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

Siker Kimbung¹, Ching-yi Chang², Pär-Ola Bendahl¹, Laura Dubois³, J Will Thompson²,³, Donald P McDonnell² and Signe Borgquist¹,⁴

¹Division of Oncology and Pathology, Department of Clinical Sciences, Lund, Lund University, Sweden
²Department of Pharmacology and Cancer Biology, Duke University School of Medicine, Durham, NC, USA
³Duke Proteomics and Metabolomics Resource, Duke University School of Medicine, Durham, NC, USA
⁴Clinical Trial Unit, Clinical Studies Sweden, Forum South, Skåne University Hospital, Lund, Sweden

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, Blanchini et al. 2002, Kitahara et al. 2011), although the molecular factors linking these phenotypes to breast cancer are not well understood.

Key Words

- 27-hydroxycholesterol
- CYP27A1
- breast cancer
- statin
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 \textit{bona fide} endogenous selective estrogen receptor modulator (SERM) that manifested partial agonist activity in ER\textsubscript{α}-positive breast cancer cells (Umetani \textit{et al.} 2007, DuSell \textit{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 \textit{et al.} 2013, Wu \textit{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 \textit{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 \textit{et al.} 2007, Wu \textit{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 \textit{et al.} 2013). In addition, the concentration of 27HC was found to be higher in ER\textsubscript{α}-positive breast tumors compared to normal breast tissue, and this was attributed to a corresponding decrease in the expression of the oxysterol 7 \textalpha-hydroxylase (CYP7B1), the enzyme responsible for metabolizing 27HC in breast tumors (Wu \textit{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 ER\textsubscript{α} 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 ER\textsubscript{α} 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 ER\textsubscript{α}-positive breast cancer, the relatively rapid development of resistance remains an impediment to durable clinical responses. This suggests that other estrogen-independent but possibly ER\textsubscript{α}-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 \textit{et al.} 2013). Furthermore, because 27HC is a SERM that manifests partial agonist activity in the breast, it is possible that it could attenuate ER\textsubscript{α} action in pre-menopausal women (Umetani \textit{et al.} 2007, DuSell \textit{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 \textit{et al.} 2007, DuSell \textit{et al.} 2008, Nelson \textit{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 ER\textsubscript{α}-positive disease (Nelson \textit{et al.} 2013). Furthermore, statins have been shown to induce apoptosis \textit{in vitro} (Spamanato \textit{et al.} 2012, Bjarnadottir \textit{et al.} 2015) and in clinical breast cancer
(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 (80mg 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
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-treatment 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 50mg/mL BSA matrix at final concentrations between 0.05 µM and 10 µM. 10 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, TQS, Waters) method monitoring the MRM transitions was generated to visualize the survival difference between patients with low vs high CYP27A1 expression and other tumor pathological factors (cohort 1). To test for associations between CYP27A1 expression and other conventional primary tumor pathological factors (cohort 2 and 3), the median expression of CYP27A1 was used to categorize patients into two groups (high; ≥ 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.

**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 ≥ 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; ≥ 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.
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 expression was associated with a decreased Ki67 index.

**Table 1** Association between breast cancer tumor pathological features and serum 27HC concentrations in cohort 1.

<table>
<thead>
<tr>
<th>Factor</th>
<th>27HC Pre-atorvastatin</th>
<th>27HC Post-atorvastatin</th>
<th>Change in 27HC*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean; µM (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤20 mm</td>
<td>20</td>
<td>0.29 (0.26–0.32)</td>
<td>0.23</td>
</tr>
<tr>
<td>&gt;20 mm</td>
<td>22</td>
<td>0.32 (0.29–0.36)</td>
<td>0.24</td>
</tr>
<tr>
<td>Estrogen receptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>37</td>
<td>0.30 (0.27–0.33)</td>
<td>0.34</td>
</tr>
<tr>
<td>Negative</td>
<td>5</td>
<td>0.32 (0.21–0.41)</td>
<td>0.24</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>0.31 (0.27–0.35)</td>
<td>0.44</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>0.31 (0.26–0.36)</td>
<td>0.24</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>0.29 (0.26–0.33)</td>
<td>0.23</td>
</tr>
<tr>
<td>Ki67</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤20%</td>
<td>19</td>
<td>0.32 (0.27–0.26)</td>
<td>0.52</td>
</tr>
<tr>
<td>&gt;20%</td>
<td>15</td>
<td>0.29 (0.25–0.32)</td>
<td>0.22</td>
</tr>
<tr>
<td>CYP27A1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>7</td>
<td>0.30 (0.25–0.34)</td>
<td>0.81</td>
</tr>
<tr>
<td>Borderline / Weak</td>
<td>15</td>
<td>0.32 (0.27–0.37)</td>
<td>0.24</td>
</tr>
<tr>
<td>Moderate / Strong</td>
<td>4</td>
<td>0.27 (0.19–0.36)</td>
<td>0.21</td>
</tr>
<tr>
<td>Change in Ki67 index*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decreased</td>
<td>20</td>
<td>0.29 (0.27–0.32)</td>
<td>P=0.87</td>
</tr>
<tr>
<td>Increased</td>
<td>14</td>
<td>0.32 (0.26–0.37)</td>
<td>0.23</td>
</tr>
</tbody>
</table>

*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.

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.

<table>
<thead>
<tr>
<th>Factor at baseline</th>
<th>CYP27A1 (all cohort 2)</th>
<th>CYP27A1 (ER-positive cohort 2)</th>
<th>CYP27A1 (Cohort 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low (%)</td>
<td>High (%)</td>
<td>P</td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50 years</td>
<td>47</td>
<td>53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>&gt;50 years</td>
<td>58</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤20 mm</td>
<td>48</td>
<td>52</td>
<td>0.29</td>
</tr>
<tr>
<td>&gt;20 mm</td>
<td>51</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Nodal status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>67</td>
<td>33</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Negative</td>
<td>44</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Estrogen receptor status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>55</td>
<td>45</td>
<td>0.001</td>
</tr>
<tr>
<td>Negative</td>
<td>46</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Molecular subtype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal A</td>
<td>53</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Luminal B</td>
<td>54</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Her2-enriched</td>
<td>65</td>
<td>35</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Basal-like</td>
<td>32</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>Normal-like</td>
<td>44</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 and 2</td>
<td>54</td>
<td>46</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3</td>
<td>42</td>
<td>58</td>
<td></td>
</tr>
</tbody>
</table>

*P*-values from Pearson Chi-Square test. n.a; not applicable. Values in bold represent statistically significant *P* values.
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.

<table>
<thead>
<tr>
<th>Patient category</th>
<th>RFS_CYP27A1 (ref. low)</th>
<th>OS_CYP27A1 (ref. low)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All tumors</td>
<td>0.76</td>
<td>0.82</td>
</tr>
<tr>
<td>All ER positive tumors</td>
<td>0.70</td>
<td>0.74</td>
</tr>
<tr>
<td>ER positive/≤50 years</td>
<td>0.53</td>
<td>0.49</td>
</tr>
<tr>
<td>ER Positive/&gt;50 years</td>
<td>0.85</td>
<td>0.93</td>
</tr>
</tbody>
</table>

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.
CHOLESTEROL AND BREAST CANCER

S Kimbung et al.

α

Mast...low-grade (grades 1 and 2) tumors, but this comparison
91% of high-grade (grade 3) tumors compared to 60% of
analyses indicated that CYP27A1 was expressed in up to
CYP27A1 was more frequently observed among high-grade
Using immunohistochemistry, Nelson and coworkers
pathological features of importance in breast cancer.
Considering these findings, it was of interest to evaluate
hepatic and plasma cholesterol levels (...

significantly reduces plasma 27HC without affecting
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
interfere with the activation of SREBP2, the master
and ABCG1. In addition, 27HC can bind to INSIG1
can function as a ligand of the liver X receptor and induce
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
Era-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 Era 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
intracellular cholesterol biosynthesis. Thus, under normal physiological function of CYP27A1 is to regulate
expression of the enzymes required for

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 Era 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 Era
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 Era-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
Era-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 Era 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

DOI: 10.1530/ERC-16-0533

Printed in Great Britain

Published by Bioscientifica Ltd.
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.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-16-0533.

**Declaration of interest**

S Borgquist has received consultant and speaker fees from Roche and Novartis. The other authors declare that they have no competing interests.

**Funding**

This work was supported by grants from the Governmental Funding of Clinical Research from the National Health Services, the Swedish Breast Cancer Organization (BRO), the Mrs Berta Kamprad Foundation, the Swedish Research Council and the Swedish Cancer Foundation.

**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.

**Acknowledgements**

The authors express their profound gratitude to Kristina Lövgren for performing the CYP27A1 IHC staining. They are also very highly indebted to the Senior Pathologist Dorthe Grabau for annotating of the CYP27A1 IHC slides.

**References**


Received in final form 13 April 2017

Accepted 25 April 2017

Accepted Preprint published online 25 April 2017