# Pepsinogen C is a New Prognostic Marker in Primary Breast Cancer

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## Abstract

Purpose: Here we evaluate in breast cancer patients the prognostic value of pepsinogen C, a proteolytic enzyme involved in the digestion of proteins in the stomach that is also synthesized by a significant percentage of breast carcinomas.

Patients and Methods: Pepsinogen C expression was examined by immunoperoxidase staining in a series of 243 breast cancer tissue sections, and results obtained were quantified using the HSCORE system, which considers both the intensity and the percentage of cells staining at each intensity. Evaluation of the prognostic value of pepsinogen C was performed retrospectively in corresponding patients by multivariate analysis that took into account conventional prognostic factors. The mean follow-up period was 48.5 months.

Results: A total of 113 carcinomas (46.5%) stained positively for this proteinase, but there were clear differences among them with regard to the intensity and percentage of stained cells. Pepsinogen C values were significantly higher in well differentiated (grade 1, 89.1) and moderately differentiated (grade II, 88.5) tumors than in poorly differentiated (grade III, 27.7) tumors ($P < .001$). Similarly, significant differences in pepsinogen C content were found between estrogen receptor (ER)-positive tumors and ER-negative tumors (85.9 v 41.2, respectively; $P < .05$). Moreover, results indicated that low pepsinogen C content predicted shorter relapse-free survival duration and overall survival duration ($P < .0001$). Separate Cox multivariate analysis for relapse-free survival and overall survival in subgroups of patients as defined by node status showed that pepsinogen C expression was the strongest factor to predict both relapse-free survival and overall survival in node-positive patients ($P < .0001$ for both) and node-negative patients ($P < .005$ and $P < .01$, respectively).

Conclusion: Pepsinogen C is a new prognostic factor for early recurrence and death in both node-positive and node-negative breast cancer. In addition, and in contrast to most studies that concern the prognostic significance of proteolytic enzymes in cancer, pepsinogen C production by breast cancer cells is associated with lesions of favorable evolution.

## Introduction

Breast cancer is the most frequent malignant tumor in the female population and represents a leading cause of death in women from Western countries. Since these carcinomas display a high variability in biologic and clinical behavior, major efforts have

been directed at finding specific factors that could reflect the characteristics of each particular tumor. Among the different biochemical markers that may be useful for this purpose, proteolytic enzymes have attracted considerable interest due to their potential role in degradation of the extracellular matrix, and thereby facilitation of tumor invasion and metastasis.[1,2] Consistent with this concept, a variety of proteinases have been found to be overproduced either by breast cancer cells themselves or by sur rounding stromal cells of host tissue. These enzymes include matrix metalloproteinases such as collagenases, stromelysins, and gelatinases,[3-5] serine proteinases such as plasminogen activators,[6] cysteine proteinases such as cathepsins B and L,[7,8] and aspartic proteinases such as cathepsin D.[9,10] Also in agreement with the proposed role for these proteolytic enzymes in the spread of cancer, several clinical studies have shown that their overexpression in breast tumors is usually associated with a poor clinical outcome of the disease.[11-13]

Recently, we found that a significant percentage of breast carcinomas have the ability to synthesize pepsinogen C, a proteolytic enzyme in which the normal function is to digest proteins in the stomach.[14] In addition, immunohistochemical analysis of a large number of breast carcinomas has shown that expression levels of this proteinase are significantly associated with the histologic grade of tumors and their receptor estrogen status.[15] Thus, higher levels of pepsinogen C are found in well-differentiated tumors than in poorly differentiated tumors. Similarly, pepsinogen C values were higher in estrogen receptor (ER)-positive tumors than in ER-negative tumors. Since both conditions confer a prognostic advantage to breast cancer patients, we have proposed that pepsinogen C expression by breast carcinomas may be a marker for favorable clinical outcome of the disease.[15] Because this proposal is in marked contrast to most studies of the prognostic significance of proteolytic enzymes in breast cancer, we were prompted to examine the potential relationship between pepsinogen C levels and tumor recurrence and patient survival rates in a group of 243 women with breast cancer. Here we confirm and extend our previous observation that pepsinogen C production by breast tumors is a factor for good prognosis, independent of a number of other prognostic variables.

## PATIENTS AND METHODS

### *Patients*

This study was performed on a group of 243 women (age range, 25 to 90 years) with histologically verified ductal-infiltrating breast cancer diagnosed and treated at Hospital de Jove (Gijón, Spain) and Hospital Central de Asturias (Oviedo, Spain) between 1980 and 1992. All of them were previously untreated and without signs of distant metastasis or any other malignant tumor at the time of diagnosis. Patients' characteristics with respect to menopausal status and clinical staging of the disease are listed in Table 1. Histologic grade of tumors was determined according to criteria reported by Bloom and Richardson,[16] whereas nodal status was assessed histopathologically. ER content was measured in cytosol extracts using a commercially available kit from Abbott Laboratories

(North Chicago, IL). Breast tumors were considered ER-positive if they contained more than 10 fmol/mg total protein.

Radical or modified radical mastectomy with axillary dissection was performed in all patients. Postoperative locoregional radiotherapy was given to 92 patients with central or medial tumors or with positive axillary lymph nodes. Adjuvant systemic therapy with cyclophosphamide, methotrexate, and fluorouracil was given to 71 patients, and adjuvant tamoxifen to 73. All patients were evaluated for disease recurrence and survival status by clinical, radiologic, and biologic examinations every 3 months for the first 2 years and once per year thereafter. The mean follow-up period was 43.7 months for patients with node-positive cancer and 53.5 for those with node-negative tumors. Of 243 patients included in this study, 95 developed tumor recurrence and 61 of them died of recurrence. In addition, nine women died of causes unrelated to breast cancer.

*Pepsinogen C Purification and Antiserum Production*

Pepsinogen C was purified from human gastric mucosa obtained at autopsy from individuals without gastric disorders as previously described.[14] Purity of the obtained zymogen was confirmed by automatic Edman degradation. Antiserum against purified antigen was raised in New Zealand white rabbits using the method described by Vaitukaitis.[17] Immunized rabbits were exsanguinated 6 weeks after protein injection, and the serum obtained was dialyzed for 24 hours at 4°C against 20 mmol/L phosphate buffer, pH 7.2. Then, the dialyzed material was chromatographed on a column of diethylaminoethyl-cellulose equilibrated and eluted in the same phosphate buffer; finally, the immunoglobulin G (IgG)-containing fractions were collected and stored at -20 0 C until used.

*Immunohistochemical Staining*

Immunohistochemical assays were performed on 6-µm, formalin-fixed, paraffin-embedded tissue sections using the avidin-biotin method.[18] Endogenous peroxidase and nonspecific binding were blocked by sequential incubation of sections in 10% hydrogen peroxide solution and in normal serum. Incubation with antiserum against gastric pepsinogen C (diluted 1:500 in 20 mmol/L phosphate buffer, pH 7.2) was performed at 40 C for 16 hours. Then, slides were incubated with the second biotinylated antibody obtained from Dako (Copenhagen, Denmark) and the avidin-biotin-complex reagent (Vector Laboratories, Burlingame, CA). After 30 minutes at room temperature, the reaction was developed with 0.06% diaminobenzidine and 0.01% hydrogen peroxide. Antiserum specificity was confirmed by Western blot analysis as previously described.[15] Specificity of staining was also determined using controls that involved incubation of tissue sections with buffer alone or with an equal amount of IgG from nonimmunized rabbits. In both cases, there was no significant staining. Furthermore, immunostaining was completely abolished by antiserum preincubation with pepsinogen C purified as

previously described.[14] Tissue sections were scored in a semiquantitative fashion according to the method reported by McCarty et al,[19] which considers both the intensity and percentage of cells staining at each intensity. Intensities were classified from 0 (no staining) to 3 (very strong staining), whereas 10% groupings were used for the percentage of cells that stained positive. For each slide, a value designated HSCORE was obtained by application of the following algorithm: HSCORE = Σ([I + 1] x PC), where I and PC represent intensity and percentage cells that stain at each intensity, respectively. All sections were evaluated by two independent observers without any knowledge of the clinical outcome of patients included in the study, and corresponding HSCOREs were calculated separately. Reproducibility of the scoring method between both observers was greater than 90%. In the remaining cases in which discrepancies had been noted, differences were settled by consensus review of corresponding slides.

*Statistical Analysis*

For analysis of data, patients were subdivided into groups based on different clinical or pathologic parameters. Analysis of differences in pepsinogen C values between two groups was performed with the Mann-Whitney U test. Relationships between more than two groups were evaluated by the Kruskal-Wallis test. In the univariate study, curves for relapse-free survival and overall survival rates were established by the Kaplan-Meier method[20] and compared with the log-rank test.[21] Cox's regression model [22] was also used to examine several combinations and interactions of prognostic factors in a multivariate analysis. The following variables were included in the analysis: patient age at diagnosis, menopausal status, tumor size, histologic grade, and nodal status. ER status was not included due to the absence of corresponding data in a significant number of tumors. Selection of prognostic variables was performed with Cox's model using the stepwise regression option from BMDP (program 2L) software.[23]

**RESULTS**

Pepsinogen C expression in breast tumor tissues was analyzed by immunohistochemical staining with an antiserum raised against protein purified from human gastric mucosa. Before analysis, purity of the antigen used to raise corresponding antibodies, as well as antiserum specificity, were extensively examined as described earlier. After performing these controls, immunostaining was performed on 243 breast cancer tissue sections and the results obtained were evaluated with the HSCORE system. HSCORE values ranged from 0 to 340, and the distribution is shown in Fig 1. A total of 113 carcinomas stained positively for pepsinogen C, although there were clear differences among them with regard to intensity and percentage of stained cells. Thus, 21 tumors were weakly stained (HSCORE < 100), 55 were moderately stained (HSCORE < 200), and the remaining 37 were strongly positive for pepsinogen C. The mean HSCORE value was 76.7. Distribution of pepsinogen C levels in relation to a series of patient and tumor characteristics, which included menopausal status, tumor size, axillary node involvement, histologic grade, and

ER status of tumors, is listed in Table 1. Statistical analysis showed that pepsinogen C values were significantly correlated with both histologic grade and ER status of tumors. Thus, pepsinogen C levels were higher in well-differentiated (grade I, 89.1) and moderately differentiated (grade II, 88.5) tumors than in poorly differentiated (grade III, 27.7) tumors ($P < 0.001$). Similarly, significant differences in pepsinogen C content were found between ER-positive and ER-negative tumors (85.9 v 41.2, respectively; $P < .05$).

These results pointed to a relationship between pepsinogen C content and favorable outcome of breast cancer. To examine this hypothesis further, the potential association between pepsinogen C immunostaining and relapse-free survival and overall survival was retrospectively evaluated in 243 women included in the present study. First, we defined an optimal cutoff value by statistical analysis of the ability of pepsinogen C values to predict the relapse-free survival of the study population. This statistical analysis showed the occurrence of a continuous association between HSCORE values and relapse rate ($P < .0001$ for HSCORE values between 0 and 150 and between 180 and 200; $P < .001$ for 160, 170, and 210; $P < .01$ for 220 and 230; and $P < .05$ for values between 240 and 260). However, as shown in Fig 2, $X^2$ analysis led us to define an HSCORE of 120 as the optimal cutoff ($X^2 = 34.7$, $P < .0001$) with ability to identify 66.7% of patients as having lower or negative pepsinogen C values. Considering this cutoff value, relapse was confirmed in 81 of 162 patients (50%) with pepsinogen C-negative carcinomas, but only in 14 of 81 (17.3%) with pepsinogen C-positive tumors. Similarly, during the study period there were 55 deaths (34%) because of recurrence in patients with pepsinogen C-negative tumors and six deaths (7.4%) in patients whose tumors showed positive immunostaining. Differences in both recurrence-free and overall survival were significant ($P < .0001$) (Fig 3). Univariate analysis showed that axillary lymph node involvement, tumor size, and histologic grade were also significantly associated with relapse and survival in our study population (Table 2). However, multivariate analysis according to Cox's model showed that pepsinogen C value was the most significant independent indicator of both relapse-free and overall survival (Table 3).

Finally, since there is a need to identify additional prognostic markers in node-negative patients, women included in this study were subdivided into two groups by node status, and the possible relationship between pepsinogen C levels and clinical outcome of disease was examined in both groups. In the node-positive group, relapse was confirmed in 55 of 89 patients (61.8%) with pepsinogen C-negative tumors and in seven of 36 (19.4%) with pepsinogen C-positive tumors. On the other hand, during the study period, there were 43 deaths (48.3%) because of recurrence in patients with tumors stained negatively for pepsinogen C and four deaths (11.1%) in those whose tumors produced this protein. These differences were significant at ($P < .0001$) (Fig 4). Similarly, in the node-negative group, relapse was observed in 26 of 73 patients (35.6%) with pepsinogen C-negative tumors, but only in seven of 45 (15.6%) with pepsinogen C-positive carcinomas. On the other hand, there were 12 deaths (16.4%) because of recurrence in patients with pepsinogen C-negative tumors and two deaths (4.4%) in patients with pepsinogen C-positive tumors. Statistical analysis showed that these differences were significant at $P$ less than .005 and $P$ less than .01 for relapse and survival, respectively

(Fig 4). Multivariate analysis confirmed that pepsinogen C was significantly associated with relapse-free survival and overall survival in both node-positive and node-negative groups (Table 3).

## DISCUSSION

In this study, we report that pepsinogen C, a proteinase usually found in the stomach, is a new prognostic factor in breast cancer. Furthermore, our results indicate that expression of this proteinase by breast carcinomas confers a prognostic advantage to breast cancer patients. To our knowledge, this is the first report to show that a gastric proteinase may be of prognostic relevance in breast cancer.

The present investigation originally aimed to extend our previous observation that pepsinogen C immunostaining in breast carcinomas was statistically associated with histologic grade and ER status of tumors.[15] Since these results pointed to a potential value of pepsinogen C as a tumor marker, studies were undertaken to examine the existence of a putative correlation between pepsinogen C expression in breast tumors and clinical outcome of the disease. Results showed a significant relationship between levels of this proteinase and both relapse-free survival and overall survival. In addition, multivariate analysis demonstrated that the ability of pepsinogen C to predict clinical outcome was independent from a number of prognostic factors including tumor size and axillary nodal status. On the other hand, and considering the current need for additional predictive markers in node-negative breast cancer patients,24 it is noteworthy that these differences in pepsinogen C levels were also significant in the subset of women without axillary lymph node involvement.

The finding of a proteolytic enzyme apparently associated with lesions that have a favorable evolution is somewhat counterintuitive and in marked contrast to most studies about prognostic significance of these enzymes in human tumors. However, several pepsinogen C properties may provide biologic support to the clinical data presented herein. Thus, the observed relationship between intratumoral levels of this proteinase and ER status seems to indicate that tumors that express pepsinogen C have an intact hormone receptor pathway. Consequently, extra-gastric expression of this proteinase may be a consequence of hormonal alterations presumably associated with breast carcinomas, without causing any direct effect on the spread of cancer. A similar explanation has been proposed to justify the association between tissue-type plasminogen activator and breast tumors with good prognosis.[25] Expression of pepsinogen C by breast carcinomas also shows an interesting parallelism with pS2 protein, a member of a family of spasmolytic peptides produced by normal stomach mucosa and by a subset of breast carcinomas, but not by normal duct mammary epithelium.[26,27] Furthermore, high levels of pS2 protein are predictive of favorable prognosis, which is probably related to the fact that this protein is a marker of estrogen responsiveness.[28,29] The putative hormonal stimulus with the ability to induce pepsinogen C synthesis by mammary epithelium is presently unknown, but several lines

of evidence point to the possibility that this mechanism is mediated by androgens rather than by estrogens, which are believed to play a major role in breast cancer.[30] Thus, studies from different groups have demonstrated that human prostate, a characteristic androgen-dependent tissue, is able to produce large amounts of a pepsin zymogen closely related or identical to pepsinogen C.[31-34] In addition, it has been recently shown that proteins present in cyst fluid from women with gross cystic breast disease are induced by androgens in breast cancer cells.[35-38] Since pepsinogen C is also present at significant levels in this pathologic breast fluid, it is tempting to speculate that androgens can also be the sex steroids involved in its overproduction by a subset of breast cancer cells. Consistent with this, recent experimental evidence from our laboratory indicates that pepsinogen C is induced by androgens in T-47D breast cancer cells (M. Balbin, C. López, unpublished results). A final consideration that could contribute to explain the fact that expression of this proteinase is not associated with lesions of poor prognosis comes from the observation that pepsinogen C is secreted as a precursor of high molecular weight that requires activation at a low pH to display proteolytic activity. [14] Since these acidic conditions are difficult to achieve in the extracellular milieu, it seems unlikely that pepsinogen C became functional as a degradative enzyme in breast cancer cells. Taken together, these data may provide an explanation for the results reported herein on the association between pepsinogen C expression in breast tumors and favorable prognosis. In relation to this, the fact that this proteolytic enzyme is not synthesized by mammary epithelium under normal conditions,[14,15] together with its restricted expression in human tissues,3 strongly suggests that pepsinogen C may provide information additional to that given by other biochemical markers currently used in breast cancer. Further studies in different populations will be required to confirm the proposed value of pepsinogen C as a specific and independent prognostic factor to predict clinical outcome of breast cancer

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**Table 1.** Pepsinogen C HSCORE in Tumor Tissues Classified According to Different Characteristics

| Patient and Tumor Characteristics | No. of Patients | HSCORE | |
|---|---|---|---|
| | | Mean ± SEM | Range |
| Total tumors | 243 | 76.7 ± 6.1 | 0-340 |
| Menopausal status | | | |
| Premenopausal | 75 | 59.6 ± 9.8 | 0-285 |
| Postmenopausal | 168 | 84.3 ± 7.6 | 0-340 |
| Tumor size | | | |
| T1 | 63 | 80.1 ± 12.3 | 0-340 |
| T2 | 118 | 73.7 ± 8.6 | 0-340 |
| T3 | 46 | 93.5 ± 14.6 | 0-300 |
| T4 | 16 | 37.5 ± 19.3 | 0-210 |
| Nodal status | | | |
| N0 | 118 | 82.1 ± 8.7 | 0-300 |
| N+ | 125 | 71.6 ± 8.6 | 0-340 |
| Histologic grade | | | |
| I | 83 | 89.1 ± 11.6* | 0-340 |
| II | 112 | 88.5 ± 9.1* | 0-340 |
| III | 48 | 27.7 ± 9.3 | 0-160 |
| ER status | | | |
| Positive | 64 | 85.9 ± 12.9 | 0-300 |
| Negative | 55 | 41.2 ± 10.1 | 0-300 |

*$P < .001$ v histologic grade III.

†$P < .05$ v ER-negative.

**Table 2**. Univariate Analysis of Association of Pepsinogen C With Relapse-Free and Overall Survival

| Patient and Tumor Characteristics | n | Relapse-Free Survival (% + SE) | | | Overall Survival (% ± SE) | | |
|---|---|---|---|---|---|---|---|
| | | 5 Years | 9 Years | *P* | 5 Years | 9 Years | *P* |
| Age, years | | | | NS | | | NS |
| < 50 | 72 | 56 ± 7 | 48 ± 8 | | 66 ± 7 | 55 ± 9 | |
| > 50 | 171 | 55 ± 4 | 46 ± 5 | | 71 ± 4 | 58 ± 5 | |
| Menopausal status | | | | NS | | | NS |
| Premenopausal | 75 | 54 ± 6 | 47 ± 7 | | 64 ± 7 | 54 ± 9 | |
| Postmenopausal | 168 | 55 ± 4 | 47 ± 5 | | 72 ± 4 | 59 ± 5 | |
| Tumor size | | | | < .005 | | | < .0001 |
| T1 | 63 | 66 ± 7 | 54 ± 8 | | 80 ± 6 | 73 ± 8 | |
| T2 | 118 | 56 ± 5 | 55 ± 5 | | 70 ± 5 | 65 ± 5 | |
| T3 | 46 | 43 ± 8 | 21 ± 1 | | 65 ± 8 | 33 ± 1 | |
| T4 | 16 | 0 | 0 | | 0 | 0 | |
| Nodual status | | | | < .0005 | | | < .0001 |
| N0 | 118 | 69 ± 4 | 63 ± 5 | | 85 ± 4 | 81 ± 4 | |
| N+ | 125 | 41 ± 5 | 28 ± 7 | | 56 ± 5 | 33 ± 1 | |
| Histologic grade | | | | < .005 | | | < .05 |
| I | 83 | 65 ± 6 | 65 ± 6 | | 71 ± 6 | 67 ± 7 | |
| II | 112 | 55 ± 5 | 41 ± 6 | | 70 ± 4 | 58 ± 7 | |
| III | 48 | 31 ± 9 | 31 ± 9 | | 63 ± 9 | 31 ± 1 | |
| Pepsinogen C (HSCORE) | | | | < .0001 | | | < .0001 |
| ≤ 120 | 162 | 38 ± 4 | 29 ± 5 | | 57 ± 4 | 39 ± 6 | |
| > 120 | 81 | 83 ± 4 | 75 ± 6 | | 91 ± 3 | 89 ± 4 | |
| Nodal status N0 | | | | < .005 | | | < .01 |
| Pepsinogen C ≤ 120 | 73 | 59 ± 6 | 48 ± 8 | | 77 ± 6 | 70 ± 7 | |
| Pepsinogen C > 120 | 45 | 82 ± 6 | 82 ± 6 | | 94 ± 3 | 94 ± 3 | |
| Nodal status N+ | | | | < .0001 | | | < .0001 |
| Pepsinogen C ≤ 120 | 89 | 22 ± 5 | 11 ± 8 | | 43 ± 6 | 11 ± 8 | |
| Pepsinogen C > 120 | 36 | 86 ± 6 | 64 ± 1 | | 87 ± 6 | 81 ± 8 | |

Abbreviation: NS, not significant.

**Table 3.** Multivariate Analysis of Association of Pepsinogen C With Relapse-Free and Overall Survival

| Tumor Characteristics | Relapse-Free Survival | | | Overall Survival | | |
|---|---|---|---|---|---|---|
| | RR | RC ± SE | *P* | RR | RC + SE | *P* |
| Tumor size | | 0.26 ± 0.12 | < .05 | | 0.51 ± 0.16 | < .005 |
| T1 | 0.75 | | | 0.58 | | |
| T2 | 0.98 | | | 0.96 | | |
| T3 | 1.28 | | | 1.61 | | |
| T4 | 1.67 | | | 2.69 | | |
| Nodal status | | 0.55 ± 0.22 | < .05 | | 1.02 ± 0.32 | < .0005 |
| N0 | 0.75 | | | 0.59 | | |
| N+ | 1.30 | | | 1.64 | | |
| Pepsinogen C (HSCORE) | | −1.48 ± 0.29 | < .0001 | | −1.88 ± 0.43 | < .0001 |
| ≤ 120 | 1.64 | | | 1.87 | | |
| > 120 | 0.37 | | | 0.28 | | |
| Nodal status N0 | | −1.17 ± 0.42 | < .005 | | −1.66 ± 0.76 | < .01 |
| Pepsinogen C ≤ 120 | 1.56 | | | 1.88 | | |
| Pepsinogen C > 120 | 0.48 | | | 0.35 | | |
| Nodal status N+ | | −1.73 ± 0.40 | < .0001 | | −2.00 ± 0.52 | < .0001 |
| Pepsinogen C ≤ 120 | 1.64 | | | 1.78 | | |
| Pepsinogen C > 120 | 0.29 | | | 0.24 | | |

Abbreviations: RR, relative risk; RC, regression coefficient.

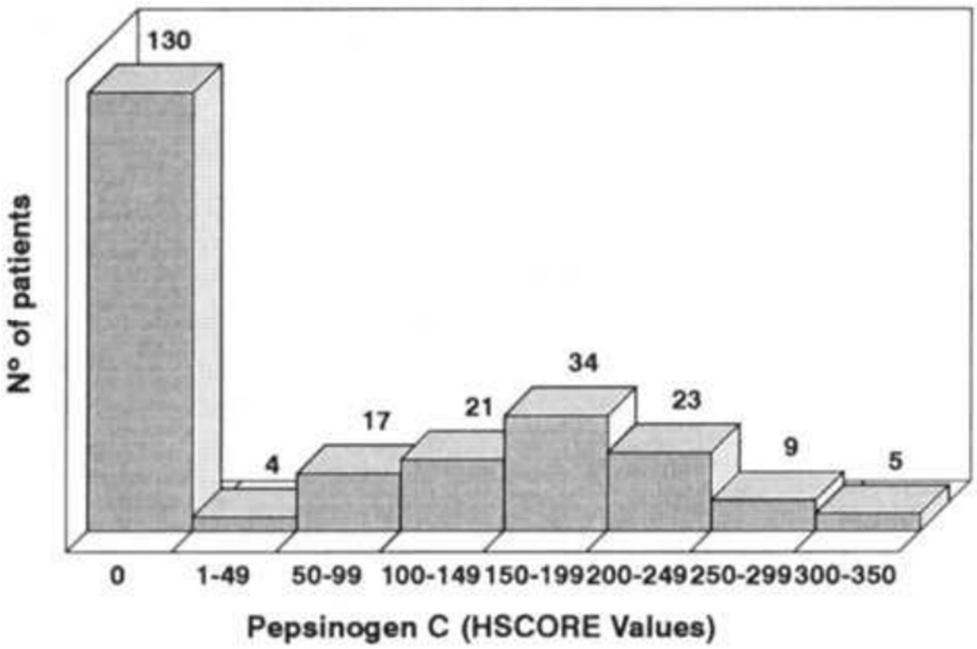


**Fig 1**. Distribution of HSCORE values obtained by immunohistochemical staining of pepsinogen C in 243 human breast carcinomas.

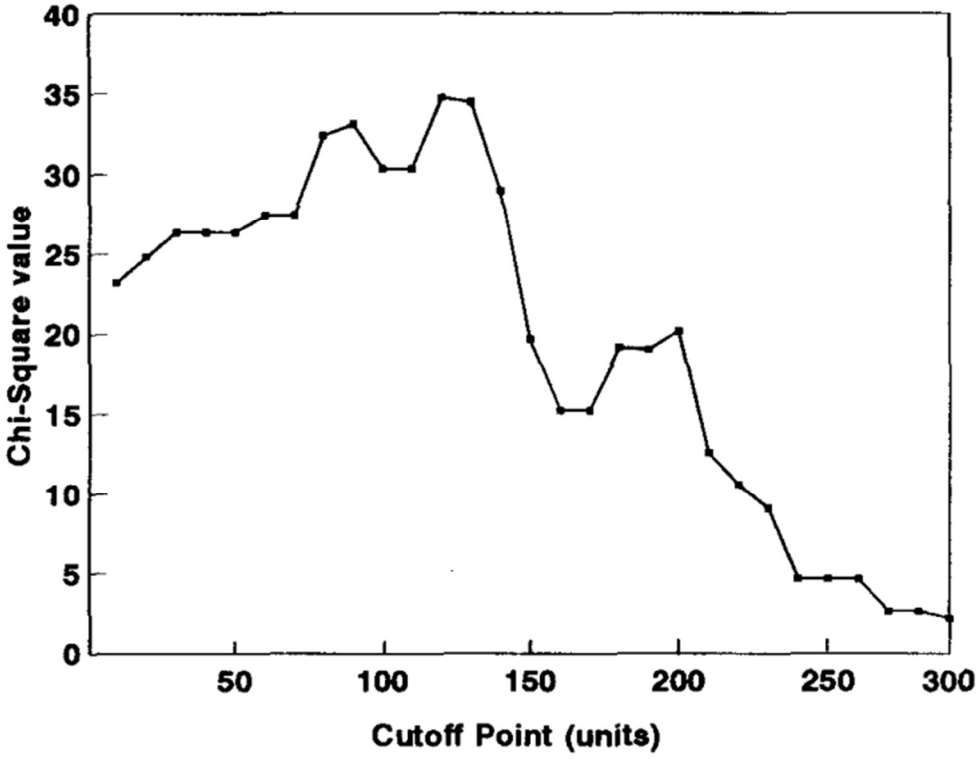


**Fig 2**. Determination of cutoff value for pepsinogen C in prediction of relapse-free survival in breast cancer. The $X^2$ values obtained for each cutoff value are plotted against the value itself.

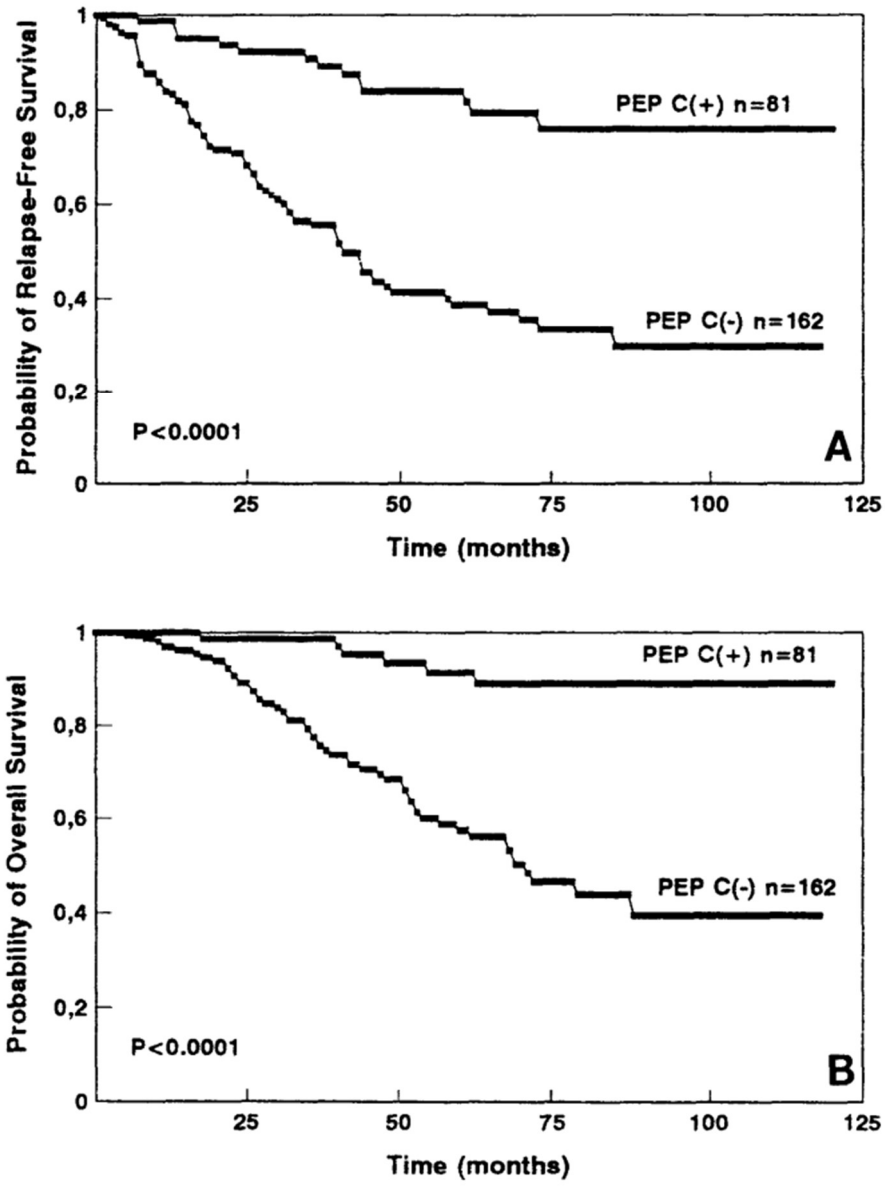


**Fig 3.** Relapse-free and overall survival as function of pepsinogen C values in patients with breast cancer. Mean follow-up period was 48.5 months. Differences in relapse-free and overall survival curves were significant at $P < .0001$.

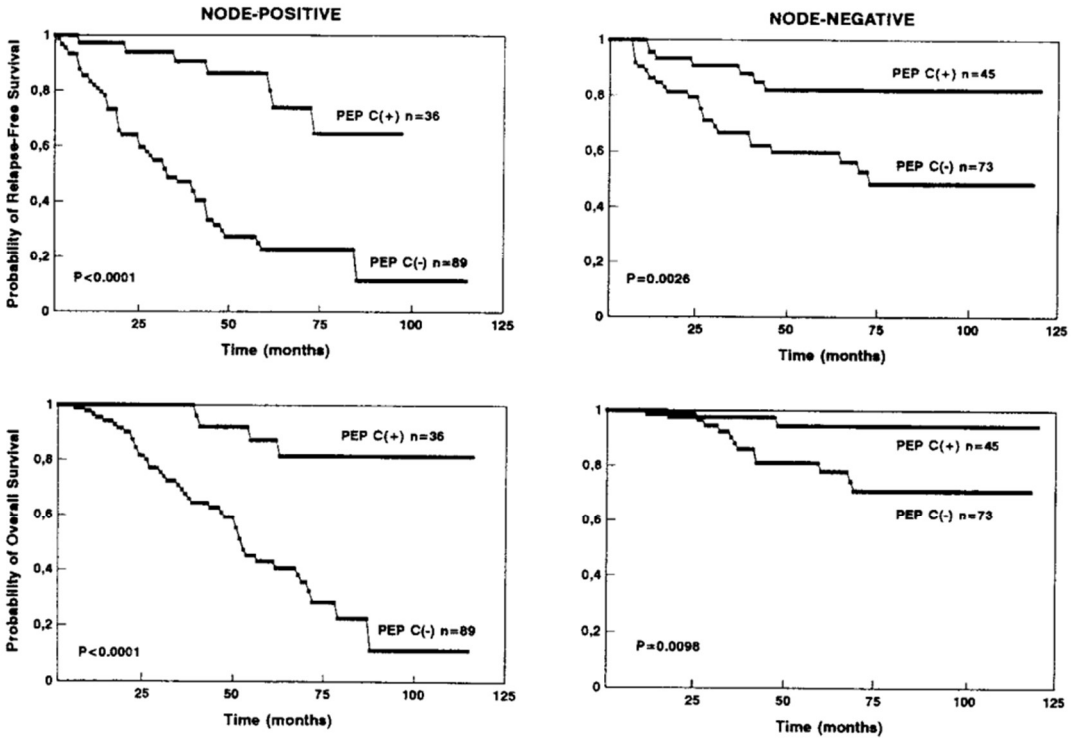


**Fig 4**. Relapse-free and overall survival as function of pepsinogen C values in breast cancer patients classified according to axillary node status. Differences in relapse-free and overall survival curves in node-positive group were significant at $P < .0001$, whereas in the node-negative group, differences in re□lapse-free survival were significant at $P < .005$, and in overall survival at $P < .01$.