useful variable for predicting the prognosis of BC patients and response to adjuvant systemic therapy. Thus, the literature and also presented study suggest that CD is not as powerful and reliable prognostic variable as we have hoped. Prospective studies addressing the predictive impact of CD and CL and other proteolytic factors in primary BC are still scarce. Ideally, a study of prognostic factors should involve only patients who have received no adjuvant systemic therapy. However, such studies have become nearly impossible to be carried out because adjuvant systemic therapy is recommended for an ever-widening range of patients with BC.

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