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Tumor specific HMG-CoA reductase expression in primary pre-menopausal breast cancer predicts response to tamoxifen

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Abstract

Introduction: We previously reported an association between tumor-specific 3-hydroxy-3-methylglutharyl-coenzyme A reductase (HMG-CoAR) expression and a good prognosis in breast cancer. Here, the predictive value of HMG-CoAR expression in relation to tamoxifen response was examined.

Methods: HMG-CoAR protein and RNA expression was analyzed in a cell line model of tamoxifen resistance using western blotting and PCR. HMG-CoAR mRNA expression was examined in 155 tamoxifen-treated breast tumors obtained from a previously published gene expression study (Cohort I). HMG-CoAR protein expression was examined in 422 stage II pre-menopausal breast cancer patients, who had previously participated in a randomized control trial comparing 2 years of tamoxifen to no systemic adjuvant treatment (Cohort II). Kaplan Meier analysis and Cox proportional hazards modelling were used to estimate the risk of recurrence free survival (RFS) and the effect of HMG-CoAR expression on tamoxifen response.

Results: HMG-CoAR protein and RNA expression was decreased in tamoxifen resistant MCF7-LCC9 cells compared to their tamoxifen sensitive parental cell line. HMG-CoAR mRNA expression was decreased in tumours that recurred following tamoxifen treatment ($P<0.001$) and was an independent predictor of RFS in Cohort I (HR 0.63, $P=0.009$). In Cohort II adjuvant tamoxifen increased RFS in HMG-CoAR positive tumours ($P=0.008$).

Multivariate Cox regression analysis demonstrated that HMG-CoAR was an independent predictor of improved RFS in cohort II (HR = 0.67, *P* = 0.010) and subset analysis revealed that this was maintained in estrogen receptor (ER) positive patients (HR = 0.65, *P* =0.029). Multivariate interaction analysis demonstrated a difference in tamoxifen efficacy relative to HMG-CoAR expression (*P*=0.05). Analysis of tamoxifen response revealed that patients with ER positive/HMG-CoAR tumors had a significant response to tamoxifen (*P*=0.010) as well as patients with ER positive or HMG-CoAR positive tumors (*P*=0.035). Stratification according to ER and HMG-CoAR status demonstrated that ER+/HMG-CoAR+ tumors had an improved RFS compared to ER+/HMG-CoAR- in the treatment arm (*P*=0.033) however this effect was lost in the control arm (*P*=0.138) suggesting that HMG-CoAR predicts tamoxifen response.

Conclusions: HMG-CoAR expression is a predictor of response to tamoxifen in both ER positive and negative disease. Premenopausal patients with tumors that express ER or HMG-CoAR respond to adjuvant tamoxifen.

Introduction

3-hydroxy-3methylglutharyl-coenzyme A reductase (HMG-CoAR) acts as a rate-limiting enzyme in the mevalonate pathway. The main product of the mevalonate pathway is cholesterol, however it also produces a number of non-sterol isoprenoid side products, which are important regulators of angiogenesis, proliferation, and migration [1, 2]. HMG-CoAR inhibitors

(statins) have demonstrated anti-neoplastic effects *in vitro* [3-5] and in xenograft models [5]. Statins have been suggested to lower the cancer incidence [6], however to date epidemiological studies have failed to confirm an association between statin use and overall breast cancer risk [7-10]. A lower incidence of estrogen receptor negative tumors has, however, been reported among statin users [11]. Furthermore, an inverse relationship between post-diagnosis statin use and breast cancer recurrence has been reported [12].

We previously demonstrated an association between tumor specific HMG-CoAR expression and improved prognosis in both breast and epithelial ovarian cancer [13-15]. Using immunohistochemistry (IHC) in 511 incident breast cancer cases within the population-based prospective cohort Malmö Diet and Cancer Study [16] we demonstrated that increased levels of HMG-CoAR protein expression were associated with favourable characteristics such as a smaller tumor size, low histological grade and estrogen receptor (ER) positivity [13]. A validation study confirmed these findings and demonstrated that HMG-CoAR was an independent prognostic marker, associated with an improved recurrence free survival (RFS) which was particularly evident in ER positive tumors [14].

Based on these findings we sought to investigate the predictive value HMG-CoAR expression in tamoxifen-treated breast cancer patients. The relationship between HMG-CoAR expression and tamoxifen response was initially examined *in vitro* using a cell line model of tamoxifen resistance [17].

HMG-CoAR mRNA expression was then examined in a gene expression dataset published by Chanrion *et al.* [18] containing 155 primary breast tumors obtained from patients treated with 5 years adjuvant tamoxifen. Finally HMG-CoAR protein expression was examined in pre-menopausal patients with stage II (pT2 N0 M0, pT1-2 N1 M0) invasive breast cancer. These patients had participated in a prospective randomized trial of two years of adjuvant tamoxifen versus no systemic treatment [19].

MATERIALS AND METHODS

Cell lines

MCF-7 cells and their tamoxifen resistant derivative LCC9 were obtained from Prof Robert Clarke (Georgetown University, Washington, DC, USA) and maintained as previously described [17].

Western Blotting

Western blot analysis was performed as previously described [20]. The primary antibody used was a polyclonal anti-HMG-CoAR antibody (Catalog # 07-457, Upstate) diluted1:500 (2ug/ml). An anti-b-actin antibody (Clone 8226, Abcam, Cambridge, UK) at a dilution of 1:5000 was used as a loading control.

Cell pellet arrays

Cell lines were fixed in 4% formalin and processed in gradient alcohols. Cell pellets were cleared in xylene and washed multiple times in molten paraffin. Once processed, cell lines were arrayed in duplicate 1.0 mm cores using a manual tissue arrayer (MTA-1, Beecher Inc, WI, USA).

Quantitative SYBR Green Real-Time PCR

Total RNA was isolated from cell lines using Trizol (Invitrogen) and reverse transcribed using SuperScript II™ Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. HMG-CoAR-F (5'-GGACCCCTTTGCTTAGATGAAA-3') and HMG-CoAR-R (5'-CCACCAAGACCTATTGCTCTG-3') primers were designed using Primer

Express software (Applied Biosystems Version 2.0) and used to amplify a HMG-CoAR-specific DNA fragment with SYBR Green PCR Master Mix (Applied Biosystems) using a 7900HT Fast Real-Time PCR System (Applied Biosystems). Relative HMG-CoAR expression levels in untreated MCF-7 cells versus LCC9 cells were calculated using the qBase real-time PCR relative quantification software [21] with all samples normalized to 18s rRNA. Negative controls included a no template control and a no reverse transcriptase control. All qRT-PCR reactions were performed in triplicate.

MTT Assay

Tamoxifen response in MCF-7 and LCC9 cells was measured using an MTT assay. 10^5 cells were plated in 96 well plates and grown for 48 hours. Fresh medium containing various concentrations of 4-hydroxy-tamoxifen (Sigma) was added to each well and cells were incubated for 5 days. On day 5, 50uL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) in PBS were added to each well. After 4 hours at 37℃, the medium was carefully removed from the wells and the remaining formazan crystals were dissolved in DMSO. The absorbance at 570 nm was read on a microplate reader.

Patients.

Cohort I consisted of a gene expression dataset published by Chanrion *et al.* [18] containing 155 primary breast tumors obtained from patients who had undergone initial surgery between 1989 and 2001 at the Cancer Research Center of Val d'Aurelle in Montpellier, the Bergonie´ Institute in Bordeaux, or

the Department of Obstetrics and Gynecology of Turin. The median follow-up time for all patients was 5.5 years. The aim of the study was to identify a gene expression signature associated with tamoxifen resistance. Eight tumors were ER negative, and six of these tumors were progesterone receptor (PR) positive. No patient received neoadjuvant or adjuvant systemic chemotherapy as a first line therapy. All patients were treated with adjuvant tamoxifen (20 mg daily) for 5 years. One hundred and twenty-one patients also received adjuvant radiotherapy. Recurrence was observed in 52 patients (48 distant metastases and 4 local recurrences) with median relapse time of 37.1 months. Raw gene expression data and clinical data were downloaded from Gene Expression Omnibus (accession number [GEO:GSE9893]) [22]. The log ratio of gene expression values was used without further transformation. For statistical analysis HMG-CoAR expression levels were analysed as a continuous variable.

Cohort II consisted 564 pre-menopausal women with primary breast cancer in the South and Southeast regions of Sweden were enrolled in a multi-centre clinical trial and randomised to either two years of adjuvant tamoxifen (*n=276*) or a control group (*n=288*) irrespective of hormonal receptor status [19]. The aim of this study was to examine the effect of tamoxifen on recurrence-free survival (RFS) and the study has been described in detail elsewhere [19]. RFS considered local, regional, distant recurrences and breast cancer-specific death, but not contralateral breast cancer. The inclusion criteria were pre-menopausal patients, or patients younger than 50 years, with stage II (pT2 N0 M0, pT1-2 N1 M0) invasive breast cancer treated by modified radical

mastectomy or breast conserving surgery with axillary lymph node dissection. Post-operative radiotherapy (50 Gy) was administered after breast – conserving surgery and all lymph node-positive patients received locoregional radiotherapy. Less than 2% of the patients received adjuvant systemic chemotherapy. The median follow-up time for patients without breast cancer events was 13.9 years. The ethics committees at Lund and Linköping Universities approved the study. Oral informed consent was registered for all patients. The results of the trial have been previously described [19] and the trial has been included in the Oxford meta-analysis [23].

Tissue microarray construction

Five hundred paraffin-embedded tumor specimens were used for tissue microarray (TMA) construction. TMAs were constructed as described previously [24]. In brief, two 0.6 mm cores were taken from areas representative of invasive cancer and mounted in a recipient block using a manual arraying device (MTA-1, Beecher Inc, WI, USA). The study was approved by the ethics committees at Linköping and Lund Universities.

Immunohistochemistry

Four μm sections from the TMAs and 3.5 μm sections from the cell pellet arrays were automatically pretreated using the PT-link system (DAKO, Copenhagen, Denmark) and then stained in a Techmate 500 (DAKO, Copenhagen, Denmark) with a polyclonal anti-HMG-CoAR antibody (Catalog # 07-457, Upstate) diluted 1:250.

For all other antibodies, heat-mediated antigen retrieval was performed using microwave treatment for 2 x 5 min in a citrate buffer before being processed either in the Ventana Benchmark system (Ventana Medical Systems Inc, AZ) using pre-diluted antibodies to ER (Anti-ER, clone 6F11), PR (Anti-PgR, clone 16) and Her2 (Pathway CB-USA, 760-2694) or in the Techmate 500 (Dako) for Ki-67 (1:200, M7240; Dako).

Cytoplasmic staining of HMG-CoAR was assessed by two investigators, one of whom is a board certified pathologist (KJ) according to intensity (negative (0), weak (1), moderate (2), strong (3). HMG-CoAR was not expressed in the nucleus. Discordant cores were reassessed jointly and a consensus reached. HER2 and Ki-67 were assessed as previously described [20]. ER and PR negativity was defined as < 10% positively staining nuclei, according to current clinical guidelines in Sweden.

Statistical analysis

Differences in distribution of clinical data and tumor characteristics between HMG-CoAR-negative and -positive tumors were evaluated using the χ^2 test. The Kaplan-Meier analysis and the log rank test were used to illustrate differences between recurrence free survival (RFS) according to HMG-CoAR expression. Cox regression proportional hazards models were used to estimate the impact of HMG-CoAR expression on RFS and overall survival (OS) in both uni- and multivariate analysis, adjusted for tumor size, age at diagnosis, ER, HER2, lymph node status and Nottingham Histological Grade (NHG) in the entire cohort. Cox proportional hazards models were used to

estimate relative hazards adjusted or not for potential prognostic factors. The model was used in cohort II to estimate the interaction effect between tamoxifen treatment and HMG-CoAR expression in order to measure any possible difference in treatment effect based on HMG-CoAR expression. For this purpose an interaction variable (TAM treatment (+/-) X HMG-COAR (+/-)) was constructed. All calculations were performed using SPSS version 15.0 (SPSS Inc, Chicago, IL). All statistical tests were two-sided and a p value < 0.05 was considered statistically significant.

Results

HMG-CoAR is Associated with Tamoxifen Response *in vitro*.

The specificity of the anti HMG-CoAR antibody was confirmed in a previous study [13]. The anti-HMG-CoAR antibody recognised a single distinct band at ~ 90kDa in MCF7 cells. HMG-CoAR protein expression was significantly decreased in the tamoxifen resistant derivative LCC9 cell line (Figure 1A). HMG-CoAR mRNA expression was also decreased in the LCC9 cells compared their MCF7 derivatives (Figure 1B). IHC performed on the same cell lines confirmed these results (Figure 1C). Finally treatment of the cell lines with tamoxifen demonstrated antiestrogen resistance in the LCC9 cells compared to their MCF7 derivatives (Figure 1D).

HMG-CoAR mRNA Expression is Associated with a Prolonged Recurrence Free Survival in Tamoxifen Treated Breast Cancer Patients

The relationship between HMG-CoAR mRNA expression and tamoxifen response was examined in cohort I. The Chanrion *et al.* dataset [18] consists of patients treated with five years adjuvant tamoxifen and contains both pre and post-menopausal patients. HMG-CoAR mRNA levels were higher in patients who remained disease free following tamoxifen treatment compared to those who developed recurrences (Figure 2A). Using a threshold of mean expression, Kaplan Meier analysis demonstrated that increased HMG-CoAR mRNA expression was associated with a prolonged RFS ($p = 0.018$) (Figure 2B). Examination of the relationship between HMG-CoAR mRNA expression and other clinicopathological parameters revealed that increased HMG-CoAR mRNA expression was associated with small ($p = 0.003$), lymph node negative ($p = 0.003$) tumors (Table 1) Univariate analysis of HMG-CoAR as a continuous variable revealed that increased levels of HMG-CoAR mRNA were associated with a prolonged RFS and OS in cohort I (Table 2). Multivariate cox regression analysis controlling for grade, age, nodal status and tumor size confirmed that HMG-CoAR was an independent predictor of RFS (HR 0.63, 95% CI 0.45-0.89, p 0.009) and OS (HR 0.49, 95% CI 0.32-0.75, p 0.001) (Table 2) suggesting that HMG-CoAR may play an important role in tamoxifen treated breast cancer patients.

HMG-CoAR Protein Expression Predicts Tamoxifen Response in Premenopausal Breast Cancer.

Having demonstrated a relationship between HMG-CoAR mRNA expression in tamoxifen-treated patients, we proceeded to examine HMG-CoAR protein expression in cohort II. Tumor samples were available from 500 of 564 patients (89%) included in the randomized study. Following antibody optimisation and staining, it was possible to evaluate the expression of HMG-CoAR protein in 422 (84.4%) of the 500 tumors represented on the TMA.

Only staining intensity was accounted for in the statistical analyses of HMG-CoAR protein expression, as when present, HMG-CoAR was generally expressed in the majority of tumor cells (> 50%), a finding consistent with previous studies [13, 14]. Two hundred and twenty three (52.7%) tumors lacked HMG-CoAR expression, 163 (38.5%) demonstrated weak, 37 (8.7%), moderate and none demonstrated a strong signal. Examples of HMG-CoAR expression are illustrated in Figure 3A.

In order to validate our previous findings [13, 14], and also to identify any differences in HMG-CoAR expression in an exclusively pre-menopausal cohort, the relationship between HMG-CoAR protein expression and established clinicopathological variables was evaluated. As demonstrated in Table 1 HMG-CoAR expression, dichotomized to absent versus any staining, was associated with low histological grade ($p = 0.009$), ER positivity ($p = 0.026$), and Her2 over-expression ($p = 0.008$). HMG-CoAR protein expression was associated with lymph node positivity in cohort II ($p = 0.009$).

Assessment of the evaluable tumours in cohort II (n = 422) revealed that HMG-CoAR protein expression was associated with a stepwise increased RFS (p = 0.028) when analysed in three groups (negative, 1+ and 2+) (Figure 3B). Dichotomization of HMG-CoAR protein expression data to absent versus any expression demonstrated an association with an improved RFS (p = 0.008) (Figure. 3C). HMG-CoAR was also associated with an improved breast cancer specific survival and overall survival (data not shown), however, as the aim of the original trial was to examine the effect of tamoxifen on RFS, this was used as the endpoint in this study. As illustrated in Table 3, multivariate Cox regression analysis revealed that HMG-CoAR expression was an independent prognostic factor in the evaluated cohort (HR – 0.67, 95% CI 0.49 – 0.91, p = 0.010).

As the aim of this study was to evaluate the potential role of HMG-CoAR expression in predicting response to tamoxifen, subset analysis of RFS was performed comparing the effect of tamoxifen versus no treatment based on HMG-CoAR expression. Figures 4A and B demonstrate that HMG-CoAR expression was associated with an improved response to tamoxifen in the entire cohort irrespective of ER status. Cox interaction analysis confirmed that HMG-CoAR expression was associated with an improved response to tamoxifen (HR 0.5, p=0.003), in the entire cohort irrespective of ER status.

Stratification according to ER and treatment status demonstrated that HMG-CoAR expression was associated with an improved RFS in all ER positive patients, irrespective of treatment status (Figure 4C) and this was confirmed

by mutltivariate Cox regression analysis whereby HMG-CoAR expression was an independent prognostic factor in ER positive patients (HR – 0.65, 95% CI 0.45 – 0.96, p = 0.029) (Table 3). This effect was maintained when tamoxifen treated ER positive patients were examined separately (Figure 4D), however HMG-CoAR expression was not associated with a prolonged RFS in untreated ER positive patients (Figure 4E), suggesting that HMG-CoAR status may predict tamoxifen response.

Based on these findings the relationship between ER and HMG-CoAR expression and tamoxifen response was examined. Three subsets were constructed: ER and HMG-CoAR negative (n = 80), ER and HMG-CoAR positive (n = 141), and ER or HMG-CoAR positive (n = 236). Analysis of these groups revealed that patients with ER and HMG-CoAR positive tumors had a significant response to tamoxifen (p = 0.010) (Figure 5A) as well as patients with ER positive or HMG-CoAR positive tumors (p = 0.035) (Figure 5B). Double negative tumors did not respond to tamoxifen. (Figure 5C). Stratification according to ER and HMG-CoAR status demonstrated that ER+/HMG-CoAR+ tumors had an improved RFS compared to ER+/HMG-CoAR- in the treatment arm (p=0.033) however this effect was lost in the control arm (p=0.138) suggesting that HMG-CoAR predicts tamoxifen response (Figures 5 D and E)

Discussion

The aim of this study was to evaluate HMG-CoAR expression as a predictive marker of tamoxifen response. Our results demonstrate a potentially important

association with tamoxifen response both *in vitro* and using two independent breast cancer cohorts, one of which encompasses a randomized control trial of premenopausal stage II disease.

A wealth of evidence supports the role of tamoxifen in the treatment of breast cancer [25], however, resistance to tamoxifen is a significant clinical problem and the Oxford meta analysis reported a relapse rate of 15%, and an 8% incidence of breast cancer specific mortality within 5 years of the commencement of therapy [25]. The introduction of aromatase inhibitors (AI) may lead to an improvement in post-menopausal women [26, 27], however resistance to AI's may become a problem over time. In pre-menopausal women tamoxifen remains the endocrine drug of choice and and both intherent and acquired resistance may be more prevalent in this population. Tamoxifen resistance in pre-menopausal women is quite hard to quantify accurately as the majority of pre-menopausal patients receive adjuvant cytotoxic chemotherapy, however Ryden *et al* [19] reported a recurrence rate of 39.7% at ten years in the absence of adjuvant chemotherapy in the trial from which cohort II was derived. An ability to identify patients who will respond to endocrine therapy prior to commencing treatment would be extremely beneficial.

To that end, the data presented here are particularly interesting. Cox regression analysis confirmed HMG-CoAR was an independent prognostic marker in both cohorts. Cohort I was a non-randomized cohort consisting of a mixture of pre and post-menopausal patients and therefore cannot be used to

validate a predictive biomarker, however it provides important information particularly as HMG-CoAR mRNA expression was measured as a continuous variable. The relationship between HMG-CoAR protein expression and tamoxifen response was examined in cohort II, a randomized exclusively premenopausal cohort, and a Cox interaction analysis revealed an interaction between tamoxifen treatment and HMG-CoAR expression. Subset analysis of cohort II revealed that tumors that expressed both ER and HMG-CoaAR were particularly sensitive to tamoxifen, however, tumors that expressed either ER or HMG-CoAR also responded to tamoxifen. In addition HMG-CoAR +/ ER + patients had a significantly improved response to tamoxifen compared to HMG-CoAR - / ER + patients.

In contrast to previous studies by our group none of the tumors expressed high levels (3+) of HMG-CoAR. This could potentially be explained by the fact that cohort II was an exclusively premenopausal cohort with stage II breast cancer, which also explains the positive association with lymph node status seen in this study. In our two previous breast cancer studies we demonstrated an inverse relationship between tumor size and HMG-CoAR [13] [14], and therefore the absence of small stage I tumors from cohort II could explain the lack of tumors expressing high levels of HMG-CoAR in this study. This argument is further strengthened by the inverse relationship between HMG-CoAR mRNA expression and tumor size demonstrated in cohort I in this study, as this cohort also included a significant number of small stage I tumors. We are unable to explain the positive association between Her2 and

HMG-CoAR seen in cohort II, however, this could potentially be explained by the association with lymph node positivity.

Such findings raise the possibility of pharmacological interventions to increase tumor-specific HMG-CoAR expression as a potential therapeutic option for breast cancer. Statin induced mevalonate depletion has been shown to result in an adaptive induction of HMG-CoAR expression in chinese hamster ovary cells [28] and MCF7 breast cancer cells [29]. Treatment of MCF7 cells with mevastatin resulted in a 10- to 15-fold induction of HMG-CoAR activity in association with a 2.5- to 3.5-fold induction of HMG-CoA reductase mRNA expression [29], suggesting that treatment with statins may increase tumor specific HMG-CoAR expression in vivo, however this remains to be fully elucidated. Given our findings that increased levels of HMG-CoAR expression are associated with an improved response to tamoxifen in ER positive tumors, a combination of tamoxifen and statins may be a new therapeutic option. It should be noted however that further studies are required to investigate the value of HMG-CoAR expression as a predictive marker of response to statin treatment.

Despite an ever-growing body of literature describing the anti-neoplastic properties of statins, epidemiologic data regarding their preventive effect against cancer in general and breast cancer in particular remain inconclusive [7, 9, 30-32]. In the adjuvant setting, a recent pre-operative window trial of ductal carcinoma in situ and stage one breast cancer was the first to demonstrate that statins can inhibit proliferation and increase apoptosis *in*

vivo [33], raising the possibility that the combination of statins and well-established chemotherapeutic and endocrine agents may be an option. A synergism between statins and trastuzumab, rapamycin and epirubicin has been demonstrated in breast cancer cell lines [34], however a synergistic relationship between tamoxifen and statins has yet to be investigated.

Conclusions

These data describe HMG-CoAR as a significant predictor of tamoxifen response in pre-menopausal breast cancer patients with both ER positive and ER negative tumors. Using a cohort of patients who had participated in a randomized control trial with long-term follow-up, we have demonstrated that tumor specific HMG-CoAR expression predicts response to tamoxifen. Tumors that express both ER and HMG-CoAR had an excellent response to tamoxifen, however tumors that express ER or HMG-CoAR also respond to tamoxifen. These findings suggest that the combination of tamoxifen and statins may be a viable and well-tolerated therapeutic option for a subset of breast cancer patients, which warrants further investigation.

Abbreviations

HMG-CoAR - 3-hydroxy-3methylglutharyl-coenzyme A reductase; IHC – immunohistochemistry; ER – estrogen receptor; RFS – recurrence free survival; PCR – polymerase chain reaction; TMA – tissue microarray; PR – progesterone receptor.

Competing Interests

DJB, KJ, MU and FP hold pending intellectual property in relation to HMG-CoAR as a prognostic biomarker in epithelial ovarian cancer and a predictive biomarker in tamoxifen treated breast cancer.

Authors' contributions

DJB conceived the study, performed statistical analysis and drafted the manuscript. HL performed western blot analysis and MTT assay, DPOC performed western blot analysis and MTT assay, SB conceived the study and drafted the manuscript, MU conceived the study and drafted the manuscript, WMG conceived the study and drafted the manuscript, FP conceived the study and drafted the manuscript, RCM provided statistical analysis, LR performed statistical analysis and drafted the manuscript, KJ conceived the study, constructed the tissue microrarrays, performed and analysed IHC and drafted the manuscript.

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Table 1. Clinical and Tumor Characteristics stratified according to HMG-CoAR protein expression in two independent cohorts

	Cohort I			Cohort II		
	HMG-CoAR Low (%) (n = 62)	HMG-CoAR High (%) (n = 93)	P value (x^2 test)	HMG-CoAR Negative (%) (n = 222)	HMG-CoAR Positive (%) (n = 200)	P value (x^2 test)
Age (Years)						
< median	34 (55)	42 (45)	0.238	36 (16)	36 (18)	0.612
> median	28 (45)	51 (55)		186 (84)	164 (82)	
Tumour Size (mm)						
0 - 20mm	21 (35)	57 (63)	0.003	75 (34)	79 (40)	0.196
> 21mm	39 (65)	33 (37)		147 (66)	120 (60)	
Missing	5					
Histological Subtype						
Ductal	49 (79)	75 (81)	0.250	181 (91)	171 (93)	0.581
Others	13 (21)	18 (19)		17 (9)	13 (7)	
Unknown				40		
Nodal status						
N0	21 (35)	57 (63)	0.003	70 (32)	42 (21)	0.009
N1+	39 (65)	33 (37)		152 (68)	158 (79)	
Unknown	5					
NHG						
I	9 (15)	12 (13)	0.535	15 (7)	28 (15)	0.009
II	38 (60)	56 (60)		85 (40)	86 (45)	
III	10 (14)	23 (27)		115 (53)	79 (40)	
Unknown	7			14		
ER status						
ER –ve				80 (38)	54 (28)	0.026
ER +ve				130 (62)	141 (72)	
Unknown				17		
PR Status						
PR –ve				78 (38)	56 (30)	0.098
PR +ve				130 (62)	133 (70)	
Unknown				25		
Ki-67						
< 10%				138 (73)	128 (71)	0.658
> 10%				52 (27)	53 (29)	
Unknown				51		
Her2 IHC						
0-2+				173 (90)	137 (80)	0.008
3+				20 (10)	35 (20)	
Unknown				57		

Abbreviations: N0= node negative, N1+ = node positive, NHG = Nottingham Histological Grade, ER = oestrogen receptor, PR= progesterone receptor, Her2 IHC = Her 2 immunohistochemistry. Values in parenthesis are percentages unless otherwise stated.

Table 2. Cox uni- and multivariate analysis of recurrence free and overall survival according to HMG-CoAR mRNA expression in cohort I

	Recurrence Free Survival						Overall Survival					
	Univariate			*Multivariate**			*Univariate*			*Multivariate**		
	HR	95% CI	P Value	HR	95% CI	P Value	HR	95% CI	P Value	HR	95% CI	P Value
HMG-CoAR mRNA (*continuous)*	0.64	0.48-0.85	0.002	0.63	0.45-0.89	0.009	0.55	0.41-0.75	<0.001	0.49	0.32-0.75	0.001
Size (*continuous)*	1.02	0.99-1.05	0.175	1.02	0.97-1.07	0.439	0.99	0.96-1.04	0.89	0.98	0.93-1.04	0.581
Grade (*I & II v's III*)	2.62	1.62-4.23	<0.001	3.13	1.80-5.44	<0.001	2.95	1.70-5.14	<0.001	4.32	2.22-8.41	<0.001
Nodal Status (*neg v's pos)*	2.80	1.44-5.42	0.002	1.13	1.10-1.21	<0.001	5.49	2.29-13.17	<0.001	1.12	1.04-1.20	0.004
Age (*continuous)*	1.02	0.99-1.04	0.23	0.99	0.96-1.03	0.700	1.01	0.98-1.04	0.599	0.99	0.95-1.03	0.566

*Adjusted for all other variables in the table.
Abbreviations: HR = Hazard ratio, 95% CI = 95% Confidence Intervals, HMG-CoAR = 3-hydroxy-3methylglutharyl-coenzyme A reductase.

Table 3. Cox uni- and multivariate analysis of recurrence free survival according to HMG-CoAR protein expression in cohort II

	All Tumors (n = 422)						ER Positive (n=270)					
	Univariate			Multivariate*			Univariate			Multivariate*		
	HR	95% CI	P Value	HR	95% CI	P Value	HR	95% CI	P Value	HR	95% CI	P Value
HMG-CoAR (*0 v's 1-2+*)	0.68	0.51-0.90	0.009	0.67	0.49-0.91	0.01	0.66	0.46-0.95	0.024	0.65	0.45-0.96	0.029
Grade (*I & II v's III*)	1.88	1.46-2.42	<0.001	1.58	1.11-2.25	0.012	1.85	1.32-2.60	<0.001	1.56	1.04-2.34	0.03
Age (*continuous*)	0.95	0.95-0.99	0.002	0.97	0.95-1.01	0.071	0.95	0.93-0.98	0.001	0.95	0.92-0.99	0.015
Nodal Status (*negative v's positive*)	1.7	1.26-2.29	0.001	2.12	1.47-3.28	<0.001	1.42	0.94-2.15	0.098	1.56	0.95-2.57	0.079
Tumor Size (*continuous*)	1.16	0.90-1.50	0.254	1.27	0.91-1.77	0.159	1.17	0.84-1.64	0.361	1.22	0.81-1.84	0.352
ER Status (0-*10% v's 11-100%*)	0.71	0.54-0.94	0.018	0.65	0.37-1.13	0.126						
PR Status (0-*10% v's 11-100%*)	0.73	0.54-0.97	0.031	1.37	0.77-2.43	0.285	0.94	0.832-1.19	0.95	2.28	0.72-7.27	0.163
Treatment (Tamoxifen *v's Control*)	0.78	0.60-0.98	0.033	0.68	0.50-0.92	0.011	0.61	0.44-0.86	0.005	0.58	0.39-0.84	0.005

*Adjusted for all other variables in the table.
Abbreviations: HR = Hazard ratio, 95% CI = 95% Confidence Intervals, HMG-CoAR = 3-hydroxy-3methylglutharyl-coenzyme A reductase, ER = Estrogen Receptor, PR = Progesterone Receptor.

Figure legends

Figure 1. HMG-CoAR Expression is Associated with Tamoxifen Response *in vitro.* (A) Western blot demonstrating increased expression of HMG-CoAR in tamoxifen sensitive MCF7 cells compared to their tamoxifen resistant derivatives LCC9. (B) qRT-PCR demonstrating increased expression of HMG-CoAR mRNA in MCF7 compared to LCC9 cells. (C) Immunohistochemistry demonstrating increased HMG-CoAR protein expression in MCF7 compared to LCC9 cells. (D) MTT assay demonstrating improved tamoxifen response in MCF7 compared to LCC9 cells.

Figure 2. HMG-CoAR mRNA Expression is Associated with Increased RFS in Tamoxifen Treated Breast Cancer Patients. (A) Mean HMG-CoAR mRNA levels were significantly higher in patients who remained disease free after tamoxifen treatment. * $p < 0.001$. (B) Kaplan Meier Using estimates of recurrence-free survival according to HMG-CoAR mRNA expression using mean HMG-CoAR expression as a threshold

Figure 3. HMG-CoAR Protein Expression in Cohort II. (A) Immunohistochemical analysis of HMG-CoAR demonstrating different levels of staining intensity. (B) Kaplan-Meier estimates of recurrence free survival according to HMG-CoAR expression in three groups. (C) Kaplan-Meier estimates of recurrence free survival according to HMG-CoAR expression defined as negative = 0 or positive = 1-3, in all patients (n = 422).

Figure 4. HMG-CoAR Protein Expression is Associated with Tamoxifen Response in Cohort II. (A) Kaplan Meier estimate of recurrence free survival comparing two years tamoxifen treatment to no adjuvant treatment in HMG-CoAR negative tumors. (B) Kaplan Meier estimate of recurrence free survival comparing two years tamoxifen treatment to no adjuvant treatment in HMG-CoAR positive tumors. (C) Kaplan Meier estimate of recurrence free survival in all ER positive tumors. (D) Kaplan Meier estimate of recurrence free survival in tamoxifen treated ER positive tumors. (E) Kaplan Meier estimate of recurrence free survival in untreated ER positive tumors.

Figure 5. Relationship between ER, HMG-CoAR and Tamoxifen Response in Premenopausal Breast Cancer. (A) Kaplan-Meier estimate of recurrence free survival based on tamoxifen treatment in ER and HMG-CoAR positive tumors (n = 141). (B) Kaplan-Meier estimate of recurrence free survival based on tamoxifen treatment in ER or HMG-CoAR positive tumors (n = 236). (C) Kaplan-Meier estimate of recurrence free survival based on tamoxifen treatment in ER and HMG-CoAR negative tumors (n = 80). (D) Kaplan-Meier estimate of recurrence free survival in tamoxifen treated ER positive patients based on HMG-CoAR expression. (E) Kaplan-Meier estimate of recurrence free survival in untreated ER positive patients based on HMG-CoAR expression.

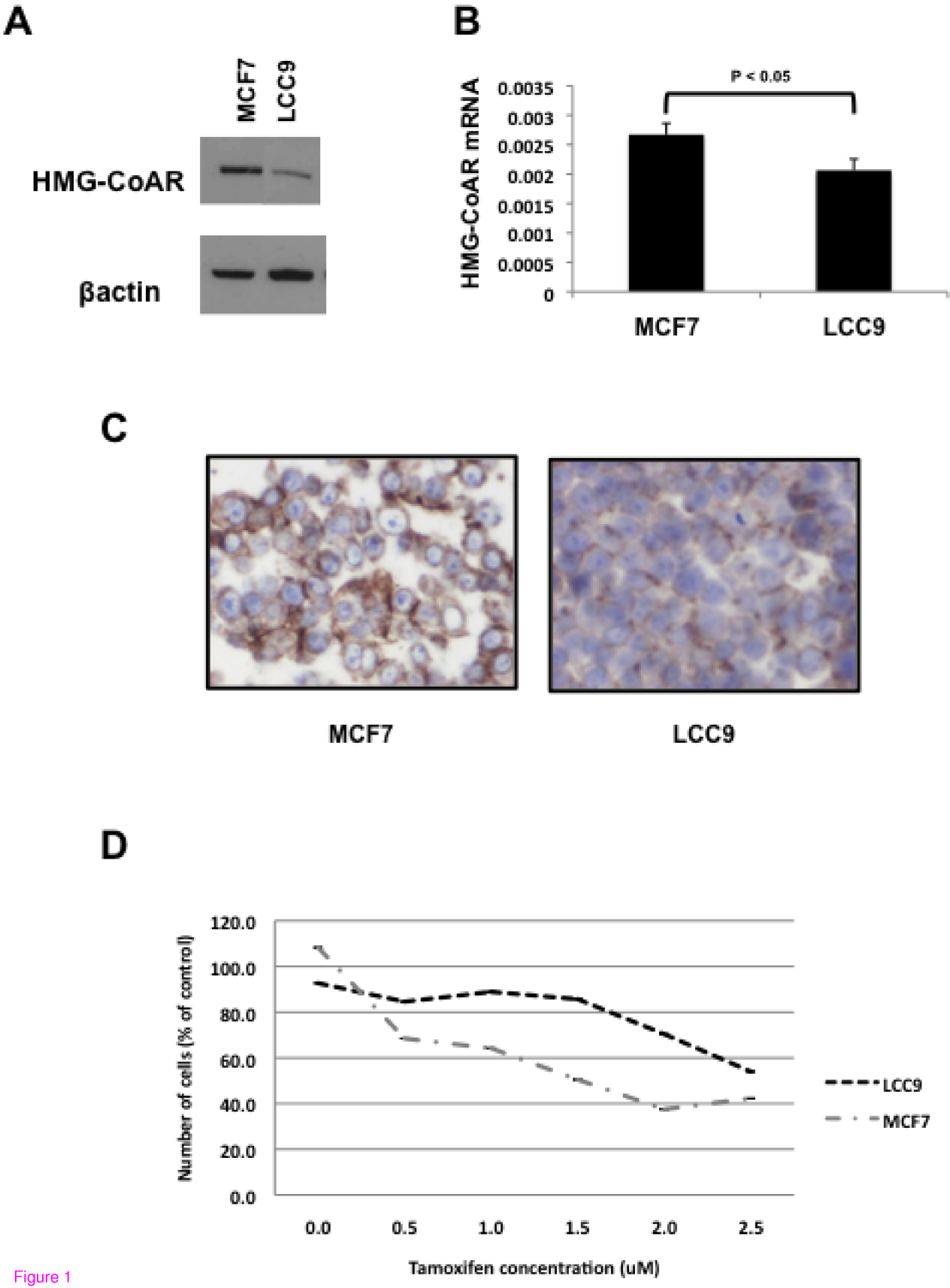
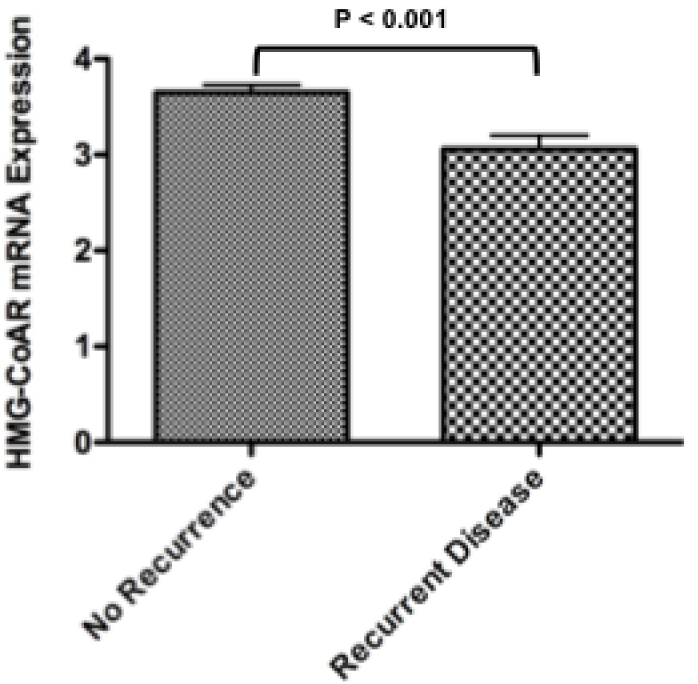

A
MCF7
LCC9
HMG-CoAR
βactin
B
HMG-CoAR mRNA
P < 0.05
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
MCF7
LCC9
C
MCF7
LCC9
D
Number of cells (% of control)
120.0
100.0
80.0
60.0
40.0
20.0
0.0
0.0
0.5
1.0
1.5
2.0
2.5
Tamoxifen concentration (uM)
LCC9
MCF7

Figure 1


P < 0.001
HMG-CoAR mRNA Expression
4
3
2
1
0
No Recurrence
Recurrent Disease

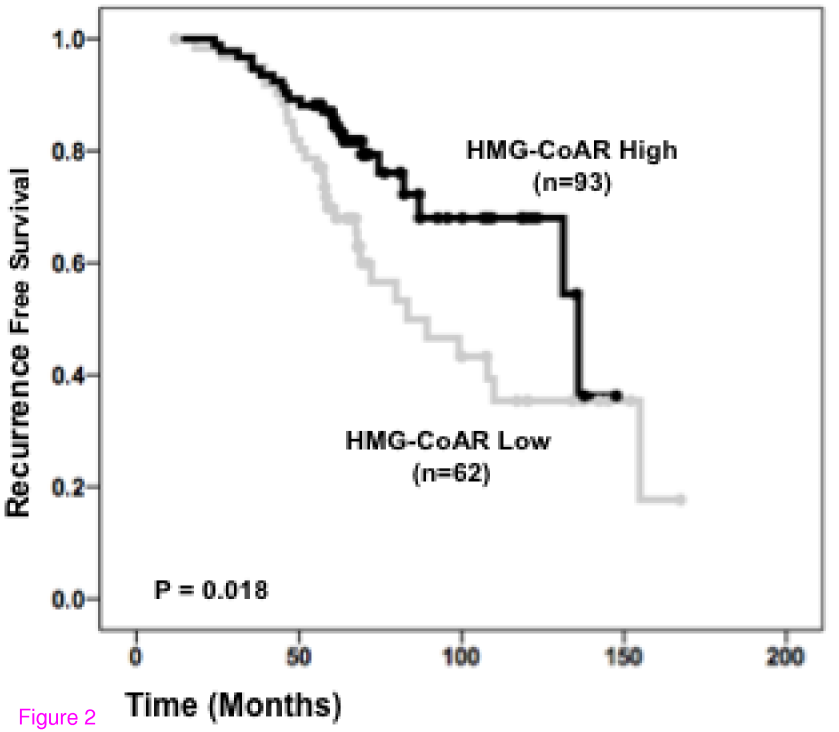
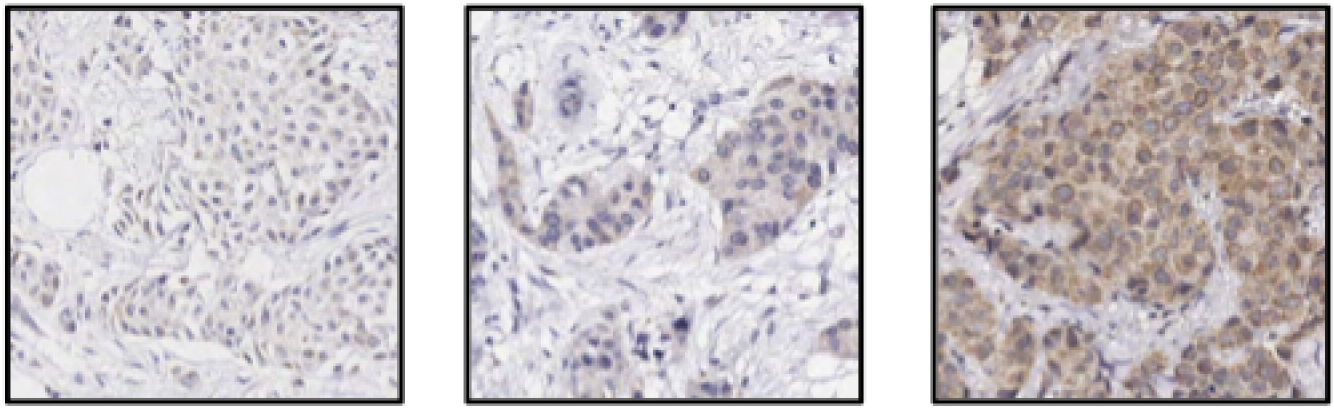
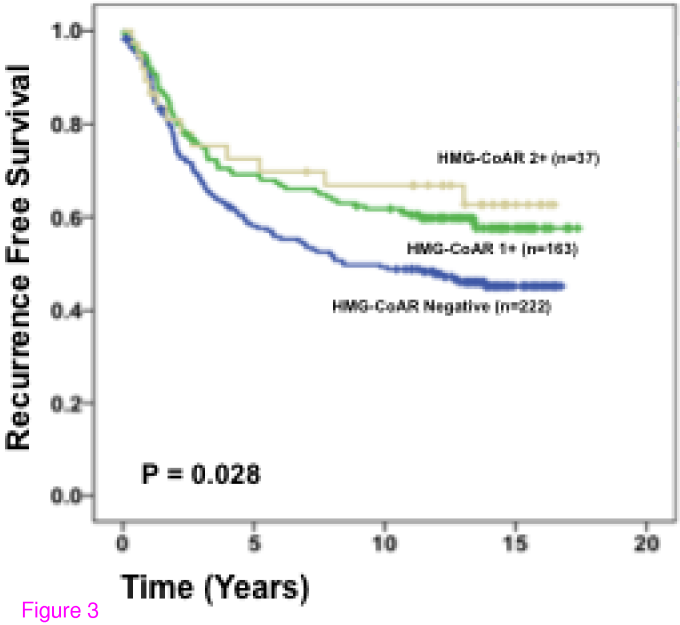

1.0
0.8
0.6
0.4
0.2
0.0
Recurrence Free Survival
HMG-CoAR High
(n=93)
HMG-CoAR Low
(n=62)
P = 0.018
0
50
100
150
200
Time (Months)

Figure 2

A



B


Recurrence Free Survival
1.0
0.8
0.6
0.4
0.2
0.0
HMG-CoAR 2+ (n=37)
HMG-CoAR 1+ (n=163)
HMG-CoAR Negative (n=222)
P = 0.028
0
5
10
15
20
Time (Years)

C

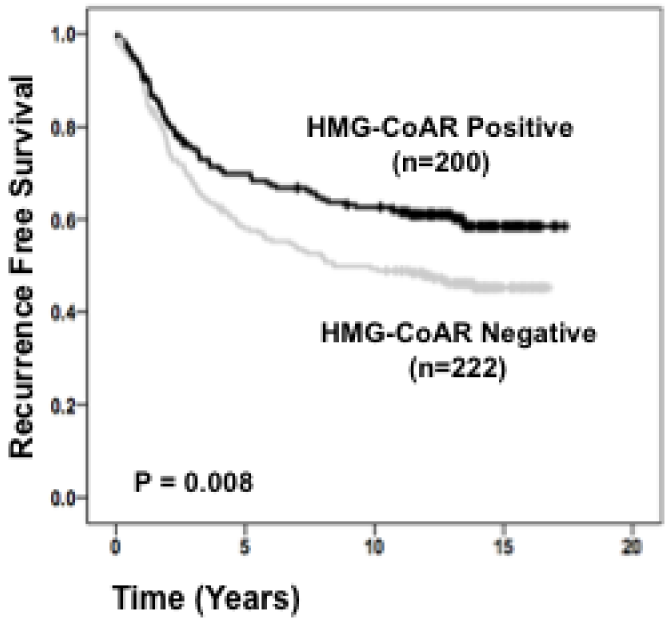

Recurrence Free Survival
1.0
0.8
0.6
0.4
0.2
0.0
HMG-CoAR Positive
(n=200)
HMG-CoAR Negative
(n=222)
P = 0.008
0
5
10
15
20
Time (Years)

Figure 3

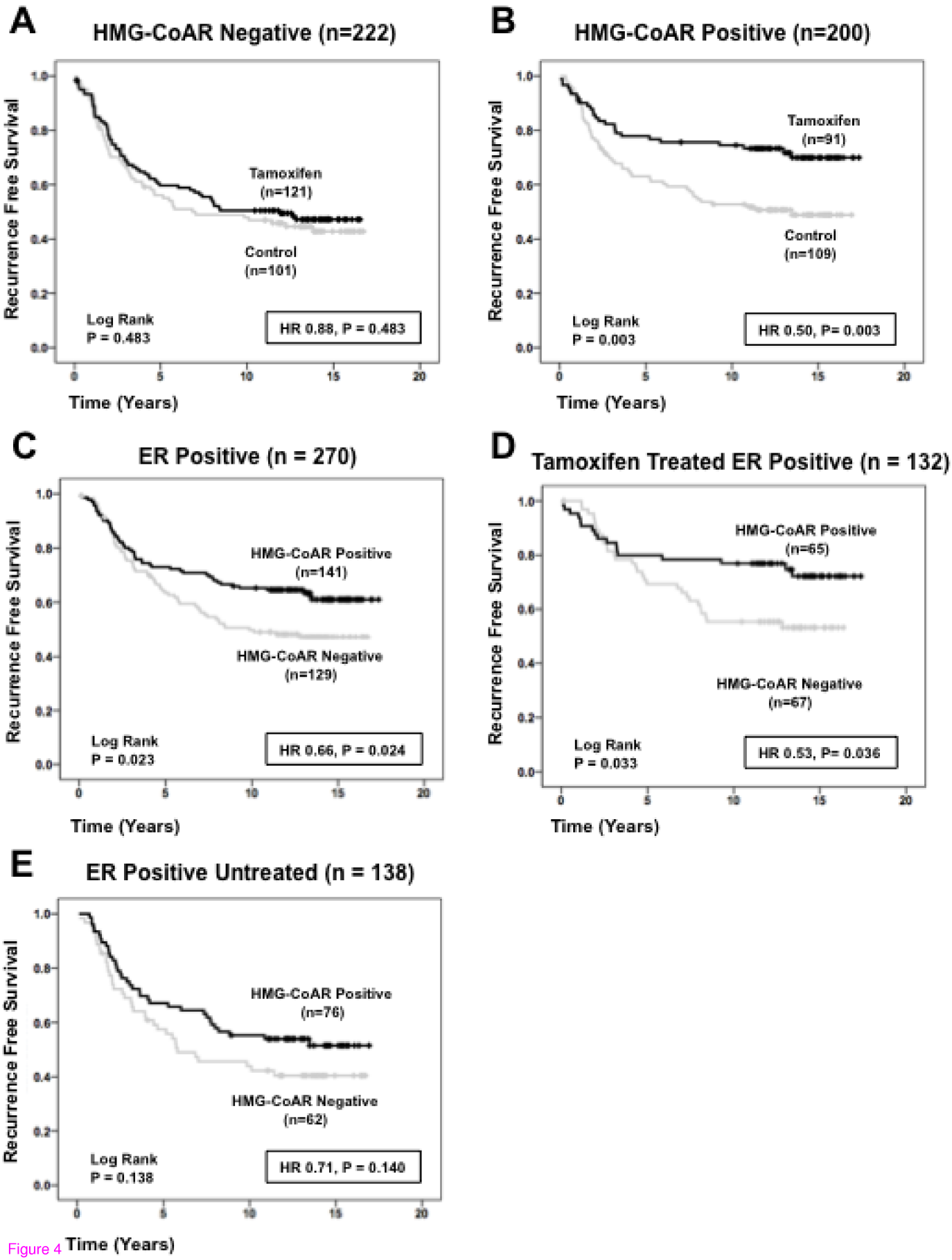

A
HMG-CoAR Negative (n=222)
Recurrence Free Survival
Tamoxifen
(n=121)
Control
(n=101)
Log Rank
P = 0.483
HR 0.88, P = 0.483
Time (Years)
B
HMG-CoAR Positive (n=200)
Recurrence Free Survival
Tamoxifen
(n=91)
Control
(n=109)
Log Rank
P = 0.003
HR 0.50, P= 0.003
Time (Years)
C
ER Positive (n = 270)
Recurrence Free Survival
HMG-CoAR Positive
(n=141)
HMG-CoAR Negative
(n=129)
Log Rank
P = 0.023
HR 0.66, P = 0.024
Time (Years)
D
Tamoxifen Treated ER Positive (n = 132)
Recurrence Free Survival
HMG-CoAR Positive
(n=65)
HMG-CoAR Negative
(n=67)
Log Rank
P = 0.033
HR 0.53, P= 0.036
Time (Years)
E
ER Positive Untreated (n = 138)
Recurrence Free Survival
HMG-CoAR Positive
(n=76)
HMG-CoAR Negative
(n=62)
Log Rank
P = 0.138
HR 0.71, P = 0.140
Time (Years)

Figure 4

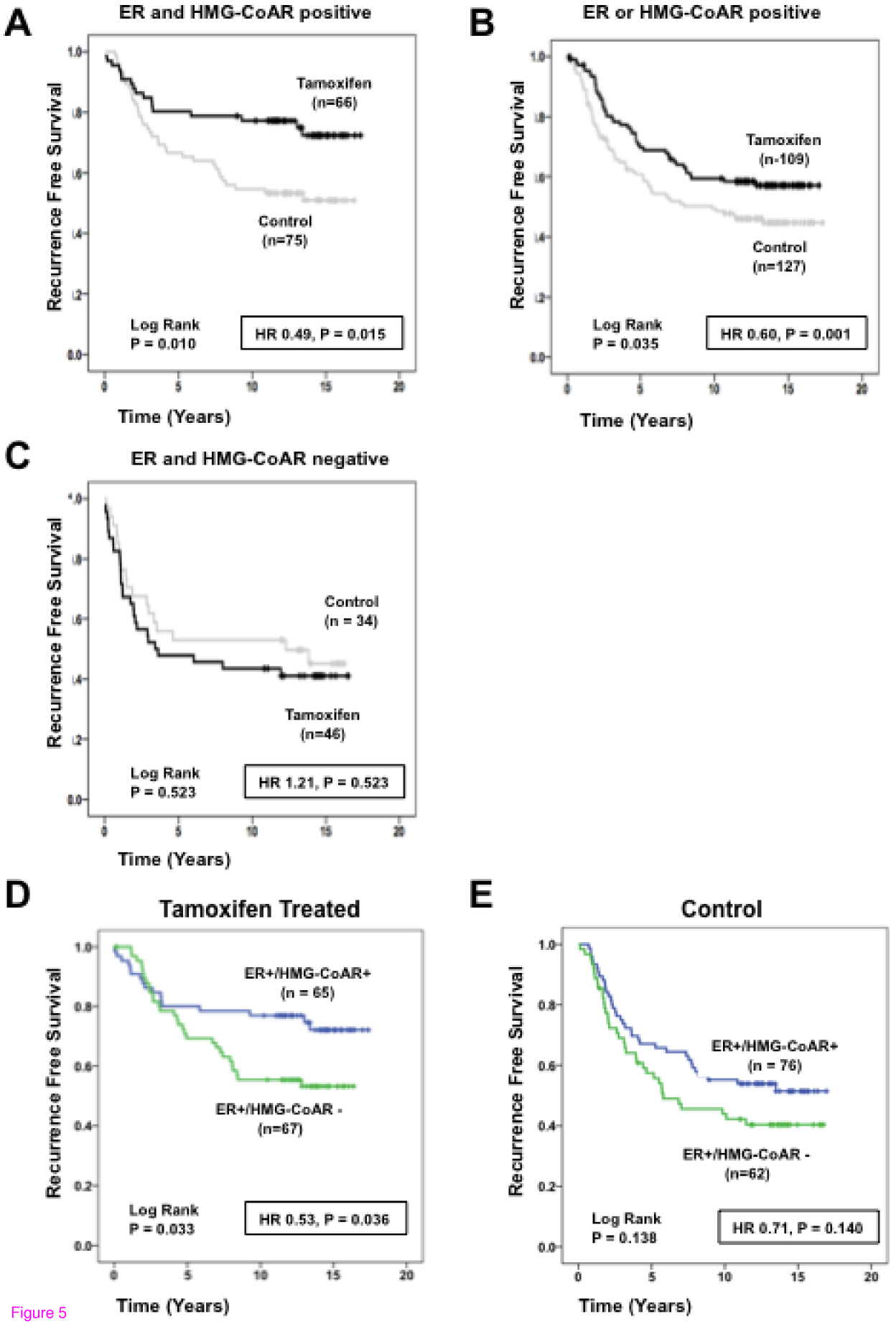

A
ER and HMG-CoAR positive
Recurrence Free Survival
Tamoxifen
(n=66)
Control
(n=75)
Log Rank
P = 0.010
HR 0.49, P = 0.015
Time (Years)
B
ER or HMG-CoAR positive
Recurrence Free Survival
Tamoxifen
(n-109)
Control
(n=127)
Log Rank
P = 0.035
HR 0.60, P = 0.001
Time (Years)
C
ER and HMG-CoAR negative
Recurrence Free Survival
Control
(n = 34)
Tamoxifen
(n=46)
Log Rank
P = 0.523
HR 1.21, P = 0.523
Time (Years)
D
Tamoxifen Treated
Recurrence Free Survival
ER+/HMG-CoAR+
(n = 65)
ER+/HMG-CoAR -
(n=67)
Log Rank
P = 0.033
HR 0.53, P = 0.036
Time (Years)
E
Control
Recurrence Free Survival
ER+/HMG-CoAR+
(n = 76)
ER+/HMG-CoAR -
(n=62)
Log Rank
P = 0.138
HR 0.71, P = 0.140
Time (Years)

Figure 5