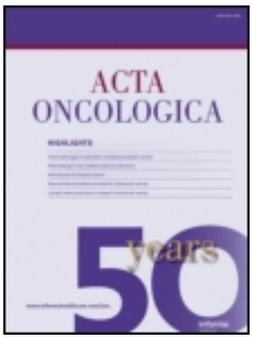

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ORIGINAL ARTICLE

Co-expression of estrogen receptor α and Apolipoprotein D in node positive operable breast cancer – possible relevance for survival and effects of adjuvant tamoxifen in postmenopausal patients

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Abstract

Background. Estrogen receptor-α (ERα) is an important prognostic and predictive marker in breast cancer. ERα signaling normally down-regulates expression of Apolipoprotein D (ApoD), a lipocalin that binds, transports or chelates lipophilic ligands, including tamoxifen (TAM). Hence, the co-expression of ApoD may therefore identify clinical relevant subgroups of ERα positive breast cancer patients. *Material and methods.* ApoD, ERα, and progesterone receptor (PR) protein expressions were determined by immunohistochemistry (IHC) in primary tumors of 290 patients with operable breast cancer. The median follow-up was 12 years. Patients were stratified according to age, nodal stage and the expression of ERα and the combined cytoplasm and nuclear staining of ApoD ($ApoD_{CN}$). *Results.* In elderly women (≥70 years) (n = 76), $ApoD_{CN}$ expression identified different prognostic subgroups in ERα positive patients (Trend: $p < 0.0001$). Multivariate analysis in this age group (n = 72), showed that the ERα-positive /$ApoD_{CN}$-negative subgroup had a better breast cancer specific survival (BCSS) compared with the ERα-positive/$ApoD_{CN}$-positive group (hazard ratio (HR) = 4.3; 95% CI = 1.6 – 11.9; $p = 0.005$). This difference was predominantly seen in the node positive patients (n = 30) (HR = 10.5; 95% CI = 2.3 – 47.6; $p = 0.002$). In a subset of postmenopausal ERα-positive/node positive patients (n = 60) previously enrolled in a trial on 2 year adjuvant TAM 20 mg vs. placebo, a better BCSS was observed in $ApoD_{CN}$ negative patients compared to placebo ($p = 0.02$). In $ApoD_{CN}$ positive patients, adjuvant TAM did not provide any survival benefit. *Discussion.* ERα and $ApoD_{CN}$ co-expression seems to be of prognostic importance in node positive elderly patients with operable breast cancer. In addition, we hypothesize that $ApoD_{CN}$ expression may be a novel marker and/or mechanism of TAM resistance in postmenopausal node positive patients. Thus, when targeting the ERα pathway in these patients, the ApoD status of the tumor may be of clinical relevance.

Estrogen receptor-alpha (ERα) has been regarded as an important initiator and promoter of tumor development [1], and a useful prognostic factor for relapse and disease survival. Determination of ERα status has been of importance for prognostication, and for prediction of tailored anti-estrogen treatment. This treatment option is of relevance in elderly patients in particular, due to a higher risk of detrimental side effects when systemic cytotoxic adjuvant treatment is employed [2]. ERα positive tumors are more commonly encountered in postmenopausal and elderly patients [3]. However, clinical effects of anti-estrogen treatment do not always parallel these higher ERα expression rates [4]. Likely, there might be various phenotypically subgroups of ERα positive tumors with different clinical courses.

The lipocalin Apolipoprotein D (ApoD = Gross Cystic Disease Fluid Protein-24 (GCDFP-24) = Progesterone Binding Cyst Protein (PBCP) [5]) thought to be a simple transport molecule for small lipophilic ligands including progesterone and

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arachidonic acid [6], has recently been associated with cytoprotection by binding or chelating toxic substances [7]. Cell line studies have shown that ApoD expression is strongly repressed by the ERα action [8]. Moreover, tamoxifen (TAM) up-regulates ApoD levels [9], probably through an indirect mechanism, by inhibition of the ERα signaling. TAM binding to ApoD [10] may indicate a triangular relationship between ApoD, ERα, and TAM [6].

In a recent paper, we report an adverse outcome in operable breast cancer patients ≥70 years with a combined expression of ApoD in the cytoplasma and nuclei ($ApoD_{CN}$) of the cancer cells [11]. We have previously suggested that effects of adjuvant TAM are related to tumor cytosol content of PBCP (=ApoD) in node positive patients with operable breast [12]. In the present study, we evaluate if $ApoD_{CN}$ expression in the invasive front of the primary tumor may be of importance for survival, and may predict effects of adjuvant tamoxifen in elderly and postmenopausal patients with ERα-positive tumors and operable breast cancer.

Material and methods

Patients

An overview of the patients included in this study is outlined in Figure 1. Between 1983 and 1987, 443 consecutive women with operable breast cancer (Stage I and II) from Western Norway were included in a study on the possible prognostic relevance of cytosol PBCP content in primary breast cancers, as reported previously [12]. From the original study population, 331 patients had confirmed invasive breast cancer (i.e. >2 mm diameter) and available tissue blocks for the recent study [11], and also for the present study. Among 112 (25%) excluded patients, 20 had ductal carcinoma in situ (DCIS I-III) or micro-invasive cancer <2 mm, four had poor quality of the histological material and the remaining 88 patients had not available tissue blocks for immunohistochemical (IHC) analysis. There were no statistically significant or clinically important differences between the original (n=443) and the present study group (n=331) with regard to age, tumor size, nodal status, clinical stage, menopausal status and progesterone receptor content (data not shown). Due to technical reasons, information on ERα and $ApoD_{CN}$ expression was available in 290 of these 331 patients. Sixty node positive postmenopausal patients from our study population were enrolled in a previous conducted randomized trial of adjuvant TAM 20 mg daily vs. placebo for 2 years [13]. The distribution of basic patients characteristics (e.g. pT status) in the tamoxifen and control arm for both the $ApoD_{CN}$ positive and $ApoD_{CN}$ negative patients was similar to the distribution in the original study population [13]. These patients were analyzed separately for possible predictive effects of ApoD expression. Postmenopausal status was defined as the clinical status of menopause. Patients ≥70 years of age were defined as elderly.

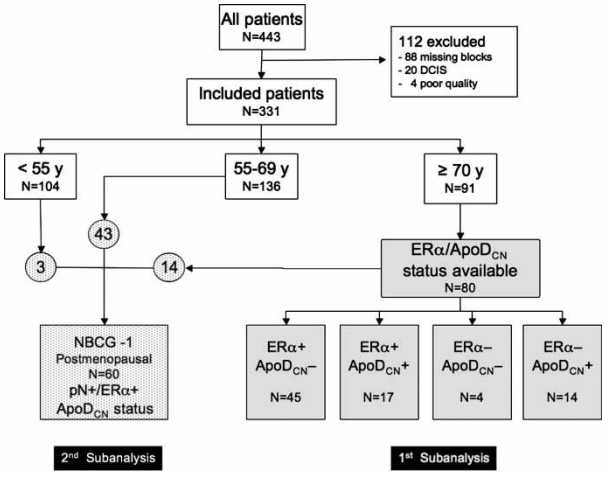

Figure 1. Flowsheet over patients included in the present paper. The 1st sub-analysis was done to evaluate a possible role of $ApoD_{CN}$ co-expression with ERα in the elderly patients ≥70 years. The 2nd sub-analysis evaluated the potential influence of $ApoD_{CN}$ status on the predictive effect of tamoxifen in the ERα positive postmenopausal patients with node positive disease included in the NBCG-1 trial [13]. DCIS, ductal carcinoma in situ; y, years; ERα, estrogen receptor alfa; $ApoD_{CN}$, Combined cytoplasmic and nuclear ApoD staining; pN+, node positive; NBCG-1, Norwegian Breast Cancer Group adjuvant study # 1 [13].

Tumor specimens

The tumor size (pT-stage) was measured in the fresh surgical breast tumor specimens and all detectable axillary lymph nodes were prepared for histology as described previously [11]. The median number of identified lymph nodes was 9 (range, 1–36). Revision of the histological classification and grading of the tumors was done by experienced pathologists (EG, JPAB) according to WHO criteria [14]. Tumor category (pT) and nodal status (pN) were reclassified according to the UICC criteria from 2002 [15], which did not alter the distribution of the patients between the pT_1/pT_2 and pN_0/pN_+ subgroups compared with the original classification of the patients [12].

Immunohistochemistry

ApoD, ERα and progesterone receptor (PR) were determined by immunohistochemistry (IHC) in

1.7 mm tissue microarray (TMA) cylinders carefully selected from the invasive front of the tumor. The invasive front of the tumor is important for the evaluation of various biomarkers in malignant tumors [16], and is recently shown to be relevant for prognostication related to ApoD expression [11]. The invasive front had to fulfill the following criteria [16]: a) most cellular area, b) in the periphery of the tumor and c) inflamed or necrotic areas or areas in contact with the epidermis should be avoided.

The antigen retrieval was performed by using a highly standardized retrieval system (ImmunoPrep, Instrumec, Oslo, Norway) and the immunohistochemical technique was based on DAKO Cytomation technology (DAKO, Glostrup, Denmark) described in details recently [11]. In short, immunostaining was performed on 4 μm sections adjacent to the H&E section used for diagnosis by using an autostainer (DAKO). The sections were incubated with the antibodies (ApoD, clone 36C6, Novocastra, 1:200 dilution; ER-α, clone SP-1, NeoMarkers, 1:400 dilution and PR, clone SP-2, NeoMarkers, 1:400 dilution) for 30 min.

The quantitation of ApoD in immuno sections has been described in detail elsewhere [17]. In brief, the amount of ApoD expressed in the cytoplasm and nuclei were semi-quantitatively assessed by the H-score method [11,17]. For ERα and PR, the percentage of positively stained nuclei was calculated by dividing the number of all positive staining intensities by the total number of counted cells.

Due to 13–14% loss of tumor tissue during the TMA production process, ApoD H-score and ERα percentage could be calculated in 290 (87%) patient samples, while all three immuno biomarkers (ApoD, ERα and PR) were available in 284 (86%) patients.

Cut-off levels

A count of $\geq 10\%$ positive cells is commonly used as a threshold for ERα and PR positivity, and was also applied in the present study. As shown recently [11], a H-score of 0 vs. >0 was found by receiver operating characteristic curve (ROC) analysis to be the optimal threshold value for both for cytoplasmic and the ApoD nuclear staining in postmenopausal and elderly women.

ERα and ApoD subgroups

The combined cytoplasmic and nuclear localization ($ApoD_{CN}$) has recently been demonstrated to be of highest prognostic relevance compared to other staining patterns [11]. We therefore made four subgroups according to the $ApoD_{CN}$ and ERα status. Except from a lower median age (53 years) in the single ERα $-$/$ApoD_{CN}-$ sub-group, various characteristics of the ERα/$ApoD_{CN}$ subgroups (n = 290) were similar (Table I).

Endpoints

Relapse free survival (RFS) was defined as the time from the primary operation until confirmation of a relapse (i.e. recurrent disease of any location, or a new malignant tumor in the contra lateral breast). Breast cancer specific survival (BCSS) was defined as the time from the primary operation until death from breast cancer. Cause of death was provided from hospital records and in few cases also by information from the patient's general physician.

Statistics

SPSS v. 13.0 for Macintosh (SPSS, Chicago, IL, USA) and MedCalc (MedCalc Statistical Software v. 9.3.7, Mariakerke, Belgium) were used for statistical calculations. Category variables were analyzed with the χ^2 test, and Fisher's exact test was applied when appropriate. Survival estimates were calculated using the Kaplan-Meier method, and univariate comparisons of survival curves were performed with the log-rank test. Stratification of age groups was done according to treatment guidelines from the Norwegian Breast Cancer Group (NBCG) [18]; and survivals were calculated and compared for these age groups. Multivariate analysis was performed with the Cox proportional hazards method using backward and forward stepwise models with the following variables: tumor size category (pT_1/pT_2), nodal status (pN_0/pN_+), histological grade (WHO grade 1–3), PR positive/negative, and the four different ERα/$ApoD_{CN}$ expression subgroups as shown in Figure 1. The "Log-minus-log" plot confirmed a constant hazard rate ratio during the observation period.

In the elderly patients, the subgroup (ERα $-$/$ApoD_{CN}-$) comprised only four patients (Figure 1), and these were therefore excluded from the survival analysis. Two-tailed p-values <0.05 were considered statistically significant.

Ethics

The Regional Ethics Committee (#151.04), the Norwegian Social Science Data Service (#11241), the Norwegian Institute of Public Health, Bio Bank Registry (#1500), and the Norwegian Data

Table I. Comparison of features between subgroups of ERα /$ApoD_{CN}$ status (n = 290).

	ERα+/$ApoD_{CN}$−	**ERα+/$ApoD_{CN}$+**	**p**	**ERα−/$ApoD_{CN}$−**	**ERα−/$ApoD_{CN}$+**	**p**
	165	62		40	23	
Age (median)	63 (21–88)	65 (34–83)	0.49	53 (25–82)	71 (27–89)	**<0.0001**
<55 y*	44	18	0.93	23	4	**<0.0001**
55–69 y	76	27		13	5	
≥70 y	45	17		4	14	
pT_1†	84	24	0.10	13	8	0.66
pT_2	79	73		25	12	
Unknown	2	1		2	3	
pN_0†	86	27	0.25	24	10	0.21
pN_+	79	35		16	13	
WHO Grade 1††	17	13	0.09	1	1	0.18
WHO Grade 2	105	34		9	10	
WHO Grade 3	43	15		30	12	
Tumor morphology††						
IDC	144	56	0.54	34	22	0.38
ILC	18	6		4	1	
Other	3	0		2	0	
PR ≥10%§	138	7	0.65	19	14	0.31
PR <10%	22	54		21	9	
Missing	5	1				
Stage I†	58	17	0.30	9	9	0.16
Stage IIa	107	44		31	14	
Unknown		1				
Pre-menopausal‖	40	12	0.46	15	4	0.09
Postmenopausal	123	48		25	19	
Unknown	2	2				

*Age distribution according to the Norwegian Breast Cancer Group [18].
†pT, pN according to UICC criteria [15].
††Histological grading and tumor morphology according to WHO criteria [14]. IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma.
§PR, progesterone receptor; immunohistochemistry determination in TMA, cutoff 10%.
‖Clinical status.

Inspectorate (#2004/1432-2) approved all aspects of this study.

Results

$ApoD_{CN}$ is inversely correlated to ERα in postmenopausal and elderly patients

An inverse correlation between ERα and $ApoD_{CN}$ expression was observed in patients ≥70 years ($r = -0.43$; $p < 0.0001$). This was also true for all postmenopausal patients ($r = -0.13$; $p = 0.045$). No correlation was found between ERα and $ApoD_{CN}$ in pre-menopausal patients. There was no correlation between PR and $ApoD_{CN}$ for any age groups. A positive correlation ($r = 0.35$; $p < 0.0001$) was found between ERα and PR for all age groups. Due to the correlation between ERα and $ApoD_{CN}$ in the elderly, interaction analysis between these two variables was done, and no interaction was found in the multivariate analysis (data not shown). Accordingly, a possible independent influence of $ApoD_{CN}$ categories on ERα subgroups was further evaluated.

Survivals

The ERα/$ApoD_{CN}$ subgroups showed prognostic relevance in patients ≥70 years, both for RFS and BCSS both in the univariate (Table II) and in the multivariate analysis (Table III). Of note is a significant different RFS of the subgroups with ERα positive tumors, related to the $ApoD_{CN}$ co-expression (p=0.004, log-rank) (Figure 2A). In addition, the ERα+/$ApoD_{CN}$+ and the ERα−/$ApoD_{CN}$+ subgroups had an almost similar RFS (Figure 2A). Regarding BCSS, a more favorable outcome was seen in elderly patients with ERα+/$ApoD_{CN}$− tumors, as compared to the ERα+/$ApoD_{CN}$+ group ($p = 0.004$, log-rank) (Figure 2B).

This prognostic association between BCSS and ERα/$ApoD_{CN}$ subgroups in elderly patients was only seen in the node positives ($p = 0.003$). In addition, the ERα+/$ApoD_{CN}$+ combination showed a HR of 10.5 (95% CI = 2.3–47.6) when compared to the reference group. No statistically significant survival differences associated to ERα/$ApoD_{CN}$ subgroups were seen in the node negative elderly patients (data not shown).

Table II. Univariate survival analysis of patients ≥70 years of age (n = 76).

	Events/ at risk	% censored	p	HR	95% CI
Relapse Free Survival (RFS)					
pN_0	17/51	66		1	
pN_+	28/40	30	<0.0001	3.2	1.2–5.8
pT_1	14/41	66		1	
pT_2	30/49	39	0.01	2.3	1.2–4.3
WHO grade 1	4/15	73		1	
WHO grade 2	26/53	51	0.16	2.2	0.8–6.2
WHO grade 3	15/23	35	0.01	4.1	1.4–12.4
PR+	29/66	56		1	
PR−	11/17	35	0.09	1.8	0.9–3.7
Overall ERα/ApoD	36/76	53	<0.0001	1.6	1.2–2.2
$ERα+/ApoD_{CN}-$	15/45	67		1	
$ERα+/ApoD_{CN}+$	11/17	35	0.007	3.0	1.3–6.6
$ERα-/ApoD_{CN}+$	10/14	29	<0.0001	5.0	2.1–11.8
Breast Cancer Specific Survival (BCSS)					
pN_0	14/51	73		1	
pN_+	24/40	40	<0.0001	3.3	1.7–6.6
pT_1	11/41	73		1	
pT_2	26/49	47	0.01	2.6	1.3–5.2
WHO grade 1	2/15	87			
WHO grade 2	22/53	59	0.05	4.2	1.0–17.9
WHO grade 3	14/23	39	0.003	9.9	2.2–43.8
PR+	23/66	65			
PR−	10/17	67	0.04	2.2	1.0–4.6
Overall ERα/ApoD	30/76	61	<0.0001	2.2	1.6–3.0
$ERα+/ApoD_{CN}-$	10/45	78		1	
$ERα+/ApoD_{CN}+$	10/17	41	0.007	3.4	1.4–8.2
$ERα-/ApoD_{CN}+$	10/14	29	<0.0001	11.1	4.2–29.0

$ApoD_{CN}$ status – a possible predictor of adjuvant effect of TAM in postmenopausal node positive patients

At the time of diagnosis and primary treatment 60 postmenopausal patients comprised in our total study population (Figure 1), were included in a prospective randomized controlled clinical trial on adjuvant TAM 20 mg daily vs. placebo for 2 years [13]. In our current evaluation, we have used the IHC-ERα information to categorize the ER status (10% cut-off). Patients with $ERα+/ApoD_{CN}+$ tumors who received adjuvant TAM had a BCSS at 15 years similar to patients receiving placebo (Figure 3A). In contrast, the estimated BCSS was improved by 30% in patients with $ERα+/ApoD_{CN}-$ tumors who received adjuvant TAM (p = 0.02) (Figure 3B).

Discussion

Our present study, based on patients with operable breast cancers, shows an inverse relationship between ERα and $ApoD_{CN}$ expression in the postmenopausal and elderly patients (i.e. ≥70 years of age). This observation is partly in line with [12], but is also contradictory to [19] previous studies. Different study populations (i.e. 33% of the patients had T_3 or T_4 tumors) and methodological differences [19] can partly explain the discrepancies.

Table III. Multivariate analysis of patients ≥70 years of age (n = 72).

	Relapse Free Survival			Breast Cancer Specific Survival		
	p	HR	95% CI	p	HR	95% CI
pN_0		1			1	
pN_+	<0.0001	4.1	1.9–8.8	0.003	3.7	1.5–8.7
WHO grade 1		1			1	
WHO grade 2	0.04	3.6	1.1–12.5	0.016	7.1	1.4–35.0
WHO grade 3	0.007	6.8	1.7–27.8	0.001	22.1	3.9–127.1
$ERα+/ApoD_{CN}-$		1			1	
$ERα+/ApoD_{CN}+$	0.01	3.3	1.3–8.3	0.005	4.3	1.6–11.9
$ERα-/ApoD_{CN}+$	0.03	3.3	1.1–9.5	0.007	5.0	1.6–15.8

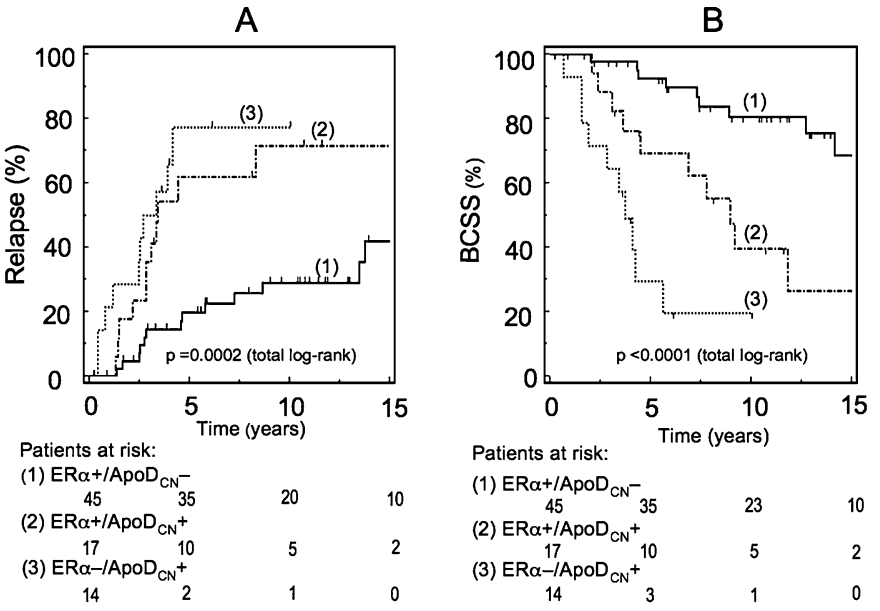

Figure 2. A. Relapse free survival (RFS; all locations) of the elderly patients ≥70 years. Grouping is based on ApoD$_{CN}$ and ERα expression in the cytoplasm. A significant difference in RFS (p = 0.004) was found between group 1 (ERα+/ApoD$_{CN}$ −) and group 2 (ERα+/ApoD$_{CN}$+). Of note is the observation of no significant difference (p = 0.39) in RFS between group 2 (ERα+/ApoD$_{CN}$+) and group 3 (ERα −/ApoD$_{CN}$+).We excluded the ERα −/ApoD$_{CN}$ − tumors from the analysis in this age group, due to a low number of patients (n = 4). B. Breast cancer specific survival (BCSS; dead of breast cancer) in the elderly patients >70 years. Stratification is based on ERα/ApoD$_{CN}$ category of the primary tumor. A statistically significant survival difference (p = 0.004) between group 1 (ERα+/ApoD$_{CN}$ −) and group 2 (ERα+/ApoD$_{CN}$+) was observed, and also between group 2 and group 3 (ERα −/ApoD$_{CN}$+) (p = 0.03). We excluded the ERα −/ApoD$_{CN}$ − tumors from the analysis in this age group, due to a low number of patients (n = 4).

Intra-tumoral heterogeneity of ApoD$_{CN}$ expression [17] may disturb correlation studies when using whole sections [19]. We have used a consistent sampling approach by TMA from the invasive front in the periphery of the tumor. Accordingly, serial TMA sections may resemble the cell line studies [8] more than whole sections do. Since ERα expression in breast cancer tissue increases with age [3], correlations between ERα and ApoD$_{CN}$ may be easier to detect in the elderly.

Detrimental aspects of ApoD$_{CN}$ co-expression in ERα+ tumors in elderly patients (Figure 2A & B)

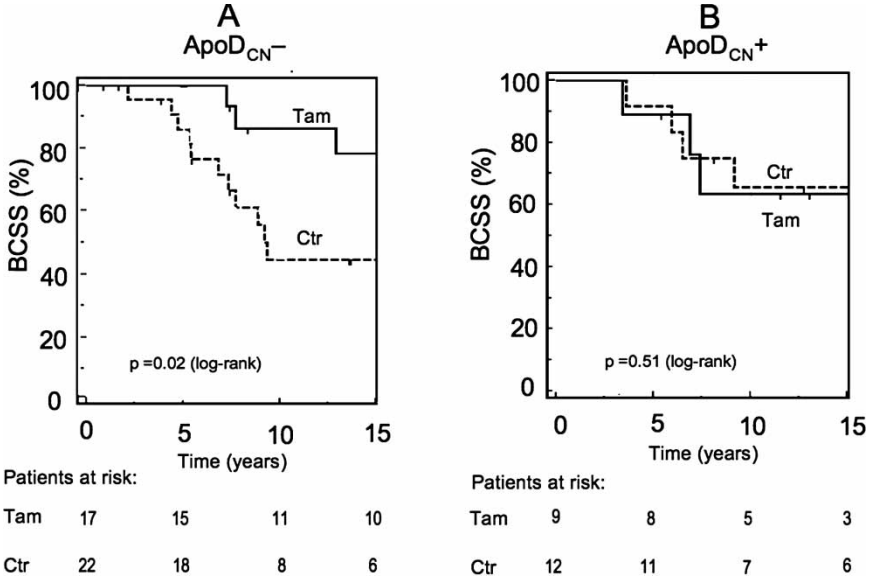

Figure 3. A. Breast cancer specific survival (BCSS) in the ApoD$_{CN}$ − tumors of postmenopausal patients enrolled in a prospective randomized adjuvant study (Adjuvant study NBCG – 1) on 20 mg tamoxifen (TAM) for 2 years vs. placebo (i.e. Control = Ctr) [13]. B. Breast cancer specific survival (BCSS) in the ApoD$_{CN}$+ tumors of postmenopausal patients enrolled in the prospective randomized adjuvant study (Adjuvant study NBCG – 1) on 20 mg tamoxifen (TAM) for 2 years vs. placebo (i.e. Control = Ctr) [13].

are of note. Previously, ApoD expression was regarded as a marker of a functional steroid-signaling pathway [8], indicating a well-differentiated tumor with a favorable prognosis [19]. However, the three strong transcriptional inhibitory ERα responsive elements in the promoter region of the *ApoD* gene [20] support an inverse relationship between ERα and ApoD expression provided by a functional ERα signal pathway. Therefore, a co-expression of $ApoD_{CN}$ and ERα may reflect a dysfunctional ERα signaling. The present observation of a shorter RFS and BCSS in elderly patients, when ERα and $ApoD_{CN}$ are co-expressed, may reflect such a dysfunctional ERα pathway. The molecular basis for this dysfunction could be mutations in the ERα gene (*ERS-1* gene) [21] or changes in various co-factors required for functional ERα signaling. In line with this suggestion, a dysfunctional pathway may lead to a phenotypically "intermediate" ERα positive tumor with a more aggressive disease in comparison with tumors harboring a normal ERα signaling. As reported recently from an animal model, the ERα signaling was damaged in MCF-7 cells that had spread to regional lymph nodes [22]. Hence, node positive breast cancers may be in a different stage of disease where ERα signaling is increasingly damaged. Effects of a dysfunctional ERα signaling may be more visible in elderly patients, rarely given chemotherapy [2,18]. Likewise, cytotoxic adjuvant treatment more commonly offered to younger age groups may also conceal these differences. Whether or not a dysfunctional ERα signaling pathway occurs more frequently in elderly node positive patients need to be evaluated.

Once expressed in the tumor cell, $ApoD_{CN}$ may act independently as a prognostic determinant through its putative protective features of the cancer cell [7], which may include binding and chelating of tamoxifen [10]. As observed previously [12], and in line with our present observations based on an extended follow-up time and improved determination methods, a possible predictive value of $ApoD_{CN}$ with regard to effects of adjuvant TAM treatment is hypothesized. Also, in line with our prognostic considerations of the ERα/$ApoD_{CN}$ co-expression, the predictive information of the $ApoD_{CN}$ may be linked to an altered function of the ERα pathway. This may be part of the complex scenario encountered in endocrine resistant breast cancers, consequently with reduced clinical effects of tamoxifen treatment [23].

In spite of our well-defined study population, comprising women with operable breast cancer, treated according to national guidelines at that time, and the use of modern and reproducible determination methods for ERα and $ApoD_{CN}$, interpretation of our observations should be done with caution. Available patients and number of events for evaluation and statistical calculations are reduced, but the previous [12] and our present study population was comparable with regard to important clinical and tumor characteristics. The concern regarding lower numbers should be partially made up for by the long follow-up time, which significantly exceeds follow-up times commonly encountered in comparable reports [19]. While this is of particular concern regarding our subgroups analysis [13], we think our observations add biological information to be further evaluated in other studies.

If ApoD expression may indicate a less than well recognized de novo mechanism of TAM resistance in breast cancer, remains to be addressed in appropriate studies. The fact that TAM is converted into different identifiable active metabolites [24] makes it necessary to focus on the possible affinity of ApoD to these metabolites in particular, before any conclusions can be made.

Our study indicates that co-expression of $ApoD_{CN}$ in ERα positive tumors may impair the prognosis in elderly women with node positive breast cancer. Improved understanding of the ERα and its signaling pathway in this particular age group is of importance. The role of $ApoD_{CN}$ expression in breast cancer is less than well elucidated and further studies to explore subcellular mechanisms and possible clinical effects are warranted.

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