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# Immunoradiometric assay of cathepsin D: Estrogen-regulated *vs*. nonestrogen-regulated cathepsin D expression in relation to clinicopathological features of breast cancer

**KEYWORDS: Breast Neoplasms; Immunoradiometric Assay; Cathepsin D**

## *ABSTRACT*

***Background:*** *Cellular biomarkers may predict tumor cell behavior in breast cancer. One of the most paradoxical biomarker in breast cancer is cathepsin D.*

***Patients and methods:*** *The study includes 152 patients with histologically verified breast carcinoma. Clinicopathological findings were classified according to classical breast carcinoma-host features (age and menopausal status) and carcinoma features (lymph node status, tumor size, type and grade). Estrogen and progesterone receptors were assayed in accordance with the recommendation of the EORTC. Cathepsin D concentrations were determined using immunoradiometric assay. The results were analyzed using non-parametric statistical methods.*

***Results:*** *All differences in the proportion of breast carcinoma classified as cathepsin D-positive and disagreements on the association of cathepsin D status with clinicopathological features of breast cancer are the result of varying cut-off values used by different authors. Using the cut-off value, which defines estrogen-regulated vs. nonestrogen-regulated cathepsin D expression, this study points to the cathepsin D status as a complementary biological information to ER and PR status, and a dependent biomarker in relation to age of patients and lymph node status.*

***Conclusion:*** *The classification of tumors according to the cathepsin D status within ER and PR status could provide more information on the association between cathepsin D status and clinical-pathological features of breast cancer.*

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## INTRODUCTION

Breast cancer is the most common and the most lethal malignancy of women worldwide. Much effort has been put into research on cell biomarkers, which may predict tumor cell behavior in breast cancer. One of the most paradoxical biomarker in breast cancer is cathepsin D. A number of studies has implicated cathepsin D in promoting tumor growth as a proteolitic enzyme (1). Others reported that the expression of cathepsin D was regulated by estrogen in breast carcinomas, with the functional integrity of the estrogen response pathway (2). Accordingly, the positive association between cathepsin D and steroid hormone receptor status is confusing, suggesting that they should provide the opposite prognostic as well as predictive information.

Our recent study (3) showed statistically significant direct correlation between cathepsin D expression and estrogen (ER) and progesterone receptor (PR) status. Baseline i.e. nonestrogen-regulated cathepsin D expression (<28.0 pmol/mg) was found in patients with ER-negative, PR-negative status and node-negative status, or carcinomas less than 2 cm.

In the current study, our aim was to assess cathepsin D status, estrogen-regulated *vs*. nonestrogen-regulated in relation to ER and PR status, and classical clinicopathological features of breast carcinomas.

## PATIENTS AND METHODS

**Patients.** This study was performed with a group of 152 consecutive patients with primary operable breast cancer. None of the patients had received preoperative radiotherapy, chemotherapy or endocrine therapy. The median age of patients was 56 years (range 24 to 78 years). A patient was considered to be premenopausal when the menstrual cycles still persisted or postmenopausal if menstruation had ceased at least six months before. The patients were categorized in younger age group (up to 45), middle-aged (between 45 and 59) and older age (60 or more).

**Histological features.** Histological specimens were reviewed and then classified according to histology type (4), TN-stages, i.e. tumor size and the presence or absence of regional lymph node metastases (5) and histology grade (6). According to histology type two groups were obtained: invasive ductal (IDC, n=85) and invasive lobular carcinomas (ILC, n=59). The remaining carcinomas - rare, mixed or unclassifiable - were not considered in histology type analysis. Further, out of these patients, 85 were found to have regional lymph node metastases, 53 were free of lymph node metastases and 14 were undefined. According to the size of tumors, 67 were smaller than 2 cm (pT1), 85 were equal or greater than 2 cm (pT2). Carcinomas were graded as follows: grade I (n=22), grade II (n=102) and grade III (n=27). A single carcinoma was not graded.

**Steroid hormone receptor assay.** Breast carcinoma samples were pulverized and homogenized with phosphate buffer. Low-salt extract, cytosol, was prepared by centrifugation at 100,000 g for 1h at 4°C. ER and PR were determined by analysis standardized under recommendation of EORTC (7). Quality assessment was confirmed (8) as recently recommended (9). The cut-off for the classification of positive receptor status was 10 fmol/mg for ER and 20 fmol/mg for PR. Cytosol protein concentration was determined by the Lowry method (10).

**Cathepsin D assay.** Cathepsin D in breast carcinoma cytosol was determined by a solid-phase immunoradiometric assay IRMA (ELSA - CathD, Cis Biointernational, Gif-sur-Yvette, France), based on two monoclonal antibodies D7E3 and M1G8, which detect the total amount of cathepsin D (52-kD, 48-kD and 34-kD). The first antibody (D7E3) was coated on the solid phase, acting as a "catcher", while the latter (M1G8), radiolabeled with $^{125}$I, was used as the "tracer". In this way, by forming a sandwich the cathepsin D molecules (antigen) are identified in the cytosol sample of breast carcinoma. The procedure was carried out exactly as described by the manufacturer. The cut-off for the classification of positive cathepsin D status was 28.0 pmol/mg as estrogen-regulated cathepsin D expression (3).

**Statistical methods.** Nonparametric statistical methods were applied to analyze the data - the Chi-square test to assess the difference in frequencies of events between subgroups, and the Spearman rank correlation test to assess the correlation between the proportion of carcinomas, classified as cathepsin D-positive, and the cut-off values used. The limit of significance was set at $p<0.05$.

## RESULTS

### Cathepsin D IRMA validation

Figure 1 shows the relative frequency distribution of cathepsin D values found in 152 breast carcinoma samples. In the majority of breast carcinomas cathepsin D was detectable, although at very low quantitative levels and in many carcinomas the values varied, but all samples contained cytosol cathepsin D protein (range 15.4-261.6; median 43.2 pmol/mg). Using IRMA to determine cathepsin D expression in this study and in previous studies (11-19), an indirect relationship was found between the percentage of cathepsin D-positive cases and the cut-off value used (Figure 2).

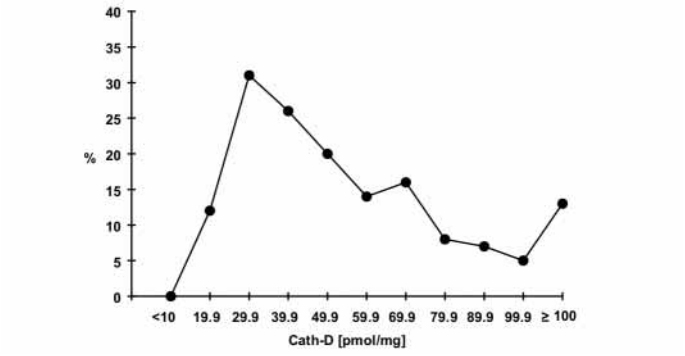


**Figure 1.** Distribution of primary breast cancers according to the cathepsin D protein values

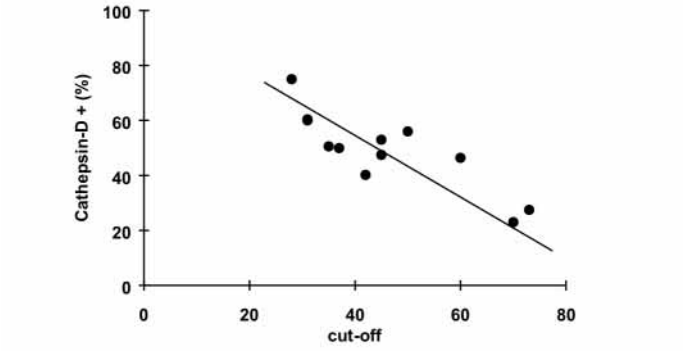


**Figure 2.** The proportion of breast carcinomas that were classified as cathepsin D-positive, in relation to the cut-off values used in different studies (Based on data in Refs. 11-19)

### ER status and PR status, and cathepsin D status

Using the biochemical method, we found 96 carcinomas (63%) with ER-positive status and 70 carcinomas (46%) with PR-positive status. One hundred and fourteen (75%) carcinomas contained estrogen-regulated cathepsin D expression, i.e. cathepsin D-positive status. Table 1 displays relationships between these findings. Fifty-six (53%) of the 105 cathepsin D-positive carcinomas displayed PR positive status. A Chi-square analysis indicates a high likelihood of an association between the cathepsin D status and PR status, $p=0.007$. This association might be more clearly defined within the segregation of the carcinomas on the basis of their ER status. Among the ER-positive carcinomas we failed to demonstrate an association between the PR status and cathepsin D status, $p=0.4$. On the other hand, among the ER-negative carcinomas, an association between the PR status and cathepsin D status was found, $p=0.008$. Eight (89%) of the 9 ER-negative, PR-positive carcinomas displayed cathepsin D-positive status.

**Table 1.** Classification of carcinomas according to determinations of ER-status, PR-status and cathepsin D status

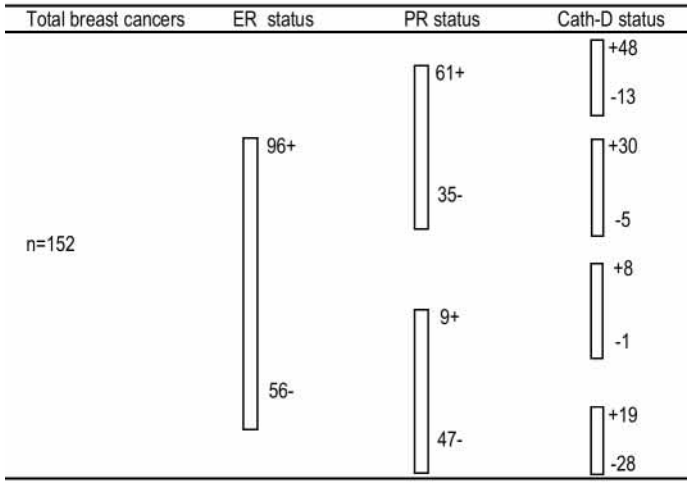


**Table 2.** Distribution of breast carcinoma - host features according to cathepsin D status

| Breast carcinoma-host features | n | Cut-off: 28 pmol/mg Cath-D+ | Cath-D- | $\chi^2$ test p |
|---|---|---|---|---|
| *Age* | | | | |
| <45 | 32 | 18 | 14 | |
| 45-59 | 55 | 42 | 13 | *0.02* |
| >59 | 65 | 54 | 11 | |
| *Menopausal status* | | | | |
| PRE | 63 | 43 | 20 | *0.1* |
| POST | 89 | 71 | 18 | |

### Cathepsin D status and clinicopathological features

Cathepsin D status was correlated to clinical features, i.e. breast carcinoma-host features and breast carcinoma features. Significant association between cathepsin D positivity and patient age was noted, $p=0.02$. Among the patients aged between 45 and 59 years and over 59 years, 76% (42/55) and 89% (54/65) were cathepsin D-positive, in contrast to 56% (18/32) of those patients younger than 45 years. A homogenous distribution of the cathepsin D status with changing of menopausal status was found, $p=0.1$ (Table 2).

**Table 3.** Distribution of breast carcinoma features according to cathepsin D status

| Breast carcinoma features | n | Cut-off: 28 pmol/mg Cath-D+ | Cath-D- | $\chi^2$ test p |
|---|---|---|---|---|
| *Lymph node status* | | | | |
| pNo | 53 | 33 | 20 | *0.03* |
| pN+ | 85 | 67 | 18 | |
| *Tumor size* | | | | |
| pT< 2 cm | 67 | 49 | 18 | *0.6* |
| pT ≥ 2 cm | 85 | 65 | 20 | |
| *Histological type* | | | | |
| IDC | 85 | 66 | 19 | *0.4* |
| ILC | 59 | 42 | 17 | |
| *Histological grade* | | | | |
| I | 22 | 12 | 10 | |
| II | 102 | 78 | 24 | *0.04* |
| III | 27 | 23 | 4 | |

No significant association was found between cathepsin D status and tumor size ($p=0.6$), or histological type ($p=0.4$). Significant associations were noted between cathepsin D positivity and lymph node status ($p=0.03$), or histologic grade ($p=0.04$). Of the lymph node-positive carcinomas, 79% (67/85) were cathepsin D-positive compared to 62% (33/53) cathepsin D

positive in lymph node-negative subgroup. Of the grade II, 76% (78/102) and grade III, 85% (23/27) were cathepsin D-positive compared to 55% (12/22) cathepsin D-positive in grade I subgroup (Table 3).

## DISCUSSION

The expression of cathepsin D may be determined by immunohistochemical or immunoradiometric methods. The immunohistochemical method indicates the special localization of cathepsin D, thus permitting the assessment of the intratumoral heterogeneity, but does not allow its precise quantification. On the contrary, with the immunoradiometric method, which is performed on the same cytosol samples prepared routinely for steroid hormone receptor determination, it is possible to quantify cathepsin D expression. A brief review of the literature indicates that the obtained distribution of cathepsin D values found in our study (Figure 1) is in accordance with previously reported results (11,12,14,17). Also, in our, as well as in those studies using immunoradiometric methods (11-19), there is an indirect relationship between percentage of cathepsin D-positive cases and the cut-off value used (Figure 2). Therefore, the differences in "positivity" of cathepsin D expression simply reflect the varying cut-off values used.

The simultaneous determination of ER and PR status and cathepsin D status, defined as estrogen-regulated vs. nonestrogen-regulated cathepsin D expression, suggested an additional functional heterogeneity in ER-positive and in ER-negative breast carcinomas.

According to the literature data on the association of cathepsin D and clinicopathological features of breast cancer it is clear that no consensus has been reached so far (13,17,20,21).

Regarding the age and menopausal status as the most important classical prognostic/predictive breast carcinoma-host features, a general agreement on a lack of association between age and cathepsin D status is evident (17,20,21). Our present data show that a statistically significant higher frequency of cathepsin D-positive status exists in middle-aged and/or older patients, than in younger patients. Further, our data showed a lack of association between menopausal status and cathepsin D status, which is in accordance with the results of Gion et al. (17), but at variance with the results of Thorpe et al. (20).

Regarding the most important classical prognostic/predictive features of breast cancer - lymph node status and tumor size - there is a general agreement on an existence of association in the former and the lack of association in the latter case. We also found significantly higher frequency of cathepsin D-positive status in node-positive, than in node-negative carcinomas, and no association between tumor size and cathepsin D status. Further, we found significantly higher frequency of cathepsin D-positive status in histological grade II and III, than in grade I carcinomas, which is in accordance with some reports (17, 20) but contradictory to others (13,21). The lack of association between histologic type of breast carcinoma and cathepsin D status, found in our study, is in agreement with the study by Gion et al. (17).

## CONCLUSION

Classification of tumors according to cathepsin D status within ER and PR status should be regularly applied in considering the association between cathepsin D status and clinicopathological features of breast cancers.

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## REFERENCES

**1.** Rochefort H. The prognostic value of cathepsin D in breast cancer, a long road to the clinic. Eur J Cancer 1996;32A:7-8.

**2.** Westley BR, May FEB. Oestrogen regulates cathepsin D mRNA levels in oestrogen responsive human breast cancer cells. Nucleic Acid Res 1987;15:3773-86.

**3.** Nikolić-Vukosavljević D, Grujić-Adanja G, Nastić-Mirić D, Branković-Magić M, Jovanović D, Polić Dj et al. Cathepsin D: Association between TN stage and steroid receptor status of breast carcinoma. Tumor Biol 1998;19:329-34.

**4.** Scarf RF, Torloni H. Histological typing of breast tumors. In: International Histological Classification of Tumors, II. World Health Organization, and Geneva; 1968:19.

**5.** UICC TNM Classification of malignant tumors. 4th ed. New York: Springer-Verlag; 1987.

**6.** Bloom MJG, Richardson WW. Histological grading of breast cancer. Eur J Cancer 1957;11:359-77.

**7.** EORTC Breast Cancer Cooperative Group. Revision of the standards for the assessment of receptors in human breast cancer. Eur J Cancer 1980;16:1513-15.

**8.** Nikolić-Vukosavljević D, Branković-Magić M, Polić Dj, Janković R. Standardization of steroid hormone receptor assay. Jugoslav Med Biochem 1998;17:391-4.

**9.** Romain S, Laine Birdon C, Martin PM, Magdalenat H on behalf of the EORTC Receptor Study Group. Steroid receptor distribution in 47,892 breast cancer. A collaborative study of 7 European Laboratories. Eur J Cancer 1995;31:411-7.

**10.** Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:165-75.

**11.** Spyratos F, Brouillet J-P, Defrenne A, Macene K, Maudelonde T, Brunet M et al. Cathepsin D: An independent prognostic factor for metastasis of breast cancer. The Lancet 1989; November, i:1115-8.

**12.** Brouillet J-P, Theillet C, Maudelonde T, Defrenne A, Simony-Lafontaine J, Sertour J et al. Cathepsin D assay in primary breast cancer and lymph nodes: Relationship with c-myc, c-erbB-2 and int-2 oncogene amplification and node invasiveness. Eur J Cancer 1990;26:437-41.

**13.** Duffy MJ, Broullet J-P, Reilly D, McDermott E, O'Huggins N, Fennelly JJ, et al. Cathepsin D concentration in breast cancer cytosols: Correlation with biochemical, histological and clinical findings. Clin Chem 1991;31:101-4.

**14.** Namer M, Ramaioli A, Fontana X, Etienne M-C, Hery M, Jourlait A et al. Prognostic value of total cathepsin D in breast tumors. Breast Cancer Res Treat 1991;19:85-93.

**15.** Spyratos F, Martin P-M, Hacene K, Romain S, Andriev C, Ferrero-Pous M et al. Multiparametric prognostic evaluation of biological factors in primary breast cancer. J Natl Cancer Inst 1992;84:1266-72.

**16.** Gion M, Mione R, Pappagallo L, Gatti C, Nascimben O, Bari M, et al. pS2 in breast cancer - alternative or complementary tool to steroid receptor status? Evaluation of 446 cases. Br J Cancer 1993; 68:374-9.

**17.** Gion M, Mione R, Dittadi M, Romanelli M, Pappagallo L, Capitanio G et al. Relationship between cathepsin D and other pathological and biological parameters in 1752 patients with primary breast cancer. Eur J Cancer 1995, 31:671-7.

**18.** Jakić-Razumović J, Romić-Stojković R, Petrovečki M, Gamulin S. Correlation of two methods for determination of cathepsin D in breast carcinoma (immunohistochemistry and ELISA in cytosol). Breast Cancer Res Treat 1997;43:117-22.

**19.** Foekens JA, Look MP, Bolt-deVries J, Meijer-vanGelder ME, van Putten WLJ, Klijn JGM. Cathepsin D in primary breast cancer: Prognostic evaluation involving 2810 patients. Br J Cancer 1999;79:300-7.

**20.** Thorpe SM, Rochefort H, Garcia M, Freiss G, Christensen IJ, Khalaf S et al. Association between high concentration of MR52,000 cathepsin D and poor prognosis in primary human cancer. Cancer Res 1989;49:6008-14.

**21.** Pujol P, Maudelonde T, Daures JP, Ravanet P, Brouillet J-P, Pujol H, Rochefort H. A prospective study of the prognostic value of cathepsin D levels in breast cancer cytosol. Cancer 1993;71:2006-12.