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Impact of 27-hydroxylase (CYP27A1) and 27-hydroxycholesterol in breast cancer

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Abstract

The impact of systemic 27-hydroxycholesterol (27HC) and intratumoral CYP27A1 expression on pathobiology and clinical response to statins in breast cancer needs clarification. 27HC is an oxysterol produced from cholesterol by the monooxygenase CYP27A1, which regulates intracellular cholesterol homeostasis. 27HC also acts as an endogenous selective estrogen receptor (ER) modulator capable of increasing breast cancer growth and metastasis. 27HC levels can be modulated by statins or direct inhibition of CYP27A1, thereby attenuating its pro-tumorigenic activities. Herein, the effect of statins on serum 27HC and tumor-specific CYP27A1 expression was evaluated in 42 breast cancer patients treated with atorvastatin within a phase II clinical trial. Further, the associations between CYP27A1 expression with other primary tumor pathological features and clinical outcomes were studied in two additional independent cohorts. Statin treatment effectively decreased serum 27HC and deregulated CYP27A1 expression in tumors. However, these changes were not associated with anti-proliferative responses to statin treatment. *CYP27A1* was heterogeneously expressed among primary tumors, with high expression significantly associated with high tumor grade, ER negativity and basal-like subtype. High *CYP27A1* expression was independently prognostic for longer recurrence-free and overall survival. Importantly, the beneficial effect of high *CYP27A1* in ER-positive breast cancer seemed limited to women aged ≤50 years. These results establish a link between CYP27A1 and breast cancer pathobiology and prognosis and propose that the efficacy of statins in reducing serum lipids does not directly translate to anti-proliferative effects in tumors. Changes in other undetermined serum or tumor factors suggestively mediate the anti-proliferative effects of statins in breast cancer.

Background

Obesity and hypercholesterolemia are associated with an increased risk of developing estrogen receptor-alpha (ERα)-positive breast cancers, especially among postmenopausal women (Boyd & Mcguire 1990, Bianchini *et al.* 2002, Kitahara *et al.* 2011), although the molecular factors linking these phenotypes to breast

cancer etiology and progression remain elusive. Recently, it was demonstrated that 27-hydroxycholesterol (27HC), an oxysterol produced by the sterol 27-hydroxylase cytochrome P450 27A1 (CYP27A1) from cholesterol, was a *bona fide* endogenous selective estrogen receptor modulator (SERM) that manifested partial agonist activity in ERα-positive breast cancer cells (Umetani *et al*. 2007, DuSell *et al*. 2008). As such, it was not surprising that 27HC was shown to increase breast cancer growth and metastatic progression in preclinical experimental models and that this activity was attenuated by genetic and pharmacological approaches (i.e. inhibition of either 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR)) that interfered with the production of cholesterol and/or the conversion of cholesterol to 27HC (Nelson *et al*. 2013, Wu *et al*. 2013). Thus, although the hydroxylation of cholesterol catalyzed by CYP27A1 is a mechanism utilized by cells to eliminate cholesterol from peripheral tissues, it can also result in the production of an ER ligand that likely contributes to breast cancer pathogenesis.

Despite the substantial amount of preclinical evidence supporting a role for 27HC in breast cancer pathobiology (Nelson *et al*. 2013), it remains to be determined if variability in serum and/or intratumoral levels of 27HC or of CYP27A1 influences clinical breast cancer tumor pathology and patient outcome. The circulating levels of 27HC and cholesterol have been reported to be significantly positively correlated, and plasma 27HC tends to be elevated with hypercholesterolemia and increasing age (Brown & Jessup 1999, Burkard *et al*. 2007, Wu *et al*. 2013). In general, high CYP27A1 expression is a characteristic of hepatic tissue and stromal cells (macrophages), but differential expression of CYP27A1 has also been shown in breast cancer, where increased tumor cell-specific CYP27A1 expression was more frequently seen among high-grade tumors (Nelson *et al*. 2013). In addition, the concentration of 27HC was found to be higher in ERα-positive breast tumors compared to normal breast tissue, and this was attributed to a corresponding decrease in the expression of the oxysterol 7 α-hydroxylase (CYP7B1), the enzyme responsible for metabolizing 27HC in breast tumors (Wu *et al*. 2013). The extent to which increased systemic vs intratumoral 27HC and/or variability in the expression of CYP27A1 expression impacts breast tumor biology has not been evaluated. The limited scope of previous studies addressing this question warrants the conduct of a more comprehensive investigation of the role of CYP27A1 in breast cancer within larger cohorts of patients for which data on conventional prognostic and treatment predictive tumor pathological and molecular features are available.

The growth of most luminal breast cancers is dependent on ERα signaling and on pathways/processes downstream of this receptor. Thus, endocrine therapies, which interfere with the production of extra-gonadal estrogens or which competitively inhibit ERα activation, remain the cornerstone of therapy in postmenopausal breast cancer. However, despite the generally positive effects of aromatase inhibitors, the preferred frontline endocrine intervention in ERα-positive breast cancer, the relatively rapid development of resistance remains an impediment to durable clinical responses. This suggests that other estrogen-independent but possibly ERα-mediated mechanisms, which are unaffected by aromatase inhibitors, may still play key roles in driving tumor progression. Considering its activity in preclinical models of breast cancer, it is likely that increased intratumoral 27HC, could bypass the inhibitory effect(s) of existing aromatase inhibitors (Nelson *et al*. 2013). Furthermore, because 27HC is a SERM that manifests partial agonist activity in the breast, it is possible that it could attenuate ERα action in pre-menopausal women (Umetani *et al*. 2007, DuSell *et al*. 2008). Thus, although high 27HC stimulates ER transcriptional activity under hypo-estrogenic conditions (i.e. among postmenopausal women), the opposite effect may occur under physiologic or high estrogenic conditions (i.e. in pre-menopausal women), where 27HC exhibits ER antagonist activity (Umetani *et al*. 2007, DuSell *et al*. 2008, Nelson *et al*. 2013). This suggests that this cholesterol metabolite may play opposing roles in the pre- and postmenopausal settings. Defining how menopausal status influences the relationship between the systemic or tissue-specific levels of 27HC and CYP27A1 and breast cancer tumor pathological features and patient survival remains to be addressed.

Statins are very effective treatments for hypercholesterolemia, which reduce the circulating levels of both low-density lipoprotein (LDL) cholesterol and total cholesterol through their actions on hepatic HMGCR, the rate-limiting enzyme in cholesterol biosynthesis (Clendening & Penn 2012). In breast cancer, the cholesterol-lowering effect of statin treatment was shown to prolong tumor latency and inhibit tumor growth in experimental models of ERα-positive disease (Nelson *et al*. 2013). Furthermore, statins have been shown to induce apoptosis *in vitro* (Spampanato *et al*. 2012, Bjarnadottir *et al*. 2015) and in clinical breast cancer

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(Garwood et al. 2010, Bjarnadottir et al. 2015). These data partially provide a molecular explanation for the clinical observation that statins reduce breast cancer recurrence and prolong overall survival in breast cancer patients (Ahern et al. 2011, Chae et al. 2011, Boudreau et al. 2014, Borgquist et al. 2016). Because statins effectively decrease plasma cholesterol in breast cancer patients (Garwood et al. 2010, Higgins et al. 2012), it has been inferred that a commensurate lowering of plasma 27HC would also occur. Further, given the established roles of 27HC in breast cancer, it has been inferred that it is the ability of statins to lower 27HC, and not cholesterol *per se*, that is responsible for the beneficial effects of this class of drugs in patients with ERα-positive tumors (McDonnell et al. 2014). Although important to show that systemic levels of 27HC are in fact lowered by statin treatment in breast cancer patients, it is also of importance to assess the impact of statin treatment on intratumoral 27HC and how this is influenced by CYP27A1 expression in tumors, and how these biochemical activities influence other pathological features of tumors.

The objective of this study was to investigate the impact of statin treatment on serum 27HC and how this correlates with total cholesterol and tumor pathological factors. Further, we aimed to assess tumor cell-specific expression of CYP27A1 in primary breast tumors from patients treated with statins as an anti-cancer agent in a pre-surgical clinical trial. In addition, we present an exploratory analysis of the impact of the heterogeneous expression of CYP27A1, measured at the transcriptional level and by immunohistochemistry, on primary breast cancer regarding clinical and pathological features and prognosis.

Materials and methods

Patients and tumors

Three independent cohorts of patients with primary breast cancer were used to test the specific hypotheses of the current investigation.

Cohort 1 This cohort comprises 42 women with primary breast cancer, who were enrolled in a pre-surgical window-of-opportunity trial conducted at the Skåne University Hospital in Lund, Sweden, evaluating the effect of two weeks of high-dose atorvastatin on breast cancer proliferation (Bjarnadottir et al. 2013). The study was registered at ClinicalTrials.gov (#NCT00816244) December 30, 2008. All patients signed informed written consent.

The present sub-study aimed to investigate the impact of statin treatment on serum 27HC and on tumor-specific CYP27A1 expression. Patients and tumor characteristics at baseline are reported in Supplementary Table 1 (see section on supplementary data given at the end of this article). This cohort was enriched with older women (83% older than 50 years) presenting with primary tumors that were mostly ERα positive (88%) and of lower histological grade (62% grades 1 and 2). In addition, the majority of patients (59%) presented with positive lymph node status at the time of diagnosis. Serum samples collected before and after the completion of two weeks treatment with high-dose atorvastatin (80 mg daily) were analyzed for total cholesterol, LDL cholesterol and high-density lipoprotein (HDL) cholesterol concentrations according to standard clinical diagnostic procedures at Skåne University Hospital, Lund, Sweden. Other tumor pathological factors have been previously assessed (Supplementary Table 1 and Bjarnadottir et al. (2013)). Serum 27HC and tumor-specific CYP27A1 expression were determined by mass spectrometry and immunohistochemistry, respectively. Paired microarray data from a subset of 25 patients (GSE63427, Bjarnadottir et al. (2015)) were also available to enable an evaluation of the impact of statin treatment on *CYP27A1* mRNA expression.

Cohort 2 This cohort included 1,881 women with primary breast cancer that was diagnosed and treated at several different institutions worldwide and was used to investigate if heterogeneity in *CYP27A1* expression correlated with conventional breast cancer tumor pathological features and prognosis. Gene expression data and the corresponding clinical and tumor pathological data were downloaded from the gene expression omnibus (GEO, 11 independent datasets), and the 11 datasets were merged and normalized to obtain a single mega dataset as previously described (Ringner et al. 2011). A summary of the distribution of tumor pathological factors in this cohort is presented in Supplementary Table 1. The median follow-up for recurrence-free survival (RFS) and overall survival (OS) were 7.2 (range 0.17–24) years and 8.3 (range 0.13–25) years, respectively.

Cohort 3 The results from the analyses performed within cohort 2 were independently verified in a subset of 661 invasive breast carcinomas from the TCGA project (update September 2013). RNAseq v2 level 3 data for the 661 tumors were processed as previously described (Cancer Genome Atlas 2012, Holm et al. 2016). Briefly, the

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gene-normalized RSEM count estimates were offset by a pseudocount of 1, log2-transformed and mean centered across tumor samples to generate relative gene expression levels for 20,531 genes. Although the distribution of age and ER status were similar between cohort 3 and cohort 2, cohort 3 was enriched for patients with positive nodes and larger tumors (Supplementary Table 1). The median follow-up for OS was 2.2 (range 0.1–19) years.

Measurement of serum 27-hydroxycholesterol

The concentration of 27HC in the pre- and post-statin-treated serum samples were determined by mass spectrometry, as previously described, with modifications to improve throughput and specificity (Ayciriex *et al.* 2012). The LipidMaps standard cholest-5-ene-3ß,27-diol (Product 110818, Avanti Polar Lipids, Alabaster, AL, USA) was used to generate a standard curve in 50 mg/mL BSA matrix at final concentrations between 0.05 µM and 10 µM. 50 µL of serum, calibration standard or quality control (QC) sample was added to a 96-well plate, spiked with 1 µM d6-27 HC internal standard in MeOH (Product LM-4114, Avanti Polar Lipids), followed by saponification with ethanolic sodium hydroxide. Saponified material was extracted with hexanes, derivatized with 4-(dimethylamino)phenyl isocyanate (Sigma-Aldrich) in dimethylformamide and triethylamine, quenched with phosphate buffer and re-extracted with hexanes. Extracts were then dried under nitrogen gas and reconstituted in 4:3:1 IPA:MeCN:H2O. 27HC was measured by a targeted UPLC-MS/MS (Xevo TQS, Waters) method monitoring the MRM transitions was quantified by calculating the ratio to internal standard, followed by linear regression with 1/x weighting against the calibration curve in Skyline v3.5.1.9942. Quantitative values were reported in micromolar concentrations. Low and high QC at 0.25 and 2.5 µM showed accuracy of 109% and 98%, respectively over three determinations.

CYP27A1 protein expression (immunohistochemistry)

The tumor cell-specific expression of CYP27A1 was determined by immunohistochemistry following a previously validated protocol (Nelson *et al.* 2013). Sections of 3 to 4 µm were cut from whole tissue FFPE blocks, de-paraffinized, treated with antigen retrieval buffer (citrate, pH 6) for 20 min, and then reacted with an anti-CYP27A1 rabbit monoclonal antibody (ab126785, Abcam) at a dilution of 1:500 for 2 h. Staining procedures were performed using the DAKO Envision horseradish peroxidase rabbit/mouse kit (DAKO) and the Dakocytomation Autostainer (DAKO). Cell nuclei were counterstained with hematoxylin. CYP27A1 positivity was detected as a granular cytoplasmic reactivity. Cell type identification and staining intensity was assessed by a board certified pathologist (DG). Only tumor cell-specific CYP27A1 expression was considered for subsequent analyses. Each sample was given a semi-quantitative intensity score: 0 (absent), 0.5 (borderline), 1 (weak), 2 (moderate) or 3 (strong). For statistical analysis, the tumors were categorized as negative (0), weak (0.5, 1) and overexpressed (2, 3). Representative cases of CYP27A1 expression in these categories are illustrated in Supplementary Fig. 4.

Statistical analyses

Associations between serum 27HC at baseline, post-treatment or the statin-induced change in serum 27HC (post-treatment level minus pre-treatment level) with other baseline tumor pathological factors and the statin-induced change in tumor proliferation were assessed using the Mann–Whitney U test for variables with 2 categories and Spearman rho's test for variables with $n \geq 3$ ordered categories. The Wilcoxon signed-rank test was applied to assess the differences in the distribution of *CYP27A1* expression in relation to statin treatment, and Fisher's exact tests were used to assess the associations between CYP27A1 expression and other tumor pathological factors (cohort 1). To test for associations between *CYP27A1* expression and other conventional primary tumor pathological factors (cohorts 2 and 3), the median expression of *CYP27A1* was used to categorize patients into two groups (high; $\geq$ median and low; < median), and chi-squared tests were implemented to identify statistically significant associations. Kaplan–Meier plots were generated to visualize the survival difference between patients with low vs high *CYP27A1*-expressing tumors and the log-rank test was used to check for significance. Cox proportional hazards models were used to evaluate if *CYP27A1* expression provided independent prognostic information beyond the conventional prognostic factors in primary breast cancer including the age at primary tumor diagnosis, nodal status, histological grade, tumor size and hormone receptor status. P values from two-sided statistical tests are reported and $P < 0.05$ was considered to be significant.

Results

Associations between serum 27HC and other serum lipids and primary tumor pathological characteristics

Initially, the distribution and relationship between 27HC and three main serum lipids: total cholesterol, LDL cholesterol and HDL cholesterol, were investigated. Forty-two patients from cohort 1 were eligible for inclusion in these analyses. Of these, 83% were aged above 50 years and only 12% presented with ERα negative tumors, indicating homogeneity of the cohort in relation to menopausal status and hormone receptor expression. Baseline concentrations of total cholesterol (mean; 6.05 mmol/L, range; 4.4–9.9 mmol/L), LDL cholesterol (mean: 3.72 mmol/L, range; 2.26–6.85 mmol/L) and HDL cholesterol (mean; 1.6 mmol/L, range; 1.0–2.8 mmol/L) were predominantly within the clinical 'normal' reference ranges defined for cardiovascular disease risk assessment, exemplifying another degree of homogeneity within this cohort.

The mean baseline concentration of 27HC in this cohort was 0.31 µM (range 0.15–0.63 µM). Serum 27HC was found to be significantly positively correlated with total cholesterol (Fig. 1A) and LDL cholesterol levels (Fig. 1B), but not with HDL cholesterol (Fig. 1C). No statistically significant association was found between baseline serum 27HC concentration and primary tumor pathological factors including ERα status, tumor size, histological grade, tumor proliferation status (Ki67) and tumor cell-specific CYP27A1 expression (Table 1, $P > 0.05$ for all comparisons).

Effects of statin treatment on serum 27HC and CYP27A1 expression in primary breast tumors

Several studies in the past have suggested an association between serum 27HC and total cholesterol although the impact of statin-dependent lowering of cholesterol on 27HC levels has never been established. In this study, statin treatment was found to effectively decrease serum total cholesterol and LDL cholesterol, and this was accompanied by a commensurate decreased in 27HC concentrations in all patients. HDL cholesterol was however not significantly altered by the treatment (Fig. 2A). Noteworthy, the absolute reduction in 27HC levels were relatively smaller than the absolute decrease in total cholesterol and LDL cholesterol levels. The reduction in serum 27HC was not associated with the previously established anti-proliferative response to statin treatment in this cohort, measured as a change in the expression of the proliferation marker Ki67 (Bjarnadottir *et al.* 2013) (Table 1, $P = 0.82$).

Having established that statin treatment effectively decreased serum 27HC, we next investigated if statins influenced CYP27A1 expression, at the protein and mRNA level, in breast tumors. A non-significant decrease in *CYP27A1* mRNA expression after statin treatment (Fig. 2B, $P = 0.09$) was noted. In contrast, CYP27A1 protein expression was found to be significantly upregulated ($P = 0.033$, Fig. 2C) following statin treatment. Regardless, the anti-proliferative effect of statin treatment (change in Ki67 index) was neither significantly correlated with changes in serum 27HC (Table 1, $P = 0.82$) nor to changes in intratumoral CYP27A1 protein expression (Supplementary Table 2, $P = 0.33$). Further, the baseline and statin-induced changes in 27HC and CYP27A1 protein expression were not significantly associated with any other baseline tumor pathological factor. However, an association approaching statistical significance ($P = 0.044$) was observed between high tumor proliferation and high CYP27A1 expression (Table 1 and Supplementary Table 2, respectively). These data indicate that although statins effectively decrease serum 27HC, and may alter CYP27A1 protein expression in breast tumors, the baseline or
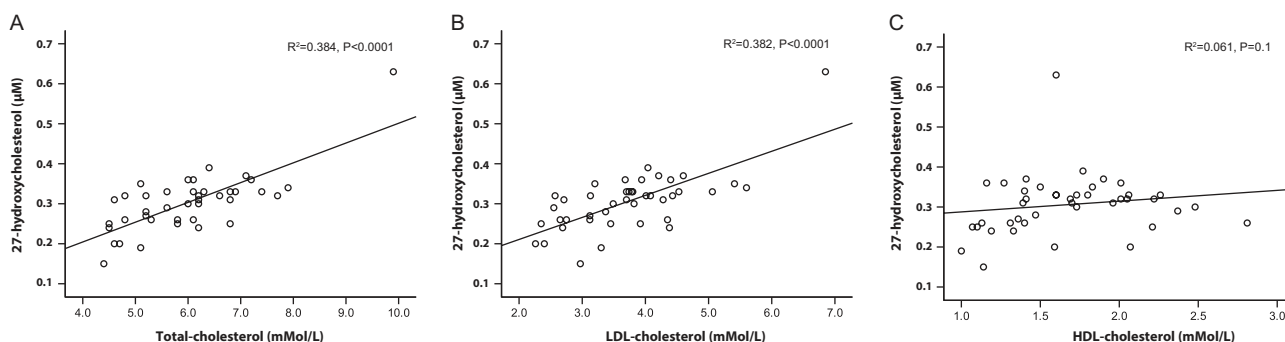

Figure 1

Relationship between 27HC and total cholesterol (A), LDL cholesterol (B) and HDL cholesterol (C) in serum of breast cancer patients before statin treatment (cohort 1). *P* values are from Spearman's rho correlation tests.

Table 1 Association between breast cancer tumor pathological features and serum 27HC concentrations in cohort 1. Correlations between baseline tumor pathological factors and 27HC levels before atorvastatin, post atorvastatin and the change in 27HC concentration following atorvastatin treatment were studied separately.

Factor	n	27HC Pre-atorvastatin		27HC Post-atorvastatin		Change in 27HC*	
		Mean; µM (95% CI)	P	Mean; µM (95% CI)	P	Mean post-pre; µM (95% CI)	P
Tumor size							
≤20 mm	20	0.29 (0.26–0.32)	0.23	0.22 (0.19–0.23)	0.22	−0.07 (−0.09, −0.05)	0.18
>20 mm	22	0.32 (0.29–0.36)		0.24 (0.22–0.26)		−0.09 (−0.10, −0.07)	
Estrogen receptor							
Positive	37	0.30 (0.27–0.33)	0.34	0.23 (0.21–0.24)	0.36	−0.08 (−0.09, −0.07)	1.0
Negative	5	0.32 (0.21–0.41)		0.24 (0.19–0.30)		−0.07 (−0.13, −0.02)	
Histological grade							
1	9	0.31 (0.27–0.35)	0.44	0.21 (0.18–0.24)	0.50	−0.10 (−0.14, −0.07)	0.10
2	17	0.31 (0.26–0.36)		0.24 (0.20–0.27)		−0.08 (−0.10, −0.06)	
3	16	0.29 (0.26–0.33)		0.23 (0.21–0.25)		−0.07 (−0.09, −0.05)	
Ki67							
≤20%	19	0.32 (0.27–0.26)	0.52	0.23 (0.20–0.26)	0.73	−0.09 (−0.11, −0.07)	0.39
>20%	15	0.29 (0.25–0.32)		0.22 (0.19–0.24)		−0.07 (−0.09, −0.05)	
CYP27A1							
Negative	7	0.30 (0.25–0.34)	0.81	0.21 (0.19–0.23)	0.72	−0.08 (−0.11, −0.06)	0.45
Borderline / Weak	15	0.32 (0.27–0.37)		0.24 (0.20–0.27)		−0.08 (−0.11, −0.05)	
Moderate / Strong	4	0.27 (0.19–0.36)		0.21 (0.17–0.25)		−0.06 (−0.11, −0.01)	
Change in Ki67 index*							
Decreased	20	0.29 (0.27–0.32)	P=0.87	0.22 (0.20–0.23)	P=1.0	−0.08 (−0.10, −0.06)	P = 0.82
Increased	14	0.32 (0.26–0.37)		0.23 (0.20–0.27)		−0.08 (−1.03, −0.06)	

P-values from Mann–Whitney U test for variables with 2 categories and Spearman rho's test for variables with n ≥ 3 ordered categories
*Effect of statin treatment expressed as the difference between the post-treatment and pre-treatment level.

statin-induced changes in 27HC or CYP27A1 do not appear to explain the anti-proliferative response to statin treatment seen in the tumors in this cohort.

Associations between *CYP27A1* expression and baseline primary breast tumor pathological characteristics and prognosis

In preclinical models, it has been shown that in addition to statins, 27HC levels can be lowered by direct inhibition of CYP27A1 (Nelson *et al.* 2013). Thus, this enzyme may be a useful drug target in breast cancer. This far, however, the expression of CYP27A1 in breast tumors has only been evaluated in small, underpowered studies. Consequently, we explored how variability in CYP27A1 expression, measured both at the transcriptional and protein levels, may influence breast cancer pathology and prognosis. Differential *CYP27A1* mRNA expression was found to be significantly associated with breast cancer tumor pathological factors. In cohort 2, high *CYP27A1*
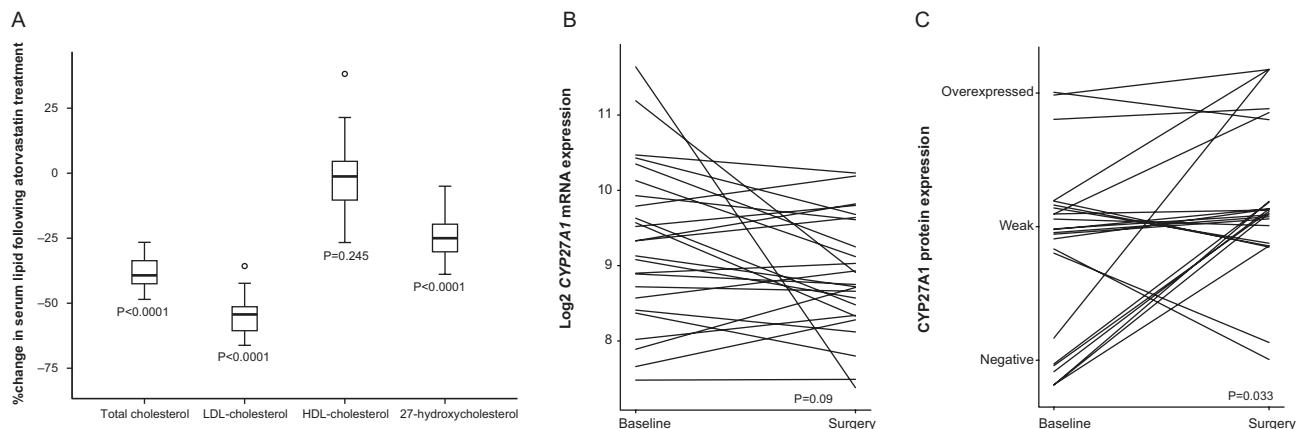

Figure 2
Statin-induced changes in serum lipids (A) and *CYP27A1* mRNA expression in tumors (B) and tumor cell specific CYP27A1 protein expression (C). P values are from the Wilcoxon signed-rank test comparing the pre-treatment (baseline) and post-treatment (surgery) measurement for each factor.

expression was more frequent in tumors among younger women (age ≤50 years), and in node-negative, ERα-negative, high-grade (grade 3), basal-like and normal-like breast cancer (Table 2). A similar association between age and nodal status with *CYP27A1* expression was observed in sub-analyses that considered ERα-positive tumors alone (Table 2). Furthermore, ERα negativity, negative nodal status and the basal-like and normal-like molecular subtypes were confirmed to be associated with high *CYP27A1* expression in the TCGA cohort (Table 2).

Next, we investigated if *CYP27A1* expression may predict primary breast cancer prognosis. In an analysis that included all patients within cohort 2, a marginal trend toward a better RFS was observed for patients presenting with high *CYP27A1* expression in the primary tumor (HR=0.82, CI=0.66–1.02, P=0.07, Supplementary Fig. 1A). This relationship was maintained when only the subset of ERα-positive tumors were considered (HR=0.76, CI=0.59–0.98, P=0.04, Fig. 3A). Interestingly, when the analysis was further stratified for age at diagnosis, the prolonged RFS associated with high *CYP27A1* expression was found to be limited to the subgroup of women ≤50 years of age (Fig. 3B and C). The predictive value of *CYP27A1* expression for OS was similar to RFS (Fig. 3D, E and F and Supplementary Fig. 1B). High *CYP27A1* expression was independently prognostic for longer RFS and OS in

multivariable analyses, especially among younger women (Table 3). Independent analyses in the TCGA cohort confirmed that high *CYP27A1* expression was associated with prolonged overall survival (Supplementary Fig. 2A, HR=0.49, CI=0.34–0.78, P=0.003), and this relationship remained significant for analyses that included only those tumors that were ERα positive (Supplementary Fig. 2B, HR=0.51, CI=0.28–0.94, P=0.03). Further stratification of ERα-positive tumors within the TCGA cohort based on age suggested that high expression of *CYP27A1* was associated with better overall survival in both age groups, but the results were not statistically significant for the subgroup of women under 50 years of age (Supplementary Fig. 2C and D).

Finally, the prognostic relevance of *CYP27A1* in ERα-negative breast cancers was investigated. Although high *CYP27A1* expression was more frequently observed among ERα-negative tumors, no significant difference in RFS and OS was seen between high and low *CYP27A1* expressing ERα-negative tumors in cohort 2 (Supplementary Fig. 3A and B). In contrast, a similar analysis in the TCGA cohort resulted in a statistically significant difference favoring a prolonged OS among high *CYP27A1*-expressing ERα-negative tumors relative to low *CYP27A1*-expressing ERα-negative tumors (Supplementary Fig. 3C, HR=0.41, CI=0.18–0.92, P=0.03).

Table 2 Associations between primary breast cancer tumor pathological factors and *CYP27A1* mRNA.

Factor at baseline	CYP27A1 (all cohort 2)			CYP27A1 (ER-positive cohort 2)			CYP27A1 (Cohort 3)		
	Low (%)	High (%)	P	Low (%)	High (%)	P	Low (%)	High (%)	P
Age at diagnosis									
≤50 years	47	53	**<0.001**	50	50	**0.002**	52	48	0.56
>50 years	58	42		60	40		49	51	
Tumor size									
≤20 mm	48	52	0.29	54	46	0.08	47	53	0.25
>20 mm	51	49		59	41		52	48	
Nodal status									
Positive	67	33	**<0.001**	68	32	**<0.001**	53	47	**0.047**
Negative	44	56		51	49		46	54	
Estrogen receptor status									
Positive	55	45	**0.001**	n.a	n.a	n.a	54	46	**0.001**
Negative	46	54					38	62	
Molecular subtype									
Luminal A	53	47		56	44		52	48	
Luminal B	54	46		57	43		67	33	
Her2-enriched	65	35	**<0.001**	68	32	**<0.001**	57	43	**<0.001**
Basal-like	32	67		32	68		31	69	
Normal-like	44	56		41	51		34	66	
Histological grade									
1 and 2	54	46	**<0.001**	60	40	**0.003**	n.a	n.a	n.a
3	42	58		50	50				

P-values from Pearson Chi-Square test. n.a; not applicable. Values in bold represent statistically significant *P* values.
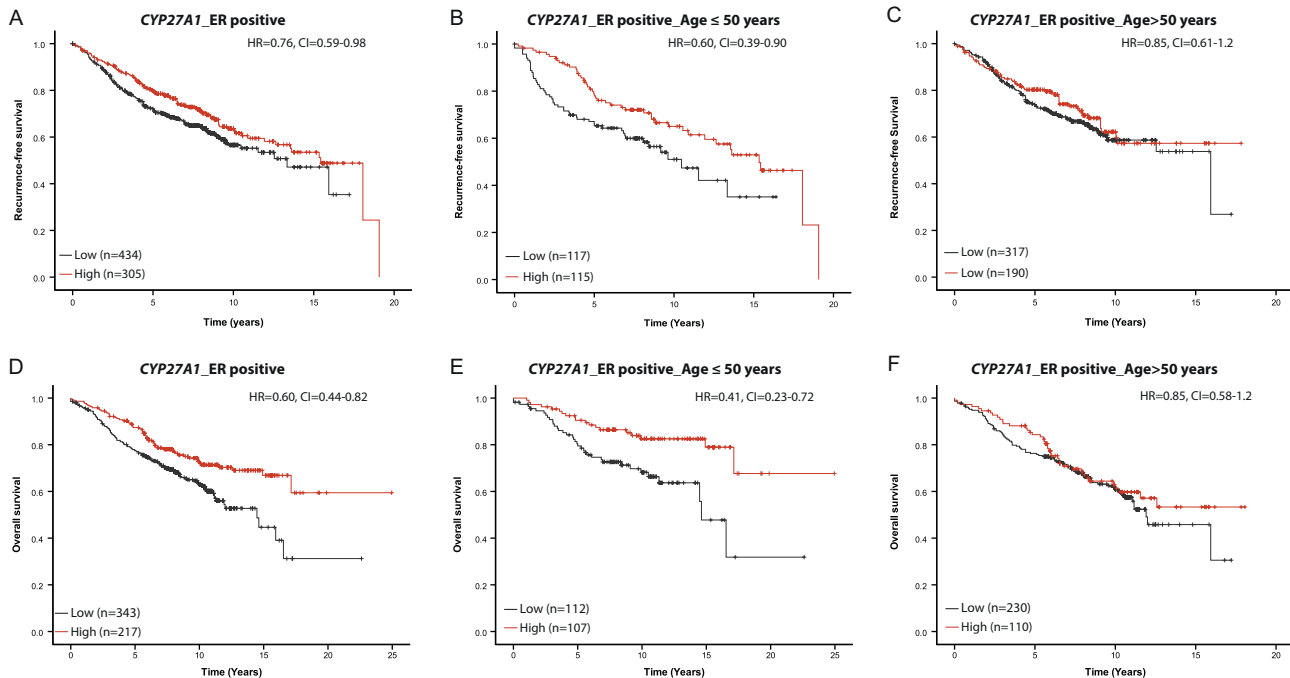
Figure 3
Associations between *CYP27A1* expression and survival in estrogen receptor-positive primary breast cancer (cohort 2). High expression of *CYP27A1* is associated with a longer recurrence-free survival, RFS **(A–C)** and overall survival, OS **(D–F)** in ERα positive breast cancer.

Discussion

The results of studies from our laboratories and others have suggested that hypercholesterolemia, secondary to its conversion to 27HC by CYP27A1, may impact breast cancer recurrence risk by activating ERα. It was inferred therefore that increased circulating 27HC and/or CYP27A1 would be associated with poorer outcomes in patients with ERα-positive tumors and that this risk could be mitigated by statins. Leveraging these findings we developed a clinical study to (a) explore the relationship between cholesterol and circulating 27HC, (b) define the impact of statins on 27HC levels and (c) define the prognostic significance of 27HC and/or intratumoral CYP27A1 on tumor pathobiology. It was anticipated that such a study would inform the future development

of a prospective adjuvant clinical trial to establish the role of lipid-lowering medications like statins in breast cancer treatment.

The results from small early-phase pre-surgical (Garwood *et al.* 2010) and biomarker discovery trials (Higgins *et al.* 2012), including the results from the window-of-opportunity trial (Bjarnadottir *et al.* 2013) presented herein, have unanimously confirmed that the compliance to statin treatment is remarkably high and that these drugs effectively decrease serum total cholesterol and LDL cholesterol. Further, in this study, we have shown for the first time that statin-induced reduction in serum cholesterol is accompanied by a corresponding decrease in serum 27HC. This finding afforded us the opportunity to explore the relationship, if any, between the beneficial effects of statins on tumor biology, noted previously, and

Table 3 Association between *CYP27A1* expression and prognosis following primary breast cancer diagnosis in cohort 2.

Patient category	RFS_CYP27A1 (ref. low)			OS_CYP27A1 (ref. low)		
	HR	CI	*P*	HR	CI	*P*
All tumors	0.76	0.60–0.96	**0.02**	0.82	0.62–1.1	0.16
All ER positive tumors	0.70	0.53–0.92	**0.01**	0.74	0.53–1.0	0.07
ER positive/≤50 years	0.53	0–33–0.83	**0.01**	0.49	0.27–0.89	**0.02**
ER Positive/>50 years	0.85	0.60–1.2	0.36	0.93	0.62–1.4	0.7

Multivariable Cox-regression analyses for recurrence-free survival (RFS) and overall survival (OS). *P* < 0.05 was considered significant.
Adjusted for age at diagnosis, ER status, nodal status, histological grade and tumor size where applicable. HR; hazard ratio, CI; confidence interval, RFS; recurrence-free survival, OS; overall survival, ref; reference, ER; estrogen receptor. Values in bold represent statistically significant *P* values.

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27HC production. However, baseline serum 27HC and the magnitude of the treatment-induced reduction in serum 27HC were neither associated with any baseline tumor pathological factor nor the anti-proliferative tumor response to statin treatment in the 42 patient cohort included in this study.

Irrespective of how it was analyzed (mRNA or protein), the change in CYP27A1 was also not associated with any baseline tumor pathological factor nor did it correlate with the anti-proliferative tumor response to statins. These results suggest that the efficacy of statin treatment in controlling breast cancer proliferation cannot be predicted from serum 27HC and that tumor cell-specific CYP27A1 expression does not inform the likely tumor response to statin treatment. However, the homogeneity within this cohort with respect to basal levels of serum lipids and patient and tumor pathological factors like age of study participants and ERα status, coupled with the small numbers of patients included in the study, may have limited the power to detect any statistically significant associations in our analyses.

Although statins effectively lower circulating cholesterol, their impact on intratumoral cholesterol content/production is likely to be less substantial. This is due in part to variation in the post-hepatic exposure level of these drugs and also to compensatory upregulation of cholesterol synthesizing enzymes and uptake machinery and decreased cholesterol efflux in cells in response to low cholesterol. Thus, significant reduction of intratumoral production of 27HC may not be accomplished by reducing circulating cholesterol. Small-molecule inhibitors of CYP27A1 have been shown to effectively reduce both circulating and intratumoral 27HC and may represent an adjunct to statins to mitigate the impact of this oxysterol in breast cancer. Interestingly, the aromatase inhibitor anastrozole was recently shown to be a potent 'off-target' inhibitor of CYP27A1 that significantly reduces plasma 27HC without affecting hepatic and plasma cholesterol levels (Mast *et al.* 2015). Considering these findings, it was of interest to evaluate the relationship between intratumoral CYP27A1 and pathological features of importance in breast cancer. Using immunohistochemistry, Nelson and coworkers (Nelson *et al.* 2013) reported that elevated expression of CYP27A1 was more frequently observed among high-grade tumors. Similarly, in this study, immunohistochemical analyses indicated that CYP27A1 was expressed in up to 91% of high-grade (grade 3) tumors compared to 60% of low-grade (grades 1 and 2) tumors, but this comparison

did not reach statistical significance in this small cohort. To address this further, we made use of well-annotated publicly available transcriptional databases for breast cancer and probed the association between *CYP27A1* expression and selected pathological factors in two independent gene expression cohorts. While confirming that high tumor grade was significantly associated with high *CYP27A1* expression, we also determined that ERα negativity, basal-like and normal-like molecular subtypes were features that were associated with high CYP27A1 expression in both primary breast tumors and in breast cancer cell lines (data not shown).

The prognostic relevance of *CYP27A1* expression in breast cancer has previously been investigated with null findings (Nelson *et al.* 2013, Wu *et al.* 2013). Interestingly, in two sizable, independent breast cancer cohorts we found, especially in ERα-positive disease, that *CYP27A1* expression in the primary tumor provided independent prognostic information beyond conventional prognostic factors. Given that 27HC may perform conflicting roles in hypo- and normo/hyper-estrogenic conditions, it is important to establish if the prognostic importance of CYP27A1 is influenced by menopausal status. Notable was the observation that both RFS and OS were improved in patients aged less than 50 years presenting with ERα-positive disease whose tumors expressed elevated *CYP27A1*. This could be explained by the fact that 27HC, being a SERM, has the potential of reducing estradiol-activated ERα to a level equivalent to its inherent partial agonist activity. Thus, in normal cycling women, it would be expected that 27HC would attenuate the actions of estradiol. Further, it has to be noted that the normal physiological function of CYP27A1 is to regulate intracellular cholesterol homeostasis by converting cholesterol to the more polar 27HC and also potentially to the bile acid precursor cholestenoic acid. 27HC itself can function as a ligand of the liver X receptor and induce the expression of the cholesterol efflux pumps ABCA1 and ABCG1. In addition, 27HC can bind to INSIG1 and interfere with the activation of SREBP2, the master regulator of the expression of the enzymes required for cholesterol biosynthesis. Thus, under normal physiological circumstances, CYP27A1 and 27HC may actually exhibit a protective effect in breast cancer by limiting the ability of the cell to accumulate cholesterol. Interestingly, the protective effect of high *CYP27A1* expression was lost in older women (presumably postmenopausal) indicating that high 27HC, consequent to high CYP27A1 expression, may play a pro-tumorigenic role in postmenopausal

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women and that decreasing 27HC by direct inhibition of CYP27A1 or indirectly with statin treatment may prolong survival in postmenopausal women with breast cancer. This result was however not verified in the TCGA cohort, but it is worth noting that the event rate (breast cancer deaths) was relatively lower in this cohort due to the limited follow-up time, the median of which was only 2.2 years, which is a time interval when more than 95% of patients with ERα-positive breast cancer are event-free after surgery and standard adjuvant therapy.

There are noteworthy caveats to this study. Restricted by insufficient tumor material, 27HC measurements were not performed for tumor samples; hence, we could not evaluate if a commensurate decrease in intratumoral 27HC levels occurs following statin treatment. The survival data in cohort 2 are derived from 11 datasets, comprising a very large cohort of nearly 2000 tumors that were heterogeneously treated. The fact that modern treatment has changed the natural history of breast cancer suggests that the prognostic implications of *CYP27A1* detected by our analyses may represent historical behavior. Although most of the results were validated in the more recent TCGA cohort, the immature follow-up data limited our investigations.

Conclusions

In summary, our study provides new insights into the pathobiological and prognostic relevance of CYP27A1 expression in breast cancer. For the first time, we have shown that statin treatment effectively decreases serum 27HC and may deregulate CYP27A1 expression in tumors, although these changes do not correlate with the anti-proliferative response of the treatment. Furthermore, our results indicate that CYP27A1 is differentially expressed in breast cancer with important consequences on the disease phenotype and prognosis. These results can have implications for selecting patients who may benefit from the addition of statins or drugs inhibiting CYP27A1 to their standard breast cancer therapeutic regimen. Our results also highlight the need for further studies to evaluate how tissue-specific and/or systemic levels of 27HC and CYP27A1 impact breast cancer pathobiology and response to endocrine and statin treatment in additional cohorts representing the clinical disease.

Authors' contribution statement
Drs Kimbung and Borgquist had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study Concept and Design: Kimbung, Chang, McDonnell, Borgquist. Acquisition of data: Kimbung, Dubois, Thompson, Borgquist. Analysis and interpretation of data: Kimbung, Chang, Bendahl, Dubois, Thompson, McDonnell, Borgquist. Drafting of the manuscript: Kimbung, Chang, McDonnell, Borgquist. Critical revision of the manuscript for important intellectual content: Kimbung, Chang, Bendahl, Dubois, Thompson, McDonnell, Borgquist. Statistical analysis: Kimbung, Bendahl. Obtained funding: Borgquist. Administrative, technical, or material support: Dubois, Thompson, McDonnell, Borgquist. Study supervision: Kimbung, Chang, McDonnell, Borgquist. All authors read and approved the final manuscript.

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