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The Role of ApoD in Tamoxifen Resistance

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Epidemiology

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The Role of ApoD in Tamoxifen Resistance

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B.A., Vanderbilt University, 2013

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An abstract of
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Abstract

The Role of ApoD Expression
By Daniella Klebaner

Background Apoliprotein D (ApoD) has been proposed as an indicator of tamoxifen resistance among ER+ patients and a predictor of recurrence.

Methods We conducted a large case-control study nested in a population of 11251 women aged 35–69 years at diagnosis with stage I–III breast cancer between 1985 and 2001 on Denmark's Jutland Peninsula and registered with the Danish Breast Cancer Cooperative Group. We identified 541 recurrent or contralateral breast cancers among women with estrogen receptor-positive (ER+) disease treated with tamoxifen for at least 1 year and 300 cancers in women with ER-negative (ER-) disease never treated with tamoxifen. We matched one control subject per case patient on ER status, menopausal status, stage, calendar time, and county, and assessed ApoD expression in the tumor cell nucleus and cytoplasm using tissue microarray immunohistochemistry (TMA IHC). We estimated the odds ratio (OR) associating ApoD expression with breast cancer recurrence and adjusted for potential confounding with logistic regression. To address bias from potential exposure misclassification of ApoD expression, we used external validation data from TMA whole sections to complete a summary-level probabilistic bias analysis using Monte Carlo simulation.

Results The frequency of cytoplasmic ApoD expression was 68% in case patients with ER+ tumors, 66% in case patients with ER- tumors, and 66% in control subjects with ER+ and ER- tumors. 39% of case patients with ER+ tumors, 29% of case patients with ER- tumors, and 39% and 26% of control subjects with ER+ and ER- tumors, respectively, had nuclear expression. In women with ER+ tumors, the associations of any cytoplasmic ApoD expression with recurrence (OR = 1.0; 95% confidence interval = 0.7 to 1.4) and increasing cytoplasmic expression with recurrence (OR = 1.0; 95% confidence interval = 1.0 to 1.0) were null, as were those for women with ER- tumors. Nuclear ApoD expression associations were similarly near-null, as were those for combined nuclear and cytoplasmic ApoD expression. All near-null associations persisted after probabilistic bias analysis, and in an analysis restricted to women with ER expression confirmed by re-assay.

Conclusion The association between ApoD expression and recurrence in tamoxifen-treated patients is likely null or weak.

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Introduction:

Breast cancer accounts for the highest number of cancer cases among women worldwide, and is the second leading cause of death; approximately 12% of women will develop breast cancer at some point in their lives,[1] or nearly 1.4 million women annually.[2] Despite successes of initial treatment plans among breast cancer patients, approximately 30% of early-stage breast cancer patients develop recurrences.[3]

More than half of all breast cancer tumors express the estrogen-receptor-alpha protein (ERα). It has been known for several decades that estrogen and estrogen-receptor positivity in breast tissue is directly linked to the development of breast cancer, but the mechanism through which this association is mediated is unknown.[4] Theories include estrogen's stimulation of the expression of certain oncogenes, genotoxic effects of downstream metabolites, and increased cellular proliferation.[5] In general, estrogen receptors regulate the cell cycle of breast epithelial cells, and cells expressing ERα typically induce expression in neighboring cells.[6]

If the potential for breast cancer progression or metastasis is indicated, treatment for breast cancer typically includes combinations of various systemic therapies, including cytotoxic, hormonal, and immunotherapeutic agents.[3] For hormone-receptor positive breast cancer, hormone therapies are generally indicated for patients who have low-risk breast tumors greater than 1 cm in size, or high-risk tumors of any size, regardless of lymph node involvement, according to NCCN guidelines.[7,8] ER+ breast cancer patients are primarily treated with anti-estrogen therapies, which typically include tamoxifen, a selective estrogen receptor modulator, or aromatase inhibitors (AI). Tamoxifen selectively binds to the ligand-binding domain of the estrogen receptor, blocking estrogen's ability to bind and induce downstream effects, such as the association of co-activators..[6,9] Both pre- and postmenopausal women are

eligible for adjuvant and extended tamoxifen therapy.[7,8] Aromatase inhibitors work through decreasing levels of estrogen in the circulatory system by interfering with conversion pathways from androgens in peripheral tissues.[10] AIs are prescribed solely for postmenopausal women due to their biologic mechanismism, which sufficiently blocks the formation of estrogen only in postmenopausal women.[7,8] Clinical trials have suggested variable conclusions as to whether AIs, such as anastrazole and exemestane, or tamoxifen is a more effective adjuvant therapy in hormone-receptor positive breast cancers. The ATAC trial demonstrated the superiority of Anastrazole, an AI, over Tamoxifen for preventing recurrence, but no measurable effect on deaths after recurrence or overall mortality.[11] An analysis of longitudinal trends of endocrine therapy utilization for breast cancer found a shift from the use of tamoxifen to AIs, which is consistent with emerging guidelines that support the use of AIs in post-menopausal women.[12]

Women remain on tamoxifen for different amounts of time even after completing other treatments. Current NCCN guidelienes vary, suggesting combinations of AIs and tamoxifen depending on menopausal status, with most protocols recommending 5-10 years of hormone therapy.[7,8] Previous guidelines varied from one, two, or five years, with some evidence supporting the superiority of a five-year protocol, which halves the rate of recurrence in women with non-metastatic breast cancer.[13] Tamoxifen use has been shown to be associated in some studies with extremely rare side effects, including venous thromboembolism and uterine cancer.[7,8,14] Numerous studies have produced different estimates regarding the net effect of tamoxifen on outcomes in ER+ patients, with most studies showing that in early-stage patients, adjuvant tamoxifen improves disease-free survival and overall survival rates.[3] One challenge in developing appropriate treatment protocols is the fact that several trials have shown that adjuvant

endocrine therapy for estrogen-receptor positive patients does not reduce the risk of recurrence once patients cease taking these medications. [14] The number of post-treatment recurrences far outnumbers recurrences that occur during treatment.[14] Given this predicament, treating women with ER+ breast cancers for longer than five years with adjuvant therapies is becoming a more pertinent option, with questions remaining regarding what combinations/protocols of therapies to adopt. The American Society of Clinical Oncology currently recommends that postmenopausal women who are considering incorporating aromatase inhibitor therapy limit their total time on AI to five years, despite the fact that optimal timing and duration of therapy is currently unresolved. RCTs have demonstrated a very limited difference between five years of AI treatment and a sequence of tamoxifen and AI treatment.[15]

Despite tamoxifen's measurable positive effect on breast cancer prognosis, 70% of all breast-cancers expressing estrogen or progesterone receptor positivity, and only half of such metastatic breast cancers, respond to endocrine therapies.[6,16] In addition, the majority of breast cancers that do initially respond to endocrine therapies eventually develop resistance to these therapies.[6] Molecular studies have demonstrated that response to hormone therapies is likely dependent upon estrogen-receptor-related pathways, although detailed mechanisms of resistance have not yet been determined despite having been studied extensively.[16,17,18] Effective use of hormone therapy will likely depend on the ability to subtype receptor-positive breast cancers based on their biomarker profiles.[16,19] To date, no biomarkers have been used to identify patients at high risk for tamoxifen resistance in clinical practice.[20]

Identifying biomarkers that modulate tamoxifen response presents a challenge due to the inherent complexity of signal transduction pathways, and the difficulties associated with isolating specific biomarker effects. Apolipoprotein D (ApoD)

expression may be predictive of resistance to tamoxifen, although its exact mechanism is unknown.[21,22,23,24] ApoD is a small glycoprotein involved in transport of hydrophobic ligands, and is ubiquitous in human tissue in all stages of development and adulthood.[25] ApoD has been associated with cytoprotection through its ability to remove toxic substances from cells. Molecular studies have demonstrated an inhibitive effect of the estrogen receptor on ApoD, with an up-regulation effect by tamoxifen, likely through blockage of ER activity.[24] As a result, combined estrogen-receptor positivity and ApoD positivity could be reflective of a of a non-functional hormone receptor pathway, resulting in ineffective tamoxifen treatment and subsequent relapse.[26] In a 1994 study, tamoxifen's improvement of relapse-free survival was limited to patients whose tumor cytosol did not express ApoD.[27]

A follow-up study in 2007 reported an association between ApoD's simultaneous presence in the cytosol and nucleus with tamoxifen resistance in postmenopausal and elderly patients.[24] Normal growing cells show ApoD localizing to the cytoplasm and not the nucleus, whereas ApoD localization to the nucleus is observed in serum starved cells. Molecular studies strongly suggest that ApoD that has already been secreted may appear in the nucleus after reentry into the cell, and implies ApoD's involvement in genetic processes such as transcription activation, involvement in the cell cycle, or triggering of apoptosis.[28] ApoD may also have a role in the inhibition of translocation of phosphorylated MAPK, a kinase involved in signal transduction, into the nucleus, and the reduction of proliferative activity in cancer cells.[26]

If ApoD has a functional role in the tamoxifen-estrogen receptor pathway, the presence of ApoD in breast tumors has the potential to be a biomarker for assessing the success of hormone therapies in breast cancer treatment.[29] Several studies have shown that ApoD levels predictably rise in response to reduced cell proliferation and

tamoxifen therapy, and the lack of such a response, or the presence of high levels of ApoD prior to therapy, is a non-invasive marker of ineffective treatment.[30]

In addition to its potential role as a biomarker for tamoxifen response, ApoD has also been proposed, but not substantiated, as a predictor of breast cancer prognosis. A 1991 study found that ApoD was an independent predictor of axillary nodal involvement, but was not a strong predictor of overall relapse-free survival.[31] A separate study proposed that ApoD expression in tumor cells was associated with a favorable prognosis, but its presence in adjacent tumor stroma was a negative sign for relapse-free survival.[26]

Only two studies have explored the direct relationship between ApoD and resistance to tamoxifen therapy.[24,25] We seek to precisely estimate this association with a larger, well-characterized population. This study takes into account different ApoD staining patterns and characteristics while exploring possible sources of bias, with the ultimate goal of producing a result that can be clinically applicable to treatment protocols.

Materials and Methods

Patients

The Danish Breast Cancer Cooperative Group (DBCG) has collected breast cancer patient information since 1977. Nearly all Danish breast cancer patients under 70 years of age are enrolled in the DBCG database, and information on their diagnostics, treatment, and follow-up is collected for ten years, making it one of the largest modern-day clinical registries of breast cancer patients.[34] Using the DBCG database, we collected information on 11,251 female residents of the Jutland Peninsula between the ages of 35-69 who were diagnosed with Stage I-III breast cancer, as designated by the Union for International Cancer Control, between 1985 and 2001.[35]

For the purposes of this study, data were collected beginning at one year from date of diagnosis, and ending at date of first breast cancer recurrence, death from any cause, loss to follow-up, after ten years, or September 1, 2006 (the end of the study's follow-up). All data were linked using Danish Civil Person Registration Numbers.

Patients were divided into two subgroups – those whose tumors showed expression of estrogen receptor-α and had been on tamoxifen therapy for at least one year, and those who whose tumors did not show expression of estrogen receptor-α, were not treated with tamoxifen, and survived one year. All patients who did not fit into these two subcategories were excluded.

Patients were also excluded because they had insufficient or invalid tissue material that could not be scored on the TMAs (tissue microarrays). Invalid tissue included tumors that were non-invasive, such as cores that only had ductal carcinoma in situ, cores that were excessively over stained, or those that were torn. After these quality control measures, 1,267 women remained in the study, with one to four valid cores available per patient on average.

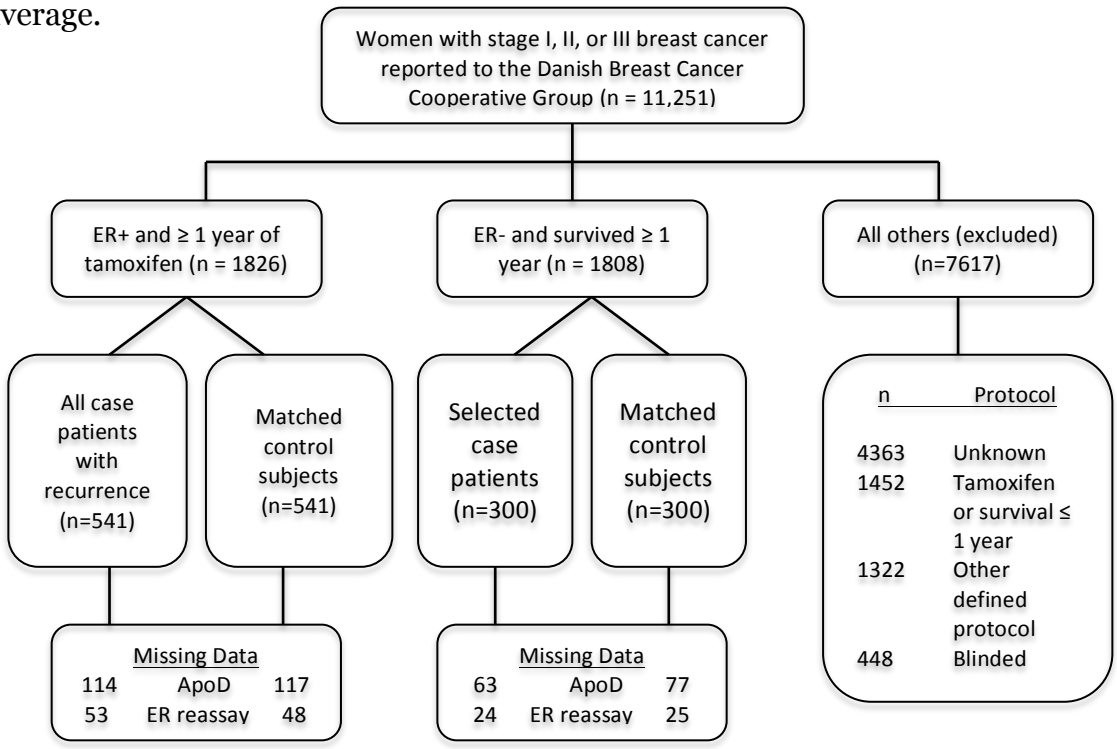


Figure 1. Design used to select study sample based on inclusion criteria. The source population consisted of 11,251 female residents fo the Jutland Peninsula in Denmark aged 35-69 years who were diagnosed with stage I, II, or III breast cancer between 1985 and 2001. Most patients (n=4363) who were excluded because their treatment protocol was unknown had Stage I breast cancer treated without a guideline protocol from the DBCG. ApoD was missing for a substantial portion of subjects due to insufficient or damaged tissue, or incorrect staining. ER-reassay results were missing for a small proportion of subjects due to unavailable tumor blocks or indeterminate assay results. Case patients and control subjects with missing data were excluded from analyses that required the variable with missing data. ER=Estrogen Receptor.

Immunohistochemistry

We used tissue microarray immunohistochemistry to determine the subcellular location of ApoD. Laboratory personnel were blinded to all clinical information, including case or control status, estrogen receptor status, and receipt of therapeutic agents. Tissues were processed from formalin-fixed paraffin-embedded tissue blocks from pathology department archives using sterile protocols designed to avoid case contamination. The antibody used for ApoD staining was a rabbit monoclonal antibody EPR2916 from Abcam.

Cylindrical samples were taken from each donor tumor and re-embedded into a paraffin block. The tissue microarrays were then constructed using the TMA Master from 3DHistech, using cores of 1 mm thickness. An asymmetrical design was used with liver and placenta tissue markers indicating the beginning and end of each TMA. Liver and placental cores were stained as controls, with liver cores as the positive controls and placental cores as the negative controls. Each donor block contributed three representative tumor cores and one marginal tissue core where possible. The TMAs were scanned at 40x magnification with the Hamamatsu Nanozoomer 2.0HT in .ndpi format. Slides were converted with a beta version converter to conform to the 3DHistech software and uploaded to Panoramic Viewer TMA Module software.

Scoring of TMA Cores

One pathologist at the Aarhus University Department of Pathology trained one epidemiology student (DK) and one medical student to read and score breast carcinoma TMA cores, stained for Apo-D expression using immunohistochemical methods. The pathologist reviewed all the TMA cores after they were initially scored by the other two raters. Eight hundred TMA cores were initially reviewed and a preliminary scoring system was developed. Initial scoring was performed and categorized into cytoplasmic staining, nuclear staining, and granularity. Cores that were marked as "uncertain" by the scorers were assessed by all three raters and discussed if further disagreement remained until a consensus was reached.

Cytoplasmic staining was grouped into four categories: negative (0), undetermined, weakly positive (1), moderately positive (2), and strongly positive (3). Frequency of cytoplasmic staining was quantified by allocating different percentages to each of the four categories for each core, adding up to 100%.

Nuclear staining was grouped into two categories, one specifying intensity and the other specifying frequency. Within the frequency category, staining was quantified as no nuclear staining (0), 1-25% of nuclei stained (1), and 25%+ of nuclei stained (2). If there was nuclear staining present, intensity was described as either light staining (1), or dark staining (2).

Similarly to nuclear staining, granularity was scored in two categories – one specifying intensity, and one specifying percentage of all staining that was granular. Within the latter category, staining was quantified as no granular staining, 1—10% of all staining granular, 11-50% of all staining granular, and 51+% of all staining granular. This percentage was assigned to both a weak granular staining category, and a strong granular staining category. For example, a TMA core displaying weak granular

staining in 75% of cell cytoplasms and strong granular staining in 5% of cell cytoplasms would be scored in the 51-100% group in one category (intensity=1), and in the 1-10% group in the other (intensity=2).

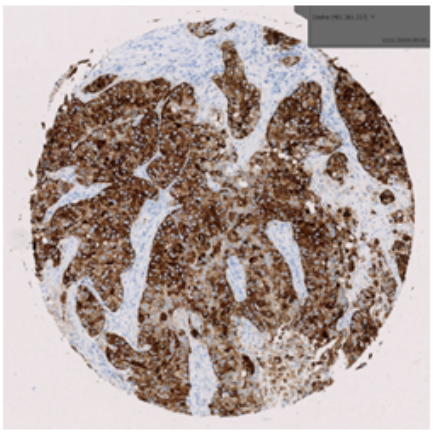


Figure A

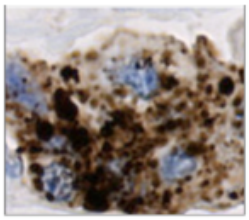


Figure B

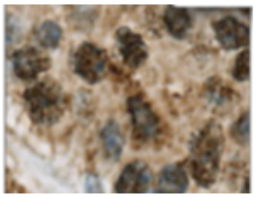


Figure C

Figures 2a-c: ApoD Staining Determination. **Figure a** depicts a TMA core that was scored: 45% weakly positive, 50% moderately positive, and 5% strongly positive. **Figure b** depicts typical strong granular staining, while **Figure c** shows diffuse cytoplasmic staining with some nuclear staining.

A semi-quantitative scoring system employing an "H-score" was used for all analyses. This score was calculated separately for nuclear staining, diffuse cytoplasmic staining, and granular staining. For diffuse cytoplasmic staining, the four staining categories ranging from negative to strongly positive, or 0 to 4, respectively, were multiplied by the respective percentages in each category. For example, a core showing 30% no staining, 20% weak positivity, 40% moderate positivity, and 10% strong positivity would receive an H-score of 0x30 + 1x20 + 2x40 + 3x10 = 130. The maximum H-score in this category was 300 (3x100%). A similar but simplified H-score was computed for nuclear staining. A cell with 10% nuclear staining (1) and all dark staining (2) would receive a score of 1x2 = 2. The maximum nuclear score is 4 (2 in each category).

As there were up to four TMA cores for patients, an H-score was computed for each core, and then the H-scores were averaged into a final H-score for each patient. A previous paper by Soiland et al. identified a threshold H-score of 0 as a significant cutoff for patients over seventy years of age.[33] This cutoff was used for all subsequent

analyses. Thus, patients with an H-score=0 were considered to have no ApoD present, while patients with an H-score>0 were considered to have ApoD present.

To assess inter-rater agreement, H-scores and N-scores were computed, averaged, and dichotomized for one hundred cores based on scoring by the three raters who were blinded to each other's scores, sixty-two of which contained valid tissue for scoring. When scored independently, agreement was generally good aside from ascertainment of nuclear scoring between two raters (Table 1). All of the cores were scored by at least two of the three raters non-independently, as this allowed for discussion of controversial cores and resulted in more consistency in scoring.

Table 1. Inter-rater agreement of ApoD staining on TMAs*

	Cytoplasmic†					
	Scored Positive Frequency (Percentage)			**Scored Negative Frequency (Percentage)**		
	Rater 1	**Rater 2**	**Rater 3**	**Rater 1**	**Rater 2**	**Rater 3**
Rater 1 Agreed	40 (100)	35 (85)	34 (81)	22 (100)	16 (76)	14 (70)
Rater 2 Agreed	35 (88)	41 (100)	40 (95)	16 (73)	21 (100)	19 (95)
Rater 3 Agreed	34 (85)	40 (98)	42 (100)	14 (64)	19 (90)	20 (100)
Total Scored	40	41	42	22	21	20

	Nuclear†					
	Scored Positive Frequency (Percentage)			**Scored Negative Frequency (Percentage)**		
	Rater 1	**Rater 2**	**Rater 3**	**Rater 1**	**Rater 2**	**Rater 3**
Rater 1 Agreed	10 (100)	10 (63)	10 (42)	52 (100)	46 (100)	38 (100)
Rater 2 Agreed	10 (100)	16 (100)	16 (67)	46 (89)	46 (100)	38 (100)
Rater 3 Agreed	10 (100)	16 (100)	24 (100)	38 (73)	38 (83)	38 (100)
Total Scored	10	16	24	52	46	38

*62 cores were independently scored by three raters, and staining was compared between each rater pair. For actual ascertainment of exposure, scoring was done non-independently to improve consistency in scoring.
†Positive and negative scores were computed based on a cutoff of >0 or =0, respectively, on the H-score or N-score scale.

Statistical Analysis

Definitions of Analytical Variables

ApoD Cytoplasmic Staining

Cytoplasmic staining was categorized as either granular or diffuse. It was quantified using an H-score as described previously, and the cutoff between negative and positive staining was H-score=0. Two variables were used for this analysis – one dichotomous variable specifying staining as negative versus positive, and one continuous variable with the H-score itself (1-300) to assess dose response.

ApoD Nuclear Staining

Nuclear staining was quantified using a specific nuclear H-score, as described above. Two variables were used for this analysis as with cytoplasmic staining – one dichotomous (negative versus positive, cutoff of 0), and one continuous (1-4) using the nuclear H-score.

Recurrence

The DCBG data provided recurrence information, defined as breast cancer, including contralateral cancer, or distant metastases diagnosed after receipt of initial treatment. For the purposes of this study, a "case" was defined as a recurrence that occurred within eleven years of the initial diagnosis.

Covariates

Cases and controls were matched on the following covariates: time of breast cancer diagnosis, age at diagnosis, Charlson comorbidity index score at diagnosis,

menopausal status at diagnosis, county of residence at diagnosis, UICC stage at diagnosis, histological grade, surgery type, and receipt of systemic adjuvant chemotherapy.

Stain Type Analysis and Variable Categorization

To determine how best to categorize cytoplasmic staining within categories of granular, diffuse, and mixed staining, boxplots were developed comparing intensity and frequency of staining within each category (negative, weak, moderate, and strong). The distribution of points was compared between each set of boxplots to ascertain whether separate categorization was necessary for the diffuse, mixed, and granular staining groups.

Conventional Analysis

All statistical analyses were performed in SAS 9.3. All analyses were performed within strata of ER+/TAM+ and ER-/TAM- to isolate the association between ApoD and tamoxifen resistance. Crude frequencies were calculated within the two strata showing the proportion of cases and controls in various categories of ApoD staining, separated into nuclear and cytoplasmic staining. Conditional logistic regression was used to calculate measures of association, with recurrence as the outcome and ApoD staining status as the exposure variable. Odds ratios estimating the association of ApoD positivity in the nuclear or cytoplasm with recurrence were computed for dichotomous categorization of staining (using negative staining as the referent group), and continuous categorization of staining, controlling for covariates. Within continuous categorization of ApoD staining status, the odds ratio was computed excluding the negative referent group to assess the presence of a dose response.

Biologic interaction with menopausal status was assessed based on a 2012 paper that stressed the need for stratified analyses of these two groups, and with age based on the results of the Soiland study, which found an association only among women over age 70.[33]

Validation Data and Quantitative Bias Analysis

Apo-D Validation

One major drawback to the use of tissue microarrays in this study was its potential to be incompletely representative of the heterogeneity of certain tumors. A 2008 paper by Soiland et al. that compares TMA IHC analysis of ApoD expression to Whole Section IHC analysis shows a strong correlation between the two methods ($p<0.0001$) with a very wide spread ($R^2=0.60$).[36] This heterogeneity is not apparent in the results, leading to potential exposure misclassification. This misclassification is likely to be non-differential, as scorers were blinded to disease status, and previous research has not shown differing heterogeneity patterns in tumors between recurrent cases and non-cases.

Data from the Soiland paper were used to determine parameters for sensitivity and specificity. In their study, TMAs were sampled only from the invasive front, and produced a sensitivity of 70% and perfect specificity when compared with whole section staining. In our study, since four TMAs were sampled from different parts of the tumor to obtain representative staining, the sensitivity was likely to be higher. Several trapezoidal distributions were assigned to the sensitivity to account for the likely under-estimation of sensitivity in the external validation data with respect to our study (Figure 3).

Positive and negative predictive values were calculated from these sensitivity

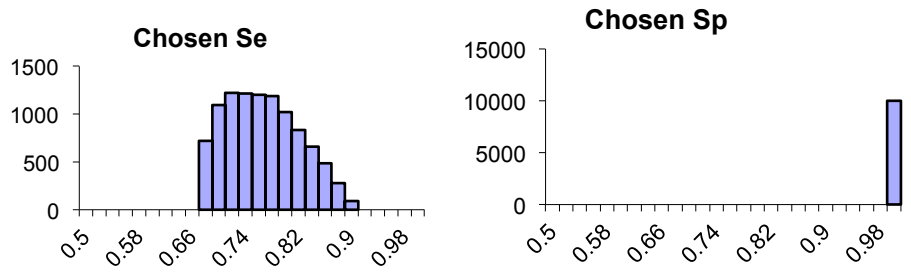
values, and incorporated into a probabilistic bias analysis. Using Monte Carlo simulation, these values and their distributions were applied to summary-level data stratified on menopausal status using an excel spreadsheet developed by Lash, Fox, and Fink in order to obtain reclassified counts for positive (>0) and negative (=0) H-scores, and bias-adjusted measures of association with a 95% simulation interval.[37,38]

A multi-dimensional bias analysis was also performed to assess the effect of selecting different sensitivity values on the bias-adjusted odds ratio. In addition to the estimated sensitivity from the Soiland paper, values of TMA IHC sensitivity relative to WS IHC were selected from a literature search, ranging from 60% to 95%.[39,40,41]

Estrogen Receptor Re-assay

Due to evolving DCBG recommendations regarding ER expression assay and the potential for inter-hospital variability, whole sections were re-assayed to assess concordance with the original diagnostic categorization. Whole sections were sampled from the original diagnostic paraffin embedded tissues, and a primary antibody against ERα was used (clone 6F11; Novocastra, Newcastle-Upon-Tyne, UK). Heat-induced epitope retrieval for ER was achieved by incubation in a Tris/EGTA buffer, pH 9 (VWR-Bie & Bertsen, Denmark) using a microwave oven. Sections were stained on a Lab Vision Autostainer (Thermo Fisher Scientific, Fremont, CA) using the EnVision™+ detection system (Dako). Sections were enhanced using copper sulphate and visualized with horseradish peroxidase and diaminobenzadine. We scored slides as positive for ER when there was clear nuclear staining of tumor cells. We scored sections as ER positive if ≥10% of tumor nuclei were positive in accordance with previous DBCG recommendations for the diagnostic period of patients included in the study.[42]

Figure 3. Example of a trapezoidal distribution for sensitivity and specificity*



*Lower mode (0.70) was the estimated sensitivity from a separate validation study of ApoD IHC TMA expression. Minimum and upper mode were the 99th percentile limits of a beta distribution, centered on 0.70 and skewed to the right. Specificity was fixed at 1.

Results

Descriptive Statistics

The majority of women were either Stage II (45%) or III (53%) at diagnosis, with only 2% designated as Stage I according to standards set by the Union for International Cancer Control. Approximately half of the women were between the ages of 55-64, with 24% in the 65-69 category, 22% in the 45-54 category, and only 3% of women between the ages of 35-44. Given the age distribution, only 6% of women were premenopausal. The study lasted from 1985-2001 with women diagnosed throughout this period; 43% were diagnosed during the earlier portion of the study from 1985-1993, with 21% and 36% diagnosed from 1994-1996 and 1997-2001, respectively.

Approximately half of the ER+ patients were initially assigned to tamoxifen treatment protocols of two years according to the DCBG registry, with the remaining half split between one and five year protocols. Medical records often indicated a longer tamoxifen protocol than the registry, as patients were likely switched to the five-year protocol as evidence emerged supporting longer adjuvant treatment times. As expected, a much greater percentage of the ER- group was assigned to systemic chemotherapy treatment, as overall prognosis for this subset of breast cancer patients is lower, and fewer adjuvant treatment options exist.

In both the ER+ and ER- strata, the percentage of women with some positive cytoplasmic ApoD expression was between 65 and 70%. Nuclear staining patterns differed somewhat between ER strata, with approximately 39% of ER+ patients exhibiting positive nuclear staining, as compared to 25-30% of ER- patients (Table 1).

Table 2. Frequency and proportion of breast cancer recurrence case patients and matched control subjects within group strata*

	ER+/TAM+, No.(%)		ER-/TAM-, No.(%)	
Patient Characteristic	**Case Patients**	**Control Subjects**	**Case Patients**	**Control Subjects**
Cytoplasmic ApoD Expression (H-Score)				
=0	135 (32)	144 (34)	80 (34)	77 (35)
>0	292 (68)	280 (66)	157 (66)	146 (66)
Missing †	114	117	63	77
Nuclear ApoD Expression (H-Score)				
=0	260 (61)	258 (61)	169 (71)	165 (74)
>0	167 (39)	166 (39)	68 (29)	58 (26)
Missing †	114	117	63	77
Joint ApoD Expressionζ				
=0	115 (44)	131 (46)	76 (54)	71 (58)
>0	147 (56)	153 (54	64 (46)	52 (42)
Missing †	279	257	160	177
Diagnosis Year§				
1985-1993	235 (43)	234 (43.3)	107 (36)	100 (33)
1994-1996	113 (21)	112 (20.7)	81 (27)	83 (28)
1997-2001	193 (36)	195 (36)	112 (37)	117 (39)
Age category at diagnosis, y				
35-44	13 (3)	12 (2.8)	52 (22)	41 (18)
45-54	92 (22)	86 (20)	94 (40)	84 (38)
55-64	221 (52)	222 (52)	67 (28)	68 (31)
65-69	101 (24)	104 (25)	24 (10)	30 (13)
Menopausal Status at diagnosis§				
Premenopausal	34 (6.3)	34 (6.3)	121 (40)	121 (40)
Postmenopausal	507 (94)	507 (94)	179 (60)	179 (60)
UICC tumor stage at diagnosis§				
I	8 (1.9)	6 (1.4)	14 (5.9)	15 (6.7)
II	194 (45)	193 (46)	128 (54)	115 (52)
III	225 (53)	225 (53)	95 (40)	93 (42)
Histological grade				
I	108 (20)	144 (27)	27 (9.0)	23 (7.7)
II	234 (43)	215 (40)	125 (42)	98 (33)

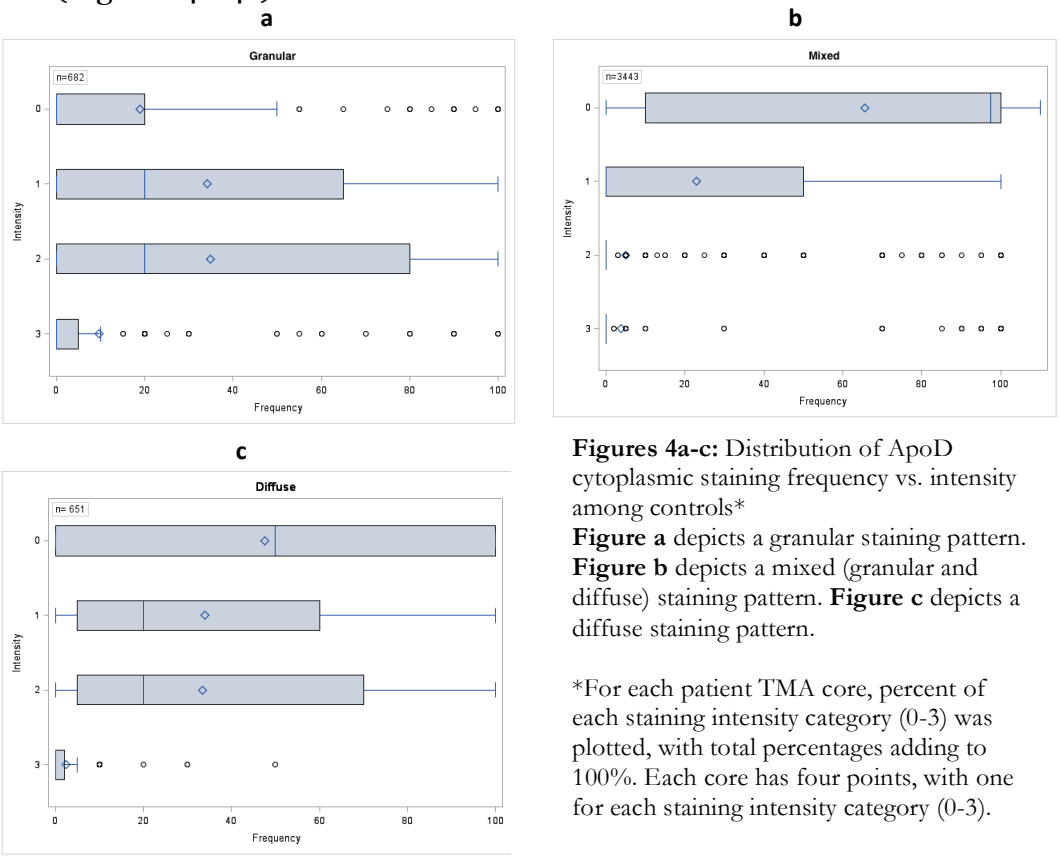
III	92 (17)	57 (11)	103 (34)	106 (35)
IV	107 (20)	125 (23)	45 (15)	73 (24)
Missing				
Surgery type				
Breast-conserving surgery	383 (90)	368 (87)	199 (84)	181 (81)
Mastectomy	44 (10)	56 (13)	37 (16)	42 (19)
Missing	0	0	1	0
Radiation therapy				
Yes	149 (35)	150 (35)	103 (44)	90 (47)
No	278 (65)	274 (65)	130 (56)	102 (53)
Missing	0	0	6	40
Tamoxifen protocol, y				
1	257 (48)	261 (48)		
2	98 (18)	92 (17)		
5	186 (34)	188 (35)		
Systemic adjuvant chemotherapy				
Yes	53 (12)	42 (9.9)	203 (86)	139 (62)
No	374 (88)	382 (90)	34 (14)	84 (38)
Current ER expression				
Positive	397 (93)	411 (97)	59 (25)	56 (25)
Negative	30 (7.0)	12 (2.8)	177 (75)	165 (74)
Not available†	0.0	1 (0.2)	1 (0.4)	2 (0.9)

*The source population consisted of 11,251 female residents of the Jutland Peninsula in Denmark aged 35-69 years who were diagnosed with Stage I, II, or III breast cancer between 1985 and 2001. Subjects were estrogen receptor positive and received at least 1 year of tamoxifen therapy (ER+/TAM+) or ER negative and never received tamoxifen therapy and survived at least 1 year after diagnosis (ER-/TAM-). ApoD=Apolipoprotein D; UICC=Union for International Cancer Control.
†No tissue available for assay or assay results indeterminate
§Variable included in risk set sampling to match control subjects to case patients.
ζJoint effect indicates combined nuclear and cytoplasmic dichotomous staining

Stain Type Analysis

Boxplots were produced comparing intensity and frequency of ApoD cell staining distributions for cytoplasmic staining. The plots appeared different between granular, diffuse, and mixed staining groups among controls. This pattern suggested that associations must be examined within subsets of staining type for patients. However, when associations were produced within homogeneous and heterogeneous staining groups, they were nearly identical to those seen across all groups (Table 3). Data were too sparse in the homogeneous category to further assess associations

between different staining types. Thus, the group-wide associations were used to retain precision as homogeneous and heterogeneous staining types produced the same results (Figures 4a-4c).



Figures 4a-c: Distribution of ApoD cytoplasmic staining frequency vs. intensity among controls*
Figure a depicts a granular staining pattern. **Figure b** depicts a mixed (granular and diffuse) staining pattern. **Figure c** depicts a diffuse staining pattern.

*For each patient TMA core, percent of each staining intensity category (0-3) was plotted, with total percentages adding to 100%. Each core has four points, with one for each staining intensity category (0-3).

Table 3. Associations between ApoD expression and breast cancer recurrence within strata of ER status and heterogeneity of cytoplasmic staining*

ApoD Expression	**ER+/TAM+ Matched OR (95% CI)**		**ER-/TAM- Matched OR (95% CI)**	
	Heterogeneous Staining*	Homogeneous Stainingξ	Heterogeneous Staining	Homogeneous Staining
Cytoplasmic H-score				
=0				
>0	1.14 (0.78, 1.68)	X	0.91 (0.55, 1.49)	X
Continuous	1.00 (1.00, 1.00)	1.03 (0.99, 1.07)	1.00 (1.00, 1.00)	X

*The source population consisted of 11,251 female residents of the Jutland Peninsula in Denmark aged 35-69 years who were diagnosed with Stage I, II, or III breast cancer between 1985 and 2001. Subjects were estrogen receptor positive and received at least 1 year of tamoxifen therap (ER+/TAM+) or ER negative and never received tamoxifen therapy and survived at least 1 year after diagnosis (ER-/TAM-).
ApoD=Apolipoprotein D; UICC=Union for International Cancer Control.

‡Estimated using logistic regression with adjustment for time to recurrence or control selection, menopausal status, stage, receipt of chemotherapy, receipt of radiation therapy, and type of surgery.
*Patients whose cores vary between granular, diffuse, or mixed staining
ξPatients whose cores were all in one category: granular, diffuse, or mixed staining
X = not enough patients in stratum to calculate estimate

Conventional Results

All matched associations between ApoD and breast cancer recurrence were near null in both the ER+/TAM+ and ER-/TAM- strata (Table 4). For cytoplasmic staining, both dichotomous coding of ApoD staining (Matched OR=1, 95% CI=0.72-1.39), as well as continuous coding assessing dose response (Matched OR=1, 95% CI=0.998-1.002) resulted in null associations. Similarly, nuclear staining also yielded near-null results both using dichotomous coding of ApoD staining (Matched OR=1.013, 95% CI=0.748-1.379), and continuous coding (Matched OR=0.989, 95% CI=0.830-1.180). There was a mildly protective joint effect when ApoD was present in both the nucleus and the cytoplasm (Matched OR=0.872, 95%CI = 0.550-1.381), although there were fewer patients with available data for both nuclear and cytoplasmic staining, and the estimate is far less precise.

The ER-/TAM- group also yielded near null associations that were almost identical to those in the ER+/TAM+ group, suggesting that recurrence among ER+ patients (and therefore ineffective tamoxifen treatment) is not associated with ApoD's presence in the tumor cytosol or nucleus. The associations estimating the effect of joint nuclear and cytoplasmic expression on recurrence were also null in both the ER+ stratum (Matched OR=0.87, 95% CI=0.55-1.38) and the ER- stratum (Matched OR=1.062, 95% CI=0.537, 2.103). Associations were examined in validated ER strata, and only negligible differences in estimates were observed.

There was no evidence for biologic interaction between menopausal status and cytoplasmic staining; there was some effect modification of the effect of joint staining

between post-menopausal (1.100, 95% CI: 0.754-1.606) and pre-menopausal (1.749, 95% CI: 0.425-7. 204) women, although the wide and overlapping confidence intervals as a result of small sample size in these strata preclude interpreting the odds ratios as different from one another (Table 5).

Table 4. Associations between ApoD expression and breast cancer recurrence within strata*

	ER+/TAM+			ER-/TAM-		
ApoD Expression	**Case patients/control subjects or mean****	**Matched OR (95% CI)†**	**Adjusted OR (95% CI)‡**	**Case patients/control subjects or mean****	**Matched OR (95% CI)†**	**Adjusted OR (95% CI)‡**
Joint ApoD Expression						
=*0*	115/131			76/71		
>*0*	147/153	0.87 (0.55, 1.38)	1.14 (0.79, 1.65)	65/52	1.06 (0.54, 2.10)	1.29 (0.72, 2.32)
Cytoplasmic H-score						
=*0*	135/144			80/77		
>*0*	292/280	1.00 (0.72,1.39)	1.19 (8.33,1.50)	157/146	0.98 (0.64,1.49)	1.14 (0.74, 1.75)
Continuous	Mean: 85.77/87.29	1.00 (1.00, 1.00)	1.00 (1.00,1.00)	Mean: 90.9/108.4	1.00 (1.00,1.00)	1.00 (1.00, 1.00)
Nuclear H-score						
=*0*	260/258			169/165		
>*0*	167/166	1.01 (0.74,1.38)	1.05 (0.78,1.40)	68/58	1.17 (0.71,1.92)	1.25 (0.79, 2.00)
Continuous	Mean: 1.390/1.396	1.00 (0.65,1.55)	1.03 (0.88,1.21)	Mean: 1.47/1.70	0.72 (0.38,1.37)	1.01 (0.80, 1.26)

The source population consisted of 11,251 female residents of the Jutland Peninsula in Denmark aged 35-69 years who were diagnosed with Stage I, r III breast cancer between 1985 and 2001. Subjects were estrogen receptor positive and received at least 1 year of tamoxifen therapy (ER+/TAM+) r ER negative and never received tamoxifen therapy and survived at least 1 year after diagnosis (ER-/TAM-). ApoD=Apolipoprotein D; JICC=Union for International Cancer Control.

Estimated using logistic regression with adjustment for time to recurrence or control selection, menopausal status, stage, receipt of chemotherapy, eceipt of radiation therapy, and type of surgery.

Mean for cases/controls provided for continuous exposure variable, whereas frequency is provided for cases/controls for dichotomous exposure ariable

Table 5. Associations between ApoD and breast cancer recurrence among ER+ patients accounting for biologic Interaction with menopausal status and age*

ApoD Expression	Adjusted OR (95% CI)‡	
	Post-Menopausal	Pre-Menopausal
Joint ApoD Expression		
=0		
>0	1.10 (0.75, 1.61)	1.749 (0.425, 7.204)
Cytoplasmic H-score		
=0		
>0	1.12 (0.82, 1.5)	1.08 (0.33, 3.53)

*The source population consisted of 11,251 female residents of the Jutland Peninsula in Denmark aged 35-69 years who were diagnosed with Stage I, II, or III breast cancer between 1985 and 2001. Subjects were estrogen receptor positive and received at least 1 year of tamoxifen therapy (ER+/TAM+) or ER negative and never received tamoxifen therapy and survived at least 1 year after diagnosis (ER-/TAM-). ApoD=Apolipoprotein D; UICC=Union for International Cancer Control.
‡Estimated using logistic regression with adjustment for time to recurrence or control selection, menopausal status, stage, receipt of chemotherapy, receipt of radiation therapy, type of surgery, and additive interaction between the covariate and ApoD expression

Bias-Adjusted Results

Bias-adjusted estimates were slightly further from the null than conventional estimates on average among ER+ women (Bias-Adjusted OR = 1.35, SI = 1.17-6.6), as well as ER- women (Bias-Adjusted OR = 1.1, SI = 1.05-2.6). Even after taking the potential for exposure misclassification into account, the association between ApoD expression and recurrence appears to be weak or null and non-differential across ER strata, suggesting that ApoD plays a minimal role in recurrence risk via the tamoxifen-ER pathway.

Multidimensional bias analysis yielded bias-adjusted ORs ranging from 1.11 when using a sensitivity of 95%, to 3.21 when using the sensitivity calculated in the Soiland paper, 70% (Table 6).

Table 6. Results of multidimensional bias analysis for varying sensitivities among ER+ patients

Sensitivity	Bias-adjusted OR*
0.70†	3.21
0.75	1.4
0.85	1.18
0.9	1.15
0.95	1.11

*Sensitivity values were applied to 2x2 tables containing exposure and outcome data
†Value calculated from external validation study conducted by Soiland et al[36]

When calculated probabilistically, bias-adjusted estimates varied depending on the trapezoidal parameters selected (Table 7). The largest bias-adjusted OR was calculated using a lower range of sensitivities, and suggests that ApoD may have a weak effect on recurrence (Bias-adjusted OR=1.35, 95%CI: 1.17-8.43). A trapezoidal distribution using the upper range of sensitivity parameters resulted in a weaker association (Bias-adjusted OR=1.22, 95% CI=1.14-1.83).

Table 7. Bias-adjusted estimates using probabilistic methods with varying distributions

Sensitivity Trapezoidal Parameters				Bias-Adjusted OR (95% CI)*	Illegal Values‡
Minimum	Lower Mode	Upper Mode	Maximum		
0.61	0.7†	0.78	0.9	1.35 (1.17-8.43)	107
0.61	0.7†	0.85	0.95	1.26 (1.14-2.26)	27
0.65	0.7†	0.85	0.95	1.26 (1.14-3.43)	30
0.65	0.75	0.85	0.95	1.24 (1.14-2.69)	14
0.7	0.75	0.85	0.95	1.22 (1.14-1.83)	0

*Adjusted estimates were calculated using summary-level 2x2 tables containing exposure and outcome data using an excel spreadsheet created by Lash, Fox, and Fink[38]
†Value calculated from external validation study conducted by Soiland et al[36]
‡Sensitivity analysis resulted in negative bias-adjusted cell values

Discussion

Despite plausible biological hypotheses, we did not observe an association between ApoD tumor expression and recurrence among ER+ patients. The estimated effect was near null in all categories of ApoD localization - nuclear, cytoplasmic or both – and in both categorizations of staining – dichotomous and continuous to assess dose-response. Though granular and diffuse cytoplasmic staining may indicate separate molecular mechanisms for ApoD localization, neither form of staining was associated with recurrence, and there was no observable difference in the effect of heterogeneously-stained cores and homogeneously-stained cores. There was little evidence for biologic interaction between menopausal status and cytoplasmic ApoD staining. Among patients with both cytoplasmic and nuclear staining, the adjusted odds ratio increased slightly, between post-menopausal (1.100, 95% CI: 0.754, 1.606) and pre-menopausal (1.749, 95% CI: 0.425, 7. 204); however, a lack of precision in this category due to missing information for either cytoplasmic or nuclear staining resulted in very wide confidence intervals, which should caution against over interpretation of this association.

Associations were near null among ER+ patients and ER- patients, with indiscernible differences in estimates between the two groups, further suggesting no association between ApoD and recurrence, as any effect dependent upon the estrogen-tamoxifen pathway would be isolated to the ER+ group. The bias analysis yielded varying estimates that were all slightly farther from the null than conventional estimates, suggesting either a near-null or weak association. Literature regarding the sensitivity of TMA IHC as compared to WS is inconclusive; a near-perfect sensitivity yielded near-null results in a multi-dimensional bias analysis (Adjusted OR = 1.11), whereas lowering sensitivity to the value defined in the external validation substudy

resulted in a strong association (Adjusted OR = 3.21). Given the persistence of conventional null results in all ApoD categorization schemes and the likelihood that our TMA cores were more representative of the whole sections than the external stubstudy, it is likely that the true bias-adjusted estimate is closer to the conventional, near-null OR than this higher value. Subsequent probabilistic bias analyses support this conclusion, yielding bias-adjusted ORs from 1.22 (95% CI: 1.14 to 1.86) to 1.35 (1.17-8.43).

This is the largest study investigating the association between ApoD and recurrence, and resulted in precise estimates for the most part. We addressed a major source of bias by accounting for exposure misclassification using probabilistic bias analysis. Selection bias was likely avoided in the design phase, as all cases and controls were selected from the DBCG registry, which contains nearly all Danish breast cancer cases under the age of 70 at diagnosis.

Tamoxifen therapy duration was often inconsistent between the DCBG registry and the patients' medical records, with the registry indicating that the patient was on a shorter duration of therapy. These patients were likely initially assigned the one or two year protocol, and then switched to longer protocols as evidence in favor of the five year protocol became more widespread. Since patients were likely to be on tamoxifen for longer periods of time than the registry indicated, their recurrences were less likely to result from a lack of therapy; as a result, this discrepancy was actually more likely to isolate the effect of ApoD as a predictor of recurrence.

There was good concordance between ER+ status at diagnosis and upon re-assay. Associations were nearly identical and near null in both original ER strata and validated ER strata. Inter-rater agreement was generally good when assessed independently, and subsequent collaborative examination of each core resulted in

improved concordance and staining categorization. Staining guidelines were designed to be clinically applicable in order to ensure that ApoD could be a consistent prognostic indicator if an association was found. A previous validation study using medical record review confirmed all recurrences, eliminating the potential for outcome misclassification, and showed perfect agreement for all covariates except one patient's menopausal status.

In certain strata, such as for assessing the joint effect of nuclear and cytoplasmic staining, the sample size was fairly small. However, given the consistency of precise null results in nearly all categories of staining, it is unlikely that these estimates would change meaningfully with an increased sample size.

One limitation of this study was the use of external validation data for the bias analysis. The external data were based on a study that sampled exclusively from the invasive front of the tumor, whereas the TMAs in our study were sampled from three representative regions of the tumor. Though we anticipated increased sensitivity in our stained cores as compared to the external validation study due to more representative sampling, these data will likely not account for bias as accurately as an internal substudy.

As is the case with other predictive biomarkers, discerning between positive and negative stains is often questionable. For assessment of estrogen receptor positivity, the current cutoff is 1% of cells expressing the estrogen receptor; there is not sufficient information to designate such a cutoff for ApoD at this stage, but results were null when assessed using dichotomous categorization, as well as dose-response continuous coding among non-zero cores.

Earlier studies that have demonstrated an association between ApoD and recurrence did so only within age-specific strata, and had smaller sample sizes. These

studies also did not account for exposure misclassification, although doing so would likely correct for a bias that is directed towards the null, strengthening their estimated associations. In order for ApoD to be prognostically relevant, it must be meaningfully associated with recurrence in largely nonspecific groups, or its stratum-specific associations must be meaningfully different. Our results are near null or weak and highly similar across different biologically relevant groups, suggesting that the true association between ApoD and tamoxifen is likely to be null, or weak. As such, the need remains for predictors of response to tamoxifen, as well as predictors of recurrence following completion of adjuvant treatment to assess the need for longer duration of therapy or alternate treatments.

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