# Expression and Prognostic Significance of Apolipoprotein D in Breast Cancer

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## Abstract

Apolipoprotein D (apo D) is a glycoprotein involved in the human plasma lipid transport system and present at large amounts in cyst fluid from women with gross cystic disease of the breast. Apo D expression in breast carcinomas was examined by immunoperoxidase staining of a series of 163 tumors. A total of 60 (36.8%) tumors were negative for apo D immunostaining, 28 (17.2%) carcinomas were weakly positive, 33 (20.2%) were moderately stained, whereas the remaining 42 (25.8%) tumors were strongly stained with the specific antibodies. No significant correlation was found between apo D content and tumor size, lymph node involvement, or biochemical parameters such as estrogen receptors, cathepsin D, or pS2 protein. However, the finding of a significant association between apo D and menopausal status of patients or differentiation grade of tumors, with apo D values being lower in tumors from premenopausal women or in poorly differentiated carcinomas, suggested a potential value of this glycoprotein as a prognostic factor in breast cancer. Preliminary analysis of relapse-free survival and overall survival in a subgroup of 152 women with a mean follow-up of 42 months confirmed that low apo D values were significantly associated to a shorter relapsefree survival and poorer survival. According to these data, we propose that apo D in combination with other well-established prognostic fac tors may contribute to more accurately identify subgroups of breast cancer patients with low or high risk for relapse and death.

## Introduction

Apolipoprotein D (apo D) is a protein component of the human plasma lipid transport system that was first isolated and characterized by McConathy and Alaupovic.[1] This glycoprotein is mainly associated with high-density lipoprotein particles, forms disulfide-linked heterodimers with other apolipoproteins,[2] and consists of a single polypeptide chain of approximately 30 kd that exhibits several isoforms by isoelectric focusing.[3] The functional role of apo D in the metabolism of plasma lipoproteins remains unclear, but the observation that this protein forms complexes with lecithin: cholesterol

acyltransferase has led to the suggestion that apo D may be involved in cholesterol esterification and transport of substrates and products of the reaction.[4,5] Further characterization of apo D as a member of the lipocalin family of proteins[5] provided additional support to the proposal that this protein plays a role in the binding and transport in plasma of different hydrophobic substances including cholesterol, cholesteryl esters, or heme-related compounds.[6]

The unexpected relationship of apo D to breast diseases has arisen after our recent finding that apo D accumulates to extremely high concentrations in cyst fluid from women with gross cystic disease of the breast,[7] a benign condition associated in several studies from different groups to an increased risk of subsequent breast cancer.[8-12] This major intracystic protein, previously designated GCDFP-24, had been characterized as a pregnenolone- and progesterone- binding protein.[13-15] Consequently, we have proposed that apo D may also be involved in steroid hormone binding and transport in human mammary tissue.[7] On the other hand, the cystic protein had been proposed as a marker of steroid action in human breast and prostate cancer cells.[16-17] Interestingly, the effects of steroids on apo D expression in both types of cancer cells are opposite to their respective specific effects on cell proliferation.[16-18] This observation together with the fact that apo D gene expression in normal human fibroblasts seems to be specific to growth arrest has led to suggest that apo D is associated with modulation of cell proliferation.[16,19]

From these considerations and taking into account that this protein is produced and secreted by a variable percentage of breast tumors,[20-23] we were prompted to examine the potential value of apo D as a prognostic marker in breast cancer. In this work, we have studied its expression in breast carcinomas by using immunohistochemical methods and correlated its levels with other prognostic factors in breast cancer and with tumor recurrence and patient survival. Taken together, the results appear to indicate that apo D production by breast tumors is a factor of good prognosis, independent of a number of other prognostic variables.

## Materials and Methods

### *Patients*

This study was performed on a group of 163 women with primary breast cancer examined at Hospital de Jove and Hospital Central, Asturias. The patient's characteristics with respect to age, menopausal status, and clinical staging of the disease are shown in Table 1. Histological grade was determined according to Bloom and Richardson.[24] Radical or modified radical mastectomy with axillary dissection was performed in all patients included in this study. To evaluate relapse-free survival and overall survival, we selected those patients without evidence of metastasis or any other malignant tumor at the time of diagnosis and with a minimum of 1 year follow-up period. In this group (n = 152), postoperative locorregional radiotherapy was given to 57 (37.5%) patients with central or medial tumors or positive axillary nodes. Adjuvant systemic therapy with

cyclophosphamide, methotrexate, and 5-fluorouracil was given to 42 (27.6%) patients and adjuvant tamoxifen to 45 (29.6%). All patients were followed up for disease recurrence and survival status by clinical, radiological, and biological studies every 3 months for the first 2 years and then yearly.

The mean follow-up for patients with node-positive cancer was 37.2 months (range 12 to 120 months) and 47.5 months (range 12 to 120 months) for those with node-negative cancer. Of the 152 patients, 54 developed tumor recurrence and 31 of them died from recurrence. In addition, 6 women died from causes unrelated to breast cancer.

*Apo D Purification and Antiserum Production*

Apo D was purified from cyst fluid from women with gross cystic breast disease according to the high performance liquid chromatography procedure described previously.[7] Type cyst fluids, characterized by the lack of significant amounts of albumin,[25] were used as starting material in the purification process. The purity of the obtained antigen was confirmed by automatic Edman degradation after treatment of the protein with pyroglutamate aminopeptidase as previously described.[7] Antiserum against the purified protein was raised in New Zealand white rabbits following the method described by Vaitukaitis.[26] The immunized rabbits were bled 6 weeks after protein injection and the obtained serum was dialyzed for 24 hours at 4 C against 20 mM phosphate buffer, pH 7.2. Then the dialyzed material was chromatographed in a column of diethylaminoethyl-cellulose equilibrated and eluted in the same phosphate buffer and finally the IgG-containing fractions were collected and stored at -20 C until used.

*Western Blot Analysis*

Proteins separated by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) were electrophoretically transferred to Immobilon Transfer Membrane (Millipore) at 40 mA for 1 hour in a Bio-Rad Trans Blot apparatus with a buffer containing 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 4 mM NaOH, and 10% methanol. After transfer, filters were blocked in phosphate-buffered saline (PBS) containing 10% bovine serum albumin for 4 hours and incubated with the antiserum for 1 hour. After washing five times with a buffer of PBS containing 0.1% Tween 20, filters were incubated with $^{125}$I protein A for 30 minutes and washed as above. Finally, the immunoreactive bands were detected by autoradiography.

*Immunohistochemical Staining*

Immunohistochemical assays were performed on 6 pm formalin-fixed paraffin-embedded tissue sections using the avidin-biotin method.[27] Endogenous peroxidase and nonspecific binding were blocked by sequential incubation of the sections in 10% hydrogen peroxide solution and in normal serum. Incubation with antiserum against apo

D (diluted 1:500 in 20 mM phosphate buffer, pH 7.2) was conducted overnight at 4 C. Then the slides were incubated with the second biotinylated antibody obtained from Dako (Dako, 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. Finally, the sections were counterstained with a modification of the formaldehydethionine method,[28] dehydrated, cleared in eucalyptol, and mounted with Eukitt.

Slides were scored in a semiquantitative fashion according to the procedure described by McCarty et al[29] that incorporates both the intensity (I) and the percentage of cells staining at each intensity (PC). Intensities were classified from 0 (no staining) to 3 (very strong staining). For each tissue section, a value designated HSCORE[29] was obtained by applying the following algorithm: HSCORE= Σ((I+1)x PC). Each tissue was evaluated by two independent observers and the corresponding HSCOREs were calculated separately. The reproducibility of the scoring method was more than 90%. Differences between observers were settled by consensus review of those slides in which discrepancies had been noticed.

*Northern Blot Analysis*

Total RNA from breast carcinomas or normal mammary tissue was isolated by guanidinium thiocyanate-phenol-chloroform extraction, according to Chomczynski and Sacchi,[30] and samples of approximately 40 pg were separated by electrophoresis in 1.4% agarose/formaldehyde denaturing gels and blotted onto Hybond-N nylon filters (Amersham). Filters were prehybridized for 3 hours at 42 C with 50% formamide, 5x standard saline citrate (SSC) (1x = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 2x Denhardt's solution (1 x = 0.02% bovine serum albumin, 0.02% polyvinylpirrolidone, 0.02% Ficoll), 0.1% SDS, and 0.1 mg/ml of denatured herring sperm DNA. Then filters were hybridized for 48 hours under the same conditions, using as a probe the cDNA coding for apo D.[5] Filters were washed with 1x SSC, 0.1% SDS at 60 C for 2 hours and exposed to autoradiography.

*Biochemical Assays*

For the biochemical determinations in breast tumor cytosols, frozen tissues were pulverized and homogenized in 50 mM Tris/HCl buffer (pH 7.4) using a Microdismembrator II (Braun-Melsungen, Germany). The homogenate was centrifuged at 105,000 x g for 1 hour at 4 C and the resulting supernatants (cytosols) were collected and stored at -70 C. Protein concentration in breast tumor cytosols was determined by the Bradford method[31] using bovine y-globulin as standard. Apo D was quantified in breast tumor cytosols by using the enzyme immunoassay previously described.[32] In this assay, apo D from test samples competes with apo D fixed to a solid phase for binding to a limited amount of antibody. Different amounts of apo D and different dilutions of

antibody were assayed to achieve linearity and optimal sensitivity. This optimal sensitivity was established in the range of 0.2 to 0.3 ng/ml. Estrogen receptors were measured by enzyme immunoassay with a commercially available kit from Abbott Laboratories (North Chicago, IL). Cathepsin D and pS2 protein were determined in breast tumor cytosols by immunoradiometric assays using kits from CIS Bio-lnternational (Gif-sur-Yvette, France).

*Statistical Analysis*

For analysis of data, patients were subdivided into groups based on different clinical or biochemical parameters. The analysis of differences between two groups was performed using Student's t-test. Relationships between more than two groups were evaluated using the one-way analysis of variance (ANOVA) test, followed by the post-ANOVA Newman-Keuls test. The relapse-free survival and overall survival curves were established by the Kaplan-Meier method[33] and compared with the logrank test.[34] Cox's regression model[35] 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's age at diagnosis, menopausal status, tumor size, nodal status, and histological grade. Estrogen receptor status, cathepsin D, and pS2 were not included in the multivariate analysis because the corresponding values for these biochemical parameters were not available in all tumors considered in this study. BMDPP (programs 1 L and 2L) software was used for all statistical calculations.[36] Significance was established at the $P < 0.05$ level.

**Results**

*Apo D Expression in Breast Tumor Tissues and Correlation with Clinical, Histological and Biochemical Parameters*

The clinical characteristics of the 163 women included in this study are indicated in Table 1. Apo D production by the corresponding breast tumors was examined by immunohistochemical staining with an antiserum raised against the protein purified from breast cyst fluid. The purity of the antigen used for immunostaining was assessed by automatic Edman degradation of the isolated protein. After unblocking its NH2-terminal end by treatment with pyroglutamate aminopeptidase, a single amino acid sequence identical to that derived from the nucleotide sequence was obtained. No additional amino acid signals were detected in the different degradative cycles. On the other hand, the antiserum specificity was confirmed by Western blot analysis of breast tumor cytosols and breast cyst fluid samples (Figure 1). A single band of the expected molecular mass (approximately 24 kd) was observed in both cases. Specificity of staining was also determined by 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. In addition, immunostaining was completely abolished by antiserum preincubation with apo D purified as previously described [7,23]

Overall, 103 of the carcinomas stained positively for apo D, although there was a wide variability in the intensity and percentage of positivity. Representative examples of positive and negatively stained tissue sections are shown in Figure 2. Semiquantitative analysis of the stained tumor tissues was performed by using the HSCORE system, which considers both the intensity and percentage of stained cells. As indicated in Table 2, HSCORE values varied from 0 to 400 with an average of 117.7. In the group of 103 apo D-positive tumors, a total of 28 were weakly stained (HSCORE <100), 33 were moderately stained (HSCORE < 200), and the remaining 42 were strongly positive.

To investigate the possible relationship between apo D and clinicopathological parameters, women included in this study were subdivided in different groups according to the evaluated parameters and apo D tumor expression was examined in each of the established groups. The distribution of apo D values in relationship to patient and tumor characteristics including menopausal status, axillary node involvement, and size and histological grade of tumors are shown in Table 2. Statistical analysis revealed that significant differences were obtained when apo D values were compared with the menopausal status of patients and with the histological grade of tumors. Thus, apo D content was higher in postmenopausal women than in premenopausal patients (130.6 versus 86.2). In addition, the average content of apo D was higher in well-differentiated carcinomas (grade I 131.5) and moderately differentiated tumors (grade II 119.3) than in those more aggressive poorly differentiated carcinomas (grade III 50.5). These differences were significant at the $P < 0.05$ level (Table 2). A similar trend toward higher apo D levels in well differentiated tumors was observed in those cases in which it was possible to quantify apo D in breast tumor cytosols (data not shown), although the limited number of tumors (n = 26) in which both tissue sections and cytosol extracts were available precluded further comparisons.

Finally, note that the correlation between apo D expression and histological grade of tumors was also confirmed at the RNA level by Northern blot analysis using a complete apo D cDNA as a probe. As shown in Figure 3, which reflects a representative example of all analyzed tissues, a single hybridizing band corresponding to a mRNA species of approximately 1 kb was identified in normal breast and in some breast carcinomas. The strongest signal was obtained in grade breast carcinomas, whereas no significant signal was obtained in most grade Ill tumors (Figure 3).

We also examined the possible relationship between apo D values and other biochemical parameters of interest in breast cancer. To do that, we compared the HSCORE apo D values with the available tumor cytosol concentration of estrogen receptors cathepsin D, and pS2 protein in the corresponding tumors and the results obtained are shown in Table 2. Statistical analysis revealed that there was no overall correlation between apo D and any of the other markers studied.

*Apo D Immunostaining and Clinical Outcome*

The possible relationship between apo D immunostaining in breast carcinoma and relapse-free survival and overall survival was evaluated retrospectively in 152 women selected from the initial population on the basis of the above mentioned criteria. The results obtained indicated that low apo D values predicted shorter relapse-free survival. Analysis of the best cutoff value of apo D for this group of patients led to definition of a 160 HSCORE value as the optimal cutoff ($X^2$ = 15.6, P = 0.0001) for relapse-free survival (Figure 4). This value identified 66.4% of the patients as having lower or negative apo D values. Considering this cutoff value, relapse was confirmed in 43 of 101 (42.6%) patients with apo D-negative carcinomas and in 11 of 51 (21.6%) with apo D-positive tumors. As shown in Figure 5A, there was a significant difference (*P* = 0.0014) in the relapse-free survival curves determined for these two group of patients. In relationship to this, it should be noted that there was no significant difference in the mean follow-up for both group of patients (38.5 months for the apo D-negative group versus 41.7 months for the apo D-positive group). On the other hand, low apo D values seemed to be also associated to a shorter overall survival. Thus, during the study period there were 25 (24.8%) deaths because of recurrence in patients with apo D-negative tumors and 6 (11.8%) deaths in patients whose tumors showed positive immunostaining. Statistical analysis revealed that these differences were also significant (*P* = 0.01) (Figure 5B).

Also note that the univariate analysis of other well established prognostic indicators in breast cancer revealed that, as expected, tumor size and axillary lymph node involvement were also significantly associated with a high relapse rate and a poorer survival (Table 3) in our study population. However, other classical prognostic factors including menopausal status, histological grading, and age did not reach statistically significant differences. Multivariate analysis according to Cox's model confirmed that apo D was significantly associated to relapsefree survival and overall survival (Table 3). It should be also mentioned that when patients were subdivided in two groups in terms of node status, apo D was still statistically significant (Table 4) for predicting relapse-free survival (Figure 6A) and overall survival (Figure 6B) in the node-positive group. The same trend was observed in the node-negative subgroup of patients, although in this case, differences were only significant to predict disease-free survival (Table 5, Figure 7A).

## Discussion

Breast carcinomas differ markedly in their biological and clinical behavior, therefore the availability of a wide panel of prognostic markers may be very helpful to identify patients at high risk for relapse and death. In this work we have found that apo D, a component of high-density lipoprotein present at large amounts in breast cyst fluid and produced by a subset of breast carcinomas, is an indicator of favorable outcome in women with breast cancer. To our knowledge, this is the first report indicating that a protein involved in the metabolism of cholesterol may be of prognostic significance in breast cancer.

Apo D expression in breast tumors was examined by immunoperoxidase staining of a series of 163 carcinomas. A total of 103 of these tumors showed immunohistochemically detectable apo D, although a wide variability in the percentage of positive cells and the intensity of staining was observed. Because these variations could be associated with the occurrence of tumors with different clinical outcome, we tried to correlate apo D tumor values with relapsefree survival and overall survival of the corresponding patients. The obtained results showed a significant relationship between apo D expression and both relapse-free survival and overall survival. In addition, multivariate analysis revealed that apo D was an independent factor for predicting clinical outcome.

Several biological aspects of apo D related to differentiation, hormone responsiveness, and cell proliferation processes could contribute to the apparent prognostic advantage conferred by the expression of this protein in breast carcinomas. In principle, and considering that previous studies from this and other laboratories have shown that apo D may be synthesized and secreted by normal breast tissue,[23,37] the finding of apo D expression by a subset of breast tumors indicates that they possess the required degree of differentiation to synthesize the protein. In support of this proposal, we have found a significant association at both protein and RNA levels between apo D and histological grade of tumors, with higher apo D levels in welldifferentiated breast carcinomas than in moderately or poorly differentiated tumors. Similarly, previous studies from Silva et al,[20] Lea et al,[21] and Soreide et al[37,38] in breast tumor cytosols have proposed that the presence of GCDFP-24 or progesterone-binding cyst protein (now identified as apo D) in breast carcinomas is a marker of a high grade of differentiation in these tumors and therefore suggestive of good prognosis. These similarities are particularly relevant if we consider that the different study populations show wide variations in the distribution of histological grade of tumors. Thus, our study includes a rather small number of histological grade IlI carcinomas whereas that of Silva et al[20] contains a relatively low number of grade tumors.

An additional possibility to explain why apo D confers a prognostic advantage to breast cancer patients is that its presence may reflect the existence of a complete hormone receptor pathway. Currently, the hormonal stimuli potentially responsible for the expression of apo D by breast tumors is unknown, but several data suggest that androgens could be the sex steroids involved in apo D overproduction. In fact, it has been recently shown that apo D is one of the few proteins that are induced by androgens in both human breast and prostate carcinoma cells.[16-18,39] In addition, the other major breast cyst fluid proteins, Zn-$\alpha_2$-glycoprotein and GCDFP-15, are also induced by androgens in breast cancer cells.[39,40] However, the recent finding of additive stimulatory action of glucocorticoids and androgens on apo D gene expression[18] suggests that other steroids could be also involved in the hormonal regulation of this gene. In relation to this, it is also remarkable that stimulation of apo D secretion by androgens and glucocorticoids coincides with inhibition of cell proliferation in both types of cells,[17,18] thus opening the possibility that apo D expression is associated with inhibition of cell growth and tumor regression. In agreement with this hypothesis, it has been recently shown that apo D transcription in human fibroblasts occurs specifically in

nonproliferating quiescent and senescent cultures.[19] Taken together, the presence of apo D in breast tumors could be a consequence of growth arrest with the subsequent favorable prognosis in the corresponding patients.

In summary, these preliminary findings appear to indicate that apo D in combination with other prognostic factors may contribute to more accurately identify subgroups of breast cancer patients with low or high risk for recurrence or death. In this context and by virtue of its specific pattern of hormone responsiveness and its potential value as a marker of growth arrest, apo D could give additional information to that provided by other well-established prognostic markers. Further studies performed in different populations and long-term follow-up of patients included in this work will be required to precisely establish the role of apo D among the increasing number of prognostic markers of potential use in breast cancer.

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**Table 1.** Characteristics of Patients and Tumors

| | |
|---|---|
| Patients | |
| Total | 163 |
| Age | |
| Mean | 59 |
| Range | 26–90 |
| Menopausal status | |
| Premenopausal | 50 |
| Postmenopausal | 113 |
| Tumors | |
| Size | |
| $T_1$ (<2 cm) | 42 |
| $T_2$ (2–5 cm) | 68 |
| $T_{3/4}$ (>5 cm) | 53 |
| Nodal status | |
| $N_0$ | 79 |
| $N_+$ | 84 |
| Metastasis at time of diagnosis | |
| $M_0$ | 155 |
| $M_1$ | 8 |
| Histological grade | |
| I | 77 |
| II | 67 |
| III | 19 |
| Estrogen receptor | |
| Positive | 60 |
| Negative | 43 |

**Table 2.** Apolipoprotein D HSCORE in Tumor Tissues Classified According to Different Characteristics

| Patient and Tumor Characteristics | No | HSCORE | |
|---|---|---|---|
| | | Mean ± SE | Range |
| Total tumors | 163 | 117.7 ± 9.6 | 0–400 |
| Menopausal status | | | |
| Premenopausal | 50 | 86.2 ± 15.8* | 0–300 |
| Postmenopausal | 113 | 130.6 ± 11.8 | 0–400 |
| Tumor size | | | |
| $T_1$ (<2 cm) | 42 | 116.5 ± 18.2 | 0–380 |
| $T_2$ (2–5 cm) | 68 | 101.2 ± 14.2 | 0–360 |
| $T_{3/4}$ (>5 cm) | 53 | 139.7 ± 18.1 | 0–400 |
| Nodal status | | | |
| $N_0$ | 79 | 127.4 ± 14.5 | 0–400 |
| $N_+$ | 84 | 110.2 ± 12.6 | 0–360 |
| Histological grade | | | |
| I | 77 | 131.5 ± 14.1† | 0–400 |
| II | 67 | 119.3 ± 15.1† | 0–380 |
| III | 19 | 50.5 ± 19.5 | 0–285 |
| Estrogen receptor | | | |
| Positive | 60 | 116.7 ± 15.1 | 0–400 |
| Negative | 43 | 128.9 ± 19.4 | 0–360 |
| Cathepsin D | | | |
| CAT.D<40 pmol/mg | 32 | 111.7 ± 22.6 | 0–360 |
| CAT.D>40 pmol/mg | 25 | 140.7 ± 22.6 | 0–320 |
| pS2 protein | | | |
| pS2>11 ng/mg | 20 | 106.5 ± 26.7 | 0–300 |
| pS2<11 ng/mg | 33 | 131.5 ± 21.63 | 0–360 |

* $P < 0.05$ *versus* postmenopausal patients.
† $P < 0.05$ *versus* histological grade III.

**Table 3.** Apolipoprotein D and Prognosis in Patients with Breast Cancer

| Factors | Relapse-Free Survival | | Overall Survival | |
|---|---|---|---|---|
| | Univariate *P* Value | Multivariate *P* Value | Univariate *P* Value | Multivariate *P* Value |
| Apolipoprotein D | 0.0014 | 0.0001 | 0.0108 | 0.0035 |
| Nodal status | 0.0009 | 0.0081 | <0.0001 | <0.0001 |
| Tumor size | 0.0055 | 0.0074 | 0.0115 | 0.0803 |
| Histological grade | 0.0771 | 0.4074 | 0.7700 | 0.4944 |
| Age | 0.0865 | 0.0645 | 0.5912 | 0.7639 |
| Menopausal status | 0.3347 | 0.3997 | 0.4762 | 0.7531 |

**Table 4.** Apolipoprotein D and Prognosis in Patients with Node-Positive Breast Cancer

| Factors | Relapse-Free Survival | | Overall Survival | |
|---|---|---|---|---|
| | Univariate *P* Value | Multivariate *P* Value | Univariate *P* Value | Multivariate *P* Value |
| Apolipoprotein D | 0.0099 | 0.0013 | 0.0234 | 0.0234 |
| Tumor size | 0.0346 | 0.0043 | 0.1728 | 0.0662 |
| Histological grade | 0.5641 | 0.9344 | 0.1370 | 0.1888 |
| Age | 0.2687 | 0.1716 | 0.5864 | 0.7247 |
| Menopausal status | 0.3160 | 0.2833 | 0.5227 | 0.6909 |

**Table 5.** Apolipoprotein D and Prognosis in Patients with Node-Negative Breast Cancer

| Factors | Relapse-Free Survival | | Overall Survival | |
|---|---|---|---|---|
| | Univariate *P* Value | Multivariate *P* Value | Univariate *P* Value | Multivariate *P* Value |
| Apolipoprotein D | 0.0115 | 0.0115 | 0.1007 | — |
| Tumor size | 0.9221 | 0.7980 | 0.7070 | — |
| Histological grade | 0.0690 | 0.1202 | 0.6949 | — |
| Age | 0.0985 | 0.2696 | 0.8954 | — |
| Menopausal status | 0.8792 | 0.7456 | 0.9688 | — |

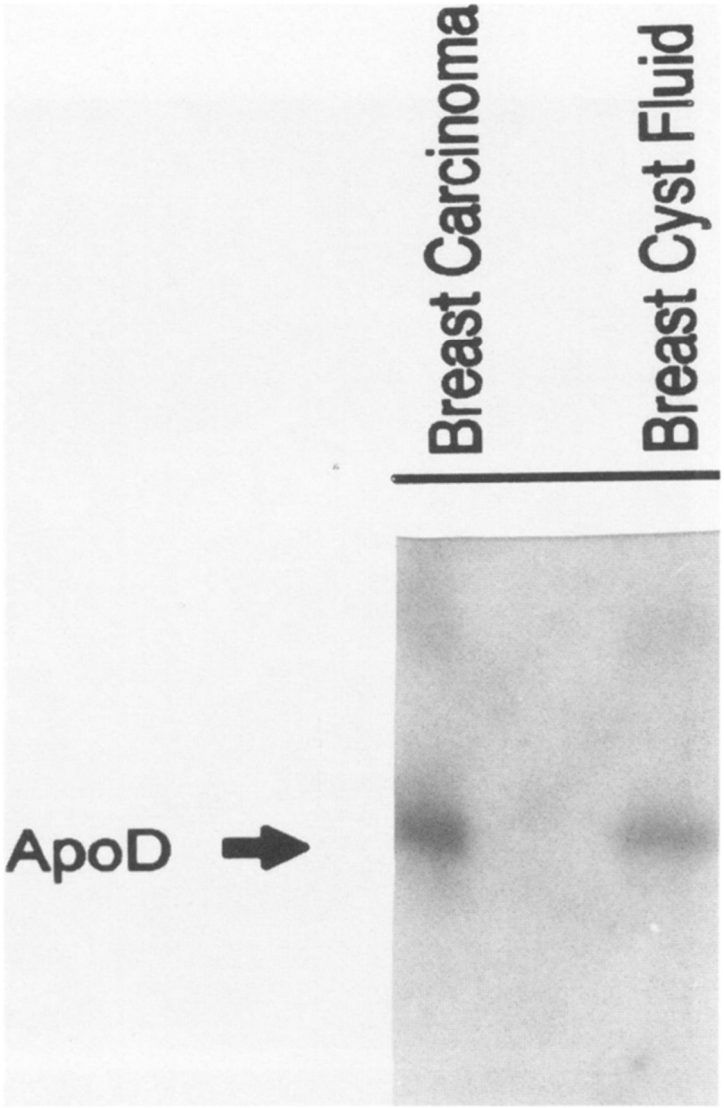


**Figure 1.** Immunoblot analysis of apo D in breast tissues. Aliquots from a breast carcinoma cytosol and breast cyst fluid were fractionated by SDS-PAGE and transferred to Immobilon TransferMembrane. Immunoblots were treated with antisenum against apo D (1:500) and developed.

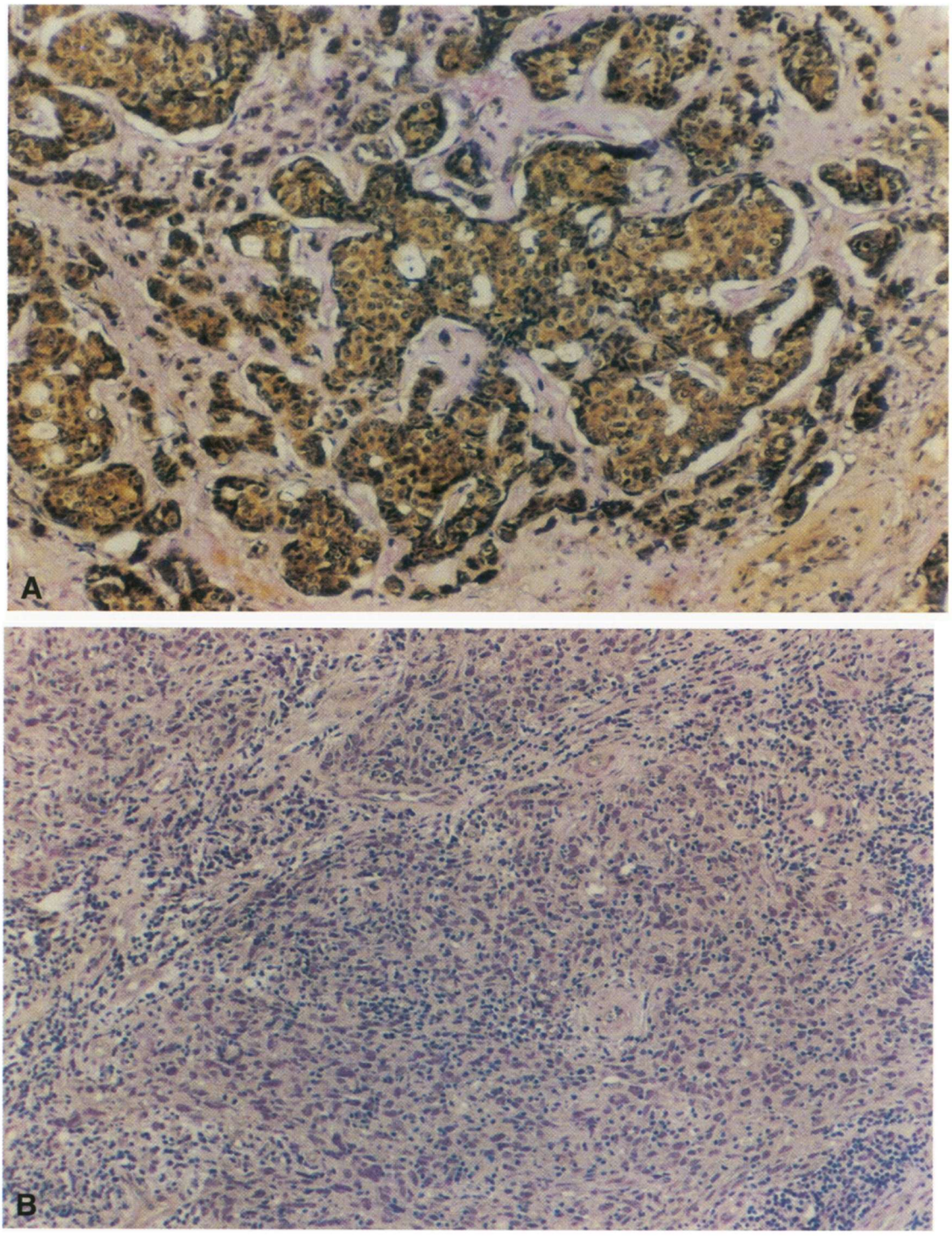


**Figure 2.** Immunohistochemical staining of apo D in human breast cancer. Positive tumor (A) and negative tumor (B) Tissue sections were immunostained with antiapo D (1:500 dilution). Sections were counterstained with formaldebyde-thionine. Original magnification X 170.

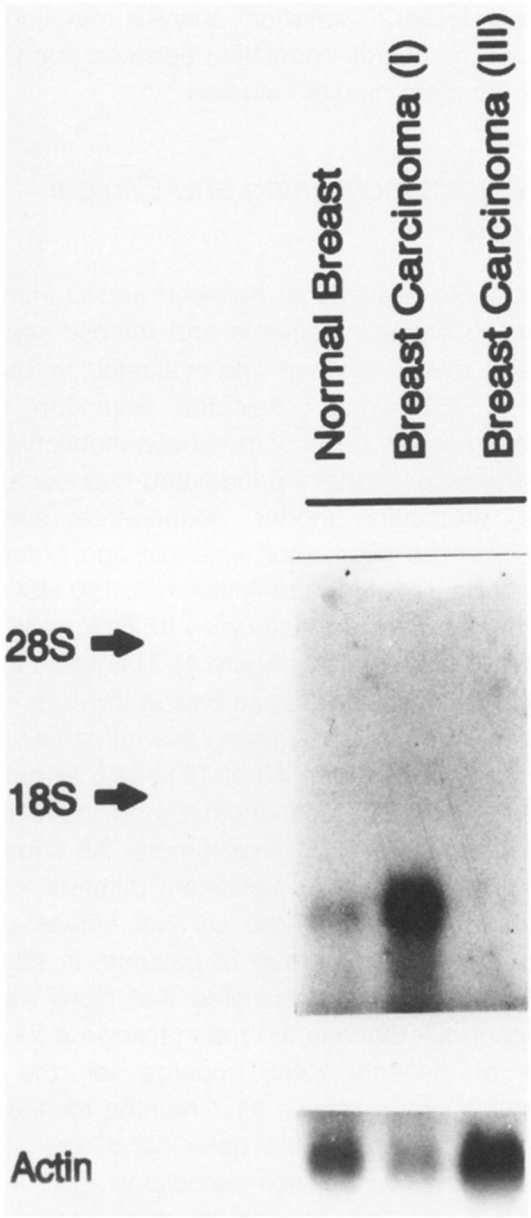


**Figure 3.** Expression analysis of apo D in mammary tissues. Samples of total RNA were obtained from two breast tumors with different histological grades (I, III) and from normal mammary tissue. RNAs were separated by electrophoresis on a 1.4% agarose-formaldehyde denaturing gel, blotted onto a nylon filter, and hybridized to the complete apo D cDNA. The positions of the 28 and 18 S ribosomal RNA bands are indicated.

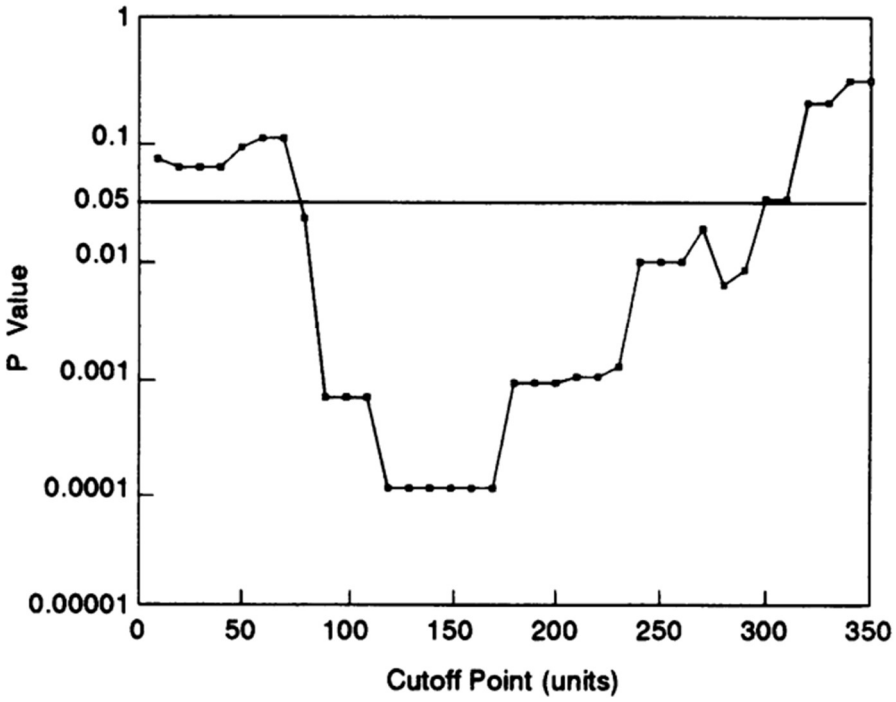


**Figure 4.** Maximal likelihood determination of the cutoff value of apo D for predicting relapse-free survival in breast cancer. *P* values obtained for each cutoff value are plotted against the value itself. Statistical significance at the $P < 0.05$ level is indicated by the horizontal line.

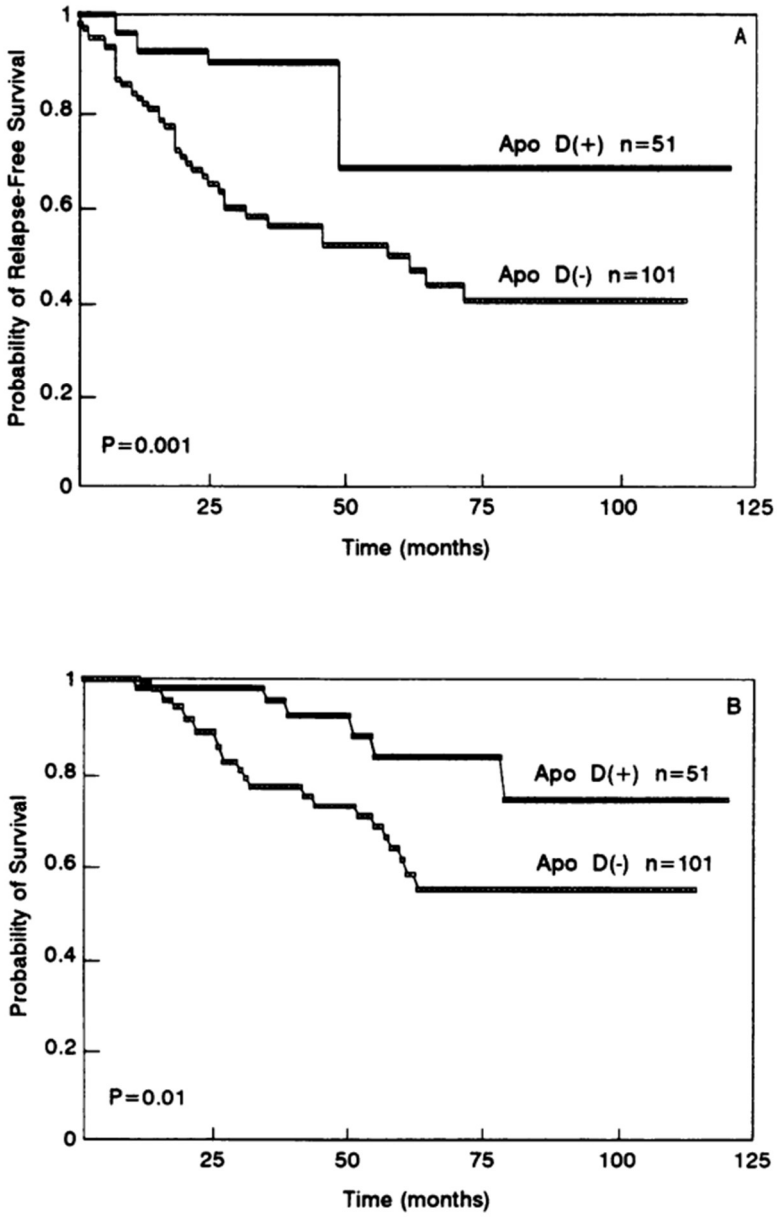


**Figure 5**. Relapse-free survival (A) and overall survival (B) as a function of apo D values in patients with breast cancer Mean follow-up was 43 months. Differences in relapse-free survival curves were significant at the *P* = 0.001 level and in overall survival at the *P* = 0.01 level.

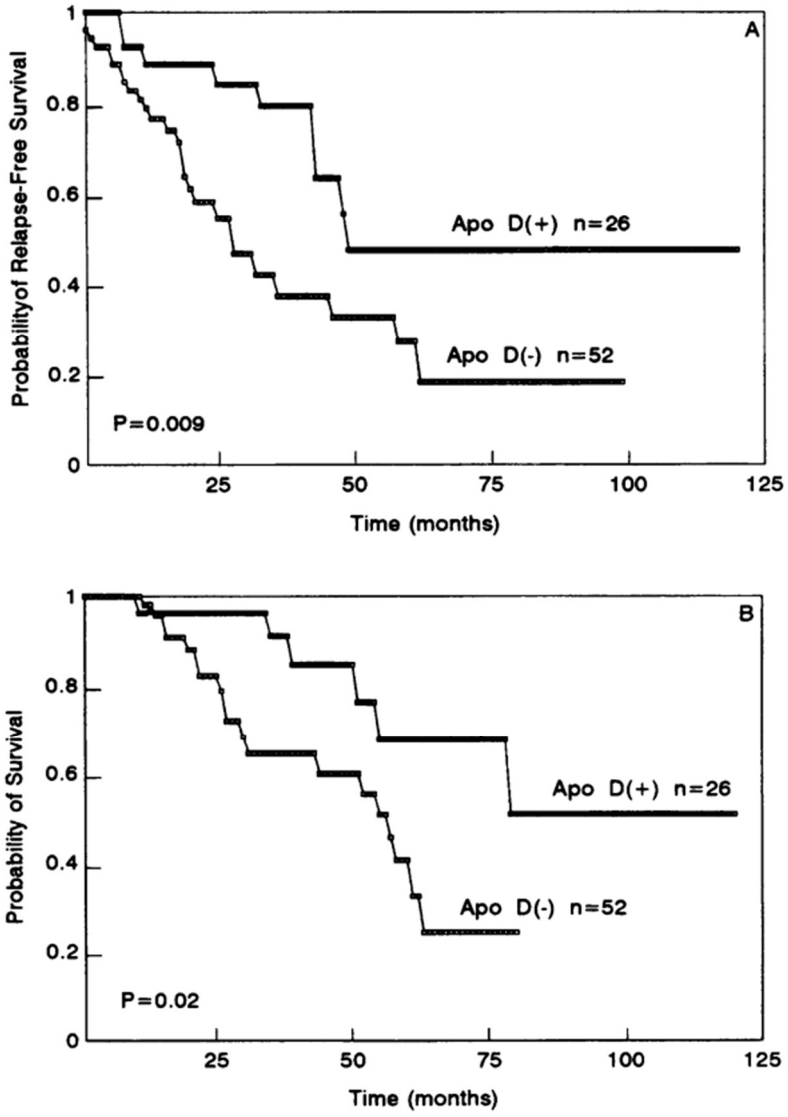


**Figure 6.** Relapse-free survival (A) and overall survival (B) as a function of apo D values in patients with node-positive breast cancer. Differences in relapse-free survival curves were significant at the *P* = 0.009 level and in overall survival at the *P* = 0.02 level.

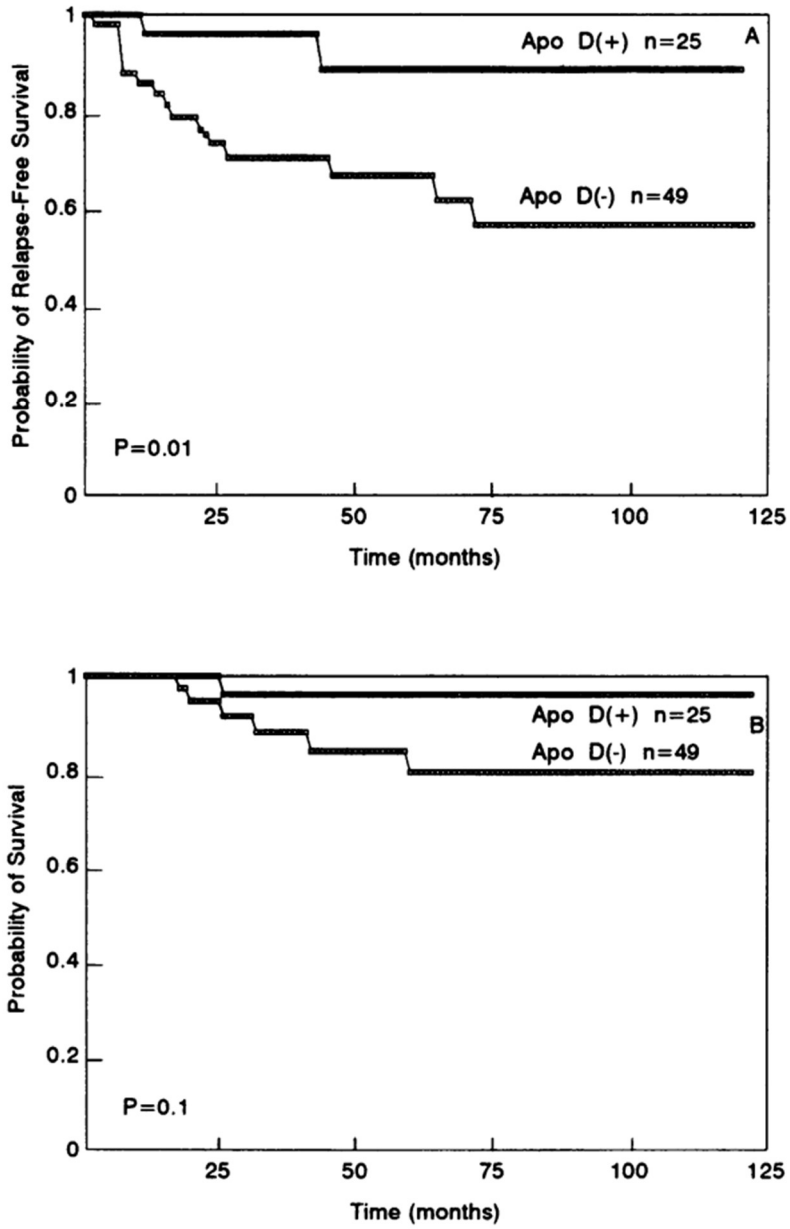


**Figure 7**. Relapse-free survival (A) and overall survival (B) as a function of apo D values in patients with node-negative breast cancer. Differences in relapse-free survival were significant at the *P* = 0.01 level.