Global transcriptional changes following statin treatment in breast cancer

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Statins induce changes in gene expression in breast cancer

120-150 words statement of translational relevance

Statins are per-oral drugs normally prescribed as cholesterol-lowering agents. Studies have shown cholesterol-independent pleiotropic effects related to cancer development. Statins purportedly exert anti-tumoral effects on breast cancer cells by decreasing proliferation and increasing apoptosis, supported by data on the protein level. However, less is known about the mechanisms of action at the transcriptional level. In this window-of-opportunity trial, 50 patients with primary invasive breast cancer were prescribed atorvastatin (80 mg/day) for two weeks pre-surgically. Global gene expression profiling was performed on the pre- and post-treatment tumor sample pairs. This study demonstrates highly significant changes in the expression of genes related to the MAP-kinase pathway and apoptosis, suggesting statin-induced cancer-inhibitory effects. The results were confirmed in vitro in breast cancer cell lines. Future phase III breast cancer trials are needed to address the potential role of statins as anti-cancer drugs in addition to current treatment guidelines.
Statins induce changes in gene expression in breast cancer

**Abstract**

**Background**

Statins purportedly exert anti-tumoral effects, but the underlying mechanisms are currently not fully elucidated. The aim of this study was to explore potential statin-induced effects on global gene expression profiles in primary breast cancer.

**Study Design and Methods**

This window-of-opportunity phase II trial enrolled 50 newly diagnosed breast cancer patients prescribed atorvastatin (80 mg/day) for two weeks pre-surgically. Pre- and post-treatment tumor samples were analyzed using Significance Analysis of Microarrays (SAM) to identify differentially expressed genes. Similarly, SAM and gene ontology analyses were applied to gene expression data derived from atorvastatin-treated breast cancer cell lines (MCF7, BT474, SKBR3 and MDA MB231) comparing treated and untreated cells. The Systematic Motif Analysis Retrieval Tool (SMART) was used to identify enriched transcription-factor-binding-sites. Literature Vector Analysis (LitVAn) identified gene module functionality, and pathway analysis was performed using GeneGo Pathways Software (MetaCore™)(https://portal.genego.com/).

**Results**

Comparative analysis of gene expression profiles in paired clinical samples revealed 407 significantly differentially expressed genes (FDR=0); 32 up-regulated and 375 down-regulated genes. Restricted filtration (fold change \(\geq 1.49\)) resulted in 21 up-regulated and 46 down-regulated genes. Significantly up-regulated genes included *DUSP1, RHOB1, GADD45B* and *RGS1*. Pooled results from gene ontology, LitVAn and SMART analyses identified statin-induced effects on the apoptotic and Mitogen-activated phosphatase kinase pathway (MAPK) pathways among others. Comparative analyses of gene expression profiles
Statins induce changes in gene expression in breast cancer in breast cancer cell lines, showed significant up-regulation of the mevalonate and pro-apoptotic pathways following atorvastatin treatment.

Conclusions
We report potential statin-induced changes in global tumor gene expression profiles, indicating MAPK pathway inhibition and pro-apoptotic events.
Statins induce changes in gene expression in breast cancer

Introduction

Statins are generally prescribed as cholesterol-lowering agents for patients with cardiovascular disease and hypercholesterolemia. Statins act by reducing de novo cholesterol synthesis through the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme of the mevalonate pathway (1). Further, HMGCR has been suggested to harbor oncogenic potential, and deregulation of the mevalonate pathway may promote transformation (2). A growing amount of literature has addressed the cholesterol-independent pleiotropic effects exerted by statins, including favorable anti-cancer effects (3-6). In vitro studies using lipophilic statins have shown reduced tumor cell proliferation, invasiveness, and survival following statin treatment (3, 4, 7). In vivo studies have confirmed statin-induced tumor growth inhibition associated with reduced tumor cell proliferation and survival (3). A previous phase II statin trial conducted by Garwood et al. showed significant changes in high-grade breast cancer tumors, including reduced proliferation and increased apoptosis (5). The complexity of the anti-cancer properties of statins also includes cholesterol-dependent effects driven by the systemic lowering of cholesterol levels, thus depriving tumor cells from their increased demand of cholesterol uptake (8).

Previous statin breast cancer trials have reported changes in single genes or proteins, but statin-mediated changes in cancer-specific whole genome expression profiles have not been reported. Gene expression profiling has predominantly been used to classify tumors, to identify biological signatures and to search for novel biomarkers (9, 10). The comparison of gene expression profiles in tumor biopsies acquired before and after a given treatment enables identification of important signaling pathways and may detect transcriptional responses to a specific therapy.
Statins induce changes in gene expression in breast cancer

We have previously reported changes in the protein expression of Ki67 and HMGCR in tumors from breast cancer patients treated with high-dose statins for two weeks prior to surgery within this phase II window-of-opportunity trial (11). In this part of the trial, the aim was to investigate statin-induced effects at the transcriptional level by comparing pre-treatment and post-treatment samples in order to deepen the insight into the molecular mechanisms of statins in breast cancer.

Materials and Methods

Trial design

The trial was designed as a phase II study using the “window-of-opportunity” design, in which the treatment-free window between a cancer diagnosis and surgical tumor resection is used to study the biological effects of a certain drug. In this trial, atorvastatin, a lipophilic statin, was prescribed to patients with primary breast cancer for two weeks pre-operatively. As a non-randomized trial, all patients received an equal daily dose of 80 mg of atorvastatin for two weeks. The trial was conducted as a single-center study at Skåne University Hospital in Lund, Sweden. All patients signed an informed consent form. The Ethics Committee at Lund University and the Swedish Medical Products Agency approved this trial. The study has been registered at ClinicalTrials.gov (NCT00816244) and adheres to the REMARK guidelines (12).

Patients

Patients were included according to the trial’s inclusion criteria, which have been reported in detail earlier (11). The trial was opened for recruitment in February 2009, and the pre-planned number of 50 patients was achieved in March 2012. Of the 50 patients enrolled in the study, a
Statins induce changes in gene expression in breast cancer
total of 42 patients completed all parts of the study. The motivations for discontinuation have been reported previously (11).

**Endpoints and tumor evaluation**

The primary endpoint of the study was statin-induced tumor response measured as change in tumor proliferation (i.e. Ki67 protein expression). The secondary endpoints included change in tumoral HMGCR expression as well as changes in gene expression following pre-surgical atorvastatin during a two-week “window-of-opportunity” period. At the time of enrollment, all participants underwent study specific core biopsies prior to the initiation of statin treatment. From each patient, one core biopsy was formalin-fixed immediately, and another core was collected and stored at -80°C. Following the two-week statin treatment, breast surgery was performed according to standard surgical procedures, and both fresh frozen and formalin-fixed tumor tissue was retrieved from the primary tumor at the Department of Pathology at Skåne University Hospital, Lund, Sweden.

**Cell lines, cell culture and treatments**

The human breast cancer cell lines MCF7, BT474, SKBR3 and MDAMB231 were purchased from the American Type Culture Collection (Rockville, MD) and maintained in culture as recommended by the vendors. Atorvastatin was purchased from Sigma and diluted in dimethyl sulphoxide (DMSO). Cells were exposed to either atorvastatin or vehicle (DMSO) for 48 hours after which total RNA was extracted and subjected to whole genome transcriptional profiling. Three independent experiments were performed per cell line.

**RNA extraction**

Total RNA was extracted from fresh frozen tumor samples and cell lines using an Allprep
Statins induce changes in gene expression in breast cancer

DNA/RNA mini kit (QIAGEN, Valencia, CA) in a QIAcube (QIAGEN) according to the manufacturer’s instructions. Prior to RNA extraction, an H/E stained section of the core needle biopsies was prepared whenever possible for determination of tumor cellularity. The tumor cellularity was greater than 50% in about 70% (14/21) of evaluable cases. The RNA integrity was assessed on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA), and RNA quantification was performed using a NanoDrop ND-1000 (NanoDrop Products, Wilmington, DE). Only samples with a RIN value ≥ 7 were included in further analyses. Labeled samples were hybridized to Human HT-12 v4.0 Expression BeadChips (Illumina Inc., San Diego, CA) in two batches at the SCIBLU Genomics Center at Lund University, Sweden (www.lu.se/sciblu). RNA extracted from cell lines was processed in one batch at a later time point. The Illumina probes were re-annotated using the R package illuminaHumanv4.db (13). The gene expression data have been submitted to the NCBI's Gene Expression Omnibus (GEO) database (GSE63427).

Microarray data analysis and statistical analysis

Microarray data were initially pre-processed and normalized using the Quantile Normalization method (14). These analyses were performed using GenomeStudio Software V2011.1. Probe sets with signal intensities below the median of the negative control intensity signals in ≥80% of the samples were excluded. Replicate probe sets were merged by the median of signal intensity values. A principal component analysis (PCA) investigating associations between technical factors with the main principal components was performed whereupon a batch effect was detected to be associated with the 7th PC. A supervised empirical Bayes method (ComBat) was unable to resolve this technical artifact but since it was not associated with main PCs, we believe the effects on the final results are minimal.
Statins induce changes in gene expression in breast cancer

A Significance Analysis of Microarrays (SAM) using 500 permutations was performed with TMeV v 4.9 software (15) to identify differentially expressed genes between paired pre- and post-treatment samples. To search for enriched transcription factor binding site (TFBS) motifs among the differentially expressed genes, the transcription factor binding analysis program, Systematic Motif Analysis Retrieval Tool (SMART), was used as previously described (16). Briefly, the promoter regions of differentially expressed genes were scanned for TFBS. Promoter regions were defined as the genomic interval between -1500 to +500 bp relative to the putative transcription start sites. Two criteria for significance were used: significantly enriched TFBS in terms of fraction of promoters and significantly present TFBS in terms of the fraction of promoters with binding sites. The identification of significant TFBS was performed by a resampling procedure in which the query gene set was compared with typically $10^5$ gene lists of similar size randomly drawn from the TFBS/promoter database. Functional annotation and pathway analysis was performed using GeneGo Pathways Software (MetaCore™), and gene module functional analysis was done using Literature Vector analysis (LitVAn) (17). A flow diagram outlining the data processing and analyses is shown in Supplementary Fig. S1.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Expression levels of *HMGCR* (exon 6-7 and exon 13-14), *DUSP1*, *RHOB*, *JUN* and *FOS* in the clinical samples were validated using RT-qPCR. Briefly, one µg of total RNA was reversely transcribed (Quantitect® Reverse Transcription Kit, Qiagen), and cDNA corresponding to five ng of total RNA was used as a template in the qPCR (Quantitect® Probe PCR Kit, Qiagen) with predesigned hydrolysis probe assays (Life Technologies). *PUM1*, *SLU7* and *PPIG* were used as endogenous reference genes. cDNA pooled from six normal breast tissue specimens served as a normalizer. All samples were run in triplicate. A
Statins induce changes in gene expression in breast cancer

standard curve was included in each run to verify the reaction efficiency, and no template controls were used. Expression ratios were calculated by the \(2^{-\Delta\Delta CT}\) formula. All assays used are listed in Supplementary Table S1. For statistical analysis of related samples, Wilcoxon signed-rank test was applied using SPSS version 19.

Results

Patients

The average age of all 50 patients at the time of inclusion was 63 years (range: 35-89 years). Forty post-treatment samples were evaluable by global gene expression analysis, but the inferior quality of several core biopsies limited the number of eligible tumor sample pairs to 25. A similar age distribution was seen among the 25 patients eligible for this study (mean 62 years, range 35-82 years). All tumors were invasive breast cancers, with tumor characteristics extensively described previously (11). Accordingly for the 25 eligible pairs, the mean pathological tumor size was 22 mm ranging from 13 to 32 mm. The majority was ER positive (96%) and sixteen percent were HER2 positive at baseline. HMGCR protein expression was present in 60% of the samples prior to treatment and 15 out of the 25 tumor pairs demonstrated a decrease in tumor proliferation following statin treatment; assessed by IHC staining for Ki67 as we previously reported.

Statin-treatment-induced gene expression alterations in paired clinical breast cancer samples

A two-class paired SAM analysis was performed to identify genes differentially expressed between paired samples (pre- and post-statin-treatment). In total, 407 genes were identified to be significantly changed (FDR=0) following statin treatment. Of these, 32 genes were up-regulated, while 375 were down-regulated (Supplementary Table S2). Following filtration of
Statins induce changes in gene expression in breast cancer genes of undefined function located at certain loci, referred to as LOC-genes 323 of the 407 genes remained. Upon further filtration, requiring a fold change of ≥1.49, a total of 67 genes remained; 21 up-regulated and 46 down-regulated (Fig. 1), which represent the genes used in all subsequent analyses. Dual-specificity phosphatase 1 (DUSP1), also identified as mitogen-activated protein kinase phosphatase-1, a well-known inhibitor of the mitogen-activated protein kinase (MAPK) pathway (18), was significantly up-regulated. Other highly significant up-regulated genes included the Ras homolog family member B (RHOB), growth arrest and DNA-damage-inducible beta (GADD45B), and the regulator of G-protein signaling 1 (RGS1).

In addition, key members of the AP-1 transcription factor complex such as FOS, FOSB, JUN and JUNB were also significantly up-regulated in the post-statin treatment samples. Gene ontology and pathway analyses revealed that GnRH signaling, immune response, PI3K/AKT, MAPK and apoptosis were among the significantly deregulated biological processes and pathways following statin treatment (summarized in Table 1).

To further explore the potential functional significance of the altered genes, we applied LitVAn, a vector-based literature search tool. The module network in Fig. 2 illustrates the connections between the significantly altered genes upon statin therapy. Interestingly, the LitVAn-derived network converged towards key genes in the MAPK signaling pathway, including MAPK, Extracellular signal-Regulated Kinase (ERK), Jun Kinase (JNK) and p38, as visualized in the network.

A sub-analysis was performed in view of identifying additional gene expression changes specific to the subset (15 out of 25) of patients, which responded to the statin treatment with a decrease in tumor proliferation (evaluated by IHC staining for the proliferation marker Ki67) as was previously reported (11). SAM followed by gene ontology analysis on the list of significantly altered genes (FDR<0.05) did not provide any additional information beyond what was observed when the entire cohort was tested (data not shown).
Statins induce changes in gene expression in breast cancer

Analysis of transcription factor binding sites enriched in genes altered by statin treatment

The TFBS analysis program SMART was applied to further investigate if genes altered upon statin treatment were potentially co-regulated by specific groups of transcription factors. The binding site for the transcription factors with significant enrichment and the percentages of motif presence are shown in Fig. 3. Among the TFBS identified with significant enrichment (<0.005) and >50% hits included cAMP responsive element binding protein 1 (CREB1), octamer transcription factor (OCT), activating transcription factor (ATF) and serum response factor (SRF), as shown in Supplementary Table S3. ATF is an important paralog of CREB1, and both ATF and CREB1 bind to cAMP response element (CRE), a sequence present in many cellular promoters (19). ATF can build a subunit dimer with Jun, a member of the bZip protein family, which shares the basic region (b) for DNA binding and leucine zipper (Zip) for dimerization (20). SRF is a 67-kDa ubiquitous protein that binds to serum response element (SRE) in the promoter region of target genes and regulates many immediate-early genes such as c-fos, thereby participating in apoptosis and cell differentiation, among other functions (21). Both SRF and ATF have been connected to the MAPK pathway (20, 21). OCT, also known as POU class 2 homeobox 1, is a member of the POU domain transcription factor family, and it binds to the octamer motif and interacts with regulatory interleukin and histone genes (22).

RT-qPCR validation of candidate genes in pre- and post-statin treated clinical samples

The results from the RT-qPCR analyses showing the expression ratios of five candidate genes: \textit{HMGCR, DUSP1, RHOB, JUN} and \textit{FOS} between the pre-treatment samples and the post-treatment samples are shown in Supplementary Fig. S2. \textit{DUSP1, RHOB, JUN} and \textit{FOS} were found to be up-regulated in the post-treatment samples by RT-qPCR, displaying...
Statins induce changes in gene expression in breast cancer relatively similar trends in fold changes as was observed by microarray profiling (Supplementary Fig. S2A). While DUSP1, RHOB, JUN and FOS were consistently up-regulated after treatment in the majority of cases, the expression of HMGCR increased in 12 of 25 cases, while it decreased upon statin treatment in the remaining 13 cases (Supplementary Fig. S2B). Consistent with the results from microarray SAM analyses, these alterations were not statistically significant (P>0.05). In analyses stratified for treatment effects in terms of any decrease in Ki67, no significant changes in HMGCR were detected in either stratum (data not shown). Similarly, no significant change in HMGCR was observed after stratification by change in protein expression of HMGCR (data not shown).

In vitro statin-treatment-induced gene expression alterations

To test if the observed gene expression changes in the clinical samples were directly associated with statin treatment, total RNA extracted four human breast cancer cell lines was subjected to whole genome transcriptional profiling following 48 hours atorvastatin treatment. Compared to vehicle (DMSO) treated controls, 48 hours of statin exposure was found to consistently and significantly up-regulate several key genes involved in the cholesterol biosynthesis pathway in all four cell lines (Fig. 4A). Furthermore, a significant down-regulation of genes involved in cell proliferation and cell cycle progression; particularly genes necessary for the G2/M-phase was noted. In addition, a significant up-regulation of many pro-apoptosis genes was noted in some cell lines. Of note, and consistent with the clinical data, a significant up-regulation of some DUSP genes (DUSP4 and DUSP6) which regulate the MAPK activity, RHOB and the AP-1 transcription factor JUN was seen following statin treatment in a subset of the cell lines. A summary of the significantly altered biological processes and pathways in the cell lines is presented in Fig. 4B.
Statins induce changes in gene expression in breast cancer

Discussion

Despite a plethora of novel anti-cancer treatments, breast cancer recurrences and mortality are still major concerns, and additional treatments are required. Statins are inexpensive per-oral drugs with few adverse effects, and suggested anti-cancer effects are currently being tested in more than 50 clinical trials, both in the preventive and adjuvant treatment settings (http://www.clinicaltrials.gov). The identification of predictive biomarkers for statin efficacy is essential, considering the heterogeneous nature of breast cancer, as demonstrated in vitro in cell lines displaying substantial differences in statin susceptibility (1, 23). Further, the mechanisms behind the anti-cancer effects induced by statins are not fully understood, and translational studies within clinical trials addressing the biological effects of statins are needed (24).

To our knowledge, this is the first report of potential global transcriptional alterations in breast cancer following statin treatment, demonstrated in both breast cancer cell lines and clinical breast cancer samples. In the clinical part of this study, we have described substantial changes in gene expression profiles following two weeks of pre-surgical statin treatment, suggesting inhibition of the mitogen-activated protein kinase (MAPK) pathway and increased apoptosis. A number of significant up-regulated genes are described: **DUSP1, RGS1, RHOB, GADD45B, FOS and JUN**. **DUSP1** acts as an inhibitor of the MAPK pathway and is involved in the regulation of cell growth and cell death (25). The downstream substrates of the MAPK pathway have been demonstrated to regulate vital cellular activities, including growth, differentiation, apoptosis, immune function and development (26).

Interestingly, in this study, the up-regulation of **DUSP1**, culminated in the induction of apoptosis as revealed by gene ontology analysis. Furthermore, an independent role of **DUSP1** was suggested in the LitVAn network analysis, indicating **DUSP1** to be a key player regulating several genes, although with limited actions by other genes towards **DUSP1**.
Statins induce changes in gene expression in breast cancer itself. In this context, several studies addressing the role of \textit{DUSP1} in cancer have reported conflicting results. In a study performed by Pervin \textit{et al}., apoptosis was triggered by nitric oxide (NO) in human breast cancer cell lines, increasing DUSP-1 expression upon NO treatment, which led to inactivation of ERK (27). In line with our results, a study on non-small-cell lung cancer cells showed that, overexpression of DUSP-1 was associated with a decrease in cell growth (28). Evidently, the effects of kinase and phosphatase activity depend on many factors such as time and duration of the activity, as well as tumor type and tumor grade (29). Improved understanding of the cross talk between substrates within the MAPK pathway and the role of the main down-stream products is necessary. In this study, the expression of \textit{DUSP1} was highly up-regulated in the clinical samples after statin treatment, whereas \textit{in vitro}, up-regulation of other DUSPs (\textit{DUSP4} and \textit{DUSP6}) was observed. Of note, \textit{DUSPs} specifically dephosphorylate threonine and tyrosine residues on MAPKs and render them inactive. Further studies are warranted to elucidate the importance of \textit{DUSPs} in breast cancer and their role in mediating the anti-cancer effects exerted by statins.

Apoptosis was found to be significantly up-regulated following statin treatment both \textit{in vivo} in clinical samples and \textit{in vitro}. \textit{GADD45B}, an important pro-apoptotic gene, was up-regulated in the clinical samples. \textit{GADD45s} play important roles in a plethora of cellular processes, including growth control and apoptosis (30). The MAP kinases p38 and JNK have complex roles in the regulation of \textit{GADD45s}, with tissue and cell-type-specific differences (30). Interestingly, gene enrichment analysis implicated the GnRH signaling pathway in response to statin treatment. GnRH analogs have been used in the treatment of endocrine dependent cancers, including breast cancer (31). In line with our results, in the identification of potential anti-proliferative target genes upon GnRH receptor activation in cell lines, \textit{DUSP1}, \textit{JUNB}, \textit{FOS} and \textit{FOSB} were among the up-regulated genes (32). Wu \textit{et al.} showed that GnRH type II induces apoptosis in human endometrial cancer cells by activating...
Statins induce changes in gene expression in breast cancer

**GADD45A** (33). Further, GADD45B has been suggested to have a novel function during Fas-induced apoptosis where it acts as an adaptor between p38 and retinoblastoma tumor suppressor (Rb) to enhance p38-mediated phosphorylation of Rb, an important step during Fas-induced apoptosis (34).

Another up-regulated gene in this study, **RHOB**, is a member of the Ras superfamily of proteins with GTPase activity that are conserved and widely expressed in different tissues. Rho protein expression and/or activity is frequently altered in many types of cancers (35). Statin induced inhibition of HMGCR, the rate-limiting enzyme in the mevalonate pathway, also causes a decrease of down-stream products such as isoprenoids. The inhibition of isoprenylation results in the inactivation of small GTPase proteins. Unlike its family members RhoA and RhoC, which are often over-expressed in different types of cancers, RhoB has been reported to be down-regulated in human cancers, but in lung and gastric cancer, its expression significantly inhibits proliferation, migration and invasion (35).

Transcription factor binding motifs for **CREB1, ATF, OCT** and **SRF** were identified in most of the differentially expressed genes upon statin treatment in this trial. Few breast cancer studies have investigated the changes in transcription factors after statin treatment, and to our knowledge, no human studies have addressed the topic. However, *in vitro* studies reported by Campbell *et al.* showed down-regulation of AP-1 and NFκB DNA binding site activity in breast cancer cell lines 48 hours after statin treatment (3). Corresponding with our results, a concomitant and significant decline in various MAP kinase proteins (p-ERK1/2, p-JNK, p-p38) was observed (3). In future studies, the up-regulated transcription factors and their proposed target genes, can preferably be validated in tissue cultures or animal models; however such validation studies were considered to be beyond the scope of this work.
Statins induce changes in gene expression in breast cancer

The mechanism of statin induced anti-proliferative and pro-apoptotic effects in cancer cells is currently not clear. Functional in vitro and in vivo studies have demonstrated mutant *p53*-dependent up-regulation of genes in the mevalonate pathway in *p53* mutant breast cancer cells (36). Furthermore, induction of RhoA-dependent retention of the transcription factor NFkB, leading to transcriptional down-regulation of the anti-apoptotic protein BCL2, and reduced production of AKT1 upon statin treatment has been reported (37). Induction of apoptosis via activation of the JNK/CHOP/DR5 pro-apoptotic pathway has also been shown (38). Koyuturk *et al.* showed that statins activate the JNK pathway, leading to apoptosis in breast cancer cell lines, whereas the ERK1/2 and p-38 MAPK pathways were not involved in the anti-cancer activity of simvastatin in that study (39). In the same study, simvastatin inhibited proliferation and induced apoptotic cell death, without altering the expression of either wild-type or mutant *p53* (39). Spamapanato *et al.* demonstrated that statins induced apoptosis in breast cancer cell lines, with increased expression of the pro-apoptotic gene *BAX* and decreased expression of the anti-apoptotic gene *BCL-2*, both at the mRNA and protein levels (7). No significant changes in the expression of the *BAX* and *BCL-2* genes were observed in the clinical samples in the present study but a significant decrease in *BCL-2* was observed in MDAMB231 cells and an up-regulation of the BNIP3 gene, which is a member of the BCL2/adenovirus E1B 19 kd-interacting protein (BNIP) family and a strong apoptosis inducer, was observed in all four cell lines.

To summarize the complex interrelationship of statin-induced transcriptional alterations, findings from the gene ontology, LitVAn and TFBS analyses were integrated, leading to the proposed statin-induced apoptosis network presented in Fig. 5. A consistent finding across all three approaches was the enrichment of genes involved in the MAPK pathway and the apoptotic process. In Fig. 5, *RHOB* activation and *DUSP1* inhibition of
Statins induce changes in gene expression in breast cancer

MAPK leads to JNK activation, which activates GADD45B resulting in the induction of apoptosis. On the other hand, RGS1 inhibition on CXCR4 inhibits cell survival.

Gene set enrichment analysis suggested an effect on the immune system, which concurs with previous, reports on statin-induced immunoregulatory effects (40). The amount of tumor infiltrating lymphocytes (TILs) in tumor tissue has received increasing scientific attention lately in terms of prognosis and treatment prediction (41, 42). Potential changes in TILs in this study would have been of interest to validate the gene set enrichment results but these analyses were restricted due to limited amount of tumor tissue.

We have previously reported that breast cancers expressing HMGCR in pre-treatment samples showed a significant decrease in Ki67 after statin treatment (11). Both microarray and RT-qPCR analyses showed no significant differences in HMGCR mRNA expression between the pre- and post-treatment samples. However, some sample pairs showed a clear up-regulation whereas others displayed down-regulation of HMGCR mRNA, suggesting that the transcriptional effects of statin treatment observed herein may be dependent upon the regulation of HMGCR in a differential manner. In the statin treated cell lines, however, a significant up-regulation of genes involved in the mevalonate pathway, including HMGCR, was observed. This up-regulation of mevalonate pathway genes corresponds with the previously reported robust homeostatic feed-back response, which is triggered upon the inhibition of HMGCR by statins leading to increased transcriptional activity of the sterol regulatory element-binding proteins (SREBP) transcription factor (1).

The diverse results on mevalonate pathway inhibition seen in the clinical samples compared to the in vitro samples may reflect the interplay between hepatic-driven levels of circulating cholesterol and intracellular cholesterol levels in the tumor in humans (1, 43).

In this window-of-opportunity phase II trial, we have reported for the first time possible statin-induced changes in global gene expression converging on the suggested
Statins induce changes in gene expression in breast cancer.

Apoptotic effects. We investigated transcriptional changes after two weeks of statin treatment, which limits the study to one time point. The treatment time required to translate transcriptional activities to a measurable biological phenotype is currently unknown. Further investigations of earlier or later transcriptional effects of statins are warranted. Stratification of analyses for ER and HER2 status would have been of interest but the relatively homogenous composition of this cohort (only one patient was diagnosed with an ER negative breast cancer and four with a HER2 positive disease) prohibited such analyses, which should be considered in larger future studies. Although previous studies addressing the interactions between cancer and statin treatment have shown promising results, additional prospective studies, preferably large, randomized, clinical phase III trials, are needed to understand statin’s putative role in future breast cancer treatment.

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Statins induce changes in gene expression in breast cancer

Disclosures

The authors declare that they have no competing interests.

References

Statins induce changes in gene expression in breast cancer

Statins induce changes in gene expression in breast cancer


Figure legends

**Fig. 1:** Heat map demonstrating the transcriptional changes in pre- and post-treatment samples after two weeks of statin therapy. Data generated from SAM analyses followed by filtering out of genes with uncertain functions (LOC genes) and fold change <1.49. Red in the heatmap represents up-regulation, and green represents down-regulation.
Statins induce changes in gene expression in breast cancer

**Fig. 2:** The suggested gene function modality affected by two weeks of statin therapy as visualized by LitVAn.

**Fig. 3:** Illustration of the binding sites for transcription factors identified by SMART analyses. Enriched TFBS were identified using the -1500,+500 portions of the promoters. Colored boxes indicate transcription factor binding sites for the respective genes from the gene expression analysis.

**Fig. 4:** *In vitro* statin induced transcriptional changes. A) Up-regulation of genes involved in cholesterol biosynthesis (mevalonate pathway) was observed in four breast cancer cell lines following 48h atorvastatin treatment. B) Summary of altered biological processes and pathways following 48h atorvastatin treatment in breast cancer cell lines.

**Fig. 5:** The hypothetical apoptosis network of combined data from the gene expression, LitVAn and SMART analyses. Red ovals are up-regulated genes from the gene expression analysis. Green squares show results from the LitVAn analysis, and blue squares show transcription factor binding sites from the SMART analysis. ➔ indicates expression/activation, and ⊥ indicates inhibition. Solid lines represent connections that have been shown in the literature, and dotted lines represent hypothetical connections.

**Supplementary Figures**

**Supplementary Fig. S1:** Flowchart of patients included in the trial and the data analyses conducted.

**Supplementary Fig. S2:** RT-qPCR validation of the expression of five selected genes. A) Expression of *HMGCR, DUSP1, RHOB, JUN* and *FOS* measured by microarray and RT-qPCR respectively. B) Change in *HMGCR* expression in all patients by RT-qPCR.
Table 1.
Gene ontology and pathway analyses identified deregulated biological processes using significantly differentially expressed genes following statin treatment as input.

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<tr>
<td>4 Immune response_IL-3 activation and signaling pathway</td>
<td>31</td>
<td>2.01536E-06</td>
<td>9.67345E-05</td>
<td>4</td>
<td>c-Jun/c-Fos, EGR1, AP-1, c-Jun, c-Fos</td>
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<td>5 Immune response_Oncostatin M signaling via MAPK in mouse cells</td>
<td>35</td>
<td>3.3261E-06</td>
<td>0.00127722</td>
<td>4</td>
<td>EGR1, AP-1, c-Jun, c-Fos</td>
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<td>6 Immune response_Oncostatin M signaling via MAPK in human cells</td>
<td>37</td>
<td>4.17812E-06</td>
<td>0.00127818</td>
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<td>EGR1, AP-1, c-Jun, c-Fos</td>
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<td>7 Immune response_Human NKG2D signaling</td>
<td>38</td>
<td>4.66002E-06</td>
<td>0.00127818</td>
<td>4</td>
<td>c-Jun/c-Fos, AP-1, c-Jun, c-Fos</td>
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<td>8 Development_Growth hormone signaling via PI3K/AKT and MAPK cascades</td>
<td>42</td>
<td>7.0081E-06</td>
<td>0.00149506</td>
<td>4</td>
<td>EGR1, JunB, c-Jun, c-Fos</td>
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<td>9 Immune response_Murine NKG2D signaling</td>
<td>42</td>
<td>7.0081E-06</td>
<td>0.00149506</td>
<td>4</td>
<td>c-Jun/c-Fos, AP-1, c-Jun, c-Fos</td>
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<td>10 Development_Activation of Erk by ACM1, ACM3 and ACM5</td>
<td>44</td>
<td>8.46451E-06</td>
<td>0.00156068</td>
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<td>c-Jun/c-Fos, Cyr61, EGR1, c-Fos</td>
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**Biological processes**

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<thead>
<tr>
<th>Biological processes</th>
<th>Total</th>
<th>P-value</th>
<th>FDR</th>
<th>In Data</th>
<th>Network objects from active data</th>
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<tbody>
<tr>
<td>1 Apoptotic process</td>
<td>1806</td>
<td>3.571E-07</td>
<td>0.000029</td>
<td>10</td>
<td>CYR61, RHOB, EGR1, IER3, GADD45B, CXCR4, JUN, CTGF, DUSP1, C8orf4</td>
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<td>2 Positive regulation of cell death</td>
<td>482</td>
<td>7.331E-08</td>
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<td>CYR61, RHOB, EGR1, GADD45B, JUN, CTGF, DUSP1</td>
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<td>3 Regulation of cell cycle</td>
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<td>0.00003646</td>
<td>0.000112</td>
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<td>RHOB, IER3, GADD45B, JUN, JUNB, CTGF, DUSP1</td>
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<tr>
<td>4 MAPK cascade</td>
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<td>5.765E-07</td>
<td>0.0000345</td>
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<td>CYR61, FOS, GADD45B, CXCR4, JUN, CTGF, DUSP1</td>
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</tbody>
</table>
Figure 4

(A) Heatmap showing gene expression changes in MCF7, BT474, MDA-MB-231, and SKBR3 cells in control and treated conditions. The expression levels are color-coded, with green representing lower expression and red representing higher expression.

(B) P-value heatmap indicating statistical significance of the gene expression changes. The color gradient represents the range of P-values, with red indicating lower P-values and blue indicating higher P-values.

Additional information:
- Cholesterol biosynthetic process
- Isoprenoid biosynthetic process
- Sterol biosynthetic process
- P53 downstream pathway
- Glycolysis gluconeogenesis
- MAPK targets Nuclear events mediated by MAP kinases
- ERK1_ERK2 MAPK pathway
- Stress-activated MAPK cascade
- Apoptotic process
- Mitotic cell cycle
- Cell division
- M phase of mitotic cell cycle
- G1/S transition of mitotic cell cycle
- Cell proliferation
- Negative regulation of cell proliferation
- Cell cycle arrest
Figure 5

Results from gene expression

Results from LitVAn

Results from SMART

Cell Survival Apoptosis
Clinical Cancer Research

Global transcriptional changes following statin treatment in breast cancer

Clin Cancer Res  Published OnlineFirst April 3, 2015.

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